An environmental metabolomics study of the effect of abiotic substances on *Pseudomonas putida* by employing analytical techniques

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# Table of contents

Table of contents ........................................................................................................... 2  
List of Figures .................................................................................................................. 7  
List of Tables .................................................................................................................... 15  
Abstract ........................................................................................................................... 16  
Declaration ....................................................................................................................... 17  
Copyrights Statement ...................................................................................................... 18  
Dedication ......................................................................................................................... 19  
Acknowledgment ............................................................................................................. 20  
Abbreviations and Acronyms .......................................................................................... 21  
Preface ............................................................................................................................... 24  
1 Chapter 1: Literature review ......................................................................................... 25  
1.1 Introduction ............................................................................................................... 25  
1.1.1 The impact of active pharmaceutical compounds on the environment .......... 25  
1.1.2 Organic solvent tolerant microorganisms ......................................................... 26  
1.1.2.1 Physiological basis of solvent toxicity and response to the toxic effects of organic compounds ................................................................. 26  
1.1.2.2 Efflux pump systems ...................................................................................... 29  
1.1.3 Metabolomics ....................................................................................................... 30  
1.1.3.1 Introduction to metabolomics ........................................................................ 30  
1.1.3.2 Techniques employed in metabolomics studies ............................................. 31  
1.1.3.3 Metabolomics experiment (metabolomics pipeline) ..................................... 34  
1.1.4 Introduction to data analysis (Chemometrics) ....................................................... 34  
1.1.4.1 Unsupervised methods .................................................................................. 35  
1.1.4.1.1 Principal components analysis (PCA) ....................................................... 35  
1.1.4.2 Supervised methods ...................................................................................... 36  
1.1.4.2.1 Discriminant function analysis (DFA) ...................................................... 37  
1.1.4.3 Model validation ............................................................................................ 38
Table of contents

1.1.5 Analytical techniques for metabolomics

1.1.5.1 Fourier-transform infrared (FT-IR) spectroscopy

1.1.5.2 Raman spectroscopy

1.1.5.3 Gas chromatography mass spectrometry (GC-MS)

1.1.5.4 Liquid chromatography mass spectrometry (LC-MS)

1.2 Research Objective

1.3 References

2 Chapter 2: Metabolomics analysis reveals the participation of efflux pumps and ornithine in the response of Pseudomonas putida DOT-T1E cells to challenge with propranolol

2.1 Abstract

2.2 Introduction

2.3 Material and methods

2.3.1 Bacterial strains and cultivation of bacteria

2.3.2 Growth curve monitoring

2.3.3 Growth in response to propranolol shock, sample collection and analysis

2.3.3.1 Growth curve measurement

2.3.3.2 FT-IR analysis

2.3.3.2.1 Sample collection

2.3.3.2.2 Sample preparation for FT-IR spectroscopy

2.3.3.2.3 FT-IR setup

2.3.3.2.4 FT-IR data analysis

2.3.3.3 GC-MS analysis

2.3.3.3.1 Sample collection

2.3.3.3.2 Metabolic quenching and metabolite extraction

2.3.3.3.3 Derivatisation process

2.3.3.3.4 GC-MS instrument setup

2.3.3.3.5 Data analysis

2.4 Results and discussion

2.4.1 Characterisation of P. putida DOT-T1E strains
Table of contents

2.4.2 Characterisation of *P. putida* DOT-T1E strains to propranolol shocks. ....... 73

2.4.2.1 Minimal inhibitory concentration (MIC) .............................................. 73
2.4.2.2 Bacterial growth in the presence of propranolol ................................. 74
2.4.2.3 FT-IR fingerprinting of cell cultures ................................................. 77
2.4.2.4 GC-MS metabolic profiling of cell cultures ....................................... 80

2.5 Conclusion ................................................................................................. 89

2.6 References ................................................................................................. 90

2.7 Supplementary information ......................................................................... 96

3 Chapter 3: The identification and quantification of toluene in *Pseudomonas putida* DOT-T1E using Raman spectroscopy and liquid chromatography .................................. 108

3.1 Abstract ....................................................................................................... 108
3.2 Introduction .................................................................................................. 109

3.3 Material and methods .................................................................................. 110

3.3.1 Bacterial strains and growth conditions ............................................... 110
3.3.2 Sample preparation .................................................................................. 110

3.3.2.1 Bacterial biomass ............................................................................... 110
3.3.2.2 Direct quenching extraction of intracellular metabolites .................... 110

3.3.3 Sample analysis ....................................................................................... 111

3.3.3.1 Raman spectroscopy ........................................................................... 111

3.3.3.1.1 Biomass analysis .............................................................................. 111
3.3.3.1.2 Liquid extracts ................................................................................ 111

3.3.3.2 High-performance liquid chromatography (HPLC) ............................ 112

3.4 Results and discussion ............................................................................... 112

3.4.1 Raman spectroscopy analysis ................................................................. 112
3.4.2 HPLC analysis ....................................................................................... 122

3.5 Conclusion .................................................................................................. 126

3.6 References .................................................................................................. 127

4 Chapter 4: Metabolic analysis of the response of *Pseudomonas putida* DOT-T1E strains to toluene using Fourier transform infrared spectroscopy and gas chromatography mass spectrometry ........................................................................................................ 130
Table of contents

4.1 Abstract .................................................................................................................. 131
4.2 Introduction ............................................................................................................ 132
4.3 Material and methods ............................................................................................ 134
  4.3.1 Bacterial strains and culture medium ................................................................. 134
  4.3.2 Cultivation of bacteria and culture conditions .................................................. 134
  4.3.3 Growth in response to toluene, sample collection and analysis ..................... 135
    4.3.3.1 Bacterial growth profiles ............................................................................. 135
    4.3.3.2 Analysis of biomass samples by FT-IR spectroscopy ................................. 135
    4.3.3.3 Metabolite profiling .................................................................................. 137
      4.3.3.3.1 Sample collection and metabolic quenching ......................................... 137
      4.3.3.3.2 Metabolite extraction ......................................................................... 137
      4.3.3.3.3 GC-MS analysis ................................................................................ 138
      4.3.3.3.4 Data analysis ..................................................................................... 138
  4.4 Results and discussion ........................................................................................... 139
    4.4.1 The effect of toluene on the Growth of P. putida strains ................................. 139
    4.4.2 FT-IR spectroscopy of collected biomass samples .......................................... 142
    4.4.3 Metabolic profiling with GC-MS ................................................................... 145
  4.5 Conclusion ............................................................................................................. 152
  4.6 References ............................................................................................................. 153
  4.7 Supplementary Information: Experimental ......................................................... 157
    4.7.1 Sampling and analysis of cell extracts by HPLC-UV ...................................... 157
  4.8 Supplementary Information: Results ................................................................. 158
5 Chapter 5: Metabolic fingerprinting of Pseudomonas putida DOT-T1E strains: understanding the influence of divalent cations in adaptation mechanisms following exposure to toluene ...................................................... 167
  5.1 Abstract ................................................................................................................. 168
  5.2 Introduction ........................................................................................................... 169
  5.3 Material and methods ............................................................................................ 171
    5.3.1 Bacterial strains and growth conditions .......................................................... 171
List of Figures

**Figure 1.1:** Schematic drawing of cellular solvent mechanisms involved in *P. putida* strains adapted from (Udaondo et al. 2012). ................................................................. 28

**Figure 1.2:** Typical FT-IR absorbance of *P. putida* DOT-T1E showing the major regions of biological interest are labelled. Where A refers to fatty acid region, B represents the amide region, C is mixed vibration from carboxylic groups of protein and PO$_2^-$ of phosphodiesters, D is the carbohydrate region and E is the fingerprint region. ......................................................... 42

**Figure 1.3:** The basic components of a FT-IR spectrometer adapted from (Stuart 1996). .... 43

**Figure 2.1:** Growth curves of all three *P. putida* DOT-T1E strains in LB medium. Symbols represent different strains. DOT-T1E is the wild type (close circles), DOT-T1E-PS28 is the mutant (closed triangles) and DOT-T1E-18 is the mutant (closed squares). A 1/10 dilution of 100 µL samples were prepared for OD measurement at 660 nm. ......................................................... 72

**Figure 2.2:** Growth curves of: (A) *P. putida* DOT-T1E, (B) *P. putida* DOT-T1E-PS28, and (C) *P. putida* DOT-T1E-18 in the absence and presence of propranolol. A 1/10 dilution of 100 µL samples were prepared for OD measurement at 660 nm. ......................................................... 76

**Figure 2.3:** PC-DFA scores plots of FT-IR data for three different strains of *P. putida* strains upon propranolol shock. Symbols represent different strains. (A) *P. putida* DOT-T1E is the wild type (stars) and ten PCs with a total explained variance (TEV) of 99.43% were used for the DFA, (B) *P. putida* DOT-T1E-PS28 is the mutant (closed triangles) and ten PCs with a TEV of 99.65% were used for the DFA, (C) *P. putida* DOT-T1E-18 is the mutant (closed circles) and ten PCs with a TEV of 99.03% were used for the DFA. Colour coding: control with no propranolol (red), cells exposed to 0.2 mg mL$^{-1}$ propranolol (black), 0.4 mg mL$^{-1}$ propranolol (brown), and 0.6 mg mL$^{-1}$ propranolol (blue). Arrows indicate the direction of shift because of the increase of propranolol concentration. (D) PC-DFA loadings plot for *P. putida* DOT-T1E. (E) PC-DFA loadings plot for *P. putida* DOT-T1E-PS28, (F) PC-DFA loadings plot for *P. putida* DOT-T1E-18. Significant loadings were assigned to bacterial proteins. ................................................................. 79

**Figure 2.4:** Box-whisker plots showing the changes in metabolite levels in control and cells exposed to propranolol for 4 biological replicates. Variable 180 was identified as propranolol. (Red line) indicates the median m/z intensity. (A) Represent the data for 3 *P. putida* strains, 4 concentrations of propranolol and 3 time points, dashed lines separate different concentration levels of propranolol and solid line separates different strains. (B) Represent the data for 3 *P. putida* strains, 3 concentrations of propranolol and 1 time point at 60 min, dashed lines separate different strains. ................................................................. 82

**Figure 2.5:** Box-whisker plot showing the changes in ornithine levels (variable id 100) in control and exposed cells to propranolol. (Red line) indicates the median m/z intensity. These plots represent the data for 3 *P. putida* strains, 4 concentrations of propranolol and 3 time points, for 4 biological replicates. Dashed lines separate different concentration levels of propranolol and solid lines separate different strains. ................................................................. 84

**Figure 2.6:** Schematic metabolic diagram of central carbon metabolism in *P. putida* DOT-T1E adapted to propranolol showing the level of metabolites for cells exposed to propranolol compared to the control. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. The median m/z intensity (red line) in the box-whisker plots was used to compare the level of metabolites. (A) Represents the level of metabolites at 10 min, while (B) the level of metabolites at 60 min. Traffic light system represents different concentration of propranolol.
Red, yellow and green represent exposed cells to 0.2, 0.4 and 0.6 mg mL\(^{-1}\) of propranolol respectively. Up-arrow, down-arrow and steady arrow indicate an increase, a decrease and no change in the level of metabolite respectively. The number of arrows represents the level of metabolites. Slight change (single arrow), medium change (double arrows) and high change (triple arrows).

**Figure 2.7:** Schematic diagram of central carbon metabolism in *P. putida* DOT-T1E-18 adapted to propranolol showing the level of metabolites for cells exposed to propranolol compared to the control ones. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. The median m/z intensity (red line) in the box-whisker plots was used to compare the level of metabolites. (A) Represents the level of metabolites at 10 min, while (B) the level of metabolites at 60 min. Traffic light system represents different concentration of propranolol. Red, yellow and green represent exposed cells to 0.2, 0.4 and 0.6 mg mL\(^{-1}\) of propranolol respectively. Up-arrow, down-arrow and steady arrow indicate an increase, a decrease and no change in the level of metabolite respectively. The number of arrows represents the level of metabolites. Slight change (single arrow), medium change (double arrows) and high change (triple arrows).

**Figure S2.1:** MB-PCA scores plot of GC-MS data for the wild type and the mutants in the absence of propranolol. Colours represent different strains. (A) *P. putida* DOT-T1E is the wild type (red), (B) *P. putida* DOT-T1E-PS28 (green), and (C) *P. putida* DOT-T1E-18 (blue).

**Figure S2.2:** MB-PCA loading plot of GC-MS data showing most significant metabolites between the wild type and the mutants in the absence of propranolol. Significant loadings were observed in the positive side of the plot.

**Figure S2.3:** Box-whisker plots of a few selected most significant metabolites between the wild type and the mutants in the absence of propranolol. (A) *P. putida* DOT-T1E is the wild type, (B) *P. putida* DOT-T1E-PS28, and (C) *P. putida* DOT-T1E-18.

**Figure S2.4:** Schematic metabolic pathway diagram of central carbon metabolism in *P. putida* DOT-T1E showing the level of metabolites for both mutants compared to the wild type. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. The median m/z intensity (red line) in the box-whisker plots was used to compare the level of metabolites. Blue and brown represent the mutant DOT-T1E-PS28 and DOT-T1E-18 respectively. Up-arrow, down-arrow and steady arrow indicate an increase, a decrease and no change in the level of metabolite respectively. The number of arrows represents the level of metabolites. Slight change (single arrow) and medium change (double arrows).

**Figure S2.5:** Validated PC-DFA models of (A) *P. putida* DOT-T1E, (B) *P. putida* DOT-T1E-PS28, (C) *P. putida* DOT-T1E-18 upon 0.2, 0.4 and 0.6 mg mL\(^{-1}\) Propranolol shock. Symbols coding: control with no propranolol (circles), cells exposed to 0.2 mg mL\(^{-1}\) propranolol (squares), 0.4 mg mL\(^{-1}\) propranolol (triangles), and 0.6 mg mL\(^{-1}\) propranolol (upside down triangles). Opened symbols represent the test set while closed symbols represent the training set.

**Figure S2.6:** MB-PCA score plot of GC-MS data showing the effect of different concentrations on *P. putida* strains. Colours represent different dosage of propranolol. (D0) exposed to 0 mg mL\(^{-1}\) propranolol (blue), (D1) exposed to 0.2 mg mL\(^{-1}\) propranolol (green), and (D2) exposed to 0.4 mg mL\(^{-1}\) propranolol (pink). (D3) exposed to 0.6 mg mL\(^{-1}\) propranolol (red).
List of Figures

**Figure S2.7:** MB-PCA loading plot of GC-MS data showing the most significant metabolites in the presence of different concentrations of propranolol. Significant loadings were observed in the positive and negative sides of the plot. ................................................................. 100

**Figure S2.8:** Schematic metabolic diagram of central carbon metabolism in *P. putida* DOT-T1E-PS28 adapted to propranolol showing the level of metabolites for cells exposed to propranolol compared to the control. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. The median m/z intensity (red line) in the box-whisker plots was used to compare the level of metabolites. (A) Represents the level of metabolites at 10 min, while (B) the level of metabolites at 60 min. Traffic light system represents different concentration of propranolol. Red, yellow and green represent exposed cells to 0.2, 0.4 and 0.6 mg mL⁻¹ of propranolol respectively. Up-arrow, down-arrow and steady arrow indicate an increase, a decrease and no change in the level of metabolite respectively. The number of arrows represents the level of metabolites. Slight change (single arrow), medium change (double arrows) and high change (triple arrows). ................................................................. 101

**Figure S2.9:** Box-whisker plots of the detected metabolites of central carbon metabolism in *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18, dashed lines separate different concentration levels of propranolol and solid lines separate different strains. The label is constructed in a format of “Aac”, “A” represents strains, and varies from A to C: A = *P. putida* DOT-T1E; B = *P. putida* DOT-T1E-PS28 and C = *P. putida* DOT-T1E-18. “a” represents 4 different concentration levels, and varies from 0 to 3: 0 = control; 1 = 0.2 mg mL⁻¹; 2 = 0.4 mg mL⁻¹ and 3 = 0.6 mg mL⁻¹ propranolol. “c” represents time points, 1 = T0 (0 min); 2 = T1 (10 min) and 3 = T2 (1 h). Such plots give a comprehensive view of how the concentration levels of the metabolite are changing under each unique combination of the factors (strains, dosage of propranolol and time). Variable 14 (alanine), Variable 20 (valine), Variable 29 (leucine), and Variable 34 (isoleucine). ................................................................. 102

**Figure S2.10:** Box-whisker plots of the detected metabolites of central carbon metabolism in *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18, dashed lines separate different concentration levels of propranolol and solid lines separate different strains. The label is constructed in a format of “Aac”, “A” represents strains, and varies from A to C: A = *P. putida* DOT-T1E; B = *P. putida* DOT-T1E-PS28 and C = *P. putida* DOT-T1E-18. “a” represents 4 different concentration levels, and varies from 0 to 3: 0 = control; 1 = 0.2 mg mL⁻¹; 2 = 0.4 mg mL⁻¹ and 3 = 0.6 mg mL⁻¹ propranolol. “c” represents time points, 1 = T0 (0 min); 2 = T1 (10 min) and 3 = T2 (1 h). Such plots give a comprehensive view of how the concentration levels of the metabolite are changing under each unique combination of the factors (strains, dosage of propranolol and time). Variable 40 (glycine), Variable 53 (threonine), Variable 54 (serine), and Variable 78 (aspartic acid). ................................................................. 103

**Figure S2.11:** Box-whisker plots of the detected metabolites of central carbon metabolism in *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18, dashed lines separate different concentration levels of propranolol and solid lines separate different strains. The label is constructed in a format of “Aac”, “A” represents strains, and varies from A to C: A = *P. putida* DOT-T1E; B = *P. putida* DOT-T1E-PS28 and C = *P. putida* DOT-T1E-18. “a” represents 4 different concentration levels, and varies from 0 to 3: 0 = control; 1 = 0.2 mg mL⁻¹; 2 = 0.4 mg mL⁻¹ and 3 = 0.6 mg mL⁻¹ propranolol. “c” represents time points, 1 = T0 (0 min); 2 = T1 (10 min) and 3 = T2 (1 h). Such plots give a comprehensive view of how the concentration levels of the metabolite are changing under each unique combination of the factors (strains, dosage of propranolol and time). Variable 81 (methionine), Variable 88 (glutamine), Variable 95 (phenylalanine), and Variable 103 (fumarate) ................................................................. 104
List of Figures

**Figure S2.12:** Box-whisker plots of the detected metabolites of central carbon metabolism in *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18, dashed lines separate different concentration levels of propranolol and solid lines separate different strains. The label is constructed in a format of “Aac”, “A” represents strains, and varies from A to C: A = *P. putida* DOT-T1E; B = *P. putida* DOT-T1E-PS28 and C = *P. putida* DOT-T1E-18. “a” represents 4 different concentration levels, and varies from 0 to 3: 0 = control; 1 = 0.2 mg mL⁻¹; 2 = 0.4 mg mL⁻¹ and 3 = 0.6 mg mL⁻¹ propranolol. “c” represents time points, 1 = T0 (0 min); 2 = T1 (10 min) and 3 = T2 (1 h). Such plots give a comprehensive view of how the concentration levels of the metabolite are changing under each unique combination of the factors (strains, dosage of propranolol and time). Variable 109 (citrate), Variable 119 (lysine), Variable 135 (tyrosine), and Variable 177 (tryptophan).................................105

**Figure 3.1:** Typical Raman spectra of unlabelled toluene (red line) compared to labelled toluene-d₃ (blue line) and toluene-d₅ (black line). Raman spectra were recorded using Raman spectroscopy with an excitation wavelength of 532 nm. .........................................................113

**Figure 3.2:** Raman spectra of *P. putida* DOT-T1E culture biomass at three different time points following 5 mM toluene stress. (A) 2 h, (B) 6 h, and (C) 12 h after the addition of toluene. Colours represent different conditions. Control-no toluene (blue line) and culture exposed to toluene (red line). Raman spectra were recorded using Raman spectroscopy with an excitation wavelength of 532 nm. .................................................................115

**Figure 3.3:** Raman spectra of *P. putida* culture biomass at four different concentrations of toluene. Colours represent different concentrations. Cell exposed to 0 mM toluene (blue line), 5 mM toluene (black line), 30 mM toluene (green line), and 100 mM toluene (red line). Raman spectra were recorded using Raman spectroscopy with an excitation wavelength of 532 nm. .................................................................116

**Figure 3.4:** Raman spectra of pure solvents and *P. putida* cultures extracts upon 20 mM toluene stress. Colour coding: *P. putida* DOT-T1E cells control with no toluene (brown line), DOT-T1E cells exposed to toluene (light blue line), DOT-T1E-PS28 cells exposed to toluene (green line), DOT-T1E-18 cells exposed to toluene (pink line), 2 M toluene (black line), pure toluene (red line) and pure methanol (dark blue line). Raman spectra were recorded using Raman spectroscopy with an excitation wavelength of 532 nm. .................................................................117

**Figure 3.5:** Raman spectra of (A) serial dilution of toluene and (B) the calibration curve of 3055 cm⁻¹ peak area which was used to calculate the limit of detection for toluene. Each point represents the median of three replicates of each concentration. Error bars are standard deviations. Raman spectra were recorded using Raman spectroscopy with an excitation wavelength of 532 nm. .................................................................118

**Figure 3.6:** Raman spectra of (A) reference solvents, and (B) serial dilution of toluene. Raman spectra were recorded using a hand-held 1064 nm Raman spectrometer. .................120

**Figure 3.7:** Calibration curve obtained from toluene ranging from 1 to 100 mM for 3 replicates using a hand-held 1064 nm spectrometer. The peak area of 790 cm⁻¹ was used to obtain the calibration curve and calculate the limit of detection for toluene. Error bars are standard deviations. .................................................................121

**Figure 3.8:** Chromatograms for (A) 1 mM toluene, (B) *P. putida* DOT-T1E (no toluene), exposed cells to 20 mM toluene (C) DOT-T1E, (D) DOT-T1E-PS28 and (E) DOT-T1E-18 obtained from HPLC-UV. Toluene is eluted with a retention time of 1.458 ± 0.003 min. .................123

**Figure 3.9:** Calibration curve obtained from toluene ranging from 0.001 to 1 mM for 3 replicates using HPLC-UV. Points are means of the 3 replicates and error bars are standard deviations. ..............................................................................................124
**Figure 3.10:** Box-whisker plot representing the toluene level in *P. putida* strains exposed to 20 mM toluene for 4 replicates. The red lines indicate the median of the peak area. DOT-T1E is the wild type, DOT-T1E-PS28 is the mutant (lacking the TtgGHI pump) and DOT-T1E-18 is the mutant (lacking the TtgABC pump). Error bars are standard deviations of 4 replicates.

**Figure 4.1:** Growth curves of: (A) the wild-type DOT-T1E, (B) the mutant DOT-T1E-PS28, and (C) the mutant DOT-T1E-18 in LB medium with and without toluene. Symbols represent different growth conditions. Control cultures - no toluene (closed diamonds), exposed cultures to 0.1% (v/v) toluene (closed squares), toluene gas (closed triangles), toluene gas and 0.1% (v/v) toluene (crosses). A 1/10 dilution of 100 µL samples was prepared in order to determine the turbidity at 660 nm. 5 h time point is the point immediately before any toluene addition.

**Figure 4.2:** PC-DFA scores plots of FT-IR data for three different strains of *P. putida* strains upon toluene stress. Symbols show different strains. (A) *P. putida* DOT-T1E (stars) and the first 10 PCs with a total explained variance (TEV) of 99.94% were used for the DFA, (B) *P. putida* DOT-T1E-PS28 (closed triangles) and PCs 1-10 with TEV of 99.93% were used for the DFA. (C) *P. putida* DOT-T1E-18 (closed circles) and first 10 PCs with TEV of 99.90% were used for the DFA. Colour codings represent different conditions: control cultures - no toluene (red), cultures exposed to 0.1% (v/v) toluene (black), toluene gas (brown), toluene gas and sudden 0.1% (v/v) toluene (blue). Arrows indicate the direction of shift because of the presence of toluene. (D) PC-DFA loadings plot for *P. putida* DOT-T1E, (E) DOT-T1E-PS28 and (F) DOT-T1E-18. Significant loadings were assigned to bacterial proteins and lipids.

**Figure 4.3:** MB-PCA scores plot of GC-MS data showing the effect of different conditions of toluene on *P. putida* strains. Colours represent different conditions of toluene. Control with no toluene (blue), cells exposed to 0.1% (v/v) toluene (T; green), toluene gas (G; pink), and toluene gas and 0.1% (v/v) toluene (GT; red).

**Figure 4.4:** MB-PCA scores plot of GC-MS data showing the effect of different time points on *P. putida* strains. Colours represent different time points. 0 min is blue which refers to the point immediately before toluene addition (blue), 10 min is (in green) and 1 h (red) refer to the points after 10 min and 60 min of the addition of 0.1% (v/v) toluene.

**Figure 4.5:** Box-whisker plot showing the alterations in ornithine levels (variable id 54) in control and cells exposed to toluene for 4 biological replicates. The red lines represent the median m/z intensity. Box plot represents the data for 3 *P. putida* strains, 4 conditions of toluene and 3 time points, dashed lines separate different conditions of toluene and solid line separates different strains. Codes: control - no toluene (C), cells exposed to 0.1% (v/v) toluene (T), toluene gas (G), and toluene gas and 0.1% (v/v) toluene (GT).

**Figure 4.6:** Schematic metabolic diagram of central carbon metabolism in *P. putida* DOT-T1E adapted to toluene. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. (A) Represents the level of metabolites at 10 min after toluene exposure, and (B) at 60 min. Box-whisker plots show the changes in metabolite levels in control and cells exposed to toluene for 4 biological replicates. The red lines indicate the median m/z intensity. Codes: control - no toluene (C), cells exposed to 0.1% (v/v) toluene (T), toluene gas (G), and toluene gas and 0.1% (v/v) toluene (GT).

**Figure S4.1:** Validated PC-DFA models of (A) *P. putida* DOT-T1E, (B) DOT-T1E-PS28, (C) DOT-T1E-18 upon toluene stress. Symbol coding: control with no toluene (circles), cells exposed to 0.1% (v/v) toluene (squares), toluene via gas phase (triangles), and toluene via gas...
List of Figures

phase and 0.1% (v/v) toluene (upside down triangles). Closed symbols represent the training set while open symbols represent the test set that was projected into the PC-DFA scores space constructed from the training set. ................................................................. 158

Figure S4.2: Effect of oxidative stress on P. putida strains growth. Symbols and colours represent different strains. (Closed black diamonds) represents the wild-type DOT-T1E, (Closed red circle) the mutant DOT-T1E-PS28, and (Closed green triangles) the mutant DOT-T1E-18. (Solid lines) represent the growth curves of the control cells, while (dotted lines) cells exposed to oxidative stress. ......................................................... 159

Figure S4.3: Schematic metabolic diagram of central carbon metabolism in P. putida DOT-T1E-PS28 adapted to toluene. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. (A) Represents the level of metabolites at 10 min after toluene exposure, and (B) at 60 min. Box-whisker plots show the changes in metabolite levels in control and cells exposed to toluene for 4 biological replicates. The red lines indicate the median m/z intensity. Codes: control - no toluene (C), cells exposed to 0.1% (v/v) toluene (T), toluene gas (G), and toluene gas and 0.1% (v/v) toluene (GT). ................................................................. 160

Figure S4.4: Schematic metabolic diagram of central carbon metabolism in P. putida DOT-T1E-18 adapted to toluene. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. (A) Represents the level of metabolites at 10 min after toluene exposure, and (B) at 60 min. Box-whisker plots show the changes in metabolite levels in control and cells exposed to toluene for 4 biological replicates. The red lines indicate the median m/z intensity. Codes: control - no toluene (C), cells exposed to 0.1% (v/v) toluene (T), toluene gas (G), and toluene gas and 0.1% (v/v) toluene (GT). ................................................................. 161

Figure 5.1: Influence of 7 mM MgSO₄ on growth of P. putida DOT-T1E strains in the presence of toluene. Growth curves of: (A) the wild-type DOT-T1E, (B) the mutant DOT-T1E-PS28, and (C) the mutant DOT-T1E-18. Symbols and colours represent different growth conditions. Control cultures with no toluene (blue closed diamonds), exposed cultures to 0.1% (v/v) toluene (red closed circles), 0.5% (v/v) toluene (yellow closed triangles), 1% (v/v) toluene (green closed square). Solid and dotted lines represent the absence and presence of metal ions in the culture respectively. A 1/10 dilution of 100 µL samples was prepared to determine the turbidity at 660 nm. ................................................................. 178

Figure 5.2: Turbidity at OD 660 nm of P. putida strains after 8 h incubation in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. Colours represent different strains: the wild-type DOT-T1E (red), the mutant DOT-T1E-PS28 (yellow), and the mutant DOT-T1E-18 (green). Bars of the means of 4 replicates and error bars are standard deviations. ................................................................. 179

Figure 5.3: PC-DFA scores plots of FT-IR data of P. putida DOT-T1E strains in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. Symbols represent different strains: P. putida DOT-T1E wild type (stars), P. putida DOT-T1E-PS28 (closed circles), and P. putida DOT-T1E-18 (closed triangles) PCs 1-30 with a total explained variance (TEV) of 99.92% were used for the DFA. Colour coding: control with no toluene (red), cells without toluene in the presence of 7mM Mg²⁺ (brown), 3mM Ca²⁺ (black), mixed 7mM Mg²⁺ and 3mM Ca²⁺ (green), cells challenged with 0.05% (v/v) toluene in the presence of 7mM Mg²⁺ (dark blue), 3mM Ca²⁺ (light blue), mixed 7mM Mg²⁺ and 3mM Ca²⁺ (grey), and cells with 0.05% (v/v) toluene in the absence of divalent cations (pink) ................................................................. 181
Figure 5.4: PC-DFA loadings plot from DF 2 of P. putida DOT-T1E strains in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. Significant loadings were assigned to bacterial lipids highlighted in yellow), proteins (blue box) and polysaccharides (red box). 

Figure 5.5: Box-whisker plot for FT-IR scaled spectra showing the ratio of saturated fatty acids (CH$_3$:CH$_2$) of P. putida DOT-T1E strains grown strain in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. Red lines indicate the median of peak area of saturated fatty acids ratio of infrared spectra. The median was used to compare the level of saturated fatty acids ratio. Red plus signs represent the outliers.

Figure 5.6: DFA scores plots of GC-MS data of P. putida DOT-T1E strains in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. (A) represents scores plot for (DF1 vs. DF2), while (B) for (DF1 vs. DF3). Colours represent different strains. P. putida DOT-T1E is the wild type (red), P. putida DOT-T1E-PS28 is the mutant (green), and P. putida DOT-T1E-18 is the mutant (blue). Symbols represent different conditions. Control with no toluene (triangles), cells without toluene in the presence of 7mM Mg$^{2+}$ (squares), 3mM Ca$^{2+}$ (circles), 7mM Mg$^{2+}$ and 3mM Ca$^{2+}$ (diamonds), cells with 0.05% (v/v) toluene in the presence of 7mM Mg$^{2+}$ (pluses), 3mM Ca$^{2+}$ (upside down triangles), 7mM Mg$^{2+}$ and 3mM Ca$^{2+}$ (stars), and cells with 0.05% (v/v) toluene in the absence of divalent cations (crosses).

Figure S5.1: Growth of P. putida DOT-T1E strains on toluene at four different concentrations. Growth curves of: (A) the wild-type DOT-T1E, (B) the mutant DOT-T1E-PS28, and (C) the mutant DOT-T1E-18. Symbols and colours represent different concentrations of toluene. Control cultures - no toluene (blue closed squares), exposed cultures to 0.1% (v/v) toluene (green closed triangles), 0.5% (v/v) toluene (red closed circles), 1% (v/v) toluene (yellow closed diamonds) and 5% (v/v) toluene (purple stars). A 1/10 dilution of 100 µL samples was prepared to determine the turbidity at 660 nm.

Figure S5.2: Influence of three different concentrations of magnesium ions on growth of P. putida DOT-T1E strains in the presence of 0.1% (v/v) toluene. Growth curves of: (A) DOT-T1E, (B) DOT-T1E-PS28 and (C) DOT-T1E-18. Symbols and colours represent different concentrations of metal ion. Control cultures - no toluene and metal ion (blue closed diamonds), exposed cells to 0.1% (v/v) in the presence of 3.5 mM Mg$^{2+}$ (red closed squares), 14 mM Mg$^{2+}$ (green closed triangles), 30 mM Mg$^{2+}$ (purple crosses). A 1/10 dilution of 100 µL samples was prepared to determine the turbidity at 660 nm.

Figure S5.3: The influence of cations and anions of metal ions on the growth of P. putida DOT-T1E strain in the absence and presence of 0.05% (v/v) toluene. Symbols and colours represent different growth conditions. Solid lines represent the absence of toluene in the culture, while dotted lines represent the presence of toluene in the culture. A 1/10 dilution of 100 µL samples was prepared to determine the turbidity at 660 nm.

Figure S5.4: FT-IR spectra collected for P. putida DOT-T1E cultures in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. (A) FT-IR raw spectra, while (B) scaled spectra using extended multiplicative signal correction (EMSC).

Figure S5.5: Box-whisker plot for FT-IR raw spectra showing the ratio of saturated fatty acids (CH$_3$:CH$_2$) of P. putida DOT-T1E strains grown in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. The red lines indicate the median of peak area of saturated fatty acids ratio of
infrared spectra. The median was used to compare the level of saturated fatty acids ratio. Red plus signs represent outlier. .......................................................... 199
List of Tables

Table 1.1: The logP values of organic solvents adapted from Sardessai and Bhosle (2002). 28
Table 1.2: Definition of terms used in metabolome analysis adapted according to (Ellis et al., 2007; Ellis and Goodacre, 2012; Fiehn, 2002; Goodacre, 2004; Kell, 2006; Mapelli et al., 2008; Dunn, 2008) ........................................................................................................................................ 33
Table 1.3: Wavenumber regions of biological interest .................................................................................................................. 42
Table 2.1: Bacteria used in this study ...................................................................................................................................................... 66
Table S2.1: Results from the propranolol MIC experiments using P. putida DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18. Culture growth was observed after overnight incubation..... 106
Table S2.2: Viability of P. putida cells 1 h later after exposure to propranolol ......................... 107
Table 3.1: Major Raman spectral shift in wave numbers detected due to deuterated atom. 114
Table 3.2: Concentrations of toluene in P. putida DOT-T1E strains ........................................................................................................ 124
Table 4.1: P. putida strains used in this study ................................................................................................................................. 134
Table S4.1: Results from the toluene MIC experiments using P. putida DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18. Culture growth was recorded after overnight incubation. 162
Table S4.2: List of the top 30 significant variables from MB-PCA loading .............................. 163
Table S4.3: List of the top 30 significant variables from N-way ANOVA test ......................... 164
Table S4.4: A list of detected metabolites by GC-MS. All identifications are based on minimum metabolite reporting standards (Sumner et al., 2007). ......................................................... 166
Table 5.1: Wavenumber regions of biological interest and assignment for P. putida DOT-T1E cells (Kim et al., 2010) ........................................................................................................................................... 182
Table S5.1: List of the significant variables from Two-way ANOVA test ............................ 199
Abstract

The University of Manchester
Ali Sayqal
Doctor of philosophy
An environmental metabolomics study of the effect of abiotic substances on *Pseudomonas putida* by employing analytical techniques
2016

An exceptionally important stress response of *Pseudomonas putida* strains to toxic chemicals is the induction of efflux pumps that extrude solvents, as well as other toxicants, into the surrounding medium. However, the bacterial tolerance mechanisms are still not fully understood, thus in this thesis metabolomic approaches were used to detect and identify metabolites involved in *P. putida* DOT-T1E tolerance to abiotic stresses, in particular focussing on the role of efflux pumps. To elucidate any metabolome alterations several strains of *P. putida*, including the wild type DOT-T1E, and the efflux pump knockouts DOT-T1E-PS28 and DOT-T1E-18, were challenged with different levels of propranolol. Fourier-transform infrared (FT-IR) spectroscopy, which provided a rapid, high-throughput metabolic fingerprint of *P. putida* strains, was used to investigate any phenotypic changes resulting from exposure to propranolol. FT-IR data illustrated phenotypic changes associated with the presence of propranolol within the cell that could be assigned to the bacterial protein components. To complement this phenotypic fingerprinting approach metabolic profiling on the same samples was performed using gas chromatography mass spectrometry (GC-MS) to identify metabolites of interest during growth of bacteria following this toxic perturbation with propranolol. GC-MS revealed significant changes in ornithine levels which can be directly linked to bacterial tolerance mechanisms, and alterations in the levels of several other metabolites which were also modified in response to propranolol exposure.

Moreover, the effect of the organic solvent toluene was also investigated using the same approach. Examination of FT-IR data indicated that protein and fatty acids were the most affected components of *P. putida* strains due to the presence of toluene within the cell. Moreover, application of GC-MS allowed for the identification and quantification of several metabolites which were differentially produced or consumed in the presence of toluene. To investigate the role of efflux pumps in *P. putida* DOT-T1E, several analytical techniques were employed including Raman spectroscopy, gas and liquid chromatography to identify and quantify the level of propranolol or toluene in *P. putida* cells. These analyses showed that propranolol and toluene accumulated in the mutant *P. putida* DOT-T1E-18 (lacking the TtgABC pump) at higher levels in comparison with the levels found in the wild-type DOT-T1E and the mutant DOT-T1E-PS28 (lacking the TtgGHI pump), indicating the key role of efflux pumps in solvent tolerance. Furthermore, the effect of Mg$^{2+}$ and Ca$^{2+}$ on the stabilisation of the toluene tolerance of *P. putida* DOT-T1E strains was examined in order to elucidate whether divalent cations interact with efflux pumps or other resistant mechanisms to improve solvent tolerance. FT-IR analysis suggested that the influence of divalent cations on the stabilisation of the toluene tolerance could be due to the contribution of metal ions towards other tolerance mechanisms such as lipopolysaccharide (LPS) instead of enhancing the activity of efflux pumps.

In conclusion, this thesis presents evidence that phenotypic fingerprinting and metabolic profiling approaches in combination with chemometric methods can generate valuable information on phenotypic responses occurring within microbial cultures subjected to abiotic stress.
Declaration

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Dedication

To my beloved Mum and Dad, my adorable wife, my lovely kids,

and my delightful brothers and sisters
Acknowledgment

First and foremost, I would like to express my gratitude to Allah (God) for providing me the blessings to complete this work.

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I thank the Royal Embassy of Saudi Arabia and Umm Al-Qura University for financial support.
### Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AMPs</td>
<td>antimicrobial peptides</td>
</tr>
<tr>
<td>ANNs</td>
<td>Artificial Neural Networks</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>APCI</td>
<td>atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>APCs</td>
<td>active pharmaceutical compounds</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>attenuated total reflectance</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge Coupled Device</td>
</tr>
<tr>
<td>CI</td>
<td>chemical ionisation</td>
</tr>
<tr>
<td>DFA</td>
<td>discriminant function analysis</td>
</tr>
<tr>
<td>DFs</td>
<td>discriminant functions</td>
</tr>
<tr>
<td>DTGS</td>
<td>deuterated triglycine sulfate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EI</td>
<td>electron ionisation</td>
</tr>
<tr>
<td>EMSC</td>
<td>extended multiplicative signal correction</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl esters</td>
</tr>
<tr>
<td>FIR</td>
<td>far-infrared</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>HCA</td>
<td>hierarchical cluster analysis</td>
</tr>
<tr>
<td>HILIC</td>
<td>hydrophilic interaction chromatography</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>Km&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>ln</td>
<td>natural logarithm</td>
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<tr>
<td>logP</td>
<td>logarithm of partition coefficient</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
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<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MATE</td>
<td>multidrug and toxic substance extrusion</td>
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<tr>
<td>MB-PCA</td>
<td>multi-block principal components analysis</td>
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<tr>
<td>MCA</td>
<td>metabolic control analysis</td>
</tr>
<tr>
<td>MCT</td>
<td>mercury cadmium telluride</td>
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<tr>
<td>MDR</td>
<td>multidrug resistance</td>
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<td>MFS</td>
<td>major facilitator superfamily</td>
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<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<td>MIR</td>
<td>mid-infrared</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<td>MSI</td>
<td>Metabolomics standards initiative</td>
</tr>
<tr>
<td>MVA</td>
<td>multivariate analysis</td>
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<tr>
<td>n</td>
<td>Normal</td>
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<td>NIR</td>
<td>near-infrared</td>
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<td>NIST</td>
<td>National Institute of Standards and Technology</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>p</td>
<td>Para</td>
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<tr>
<td>PCA</td>
<td>principal components analysis</td>
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<td>PCR</td>
<td>principal components regression</td>
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<tr>
<td>PCs</td>
<td>principal components</td>
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<tr>
<td>PLSR</td>
<td>partial least squares regression</td>
</tr>
<tr>
<td>PR</td>
<td>pattern recognition</td>
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<tr>
<td>QC</td>
<td>quality control</td>
</tr>
<tr>
<td>Rif&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Rifampin</td>
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<tr>
<td>RND</td>
<td>root-nodulation-division</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reversed phase high performance liquid chromatography</td>
</tr>
<tr>
<td>RPLC</td>
<td>reversed phase liquid chromatography</td>
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<tr>
<td>rpm</td>
<td>round per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Sm</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>SMR</td>
<td>small multidrug resistance</td>
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<tr>
<td>STPs</td>
<td>sewage treatment plants</td>
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<td>TCA</td>
<td>Tricarboxylic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>TEV</td>
<td>total explained variance</td>
</tr>
<tr>
<td>TOD</td>
<td>toluene dioxygenase</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
</tr>
<tr>
<td>Tol</td>
<td>toluene</td>
</tr>
<tr>
<td>Ttg</td>
<td>toluene tolerance genes</td>
</tr>
<tr>
<td>TVC</td>
<td>Total Viable Count</td>
</tr>
<tr>
<td>UHPLC</td>
<td>ultra-high-performance liquid chromatography</td>
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</table>
Preface

Metabolomics is an emerging field that is complementary to genomics, transcriptomics and proteomics and is an area that is gaining increasing interest across all disciplines including medical science, environmental science, microbiology, plant science and food nutrition. The purpose of this thesis is to further the application of metabolomics to the area of solvent shock response in microorganisms and in particular to study the efflux pump systems in *Pseudomonas putida* strains. Launch of this research was to expand our understanding of the adaptation responses of microorganisms subjected to pharmaceutical or organic solvent stresses, and to develop further methods to identify and quantify the presence of these toxic solvents in bacterial cells. During this study, the outcomes of preliminary works led to establish a foundation where the metabolomics experiments can be used to measure the metabolites under certain conditions. Modern analytical techniques in metabolomics such as FT-IR spectroscopy, Raman spectroscopy, HPLC and GC-MS were used in this thesis. These instruments generate large amounts of information in a relatively short time which were used to address the objectives of this study. While the work presented here focused only on *P. putida* DOT-T1E, with two efflux pump knockouts, and the effect of propranolol and toluene, the study can be extended to investigate other microorganisms or abiotic stresses.

This thesis contains six chapters: a general introduction to pharmaceuticals in the environment, organic solvent tolerant microorganisms and the field of metabolomics, this is then followed by four results chapters and a conclusion and future work chapter. In addition, chapters 2, 4 and 5 have been submitted for publication. The results chapters are the outcome of creative collaboration with colleagues and a credit is given at the start of each chapter to all collaborators for their valuable contribution.

The work presented here addressed many challenges which were found to be highly educational and this has improved my knowledge and skills, and this will benefit my future academic career and research in numerous positive ways. Finally, I believe that the work undertaken in this project has contributed, to some extent, to provide further insight into the adaptation responses of microorganisms subjected to abiotic stress. Surely there is a future, and it is hoped that other researches can use the finding of this work to expand and build a future vision for science and research.

*Ali Sayqal*
Chapter 1: Literature review

1.1 Introduction

1.1.1 The impact of active pharmaceutical compounds on the environment

One of the major issues in the environment is the presence of active pharmaceutical compounds (APCs) (Boxall, 2004) and a broad range of APCs have been detected in surface water (Hirsch et al., 1999; Monteiro and Boxall, 2010). There are several routes by which APCs can be released into the environment via the general population and directly from manufacturers (Daughton and Ternes, 1999; Kolpin et al., 2002). It has been stated that drugs are not only being introduced into the environment after consumption, but also via the disposal of unused or expired pharmaceuticals (Breton and Boxall, 2003). Thus, these active compounds are produced enter the population and eventually wind up in the aquatic environment via sewage treatment plants (STPs) and/or wastewater systems. Various studies (Ternes, 1998; Ternes et al., 2001; Kolpin et al., 2002; Hummel et al., 2006) have demonstrated the ubiquitous presence of pharmaceutical residues in the aquatic environment, and nearly a hundred different classes of drugs and their metabolites have been detected in rivers, seawater, groundwater, treated wastewater and even in potable water. A study in the United Kingdom by Ashton and co-workers (2004) revealed that the presence of the β-blocker propranolol in STP effluents was highly likely at (median level 76 ng L⁻¹), whereas diclofenac (median 424 ng L⁻¹), ibuprofen (median 3086 ng L⁻¹), mefenamic acid (median 133 ng L⁻¹), dextropropoxyphene (median 195 ng L⁻¹), and trimethoprim (median 70 ng L⁻¹) were found in 86%, 84%, 81%, 74%, 65% of their samples respectively.

Levels of pharmaceuticals in the environment are increasing and could pose a potential hazard for humans, mammals, fish, and microorganisms within, and associated with, aquatic ecosystems. One case in point is oestrogen, used for birth control, which could cause the reduction in the number and motility of sperm in human males, and the early onset of puberty in females (Buhner, 2002). In addition, this synthetic oestrogen has been observed to lead to the feminisation of male fish.
causing significant population crashes (Kidd et al., 2007). A further disadvantage of the presence of APCs in the environment is an increase in antimicrobial resistance that poses huge potential risk for the future, making the treatment of infections very difficult to cure. There are several studies have described the link between effluent exposure and bacterial antibiotic resistance (Zhang et al., 2011; Liu et al., 2012; Johnning et al., 2013; Marathe et al., 2013). Several studies have reported various effects of antibiotic-resistant bacteria in various aquatic environments (Kummerer, 2004; Kim and Aga, 2007; Schlueter et al., 2007; Watkinson et al., 2007; Caplin et al., 2008). More than 90% of bacterial strains in seawater have resistance to more than one antibiotic, whereas 20% have multiple antibiotic resistance to at least five different types of antibiotic (Martinez, 2003). These results suggest that the presence of pharmaceuticals in the aquatic environment leads to an increase in the number of resistant bacteria that pose highly significant future impact on human and animal health. Therefore, it is important to consider antimicrobial resistance as a major global problem requiring immediate guidance and action particularly for risk management and antibiotic stewardship, in order to reduce human and animal exposure to resistant bacteria as well as develop new antibiotics (Finley et al., 2013).

1.1.2 Organic solvent tolerant microorganisms

1.1.2.1 Physiological basis of solvent toxicity and response to the toxic effects of organic compounds

Aromatic hydrocarbons such as benzene, toluene, styrene and xylenes with a logP (the logarithm of its partition coefficient of the given solvent in a defined octanol and water mixture) between 1.5 and 3.5 are highly toxic for microorganisms (Inoue and Horikoshi, 1989). A previous study has revealed that the toxicity of a solvent is well correlated with its logP value (Table 1.1). The active site of organic solvents on microorganisms is the cell membrane that contains within it various transporter proteins and enzymes, which play a vital role to transport solutes, regulate intracellular status, and maintain the energy status of the cell and other processes. Thus, these solvents intercalate into the phospholipid bilayer of the cell membrane thereby disorganising its structure and impairing cell membrane function, ultimately leading to cell death (Inoue and Horikoshi, 1989; Sikkema et al., 1994; Sikkema et
al., 1995; Sardessai and Bhosle, 2002). As most organic solvents are able to inhibit the growth of microorganisms, toluene has been used for several years in order to sterilise bacterial cultures and maintain solutions in sterile conditions (Horikoshi et al., 2011). The impacts of organic solvents on the membrane composition and integrity of the cell have been studied extensively (Buttke and Ingram, 1980; Inoue and Horikoshi, 1989; Sikkema et al., 1995; Bernal et al., 2007). However, each organism has a different intrinsic tolerance level for an organic solvent, which can be influenced by environmental factors and can also be determined genetically (Kobayashi et al., 1998).

On the other hand, bacteria can adapt and reduce the activity of toxic substances by the employment of several resistant mechanisms. These mechanisms involve altering lipid composition, induction of Kerbs enzymes related to energy production, solvent extrusion and other mechanisms (Figure 1.1). First, cis-to-trans isomerisation of unsaturated fatty acid alteration is considered as a short term response to solvent exposure. Many studies have shown that various Pseudomonas putida strains respond to organic solvent compounds by shifting their cis-to-trans ratio once exposed to these chemicals, for example, toluene, xylenes, phenol, or varying lengths of alcohol chains (Keweloh et al., 1990; Heipieper and Debont, 1994; Weber et al., 1994; Pinkart et al., 1996; Ramos et al., 1997). Second, the engagement of energetic processes for coping with solvent and other chemical stress (e.g. the usage of efflux pump) is a very important process in dealing with and reducing the level of toxic substances in the cell. In one proteomic study, Wijte et al. (2011) reported that a number of proteins related to energy metabolism were up-regulated in the presence of toluene. Furthermore, energy-dependent processes in solvent tolerance in P. putida strains have been reported (Segura et al., 2005; Neumann et al., 2006; Ray and Peters, 2008; Volkers et al., 2009). Third, in Gram-negative bacteria such as P. putida and E. coli efflux pump transporter systems play a crucial role in solvent tolerance (Aono et al., 1995). The function of these pumps is the removal of toxic chemicals such as solvents, antibiotics and other drug molecules across the inner and outer membranes and into the extra-cellular environment (Zgurskaya and Nikaido, 1999). In Pseudomonas strains, various studies linked the solvent and antibiotic tolerance to the action of several efflux pumps (Li et al., 1998; Ramos et al., 1998; Wijte et al., 2011; Molina-Santiago et al., 2014). Thus, an understanding of bacterial tolerant
mechanisms is very important in order to enhance the resistant systems for non-pathogenic strains and create altered strains with superior tolerance characteristics for bioprocessing.

Table 1.1: The logP values of organic solvents adapted from Sardessai and Bhosle (2002)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Log P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Decane</td>
<td>5.6</td>
</tr>
<tr>
<td>Hexane</td>
<td>3.5</td>
</tr>
<tr>
<td>p-Xylene</td>
<td>3</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.5</td>
</tr>
<tr>
<td>Benzene</td>
<td>2</td>
</tr>
<tr>
<td>n-Butanol</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Figure 1.1: Schematic drawing of cellular solvent mechanisms involved in *P. putida* strains adapted from (Udaondo *et al.*, 2012)
1.1.2.2 Efflux pump systems

An interesting stress response of Gram-negative bacteria to toxic chemical substances is the induction of efflux pumps, which pump out the toxic chemical substances from the bacterial cell before they reach their target. This mechanism is probably the most important process that plays an important role in bacterial tolerance, and these pumps are located in the cell envelope. In the late 1970s, antibiotic efflux as a resistance mechanism was first reported for tetracycline (Ball et al., 1977; Levy and McMurry, 1978; McMurry et al., 1980) and was later reported in many bacteria resistant to organic solvents (Aono et al., 1992). Bacterial drug efflux transporters are divided or classified into five major families (Piddock, 2006b): (1) the small multidrug resistance (SMR) family such as *E. coli* EmrE (Paulsen et al., 1996); (2) the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily transporters such as *Lactococcus lactis* LmrA (van Veen and Konings, 1998); (3) the major facilitator superfamily (MFS) such as *Staphylococcus aureus* NorA (Pao et al., 1998; Marger and Saier, 1993); (4) the multidrug and toxic substance extrusion (MATE) family such as *Vibrio parahaemolyticus* NorM (Brown et al., 1999); (5) the root-nodulation-division (RND) superfamily such as *E. coli* AcrAB-tolC (Saler et al., 1994). Most Gram-negative bacteria belong to the RND family that is considered as the most important family which plays a crucial role in solvent extrusion. In addition, these transporters are made up of three components spanning both the inner and outer membranes: a cytoplasm transporter, a membrane fusion protein (located in the periplasm that forms a channel with other complex proteins), and outer-membrane proteins. These three components associate to form the efflux system, and all these pumps extrude toxic compounds chemically unaltered in an energy-dependent manner by employing either ATP or an ion gradient (H+ or Na+) (Piddock, 2006a).

In *P. putida* DOT-T1E cells, three efflux pumps, which are genome-encoded, have been identified, termed TtgABC, TtgDEF and TtgGHI. These pumps prevent the accumulation of organic solvents in the cell (Ramos et al., 1998; Mosqueda and Ramos, 2000; Rojas et al., 2001). The TtgABC and TtgGHI pumps expel both organic solvents and some antibiotics (Ramos et al., 1998; Rojas et al., 2001), whereas the TtgDEF pump has been shown to be induced only by aromatic hydrocarbons (Mosqueda and Ramos, 2000). In addition, the TtgDEF genes are linked to the chromosomal tod gene, which allows the growth of strain with toluene
as the sole C source. The TtgGHI pump confers a certain basal resistance to several numbers of aromatic hydrocarbons, and this pump is found to be of significant importance for solvent tolerant bacteria (Rojas et al., 2001).

Ramos (1998) examined the sudden shock of 0.3% (v/v) toluene supplied via the gas phase to the wild type *P. putida* DOT-TIE strain and found that almost 100% of the initial cell numbers survived when the strain was pre-grown in the presence of a low concentration of toluene. By contrast, only 0.01% of the initial population survived once the cells were pre-grown without toluene. Furthermore, one such mutant, *P. putida* DOT-T1E-18, which in contrast to wild type strain, was very sensitive and could not tolerate a sudden toluene shock when cells were pre-grown in the absence of toluene, whereas only 0.0001% survived when the culture was pre-induced with toluene. Additionally, the mutant *P. putida* DOT-T1E-PS28 was not able to survive the toluene shock without induction, whereas when cultures were pre-growth on toluene resulted in survival of a small fraction of initial number (Rojas et al., 2001). Survival analysis of *P. putida* DOT-T1E cells upon sudden exposure to toluene revealed that the presence of the TtgGHI pump is absolutely necessary for survival in the presence of 0.3% (v/v) toluene (Rodriguez-Herva et al., 2007; Molina et al., 2011).

### 1.1.3 Metabolomics

#### 1.1.3.1 Introduction to metabolomics

In 1998 the term ‘metabolomics’ (Oliver et al., 1998) was introduced to the ‘-omics’ field, complementing genomics (gene function) and proteomics (protein regulation). The metabolome, which represents the final downstream product of the genome, can be defined as the complete quantitative collection of small molecules (metabolites) present in a cell that participate in general metabolic reactions needed for growth, maintenance, and other normal function within a biological system (Oliver et al., 1998; Goodacre et al., 2004; Dunn et al., 2005). The molecular weight of these metabolites are typically less than 1000 Da, and thus they are smaller than genes, transcripts and proteins (Raamsdonk et al., 2001; Winder et al., 2011). Metabolome analysis can provide a comprehensive estimation of the physiological state of an
organism linked with unique insight into certain biochemical processes (Oliver et al., 1998; Fiehn, 2001; Fiehn, 2002).

Generally, the number of metabolites is fewer than those for genes, and the evaluated size of the metabolome varies depending on the type of organism studied. Additionally, even when any alterations in the other protein or transcript concentrations are small, changes in the metabolite concentrations can be observed and detected by the application of metabolic control analysis (MCA) (Kell and Mendes, 1999). The chemical and physical properties of the metabolome are more diverse than those for the proteome or transcriptome owing to the greater variation in atomic arrangements (Dunn et al., 2005). Thus, metabolomic studies include the analysis of a broad range of chemical species, from low molecular-weight polar volatiles, for example ethanol, to high molecular-weight polar metabolites such as glucosides, non-polar lipids and inorganic species (Dunn et al., 2005; Lahner et al., 2003). In addition, the analysis of the metabolome is more complex compared to that of the proteome or genome because the range of metabolite concentrations can vary over nine orders of magnitude (pM-mM) (Dunn et al., 2005).

Metabolomics is a complex science that covers various disciplines; for instance, organic and analytical chemistry, chemometrics, informatics and the biosciences (Krastanov, 2010). The field of applications of metabolomics is varied and includes medical science, plant sciences, microbiology, pharmaceutical research, food and plant nutrition as well as others (Fiehn et al., 2000; Viant et al., 2003; Winder and Goodacre, 2004; Salek et al., 2008; Biais et al., 2009; Kim et al., 2010).

1.1.3.2 Techniques employed in metabolomics studies

The qualitative and quantitative measurements of the cellular metabolites of an organism can reveal its biochemical status and these data can be used to monitor and determine the function of genes (Fiehn, 2002; Fiehn, 2001). To date, no individual extraction method or analytical instrument can detect the entire metabolome (Hall, 2006). As a result, several targeted extraction procedures and analytical technologies are employed (Weckwerth, 2003; Sumner et al., 2003). The most widely employed analytical technologies in metabolomics include vibrational spectroscopies (e.g. Fourier transform infrared (FT-IR) spectroscopy and Raman microspectroscopy),
Nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), chromatographic and other techniques (Ellis et al., 2007; Dunn et al., 2005).

A range of different analytical strategies have been employed due to various metabolomics approaches and these are shown in (Table 1.2). Metabolomics studies might employ a broad range of metabolic fingerprinting and profiling approaches. Metabolic fingerprinting approaches comprise either the high-throughput analysis of crude samples, or sample extracts, and provide a comprehensive metabolite fingerprint (Ellis et al., 2012). These techniques are generally used for the classification or screening of samples (Winder et al., 2006; Winder et al., 2004; Johnson et al., 2003). Nevertheless, in this method the identification and quantification of metabolites are not generally performed (Dunn et al., 2005). Fingerprinting approaches, which are considered as non-targeted analyses, represent relatively inexpensive means of screening a biological system prior to metabolic profiling methods that employed more expensive techniques (Kaderbhai et al., 2003; Ellis et al., 2007). Metabolic profiling approaches normally employ separation techniques linked to mass spectrometry to identify, quantify and detect the target of metabolites within a biological system (Dunn et al., 2005; Bino et al., 2004).
Table 1.2: Definition of terms used in metabolome analysis adapted according to (Ellis et al., 2007; Ellis and Goodacre, 2012; Fiehn, 2002; Goodacre, 2004; Kell, 2006; Mapelli et al., 2008; Dunn, 2008)

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolome</td>
<td>The complete set of all small-molecular (metabolites) present in a biological sample/system (i.e., a single organism). The metabolome includes the endo-metabolome and exo-metabolome.</td>
</tr>
<tr>
<td>Metabolomics</td>
<td>The non-biased identification and quantification of the metabolome. The employed analytical techniques are required to be highly selective and sensitive. It is not currently possible to determine the entire metabolome in microbial, plant or mammalian systems by any single analytical technique.</td>
</tr>
<tr>
<td>Metabolic profiling</td>
<td>Analysis to identify and quantify a group of pre-defined metabolites, which share similar chemical and physical properties or metabolic pathways. Normally, chromatographic separation is used before detection.</td>
</tr>
<tr>
<td>Metabolite target analysis</td>
<td>Qualitative and quantitative determination of one or several metabolites of interest related to a specific metabolic pathway, ignoring all the non-target peaks existing in the sample. This method uses chromatographic separation and sensitive detection.</td>
</tr>
<tr>
<td>Metabolic fingerprinting</td>
<td>Spectra from rapid and high-throughput approach to provide sample classification, and also used as screening tool to differentiate between samples from various biological origin (i.e., disease/health).</td>
</tr>
<tr>
<td>Metabolic footprinting</td>
<td>Global analysis of the exometabolites that are excreted from the intra-cellular to extra-cellular environment by an organism. Rapid quenching and time consuming extraction of intra-cellular metabolites is not required for this method.</td>
</tr>
<tr>
<td>Lipidomics</td>
<td>The identification and quantification of all lipids within biological systems in order to study the interactions, structures and functions occur during perturbation of the system.</td>
</tr>
<tr>
<td>Fluxomics</td>
<td>Bacterial, yeast or tissue cultures are fed with specific labelled ($^{13}$C or $^{15}$N) substrates in order to quantify the rates of metabolic reaction within metabolic pathways.</td>
</tr>
</tbody>
</table>
1.1.3.3 Metabolomics experiment (metabolomics pipeline)

Collaboration within a multidisciplinary team of scientists in a workflow is required to undertake a metabolomics experiment. The process of a metabolomics experiment consists of different stages: experimental design, sample collection, sample preparation, sample analysis, pre-processing of data, and chemometric analysis of the processed data (Brown et al., 2005).

In order to achieve valid datasets and subsequently valid experimental conclusions and hypotheses, all experimental stages must be carefully designed and carried out. Thus, close attention is required at all stages of a workflow in terms of the number of samples, preparation of samples, proper selection of analytical techniques and strategies for data processing.

1.1.4 Introduction to data analysis (Chemometrics)

At present, large amounts of information (features or variables) for a vast number of samples (objects), can be generated by modern analytical instrumentation in a relatively short time. This requires established methods such as multivariate data matrices, which utilize mathematical and statistical methods, in order to extract the most important and maximum useful information from these data (Berrueta et al., 2007). In metabolomics investigations, large amounts of multivariate data are generated, and it can be difficult to distinguish between samples via visual inspection alone. Furthermore, not all of these data points are required to describe the problem sufficiently, only a few of them (Goodacre et al., 2004). In other words, it is important to extract the most essential elements of these data in order to generate new knowledge in a robust and interpretable manner (Eriksson et al., 2004; Goodacre et al., 2004). Metabolomics data require tools such as multivariate analysis (MVA) in order to analyse the highly complex spectral information and also to reduce and simplify the dimensionality in multivariate hyperspace to fewer numbers of components. The complexity of the datasets generated in metabolomics experiments can be reduced by employing pattern recognition (PR) strategies (Goodacre et al., 2004). In addition, the identification and interpretation of non-random behavior in complex systems which can be obscured by random variations or noise within a biological system, can be obtained by applying PR algorithms (Lindon et al., 2001).
Chapter One

There are number of methods for analysis of metabolomics data including unsupervised and supervised methods and these will be explained below in more detail.

1.1.4.1 Unsupervised methods
In general, unsupervised methods are used to determine the general relationship between data, and these algorithms may be useful to retrieve an answer for question such as: “do metabolite profiles from one patient taken at various times of day differ”? This question requires an algorithm where the metabolite data can be clustered into groups. This optimisation process achieves the dimensionality reduction for MVA, and hence a small number of components are used to minimize a large amount of metabolite data (X-data) with minimum loss of information. This can be obtained by employing one of two methods such as principal components analysis (PCA) and hierarchical cluster analysis (HCA). When the clustering is complete and either one of the dendograms or ordination score plots are generated, plots can be analyzed and interpreted manually.

1.1.4.1.1 Principal components analysis (PCA)
PCA is widely applied and utilises clustering methods to determine the similarity in a dataset based on their metabolite profiles (Joliffe, 1986; Goodacre et al., 2004), as well as a starting point of most multivariate data analysis (Boccard et al., 2010). Thus, a complex dataset is reduced to principal components (PCs), which explain the majority of the variance. The purpose of the data compression is to generate a set of new PCs that describe most of the variance in the original dataset. Decreasing the dimensionality of the data facilitates the interpretation of the variation within a dataset, and thus the identification of groups, trends, and outliers within a sample set might be achieved. In many cases, correlations between variables such as metabolites take place as they change according to some systematic underlying common factors such as genetic modification. PCA can detect these factors and also compress the information according to these factors.

PCs are new variables and they are generated by the linear combinations of the starting variables with the suitable weighting coefficients (Nicholson et al., 1999). In
addition, a specific linear combination of the PCs can be employed to explain the
original variables of metabolite concentrations, so a small number of PCs often have
the ability to describe more than 90% of the total explained variance (TEV). Each
PC is not correlated (orthogonal) with all the other PCs and made up of one score
vector, which may be described as the new variable, and one loading vector, which is
considered as a link between the score vector and the original variables. The score
represents a linear combination of the original variables within the dataset, while the
loadings are the influence (i.e., weighting) of the original variables on the scores
(Joliffe, 1986).

The first PC has the highest variance and the second PC is lower and so on, but at
some point the PCs would contain only data noise (Lindon et al., 2001; Nicholson et
al., 1999). Generally, the first two or three PCs would show the maximum
information content of a dataset in two dimensions (Wold et al., 1987). Therefore, a
rapid method for visualising and comparing the variance within a dataset is offered
by PCA.

1.1.4.2 Supervised methods
In general, these techniques use the class membership information of samples to a
particular group (category or class) in order to classify new unknown samples in to
one of the known classes based on its pattern of measurements (Massart et al., 1997).
Supervised pattern recognition methods use a well-known strategy whichever
algorithm is applied, so that samples are being classified by the correlation of
unknown samples with already known classes, and hence the algorithm knows which
samples should belong to which experimental class.

In supervised techniques a training set with objects of known categories is required in
order to derive a model for the identification of unknown samples. As a result, it is
essential to first establish whether chemical measurements are good enough to fit into
predetermined classes, since pattern recognition techniques are unable to compensate
either for inadequate experimental data or poorly designed experiments (Brereton,
2003). Additionally, a number of supervised techniques can be used in pattern
recognition, for instance, discriminant function analysis (DFA), partial least squares
regression (PLSR) and principal components regression (PCR). These methods seek
to extract multivariate data from metabolite profiles and convert them into something of biological interest under the guidance of a “teacher” (Goodacre et al., 2004). Two types of data are present within these techniques: inputs that are the input variables (metabolic concentrations) and targets (also referred to as outputs).

The aim of supervised learning is to find a “model” in which these data are correlated to each other correctly (Goodacre et al., 2004), and any error between the already established target and the response of the model (i.e. the output) needs to be as small as possible, and this can be obtained via one of these methods such as DFA, PLSR or ANNs (Goodacre, 2007). In addition, it is essential to perform a validation step on the model because during interpreting of such models, they are easily over trained and will fit the data to the a priori classes even on the basis of information, which can be regarded as background noise.

1.1.4.2.1 Discriminant function analysis (DFA)

Discriminant function analysis (DFA; (Manly, 1994)) is a supervised projection method, which is usually used to reduce the dimensionality of data based upon PCs. Datasets that contain collinear variables or too many variables cannot be used in the analysis. Thus, it is important to apply a data compression such as PCA, prior to the analysis of a multivariate dataset, and this combination is known as PC-DFA (Goodacre et al., 1998). A priori knowledge of class structure is used to obtain the supervision of the algorithm. Each group of samples is mean centered to each of the group means. PC-DFA generates measures of the difference both between classes and within classes by using what is known about the class structure. DFA attempts to maximise between class differences whilst minimising within class differences, according to the trained experimental class structure (Johnson et al., 2003; Kaderbhai et al., 2003).
1.1.4.3 Model validation

Model validation is an essential step in multivariate data analysis, and is performed to ensure both that the model quality is of a high standard, and that the obtained subsequent conclusions drawn from the data are valid. Models might over-fit the data if appropriate model validation techniques are not applied. So that the model finds relationships between the data and the dependent variables, which do not hold for subsequent analyses, resulting in a lack of generalisation (Picard and Cook, 1984).

The purpose of a common method of validation is to divide the data into three sets: training set, validation set, and a test set. First, the training set is utilised for training the model. Second, the validation set is employed to stop over training of the data. Finally, the test set is used to examine the ability of the model to generalise. Close attention needs to be paid when splitting the data because the groups ought to represent the spread of the variance within the dataset (Picard and Berk, 1990; Brown et al., 2005). Some techniques such as PC-DFA and PLSR use the training dataset to generate the models, and the test set is then projected into the same ordination space. Where the validation data falls within the bounds of the training and test dataset, the optimum number of the latent variables or PCs can be chosen for the model (Kaderbhai et al., 2003; Handl et al., 2005).

1.1.5 Analytical techniques for metabolomics

The qualitative and quantitative measurements of cellular metabolites of an organism can reveal the biochemical status of the organism, and these data can be used to monitor and determine gene function (Fiehn, 2001; Fiehn, 2002). As mentioned above, there is no individual analytical instrument that can be applied to detect all of the metabolites (Hall, 2006), as a result, a range of analytical techniques are employed. For example, there are some analytical instruments such as FT-IR spectroscopy and NMR spectroscopy which can be used to generate metabolic fingerprints, while metabolite profiles can be generated through the employment of chromatography linked to MS (e.g. GC-MS and HPLC-MS).
1.1.5.1 Fourier-transform infrared (FT-IR) spectroscopy

Infrared (IR) spectroscopy is a very useful technology that is employed for the determination of the structure and identification of various compounds, and the most important advantage of this technique is its ability to analyze the sample in almost any given state (Stuart, 1997). During the 1940s, the first IR spectrometers were introduced commercially to obtain an IR absorbance spectrum. These technologies used grating monochromators and a traditional prism made up of material such as sodium chloride, and each wavelength within the IR region would be determined sequentially. Thus, this technology was historically associated with several disadvantages such as being time consuming, as well as having low sensitivity and poor reproducibility. In order to reduce these drawbacks, a significant development has been undertaken by introducing FT-IR spectrometers, which provide improved spectral quality and rapid simultaneous analysis of the whole IR spectrum (Banwell and McCash, 1994). Up until relatively recently, the field of FT-IR spectroscopy was only employed to differentiate between regions of biological systems such as proteins, fats, carbohydrates, cells and tissues from animal and plants samples (Naumann et al., 1991; Stuart, 1997).

IR spectroscopy relies on the various vibrations of chemical bonds at specific frequencies that occur between the atoms and molecules. When the light is produced at a specific wavelength and focussed onto a sample, the functional groups within the sample would absorb the light and vibrate in bending, twisting and stretching manners, which depend on the shape and number of atoms of the molecules (Karoui et al., 2010). As a result, these vibrations cause alterations in the dipole moment of that bond. In this technique the frequency and intensity of the radiation, which is absorbed by a sample, are measured and hence the resultant IR absorbance of these various vibrations will be represented as an infrared spectrum (Banwell and McCash, 1994). This IR absorbance spectrum can be used for the identification of molecular contents of unknown species and hence the compound itself (Banwell and McCash, 1994; Yang and Irudayaraj, 2003; Colthup, 2012).

The infrared spectrum can be split into three different regions according to the wavenumbers of the region. These are the near-infrared (NIR) region, the mid-infrared (MIR) region and the far-infrared (FIR) region which have electromagnetic
Chapter One

spectrum regions that lie between 14000-4000 cm$^{-1}$, 4000-400 cm$^{-1}$ and 400-10 cm$^{-1}$, respectively. The far-IR region is more difficult to measure than near-IR and mid-IR regions because of the weakness of the source and detector employed. Although the employed source for the measurement of the FIR region is generally a mercury lamp, the signal-to-noise ratio is greater than that of MIR or NIR regions. Therefore, the MIR or NIR regions are used for the majority of infrared applications. Information of the absorption characteristics of CH, OH, and NH groups can be obtained in the NIR region (Belton et al., 1987; Dunn et al., 2005). The spectra generated from the NIR require high levels of statistical manipulation because it contains broad features that strongly overlap (Belton et al., 1987).

Currently, the MIR region is the most favourite for metabolomics applications (Ellis et al., 2003; Johnson et al., 2004; Dunn et al., 2005), since this region provides chemical and structural information of a sample which can be interpreted directly and easily (Griffiths and De Haseth, 2007). In terms of biology, the mid-IR region has been divided into five main regions (Forster et al., 2003) see Figure 1.2. These regions are called the fatty acid region (3000-2800 cm$^{-1}$), the amide I and amide II regions (1700-1500 cm$^{-1}$), carboxylic groups of proteins (1450-1200 cm$^{-1}$), PO$_2^-$ of phosphodiester (1250-1200 cm$^{-1}$), the polysaccharide region (1200-900 cm$^{-1}$) and the fingerprint region (1500-600 cm$^{-1}$), that contains a number of weak spectral features (Table 1.3) (Naumann et al., 1996; Stuart, 1996; Dunn et al., 2005; Ellis et al., 2012). The acquired information from these regions allows the identification of specific compounds even when the differences of chemical structure of the compounds are small (Banwell and McCash, 1994).

The presence of water in a sample is a major problem as it is highly absorbed within the mid-IR region, producing wide bands in the spectra that may mask the information of a biological fingerprint. Fortunately, there are several methods which can be employed to remove the effect of water from FT-IR spectra. The most well-known method is drying the sample in a suitable sample plate prior to analysis, and these plates are usually zinc selenide or silicon (Curk et al., 1994; Timmins et al., 1998; McGovern et al., 1999; Schuster et al., 2001; Johnson et al., 2003; Patel et al., 2008). In addition, this problem can be overcome with other potential approaches such as attenuated total reflectance (ATR) (Ellis et al., 2002).
Chapter One

The basic components of an FT-IR spectrometer are highlighted in Figure 1.3. First, the source emits an infrared beam which passes the interferometer and then through a sample. The sample absorbs the IR radiation before reaching the detector such as a deuterated triglycine sulfate (DTGS) pyroelectric detector and mercury cadmium telluride (MCT) detector. Next, the amplifier amplifies the signal before converting it to a digital form by an analogue to digital converter. The signal is then transferred to a computer where Fourier transformation is performed and the data are converted to an IR spectrum (Stuart, 1996).
Figure 1.2: Typical FT-IR absorbance of *P. putida* DOT-T1E showing the major regions of biological interest are labelled. Where A refers to fatty acid region, B represents the amide region, C is mixed vibration from carboxylic groups of protein and PO$_4$ of phosphodiester, D is the carbohydrate region and E is the fingerprint region.

<table>
<thead>
<tr>
<th>Wavenumbers (cm$^{-1}$)</th>
<th>Specific function group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000-2800</td>
<td>CH$_x$ stretches in fatty acids</td>
</tr>
<tr>
<td>1700-1500</td>
<td>Proteins</td>
</tr>
<tr>
<td>(1700-1600)</td>
<td>C=O from amide I</td>
</tr>
<tr>
<td>(1600-1500)</td>
<td>C-N and C-N-H from amide II</td>
</tr>
<tr>
<td>1450-1200</td>
<td>COOH of proteins, free amino acids, polysaccharides</td>
</tr>
<tr>
<td>(1250-1200)</td>
<td>P-O from RNA/DNA, phospholipids</td>
</tr>
<tr>
<td>1200-900</td>
<td>C-O or O-H from polysaccharides</td>
</tr>
<tr>
<td>1500-600</td>
<td>Fingerprint region</td>
</tr>
</tbody>
</table>
1.1.5.2 Raman spectroscopy

In 1928, the Indian physicists C.V. Raman and Krishnan observed the Raman Effect which can be defined as the light phenomena of inelastic scattering (Raman and Krishnan, 1928). As a result, this effect was exploited to build and develop instrumentation in order to study the structural properties of solids, liquids and gases, as well as to analyse the natural and synthetic materials (Zhu et al., 2014). Raman spectroscopy is widely recognised as a powerful technique in environmental, biological, medical and pharmaceutical applications since it provides information on both chemical composition and the structure of biological molecules (Petry et al., 2003; Ashton et al., 2011; Ellis et al., 2013). In addition, several studies have established the potential of Raman spectroscopy as a non-invasive, non-destructive and rapid identification technique for microorganisms (Jarvis and Goodacre, 2008; Efrima and Zeiri, 2009; Huang et al., 2010).

Raman spectroscopy offers benefits in several research areas. Since it requires no sample preparation, this makes it relatively inexpensive, and it can also facilitate the direct analysis of products in their packaging (e.g. glass bottles). Further, this technique does not suffer from the presence of water because water has a very weak Raman signal, therefore, cell samples can be analysed and investigated in aqueous solutions (Matthaeus et al., 2008; Ashton et al., 2011). In addition, its high sensitivity to biochemical changes is one of the greatest benefit of this technique (Baena and Lendl, 2004). Therefore, the qualitative analysis of individual compounds can be achieved so Raman spectroscopy can provide a metabolic fingerprint. However, the collection times are relatively long as only 1 in $10^6-10^8$ photons undergo an inelastic light scattering event. Furthermore, the Raman spectra collected with a visible laser
from biological samples can be associated with fluorescence which would dominate the spectra and mask the sharp Raman peaks (Ashton et al., 2011).

In spectroscopy, there are two types of light scattering which are Rayleigh and Raman scattering. The former is the Rayleigh scattering (elastic scattering), which is not accompanied by a shift in the photon frequency. In contrast, during the Raman scattering (inelastic scattering) there is a change in the frequency of the photon (Zhu et al., 2014). Basically, a Raman spectrometer consists of four major components; that is, the source for excitation, the light collection system, monochromator and detector. The employment of lasers in Raman spectroscopy is required in order to produce a large number of photons for the excitation of Raman spectra as the scattering phenomena are far less likely to occur than absorption in infrared spectroscopy for example (Matthaeus et al., 2008). In fact, the ability of Raman spectroscopy to be used in various applications relies on the type of laser and the way the Raman scattering is detected and analysed (McCreery, 2000).

1.1.5.3 Gas chromatography mass spectrometry (GC-MS)
Gas chromatography (GC) linked to mass spectrometry (MS) provides an excellent system that has been widely applied in metabolome analysis due to its high separation, high-resolution efficiency and sensitivity. In addition, GC-MS has the ability to determine and identify a huge number of metabolites in a single analysis (Kopka, 2006; Jousse and Pujos-Guillot, 2013). Another advantage of this technique is the identification of metabolites can be achieved by the availability of compound libraries which can be shared between investigators (Halket et al., 2005). The analyte being analysed in GC-MS is required to be volatile and thermally stable and some metabolites meet these requirements including short chain alcohols and low molecular weight hydrocarbons and lipids (Bedair and Sumner, 2008). GC-MS has some limitations in the analysis of many interesting compounds such as amino acids, sugars and nucleosides because of their polarity and low volatility. However, the volatility and stability of these analytes can be increased by derivatising the samples prior to separation by GC (Lenz and Wilson, 2007; Iwasaki et al., 2012). Moreover, in order to separate the majority of metabolites before they are introduced to the mass spectrometer for detection, long analysis times may be required due to a high number
of metabolites and derivatisation products present in the sample (Jousse and Pujos-Guillot, 2013).

Generally, gas chromatography is a powerful technique that permits the separation, identification, and quantification of unknown compounds in complex mixtures. In metabolomics, GC is a popular analytical platform for the analysis of non-polar and volatile compounds. Chromatographic separation may be influenced by several factors including injection conditions, temperature programme and column properties such as stationary phase, length and internal diameter. In GC, samples are introduced to the system through a heated injector which is usually heated to 200-250°C. The samples can be injected in split or splitless mode relying on the sensitivity required. A large amount of sample is introduced into the column in a splitless system, while only a small fraction of the sample is introduced in split mode. In metabolomics, the split system is often preferred as a wide range concentration of metabolites is presented and it allows for the analysis of volatile compounds which elute near the solvent peak (Jousse and Pujos-Guillot, 2013).

GC systems apply two types of columns; packed and capillary columns. Capillary columns are commonly used in GC-MS based metabolomics and are made of fused silica. These columns resist high temperature and allow for good chromatographic resolution. The internal diameter of capillary columns is small (0.25 mm) which limit the sample capacity to between 50-100 ng. Columns with various properties including length, polarity and chemical composition of the stationary phase have been applied for metabolome analysis (Jousse and Pujos-Guillot, 2013). Therefore, GC parameters such as injection conditions, temperature programming and flow rate are required to be optimised in order to enhance peak capacity production (Wilson et al., 2012).

MS is considered to be the most crucial detection system in biotechnology as it offers tremendous opportunities for metabolome analysis, and it is one of several detectors that can be combined with chromatographic techniques. This powerful, versatile and accessible technologies can solve many of the analytical and research problems in metabolomics (Villas-Boas et al., 2005). The main function of MS is the detection of mass-to-charge ratio ($m/z$) and abundance of various analytes that are produced during sample ionisation. MS mainly consists of three components that are an
ionisation source, a mass analyser and a detector, and it is necessary to keep these three components maintained under vacuum in order to optimise the ion transmission to the mass analyser and detector (Niessen, 1998; Gross, 2004).

Firstly, the ionisation source is where the production of ions takes place. This is a key step, as ions are more simply manipulated than neutral molecules (Allwood and Goodacre, 2010). Two ionisation sources are generally used in GC-MS based metabolomics: electron ionisation (EI) is the most commonly used method since it is performed in a high-vacuum ion source where analytes in the gas state are bombarded with electrons at 70 eV. This allows many fragment ions that are characteristic of particular molecules and hence this method permits the use of spectral libraries. However, chemical ionisation (CI) is a softer ionisation technique which produces less fragmentation in comparison with EI. It has also been employed in some specific metabolomics studies as it allows the generation of molecular ions from metabolites that cannot be produced in EI (Jousse and Pujos-Guillot, 2013).

Second is the mass analyser in which ions with different masses are separated by their trajectories in a magnetic field. Many mass analysers (e.g. quadrupole, time of flight (TOF)) are employed in GC-MS for metabolomics studies (Fiehn et al., 2000). The single quadrupole offers a large dynamic range and high sensitivity but is inappropriate as it operates with lower resolution and slower scan rates compared to TOF systems. The introduction of GC-triple quadrupole MS/MS solves the identification and quantification problem associated with co-eluting analytes in complex matrices (Jousse and Pujos-Guillot, 2013). However, GC-TOF/MS is increasingly employed for metabolite profiling since it provides higher mass resolution and sensitivity compared to quadrupoles (Bedair and Sumner, 2008; Almstetter et al., 2012). In addition, the ideal detector for metabolomic analysis of narrow chromatographic peaks is TOF/MS due to its fast scan speeds which make it useful for an accurate deconvolution of overlapping peaks (Davis et al., 1999; Allwood and Goodacre, 2010; Jousse and Pujos-Guillot, 2013). Finally, the ions reach the detector which records and translates the passage of specific mass ions into meaningful signals (Gross, 2004; Allwood and Goodacre, 2010).
Chapter One

1.1.5.4 Liquid chromatography mass spectrometry (LC-MS)

Liquid chromatography mass spectrometry (LC-MS) is a crucial tool in metabolomics as known and unknown compounds (e.g. polar, less-polar and natural metabolites) can be generally detected and identified even at very low concentration in a complex matrices. LC-MS does not require the prior derivatisation of samples in order to make analytes of interest amenable to detection and it has the ability to determine selected metabolites in relatively short times (Villas-Boas et al., 2005; De Vos et al., 2007; Allwood and Goodacre, 2010). Another advantage of LC-MS in metabolomics is its ability to analyse small and large molecular weights exceeding 600 Da. As a result, it is useful in the analysis of polar and non-polar metabolites (Dunn, 2008).

LC can be defined as the separation of components in a liquid mixture according to different partitioning of each compound between stationary and mobile phase. The separation of a mixture of components is achieved since each compound has different affinity for the stationary and mobile phase. In other words, compounds that have higher affinity to the mobile phase will elute more quickly than those have higher affinity to the stationary phase as they will be retained and hence have a longer retention time (Allwood and Goodacre, 2010). There are several types of liquid chromatography which widely rely on the chemistry of the stationary phase. In metabolomics, reversed phase high performance liquid chromatography (RP-HPLC) is generally used and the components of basic HPLC system are solvent reservoir, high pressure pump, solvent partitioning valve, column and detectors. To achieve better separation in HPLC, three factors should be considered which are the polarity of the sample, the composition of the mobile phase and the chemical properties of the stationary phase. Nevertheless, many other parameters (e.g. column length, column particle size and pump pressure) are important and were used in the development of ultra-high-performance liquid chromatography (UHPLC) (Romanyshyn and Tiller, 2001; Nguyen et al., 2006; Novakova et al., 2006; Allwood and Goodacre, 2010). A disadvantage of RPLC however, is that it cannot adequately retain more highly polar compounds. This limitation was overcome with hydrophilic interaction chromatography (HILIC) which was developed to separate and quantify extreme polar compounds in biological samples (Iwasaki et al., 2012; Alpert, 1990). The analysis of liquid samples is widely used in metabolomics especially through the
combination of separation systems with mass spectrometry techniques such as LC-MS (Allen et al., 2003; Bajad et al., 2006; Chen et al., 2006).

MS is one of several detectors that can be coupled with separation techniques such as HPLC. Two powerful soft ionisation sources are generally utilised in LC-MS based metabolomics: electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) (Wolfender et al., 1995). The most commonly applied method for sensitive analysis is ESI which is suited to the ionisation of a broader range of metabolites such as drug compounds, sugars, amino and organic acids, phospholipids and fatty acids (Tolstikov and Fiehn, 2002; Tolstikov et al., 2003; Allwood et al., 2006; Chen et al., 2007). However, APCI sources are suitable for a wide range of metabolites especially for the ionisation of less polar and non polar metabolites including phospholipids, fatty acids, sterols and steroids (Dachtler et al., 2001; Schweiggert et al., 2005). The basic principle of ESI begins with passing the liquid through a capillary in front of the mass spectrometer slot, which is then sprayed with a coaxial flow of nitrogen gas in a strong electric field to create a fine aerosol of charged particles. The spray of fine droplets undergoes a coulomb explosion in order to form microdroplets carrying a high charge. Finally, further solvent evaporation in the nitrogen stream results in the delivery of charged analytes at the mass spectrometer orifice (Harris, 2007).

There are many different variations of mass analysers linked to liquid chromatography. Quadrupoles were one of the first types of mass analyser used in metabolomics investigations to be applied to LC-MS (Tolstikov and Fiehn, 2002), and TOF mass separators are also employed. However, it has been reported that the sensitivity, accuracy and robustness of LC-MS for metabolomics can be improved when a LTQ-Orbitrap is linked to an LC system that operate with sub-2 µm particles (Dunn, 2008). Basically, orbitrap systems are employed for mass determination as they detect the orbital frequencies of ions. In this technique, ions cycle around the central electrode on elliptical trajectories at ultra high vacuums although in the absence of high magnetic fields (Dunn, 2008; Perry et al., 2008). There are many advantages of the orbitrap over most other mass analysers, including high mass accuracy, high mass resolution, large space capacity and a broad dynamic range (Hu et al., 2005; Lim et al., 2007; Seigelova and Makarov, 2006). Therefore, this mass analyser can be utilised to detect small peaks with a high level of accuracy.
1.2 Research Objective

The aim of this study was to investigate adaptation mechanisms involved in the response of *P. putida* DOT-T1E strains upon exposure to abiotic stress at the metabolome level. Changes identified in the metabolome can be considered to be hypothesis generating and as such can inform our biochemical knowledge. Observed metabolite changes can prove to be indicative of novel adaptation mechanisms or may support postulated adaptation mechanisms for which there is little evidence up to date. The aims of the research were addressed through the following objectives:

- To investigate the effect of propranolol on the metabolome(s) of *P. putida* DOT-T1E strains.
- To identify and quantify the accumulation level of toluene in *P. putida* strains using Raman spectroscopy.
- To study the use of FT-IR spectroscopy and GC-MS to identify phenotypic and metabolome changes in *P. putida* DOT-T1E cultures exposed to toluene.
- To investigate the use of FT-IR spectroscopy and GC-MS to identify the effect of cation metal ions on *P. putida* strains exposed to toluene.
1.3 References


Chapter One


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Chapter One


Chapter One


**Chapter 2:** Metabolomics analysis reveals the participation of efflux pumps and ornithine in the response of *Pseudomonas putida* DOT-T1E cells to challenge with propranolol

The work presented in this chapter has been published:


Contributing authors and their roles:

Ali Sayqal is the main author. Yun Xu contributed to GC-MS data analysis and Drupad Trivedi participated in data processing. Najla AlMasoud participated in quenching process and David Ellis provided assistance in reporting the study. Nicholas Rattray helped with preliminary analysis and Royston Goodacre contributed to this work through supervision and guidance of the study.
2.1 Abstract

Efflux pumps are critically important membrane components that play a crucial role in strain tolerance in *Pseudomonas putida* to antibiotics and aromatic hydrocarbons that result in these toxicants being expelled from the bacteria. Here, the effect of propranolol on *P. putida* was examined by sudden addition of 0.2, 0.4 and 0.6 mg mL\(^{-1}\) of this β-blocker to several strains of *P. putida*, including the wild type DOT-T1E and the efflux pump knockout mutants DOT-T1E-PS28 and DOT-T1E-18. Bacterial viability measurements reveal that the efflux pump TtgABC plays a more important role than the TtgGHI pump in strain tolerance to propranolol. Mid-infrared (MIR) spectroscopy was then used as a rapid, high-throughput screening tool to investigate any phenotypic changes resulting from exposure to varying levels of propranolol. Multivariate statistical analysis of these MIR data revealed gradient trends in resultant ordination scores plots, which were related to the concentration of propranolol. MIR illustrated phenotypic changes associated with the presence of this drug within the cell that could be assigned to significant changes that occurred within the bacterial protein components. To complement this phenotypic fingerprinting approach metabolic profiling was performed using gas chromatography mass spectrometry (GC-MS) to identify metabolites of interest during the growth of bacteria following toxic perturbation with the same concentration levels of propranolol. Metabolic profiling revealed that ornithine, which was only produced by *P. putida* cells in the presence of propranolol, presents itself as a major metabolic feature that has important functions in propranolol stress tolerance mechanisms within this highly significant and environmentally relevant species of bacteria.
2.2 Introduction

Active pharmaceutical compounds (APCs), in their original states or their metabolites, are ubiquitous in the environment (Escher et al., 2005), and the levels of APCs in the aquatic ecosystems (e.g., lakes, rivers, seawater and estuaries) are a growing concern (Fent et al., 2006). Pharmaceuticals are not only being introduced into the environment after consumption, but also via the disposal of unused or expired pharmaceuticals (Breton and Boxall, 2003). The levels of many pharmaceuticals in sewage treatment plants (STPs) have been detected at low concentrations in the range of ng L\(^{-1}\) to µg L\(^{-1}\) (Ashton et al., 2004; Thomas and Hilton, 2004; Carlsson et al., 2006a; Escher et al., 2005). A study in the United Kingdom revealed that the β-blocker propranolol is widely used, and for instance, around 12 tonnes of propranolol are consumed each year (Ashton et al., 2004; Carlsson et al., 2006a; Carlsson et al., 2006b). In addition, Ashton and co-workers (2004) showed that the presence of the β-blocker propranolol in STP effluents was highly likely at 76 ng L\(^{-1}\) (median level) (Ashton et al., 2004). Despite the fact that APCs are designed to have specific modes of action in the organism they were designed for, similar targets might control different metabolic processes in different species for which the original APC was not designed for (Seiler, 2002). In addition, the modes of action of the drugs within microbial systems are not fully understood. Thus, we and others believe it is necessary to increase our knowledge of the biological effects and fate of pharmaceuticals on microorganisms in the environment to appreciate the risks (Huggett et al., 2002). Indeed, bacterial communities inhabiting the benthic environment of riverbeds can be exposed to higher levels of APCs than expected, as it is known that these compounds can become concentrated in these areas (Halling-Sorensen et al., 1998; Poulquen et al., 1992; Thacker, 2005). Additionally, pharmaceuticals tend to bioaccumulate and induce impacts in aquatic and terrestrial environments due to their intrinsic pharmacokinetic properties (Halling-Sorensen et al., 1998). A major adverse side effect of the presence of APCs in the environment is an increase in antimicrobial resistance that poses huge potential risk for the future, making the treatment of infections very difficult to cure, and there are several studies that have eloquently described the link between exposure to effluent and antimicrobial resistance (Zhang et al., 2011; Liu et al., 2012; Johnning et al., 2013; Marathe et al., 2013).
Bacteria can adapt the activity of toxic substances by the employment of several resistant mechanisms including altering lipid composition, energy production, efflux pumps as well as other processes (Keweloh et al., 1990; Ramos et al., 1997; Volkers et al., 2009; Wijte et al., 2011). Efflux pumps, which transport toxic chemicals (usually waste products from normal metabolism) from the bacterial cell into the extra-cellular environment, are probably the most highly significant process which plays an important role in bacterial tolerance. One of these mechanisms is controlled by the ATP-binding cassette (ABC) transporters via the hydrolysis of ATP, whereas the transmembrane electrochemical gradient, particularly the proton motive force, is used by secondary transporters in order to drive drug efflux (Paulsen et al., 1996; Putman et al., 2000). In Pseudomonas putida DOT-T1E cells, three efflux pumps, which are genome-encoded, have been identified, and are termed TtgABC, TtgDEF, and TtgGHI. The TtgABC and TtgGHI pumps remove both organic solvents and some antibiotics, whereas the TtgDEF pump has been shown to be induced only by aromatic hydrocarbons (Ramos et al., 1998; Mosqueda and Ramos, 2000; Rojas et al., 2001). Many studies have found that an enormous number of multidrug resistance (MDR) transport proteins are involved in the export of a wide range of antimicrobial compounds (Paulsen et al., 1996; Putman et al., 2000; Saier and Paulsen, 2001). In Pseudomonas species, various studies linked solvent and antibiotic tolerance to the action of several efflux pumps (Li et al., 1998; Ramos et al., 1998; Wijte et al., 2011; Molina-Santiago et al., 2014). Moreover, solvent-tolerant microorganisms (e.g. P. putida DOT-T1E) play a crucial role in several biotechnological applications such as bioremediation, biocatalysis and agriculture (Rojas et al., 2004; Pandey et al., 2009; Garcia et al., 2010; Nicolaous et al., 2010). Thus, an understanding of bacterial tolerant mechanisms is very important, in order to enhance the resistant systems for non-pathogenic strains and create altered strains with superior tolerance characteristics for industrial bioprocessing.

The qualitative and quantitative measurements of the metabolome of an organism can reveal its biochemical status and these data can be used to monitor and determine the function of genes (Fiehn, 2002; Fiehn, 2001). Metabolomics enables the identification and quantification of endogenous biochemical reaction products of cellular regulatory pathways and metabolite levels can be regarded as the ultimate response of biological system to environmental alterations and/or genetic factors.
Metabolome analysis provides relevant information about specific cell types under different conditions that is important for a more holistic understanding of cell functions and properties (Fiehn, 2002). A comprehensive assessment of the alteration in the metabolite levels in *P. putida* strains can be acquired using a combination of metabolic profiling and multivariate data analysis approaches. The interpretation of metabolic data is complicated, thus a wide range of different analytical strategies have been employed to measure the metabolome (Westerhoff *et al.*, 2009; Daran-Lapujade *et al.*, 2007). By understanding metabolomics data the effect of stress on lowest molecular levels is revealed. This enables better understanding of altering metabolic pathways that are directly affected by change in bacterial genome.

In order to investigate the effects of propranolol on biological systems, we have employed Fourier-transform infrared (FT-IR) spectroscopy to acquire metabolic fingerprints (Ellis *et al.*, 2007; Ellis *et al.*, 2003; Ellis and Goodacre, 2006). FT-IR spectroscopy involves the observation of bond vibrations from within molecules when a sample is excited by a beam from the mid-infrared region of the electromagnetic spectrum. Briefly, the infrared beam is transmitted through or reflected from a sample, with some of the infrared radiation being absorbed at particular wavelengths within the sample, and the remainder continuing on to a detector, before being Fourier transformed and analysed via a computer. This results in an infrared absorbance spectrum which can be referred to as a metabolic “fingerprint” as it is characteristic of any chemical or biochemical substance. The fundamentals of FT-IR have been described in far greater detail elsewhere (Ellis *et al.*, 2003; Baker *et al.*, 2014) but its main advantages are that it is very rapid (taking seconds per sample), high-throughput, with 96 and 384 well sampling plates, reagentless, and non-destructive. FT-IR has been applied to a very wide-range of biological studies including clinical (Ellis and Goodacre, 2006; Baker *et al.*, 2016) and microbiological (Naumann *et al.*, 1991) analyses since the very early 1990s when Dieter Naumann and co-workers demonstrated its potential use for bacterial characterization (Helm *et al.*, 1991). Metabolic profiling approaches are powerful in that in contrast to FT-IR spectroscopy they can be used to identify, quantify and detect the metabolites within the biological system, and gas chromatography mass spectrometry (GC-MS) is currently a very popular method for analysing central carbon and nitrogen metabolism (Dunn *et al.*, 2005; Bino *et al.*, 2004; Broadhurst and
Kell, 2006). Changes identified in the metabolome can be considered to be hypothesis generating and as such can inform our biochemical knowledge (Kell and Oliver, 2004; Goodacre et al., 2004). With respect to bacterial strain tolerance we believe that the observed metabolite changes can prove to be indicative of novel adaptation mechanisms or may support postulated adaptation mechanisms for which there is little evidence to date.

The aim of this study was to investigate the changes in metabolite levels within P. putida DOT-T1E strains in the presence and absence of propranolol and determine if these changes were associated to efflux pumps or other adaptation mechanisms within these bacteria. To enable this, FT-IR spectroscopy was utilised as a rapid, high-throughput screening tool in order to identify phenotypic alterations in bacterial cultures exposed to propranolol, and metabolic profiling using GC-MS was employed to examine the change in metabolites at specific time points before and after challenge with propranolol.

### 2.3 Material and methods

#### 2.3.1 Bacterial strains and cultivation of bacteria

Three bacterial strains of P. putida DOT-T1E were used in this study. Their relevant characteristics, and references for further information on each strain are listed in Table 2.1. All strains were sub-cultured in triplicate to obtain axenic cultures. Individual colonies were then picked and transferred from plates into 250 mL flasks containing 50 mL of autoclaved Lysogeny broth (LB) medium and incubated at 24 h at 30°C in an orbital incubator (Infors HT Ltd, UK) shaking at 200 rpm.
Chapter Two

Table 2.1: Bacteria used in this study

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Relevant characteristics$^a$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. putida</em> DOT-T1E</td>
<td>Ap$^r$ Rif$^r$ Tol$^r$</td>
<td>(Ramos et al., 1995)</td>
</tr>
<tr>
<td><em>P. putida</em> DOT-T1E-PS28</td>
<td>Rif$^r$ Sm$^r$ ttgH::VSm</td>
<td>(Rojas et al., 2001)</td>
</tr>
<tr>
<td><em>P. putida</em> DOT-T1E-18</td>
<td>Rif$^r$ Km$^r$ ttgB::phoA-Km</td>
<td>(Ramos et al., 1998)</td>
</tr>
</tbody>
</table>

$^a$ Resistance to Ap$^r$: ampicillin, Rif$^r$: rifampin, Sm$^r$: streptomycin, Km$^r$: kanamycin and Tol$^r$: toluene

2.3.2 Growth curve monitoring

Bacterial growth curves were monitored manually using an orbital incubator and UV instrument at 660 nm (Biomate 5, CarePlanTM, UK). All samples were normalised to an optical density (OD) of 0.02 in 250 mL flasks containing 50 mL LB medium. *P. putida* DOT-T1E cultures were incubated at 30°C and 200 rpm. During the 24 h time course, 100 µL samples were taken at various time points (0, 2, 4, 6, 8, 10, 12 and 24 h) for OD measurement.

2.3.3 Growth in response to propranolol shock, sample collection and analysis

Cells were grown in 50 mL of LB medium for 5 h at 30°C and 200 rpm. Once cell cultures reached the mid-exponential phase, samples were divided into two groups. One group was kept as a control, and to the second group propranolol was added at three different concentrations (0.2, 0.4 and 0.6 mg mL$^{-1}$). These cultures were then incubated for an additional 8 h.

2.3.3.1 Growth curve measurement

At various time points (0, 1, 3, 5, 7, 9, 11 and 13 h) before and after the addition of propranolol, a 100 µL sample was taken for OD measurement. Growth was recorded as an increase or decrease in turbidity at 660 nm. This work was undertaken in biological triplicates.
2.3.3.2 FT-IR analysis

2.3.3.2.1 Sample collection

After 60 min of the addition of propranolol, an aliquot (2 mL) from each sample was transferred to a 2 mL tube, and the ODs of the samples were recorded for normalisation. All measurements were performed in triplicate.

2.3.3.2.2 Sample preparation for FT-IR spectroscopy

An aliquot (2 mL) from each flask was transferred to a 2 mL tube and centrifuged at 11500 ×g for 5 min at 4°C. The supernatant was removed and discarded. The remaining pellet was washed twice with 2 mL of physiological saline solution (0.9% NaCl) and centrifuged (11500 ×g, 5 min, 4°C), the supernatant discarded. The remaining cell pellets were stored at -80°C until required.

A 96-well silicon FT-IR plate (Bruker Optics, Banner Lane, Coventry, UK) was cleaned with 5% sodium dodecyl sulfate (SDS) and rinsed with deionised water and allowed to dry at room temperature. Cell pellets were then removed from -80°C and allowed to thaw on ice. Samples were normalised according to OD at 660 nm, resuspended in physiological saline and gently vortexed. Aliquots (20 µL) of each sample were randomised and spotted in triplicate onto a silicon FT-IR plate. The prepared plates were then dried on a desiccator at ambient temperature for 7 h. This step was applied to minimise any signal arising from water absorbance in the mid-IR region.

2.3.3.2.3 FT-IR setup

The prepared silicon sample plate was loaded onto a motorised microplate module HTS-XT™ under the control of a PC programmed with OPUS software version 4. Spectra were acquired using a Bruker Equinox 55 FT-IR spectrometer (Bruker Optics, Banner Lane, Coventry, UK) in transmission mode as described previously (Winder et al., 2006), with a deuterated triglycine sulfate (DTGS) detector over the wavenumber range 4000-600 cm⁻¹, with a resolution of 4 cm⁻¹. A total of 64 scans were combined and averaged in order to improve the signal-to-noise ratio. Three
technical replicates were obtained from each sample, and a total of 324 spectra were collected.

### 2.3.3.2.4 FT-IR data analysis

FT-IR data were converted to ASCII format using OPUS reader software and analysed using Matlab version 2012 (MathWorks, Natick, MA). All FT-IR spectra were CO$_2$ corrected by replacing the region from 2400 to 2275 cm$^{-1}$ with a linear trend and then scaled using extended multiplicative signal correction (EMSC) (Martens et al., 2003).

Statistical analysis of the preprocessed data was performed using principal component analysis (PCA) (Wold et al., 1987) and discriminant function analysis (DFA). PCA was used to generate the set of latent variables (PCs) which retain the major variance of the data whilst decreasing the dimensionality; DFA was then used to create a set of discriminant functions (DFs) based on PCs which maximise the differences between the known groups (classes) (Macfie et al., 1978; Johnson et al., 2003). PC-DFA was performed using 10 PCs and 3 DFs, and the class structure for the DFA algorithm was based on the biological replicates of samples of the same conditions.

### 2.3.3.3 GC-MS analysis

#### 2.3.3.3.1 Sample collection

15 mL samples were quenched at three time points 0, 10 and 60 min before and after the addition of propranolol (0 min refers to the point immediately before the addition of propranolol). This procedure was performed with four biological replicates.

#### 2.3.3.3.2 Metabolic quenching and metabolite extraction

Generally, a rapid inactivation of metabolism is achieved by alteration in pH or temperature (Villas-Bôas et al., 2005). Thus, in order to halt metabolism culture samples (15 mL) were plunged into a double volume of 60% cold methanol (-50°C) in a 50 mL tube. The quenched culture mixture was centrifuged (3000 ×g, 10 min, 1°C), and then the supernatant was discarded, while the cell pellets were stored at -80°C until required for metabolite extraction (Winder et al., 2008).
The biomass pellets were resuspended in 750 µL of freshly prepared cold methanol (80%). The solution was then transferred to a 2 mL Eppendorf tube. This was followed by three freeze-thaw cycles in order to extract the intracellular polar metabolites from the cells. Samples were centrifuged at (13500 ×g, 3 min, 4°C) and the supernatant was transferred to new tubes and stored on dry ice. The extraction was performed again on the remaining pellet, both supernatants were combined and again stored on dry ice. A final aliquot (1400 µL) of metabolite extracts were normalised using 80% methanol according to OD at 660 nm. A quality control (QC) sample (Fiehn et al., 2008; Dunn et al., 2011) was prepared by transferring 100 µL from each of the samples to a new (15 mL) centrifuge tube. This was followed by the addition of (100 µL) of internal standard solution (0.2 mg mL⁻¹ glycine-d₅, 0.2 mg mL⁻¹ benzoic-d₅ acid, 0.2 mg mL⁻¹ lysine-d₄, and 0.2 mg mL⁻¹ succinic-d₄ acid) to all samples. The samples were lyophilised for 16 h by speed vacuum concentrator (concentrator 5301; Eppendorf, Cambridge, UK), and then the pellet was stored at -80°C for further analysis.

2.3.3.3 Derivatisation process
Samples were derivatised prior to GC-MS analysis in two stages as described previously by Wedge and co-workers (Wedge et al., 2011). The first step, (50 µL) of O-methoxylamine hydrochloride diluted in pyridine (20 mg mL⁻¹) was added to the samples and then samples were heated on a heating block at 65°C for 40 min. The second step, (50 µL) of MSTFA (N-methyl-trimethylsilyltrifluoroacetamide) was added to the samples followed by heating for 40 min. At the end of second step, 20 µL of retention index was added. After each addition in all three steps described above samples were vortexed for 10 s and centrifuged at 13500 ×g for 15 min.

2.3.3.4 GC-MS instrument setup
Samples were randomised and analysed by gas chromatography electron ionisation time-of-flight mass spectrometry (GC-TOF-MS) using an Agilent 6890 GC instrument coupled to a LECO Pegasus III TOF mass spectrometer (Leco, St. Joseph, MI, USA), as described previously (Begley et al., 2009; Dunn et al., 2011; Wedge et al., 2011). GC column (VF-17MS column, 0.25 mm ID × 30 m × 0.25 µm film thickness, Varian, cat. no. CP8982) was employed at a constant helium carrier gas
flow of 1 mL min\(^{-1}\), with a temperature program that starts at 70°C and ends at 300°C. The mass spectrometer source is operated at a temperature of 250°C in electron ionization (EI) mode, with an electron energy of 70 eV and the detector is operated in the range 1400-1800 V. Raw data processing was undertaken using LECO ChromaTOF v3.26 in order to construct a data matrix of metabolite peak vs. sample and infilled with peak areas for metabolites that were detected. A reference database was prepared that contained retention times, quant mass, peak area, retention index value and peak number related to each peak by analysing QC samples. The identification of analytes was based on both spectral similarity and matched with retention indices. An in-house library as well as the NIST library was used for identification, and we followed MSI guidelines for metabolite identification (Sumner et al., 2007).

2.3.3.3.5 Data analysis

For statistical analysis multi-block PCA (Smilde et al., 2003) was used with three different types of blockings. The first type of blocking is strain | time×dosage blocking. This blocking partitioned the data into 9 blocks. Each block contained all the samples from the same time point with the same dosage of propranolol, e.g. all the samples with 0.2 mg mL\(^{-1}\) propranolol, collected at 0 min were assigned to one block, those with 0.4 mg mL\(^{-1}\) propranolol, collected at 10 min were assigned to another block and so on. Across different blocks, the strains were matched so that in every block the first 4 samples were \textit{P. putida} DOT-T1E, the next 4 samples were \textit{P. putida} DOT-T1E-18 and the last 4 samples were \textit{P. putida} DOT-T1E-PS28. Based on the same principle, dosage | strain×time blocking partitioned the data into 6 blocks (samples at 0 min were not included for this type of blocking as this time point refers to the point immediately before the addition of propranolol), each block had the samples of the same strain and same time points, the dosage of propranolol were matched. Such blocking allowed MB-PCA to detect the effects of each of the factors of interest (i.e., strain, time and dosage of propranolol) separately without the inference from others (Xu and Goodacre, 2012). A total of 200 features were detected by GC-MS. The natural logarithm (ln) was first applied on the peak area of the detected peaks. Data were mean centred, auto-scaled then subjected to MB-PCA. The potentially most significant variables were identified by selecting the most predominant averaged block loadings. Finally, box-whisker plots were used to
visualise the data. These analyses were conducted using in-house scripts under the Matlab 2014a (Mathworks, Natick, MA) environment.
2.4 Results and discussion

2.4.1 Characterisation of \( P. \text{ putida} \) DOT-T1E strains

Growth curve experiments were undertaken for \( P. \text{ putida} \) strains to determine the optimum points to induce abiotic stress using propranolol. The resultant growth curves are displayed in (Figure 2.1) and these show that there were no significant differences in the pattern of growth between the wild type DOT-T1E and the mutant DOT-T1E-PS28 (lacking the TtgGHI pump) over the 24 h incubation period. Whilst under the same conditions, the mutant DOT-T1E-18 (lacking the TtgABC pump) grew slightly poorly in comparison to the other strains. This result was in agreement with previous observations which show that \( P. \text{ putida} \) DOT-T1E-PS28 grew on LB medium and had similar growth generation time to the wild type (Rojas et al., 2001). However, the mutant DOT-T1E-18 showed less growth compared to the wild-type and this could be a result of the waste products made during cellular metabolic processes accumulating to toxic levels due to the lack of TtgABC pump, resulting in slower growth.

![Figure 2.1: Growth curves of all three \( P. \text{ putida} \) DOT-T1E strains in LB medium. Symbols represent different strains. DOT-T1E is the wild type (close circles), DOT-T1E-PS28 is the mutant (closed triangles) and DOT-T1E-18 is the mutant (closed squares). A 1/10 dilution of 100 µL samples were prepared for OD measurement at 660 nm.](image-url)
Chapter Two

To be able to investigate the metabolome changes between the wild type and the two mutants, cells were cultured in the absence of propranolol, GC-MS analysis was performed and this was followed by chemometrics. MB-PCA of all *P. putida* strains was carried out and the result showed an obvious clustering pattern as can be seen in (Figure S2.1). It was clear from this analysis that *P. putida* DOT-T1E-18 was very different to the other two strains although weak separation can also be observed between *putida* DOT-T1E and *P. putida* DOT-T1E-PS28. In addition, MB-PCA loading plots were plotted in order to investigate the significant metabolites associated with the different growth behaviour (Figure S2.2). It can be seen that many metabolites were most abundant in *P. putida* DOT-T1E-18 and least abundant in *P. putida* DOT-T1E. Box-whisker plots were generated and these generally supported the increased metabolite levels in DOT-T1E-18 (Figure S2.3). During the growth of the three *P. putida* strains, a number of metabolites detected by GC-MS were compared (e.g. carbon and nitrogen metabolism; *viz.*, sugars, sugar phosphates, amino acids, organic acids).

A schematic summary of the detected metabolites by GC-MS of central metabolic pathways in *P. putida* DOT-T1E strains is shown in Figure S2.4. It can be seen that the level of a total of 9 metabolites were similar in the mutant DOT-T1E-PS28 compare to the wild-type DOT-T1E, while only 3 metabolites had similar levels in the mutant *P. putida* DOT-T1E-18 in comparison to the wild type. These results would suggest that the TtgABC pump is involved in the removal of toxic metabolites produced during the log phase. In addition, the accumulation of toxic products might result in changes in the level of amino acids due to the activation of other metabolic pathways to deal with waste products.

### 2.4.2 Characterisation of *P. putida* DOT-T1E strains to propranolol shocks.

#### 2.4.2.1 Minimal inhibitory concentration (MIC)

In order to study the effect of propranolol on *P. putida* DOT-T1E cultures, it was necessary to establish the MIC of each bacterial strain when cultured in LB media and challenged with different levels of propranolol and the results are recorded in
Table S2.1. The visible growth of the wild-type DOT-T1E, mutant DOT-T1E-PS28 and mutant DOT-T1E-18 were inhibited at 1.5, 1.5 and 0.8 mg mL\(^{-1}\) of propranolol respectively. The resistance of DOT-T1E-PS28 to propranolol was the same as the wild-type. However, it was reduced for the mutant DOT-T1E-18, suggesting that the extrusion of propranolol by the TtgABC pump could play a more crucial role than TtgGHI pump. Observations similar to these findings have been reported by Rojas and co-workers (Rojas et al., 2001) testing MIC of several antibiotics for \(P.\ putida\) DOT-T1E strains, in which the DOT-T1E-18 mutant was more sensitive to those antibiotics than DOT-T1E. Nevertheless, the DOT-T1E-PS28 mutant showed similar sensitivity to the wild-type.

### 2.4.2.2 Bacterial growth in the presence of propranolol

From interpretation of the growth curves (Figure 2.1), it was decided to induce propranolol stress after 5 h (once the cultures reached their mid-exponential phase) at three different concentrations of propranolol (0.2, 0.4 and 0.6 mg mL\(^{-1}\)) below the MIC. The effect of propranolol on \(P.\ putida\) cells was then studied in liquid culture medium after cells had been pre-grown on LB liquid medium, and following challenge with propranolol. Growth curve results from \(P.\ putida\) cultures are shown in (Figure 2.2A-C). In general, slight variations were noted in the growth patterns between \(P.\ putida\) DOT-T1E and DOT-T1E-PS28 species exposed to 0.2 and 0.4 mg mL\(^{-1}\) propranolol, though considerable effects on the same cultures were observed when cultures were exposed to 0.6 mg mL\(^{-1}\) propranolol across a 13 h growth period. By contrast, a marked effect was observed in \(P.\ putida\) DOT-T1E-18 when exposed to 0.4 and 0.6 mg mL\(^{-1}\) concentrations of propranolol. Strain tolerance is an energy intensive process, and it was noted that the growth yields of \(P.\ putida\) DOT-T1E cultures in the presence of 0.6 mg mL\(^{-1}\) were reduced by five-fold compared to the control cultures. This decrease in the growth yield might result in consumption of energy by various mechanisms in order to protect the cells from further damage. One study examined the growth yields of \(Pseudomonas\) upon sub-lethal toluene dosages and it was found that the presence of toluene led to lower yields and that the growth yield reduced linearly with increasing toluene concentrations (Isken et al., 1999). This report deduced that the decrease in yield associated with the presence of toluene
could be due to energy-consuming adaptation mechanisms initiated to protect cells from excessive damage.
Figure 2.2: Growth curves of: (A) *P. putida* DOT-T1E, (B) *P. putida* DOT-T1E-PS28, and (C) *P. putida* DOT-T1E-18 in the absence and presence of propranolol. A 1/10 dilution of 100 µL samples were prepared for OD measurement at 660 nm.
Chapter Two

To assess bacterial membrane integrity during the growth of bacteria following propranolol perturbation a LIVE/DEAD BacLight bacterial viability assay was used, and the green and red fluorescence emissions were measured using a Flexstation 3 Microplate Reader (Molecular Devices, USA). The ratio of green to red fluorescence and the percentage of live cells from TVC plates estimations in the *P. putida* suspension are shown (Table S2.2). It was clear from these measurements that cell viability decreased linearly with increasing propranolol indicating the toxic effect of propranolol on *P. putida* DOT-T1E strains.

### 2.4.2.3 FT-IR fingerprinting of cell cultures

FT-IR spectroscopy was employed to investigate whether the phenotype of an organism had changed by exposing it to gradient levels of propranolol. PC-DFA scores plots were produced in order to visualise the distribution of samples based on their IR metabolic fingerprints (Figure 2.3A-C). From inspection of the PC-DFA scores plots of the biomass samples, it was possible to determine that there was an obvious separation between the different culture conditions. There was also a clear trajectory based on concentration (annotated with arrows) with samples from control cultures following a trend from right to left across the plot space due to the increase of propranolol concentrations. This clustering pattern was anticipated and suggests that propranolol stress has had a clear additive effect on the bacterial cells and this is reflected in the FT-IR results. In other analyses these PC-DFA models were validated by test set projection (Figure S2.5) and these ensure that the model quality is of a high standard, and that the obtained subsequent conclusions drawn from the data are valid and robust.

To assess the relevant metabolites causing these separations in PC-DFA scores plots, the loadings plots for the first discriminant functions were plotted (Figure 2.3D-F). Multiple changes occur within these loadings plot with the largest variances being observed between wavenumbers 1700-1600 cm\(^{-1}\). In this region of the mid-infrared the majority of vibrational bands are associated with protein components of the sample; most notably amide I (C=O stretching at 1690-1620 cm\(^{-1}\)) and amide II (combination of C-N stretching and N-H bending). These results suggest that the most significant effect over the duration of the 1 h incubation period following drug
shock is associated with alterations to proteinaceous components of bacteria. The profile of proteins in different *P. putida* strains – T1E and S12 – upon exposure to toluene has been investigated previously, and it was revealed that almost 90 proteins were up-regulated as a result of an exposure of strains to toluene in which some of these proteins relate to efflux pump systems (Segura *et al*., 2005; van der Werf *et al*., 2008; Wijte *et al*., 2011). Therefore, it is perhaps not surprising that the most significant changes observed from the interpretation of infrared spectra were in the vibrational frequency of the proteins components, and we can infer from this that some proteins were up-regulated to cope with the presence of propranolol.
Figure 2.3: PC-DFA scores plots of FT-IR data for three different strains of *P. putida* strains upon propranolol shock. Symbols represent different strains. (A) *P. putida* DOT-T1E is the wild type (stars) and ten PCs with a total explained variance (TEV) of 99.43% were used for the DFA, (B) *P. putida* DOT-T1E-PS28 is the mutant (closed triangles) and ten PCs with a TEV of 99.65% were used for the DFA, (C) *P. putida* DOT-T1E-18 is the mutant (closed circles) and ten PCs with a TEV of 99.03% were used for the DFA. Colour coding: control with no propranolol (red), cells exposed to 0.2 mg mL$^{-1}$ propranolol (black), 0.4 mg mL$^{-1}$ propranolol (brown), and 0.6 mg mL$^{-1}$ propranolol (blue). Arrows indicate the direction of shift because of the increase of propranolol concentration. (D) PC-DFA loadings plot for *P. putida* DOT-T1E. (E) PC-DFA loadings plot for *P. putida* DOT-T1E-PS28. (F) PC-DFA loadings plot for *P. putida* DOT-T1E-18. Significant loadings were assigned to bacterial proteins.
2.4.2.4 GC-MS metabolic profiling of cell cultures

Recently, attention has been focused on studying the stress responses in bacteria employing metabolomics-based approaches (Kol et al., 2010; Brito-Echeverria et al., 2011; Allwood et al., 2015), and this has involved a wide range of disciplines such as drug discovery, metabolic engineering and medical sciences (Mashego et al., 2007; Anton et al., 2013; Kim et al., 2014; Dunn et al., 2015; Ellis and Goodacre, 2012). In this study, we employed GC-MS to create metabolic profiles of bacterial stress to propranolol, as the knowledge of variations within the metabolome following chemical perturbation could lead to a more in-depth understanding of strain specific stress responses within these bacteria.

As there are multiple potentially interacting factors that we have in our experiment with respect to propranolol dose, bacterial strain, as well as time, MB-PCA was used for analysis. MB-PCA with dosage | strain×time blocking (see materials and methods) was undertaken and a gradient effect corresponding to differing dosages of propranolol can be seen on the resultant scores plot (Figures. S2.6 and S2.7). We observed nine metabolites that were differentially expressed between control and different dosages of propranolol and these were statistically significant. However, four metabolites (cystathionine, glutamine and two unknowns) decreased with increase in dosage of propranolol, four metabolites (ornithine, propranolol and two unknowns) increased with dosage whereas no clear pattern was seen for one metabolite (unknown).

Interestingly, it was found that two of these metabolites (variables 180 and 100) were only detected following the exposure of *P. putida* strains to all three concentrations of propranolol groups but not in the control. Variable 180 was identified by an in-house database as propranolol itself, and Figure 2.4A shows that exposure of cells to propranolol resulted in the accumulation of propranolol in comparison to non-exposed cells. These data also show that the level of propranolol in *P. putida* stains were detected at both time points at 10 and 60 min, and it was noticed that the accumulation of propranolol in the exposed cells increased as the concentration of the propranolol increased. In addition, comparing the level of propranolol between the wild-type and the mutants only at 60 min (Figure 2.4B), it was observed that *P. putida* DOT-T1E (wild-type) and *P. putida* DOT-T1E-PS28 (lacking the TtgGHI pump) showed high similarities in the level of propranolol at all tested
concentrations. By contrast, the amount of propranolol accumulating in the *P. putida* DOT-T1E-18 (lacking the TtgABC pump) was higher than the other strains. This could be further evidence for the activity of efflux pump system in *P. putida* cells due to the presence of propranolol at different levels. In addition, these results would suggest that the TtgABC efflux pump is the main extrusion pump for propranolol and that it plays a more important role than the TtgGHI pump. These findings, which agree well with other studies, show that the TtgABC pump in *P. putida* DOT-T1E is the main antibiotic extrusion pump, and it has the ability to extrude flavonoids, tetracycline, chloramphenicol and ampicillin in addition to other solvents such as toluene (Teran et al., 2003; Duque et al., 2007; Roca et al., 2008).
Figure 2.4: Box-whisker plots showing the changes in metabolite levels in control and cells exposed to propranolol for 4 biological replicates. Variable 180 was identified as propranolol. (Red line) indicates the median m/z intensity. (A) Represent the data for 3 P. putida strains, 4 concentrations of propranolol and 3 time points, dashed lines separate different concentration levels of propranolol and solid line separates different strains. (B) Represent the data for 3 P. putida strains, 3 concentrations of propranolol and 1 time point at 60 min, dashed lines separates different strains.
Interestingly, the other significant variable, 100, was identified as ornithine (ChEBI ID 15729) again from an in-house library generated on the same instrument (Sumner et al., 2007). Ornithine production was detected within 10 min after exposure to propranolol, and the level of ornithine in the wild-type DOT-T1E and mutant DOT-T1E-PS28 shows an increase at 0.2 mg mL\(^{-1}\) propranolol and a further almost linear increase in the presence of 0.4 and 0.6 mg mL\(^{-1}\) propranolol (Figure 2.5). By contrast, the production of this metabolite in the mutant DOT-T1E-18 exhibits an increase at 0.2 mg mL\(^{-1}\) propranolol followed by a further increase at 0.4 mg mL\(^{-1}\) followed by a decrease toward 0.6 mg mL\(^{-1}\) propranolol. Furthermore, the level of ornithine was further decreased, from 10 to 60 min at 0.6 mg mL\(^{-1}\) propranolol for both \textit{P. putida} DOT-T1E and DOT-T1E-PS28, while it was increased for \textit{P. putida} DOT-T1E-18 under the same conditions. This metabolite is very important, as it is only produced by the \textit{P. putida} cells in the presence of propranolol and our data suggest that this is linked to bacterial tolerance mechanisms, further studies are needed in order to understand this role and comprehend whether this is a cause or effect relationship.

In addition, in \textit{P. putida} T1E and S12 proteomic analysis found that several proteins of the TCA cycle involved in energy production were up-regulated upon toluene exposure, indicating a requirement for enhanced metabolism and high energy demands because of toluene exposure in order to power efflux pumps that extrude solvent from the cells (Segura et al., 2005; Wijte et al., 2011), which is in agreement with several proteomics, and transcriptomics studies (Eaton, 1996; Segura et al., 2005). The up-regulation of several terminal oxidase genes upon solvent stress in \textit{P. putida} T1E suggests that demands on energy consumption are necessary to cope with the presence of solvents, in particular due to high activity of efflux pumps (Rojo, 2010). Ornithine can be synthesised via the TCA cycle in which glutamate is converted into ornithine, as previously reported for \textit{P. putida} (Antonia Molina-Henares et al., 2010). The production of ornithine in the presence of propranolol is interesting, as this observation would suggest that \textit{P. putida} DOT-T1E may use this amino acid for energy production to power efflux pumps, or in order to activate other metabolic pathways that are important in bacterial tolerance to propranolol.
In addition, the primary building block of biological membranes mainly consists of glycerophospholipids such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and cardiolipin (CL); however, other lipids classes (e.g. ornithine lipids) have been described as well, which contain a 3-hydroxy fatty acyl group attached in amide linkage to the α-amino group of ornithine. This lipid can be formed only by specific groups of bacteria or under certain stress conditions (Vences-Guzman, 2015); although these have not yet been reported in *P. putida*. It is possible that the ability to produce ornithine under propranolol stress in *P. putida* strains is linked to lipid production, however we have no direct evidence for this yet.

![Box-whisker plot showing the changes in ornithine levels](image)

**Ornithine**

*Variable id. = 100*

Figure 2.5: Box-whisker plot showing the changes in ornithine levels (variable id 100) in control and exposed cells to propranolol. (Red line) indicates the median m/z intensity. These plots represent the data for 3 *P. putida* strains, 4 concentrations of propranolol and 3 time points, for 4 biological replicates. Dashed lines separate different concentration levels of propranolol and solid lines separate different strains.
Chapter Two

It is clear from the above that there are changes in central metabolism in response to propranolol exposure. Therefore, we investigated whether the levels of metabolites in the central metabolic pathways of *P. putida* strains were significantly altered or not between control and propranolol challenged samples for each bacterial strain independently. Metabolic pathways that were changed during propranolol stress were identified utilising untargeted GC-MS analysis. A comparative summary of central metabolic pathways between control and propranolol challenged cells for 10 or 60 min in *P. putida* DOT-T1E (Figure 2.6), *P. putida* DOT-T1E-18 (Figure 2.7) and *P. putida* DOT-T1E-PS28 (Figure S2.8) were generated and large effects were seen in amino acid biosynthesis. In total, 17 metabolites were differentially produced or consumed in the presence of 3 different concentrations of propranolol, compared to the control sample at two time points. Major metabolites that were changed significantly during propranolol stress were serine, glycine, tryptophan, phenylalanine, tyrosine, alanine, valine, leucine, citrate, fumarate, glutamine, ornithine, aspartic acid, lysine, methionine, threonine and isoleucine, and box-whisker plots of these metabolites show the changes in these metabolite levels (Figures S2.9-12). In *P. putida* DOT-T1E, 10 metabolites were found to be consumed, 4 metabolites produced and 3 metabolites did not change at 10 min, while 4 metabolites were down-regulated and 13 metabolites up-regulated at 60 min. In the mutant DOT-T1E-18, 10 min following exposure to propranolol the levels of 2 metabolites increased, 8 metabolites were consumed and 7 metabolites remained constant. After 60 min following exposure to propranolol, the levels of 11 metabolites were increased, 3 metabolites were consumed and 3 remained constant.

In *P. putida* DOT-T1E-PS28, although similar patterns in the level of metabolites was observed compared to the wild-type in the absence of propranolol, different patterns were observed in the presence of propranolol. Both mutants showed different metabolic profiles compared to the wild type and this could be due to the lack of the pump leading to over-expression of certain amino acids that are important to activate specific pumps or other metabolic pathways to cope with the stress.

Pathway analysis also revealed that glutamine and ornithine, which shows similar metabolic changes in all *P. putida* DOT-T1E strains, as major pathways impacted by propranolol stress. It is possible that glutamine could be being consumed by the cells in order to respond to high energy demands due to propranolol exposure. Another
possible suggestion is that the decrease in the level of glutamine may be due to the biosynthesis of ornithine which could be the key stress-responsive metabolite involved to cope with stress following perturbation by propranolol. Therefore, cells may convert glutamate into ornithine instead of glutamine, resulting in a decrease in the level of glutamine. In contrast, in comparison to the wild type both mutants undergo different metabolic changes in other detected metabolites, mainly aliphatic amino acids, aromatic amino acids, and the aspartate family. This might be explained by the lack of the efflux pump in each mutant leading to the induction of certain metabolic pathways resulting in the production or consumption of certain amino acids associated with specific pumps.
Figure 2.6: Schematic metabolic diagram of central carbon metabolism in *P. putida* DOT-T1E adapted to propranolol showing the level of metabolites for cells exposed to propranolol compared to the control. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. The median m/z intensity (red line) in the box-whisker plots was used to compare the level of metabolites. (A) Represents the level of metabolites at 10 min, while (B) the level of metabolites at 60 min. Traffic light system represents different concentration of propranolol. Red, yellow and green represent exposed cells to 0.2, 0.4 and 0.6 mg mL⁻¹ of propranolol respectively. Up-arrow, down-arrow and steady arrow indicate an increase, a decrease and no change in the level of metabolite respectively. The number of arrows represents the level of metabolites. Slight change (single arrow), medium change (double arrows) and high change (triple arrows).
Figure 2.7: Schematic diagram of central carbon metabolism in *P. putida* DOT-T1E-18 adapted to propranolol showing the level of metabolites for cells exposed to propranolol compared to the control ones. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. The median m/z intensity (red line) in the box-whisker plots was used to compare the level of metabolites. (A) Represents the level of metabolites at 10 min, while (B) the level of metabolites at 60 min. Traffic light system represents different concentration of propranolol. Red, yellow and green represent exposed cells to 0.2, 0.4 and 0.6 mg mL⁻¹ of propranolol respectively. Up-arrow, down-arrow and steady arrow indicate an increase, a decrease and no change in the level of metabolite respectively. The number of arrows represents the level of metabolites. Slight change (single arrow), medium change (double arrows) and high change (triple arrows).
2.5 Conclusion

Here we have shown that propranolol had a measurable biological effect on all three strains of bacteria studied. The results demonstrated that the mutant *P. putida* DOT-T1E-18 was more hypersensitive to propranolol than the other strains analysed due to the lack of TtgABC pump. With respect to exposure to propranolol, data from FT-IR revealed that propranolol had an effect on protein components of the bacterial cells. The investigation of the characterisation of the metabolome of *P. putida* DOT-T1E strains upon exposure to propranolol revealed the important role of efflux pump activity and the production of ornithine as major key elements for adaptation mechanisms. This information can be useful in bioengineering to create engineered *P. putida* strains or even other bacteria with superior tolerance characteristics for bioprocesses. This information can be useful in bioengineering to create engineered *P. putida* strains or even other bacteria with superior tolerance characteristic for bioprocesses, which in turn can help to remediate simple or complex mixtures of pollutants from environment. Similar to the case where lactate tolerance was improved in an engineered strain producing ascorbic acid, a well-known reactive-oxygen species scavenger (Abbott *et al.*, 2009). Furthermore, both screening tools and metabolic profiling in combination with multivariate statistical methods, seem ideally suited to monitoring the phenotypic responses occurring within microbial cultures under different growth conditions and subjected to abiotic stress.
Chapter Two

2.6 References


Chapter Two


2.7 Supplementary information

Figure S2.1: MB-PCA scores plot of GC-MS data for the wild type and the mutants in the absence of propranolol. Colours represent different strains. (A) *P. putida* DOT-T1E is the wild type (red), (B) *P. putida* DOT-T1E-PS28 (green), and (C) *P. putida* DOT-T1E-18 (blue).

Figure S2.2: MB-PCA loading plot of GC-MS data showing most significant metabolites between the wild type and the mutants in the absence of propranolol. Significant loadings were observed in the positive side of the plot.
Figure S2.3: Box-whisker plots of a few selected most significant metabolites between the wild type and the mutants in the absence of propranolol. (A) *P. putida* DOT-T1E is the wild type, (B) *P. putida* DOT-T1E-PS28, and (C) *P. putida* DOT-T1E-18.
Figure S2.4: Schematic metabolic pathway diagram of central carbon metabolism in *P. putida* DOT-TIE showing the level of metabolites for both mutants compared to the wild type. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. The median m/z intensity (red line) in the box-whisker plots was used to compare the level of metabolites. Blue and brown represent the mutant DOT-TIE-PS28 and DOT-TIE-18 respectively. Up-arrow, down-arrow and steady arrow indicate an increase, a decrease and no change in the level of metabolite respectively. The number of arrows represents the level of metabolites. Slight change (single arrow) and medium change (double arrows).
Figure S2.5: Validated PC-DFA models of (A) *P. putida* DOT-T1E, (B) *P. putida* DOT-T1E-PS28, (C) *P. putida* DOT-T1E-18 upon 0.2, 0.4 and 0.6 mg mL$^{-1}$ Propranolol shock. Symbols coding: control with no propranolol (circles), cells exposed to 0.2 mg mL$^{-1}$ propranolol (squares), 0.4 mg mL$^{-1}$ propranolol (triangles), and 0.6 mg mL$^{-1}$ propranolol (upside down triangles). Opened symbols represent the test set while closed symbols represent the training set.
Chapter Two

Figure S2.6: MB-PCA score plot of GC-MS data showing the effect of different concentrations on *P. putida* strains. Colours represent different dosage of propranolol. (D0) exposed to 0 mg mL\(^{-1}\) propranolol (blue), (D1) exposed to 0.2 mg mL\(^{-1}\) propranolol (green), and (D2) exposed to 0.4 mg mL\(^{-1}\) propranolol (pink). (D3) exposed to 0.6 mg mL\(^{-1}\) propranolol (red).

Figure S2.7: MB-PCA loading plot of GC-MS data showing the most significant metabolites in the presence of different concentrations of propranolol. Significant loadings were observed in the positive and negative sides of the plot.
Figure S2.8: Schematic metabolic diagram of central carbon metabolism in *P. putida* DOT-T1E-PS28 adapted to propranolol showing the level of metabolites for cells exposed to propranolol compared to the control. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. The median m/z intensity (red line) in the box-whisker plots was used to compare the level of metabolites. (A) Represents the level of metabolites at 10 min, while (B) the level of metabolites at 60 min. Traffic light system represents different concentration of propranolol. Red, yellow and green represent exposed cells to 0.2, 0.4 and 0.6 mg mL\(^{-1}\) of propranolol respectively. Up-arrow, down-arrow and steady arrow indicate an increase, a decrease and no change in the level of metabolite respectively. The number of arrows represents the level of metabolites. Slight change (single arrow), medium change (double arrows) and high change (triple arrows).
Figure S2.9: Box-whisker plots of the detected metabolites of central carbon metabolism in *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18, dashed lines separate different concentration levels of propranolol and solid lines separate different strains. The label is constructed in a format of “Aac”, “A” represents strains, and varies from A to C: A = *P. putida* DOT-T1E; B = *P. putida* DOT-T1E-PS28 and C = *P. putida* DOT-T1E-18. “a” represents 4 different concentration levels, and varies from 0 to 3: 0 = control; 1 = 0.2 mg mL\(^{-1}\); 2 = 0.4 mg mL\(^{-1}\) and 3 = 0.6 mg mL\(^{-1}\) propranolol. “c” represents time points, 1 = T0 (0 min); 2 = T1 (10 min) and 3 = T2 (1 h). Such plots give a comprehensive view of how the concentration levels of the metabolite are changing under each unique combination of the factors (strains, dosage of propranolol and time). Variable 14 (alanine), Variable 20 (valine), Variable 29 (leucine), and Variable 34 (isoleucine).
Figure S2.10: Box-whisker plots of the detected metabolites of central carbon metabolism in *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18, dashed lines separate different concentration levels of propranolol and solid lines separate different strains. The label is constructed in a format of “Aac”, “A” represents strains, and varies from A to C: A = *P. putida* DOT-T1E; B = *P. putida* DOT-T1E-PS28 and C = *P. putida* DOT-T1E-18. “a” represents 4 different concentration levels, and varies from 0 to 3: 0 = control; 1 = 0.2 mg mL\(^{-1}\); 2 = 0.4 mg mL\(^{-1}\) and 3 = 0.6 mg mL\(^{-1}\) propranolol. “c” represents time points, 1 = T0 (0 min); 2 = T1 (10 min) and 3 = T2 (1 h). Such plots give a comprehensive view of how the concentration levels of the metabolite are changing under each unique combination of the factors (strains, dosage of propranolol and time). Variable 40 (glycine), Variable 53 (threonine), Variable 54 (serine), and Variable 78 (aspartic acid).
Figure S2.11: Box-whisker plots of the detected metabolites of central carbon metabolism in *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18, dashed lines separate different concentration levels of propranolol and solid lines separate different strains. The label is constructed in a format of “Aac”, “A” represents strains, and varies from A to C: A = *P. putida* DOT-T1E; B = *P. putida* DOT-T1E-PS28 and C = *P. putida* DOT-T1E-18. “a” represents 4 different concentration levels, and varies from 0 to 3: 0 = control; 1 = 0.2 mg mL\(^{-1}\); 2 = 0.4 mg mL\(^{-1}\) and 3 = 0.6 mg mL\(^{-1}\) propranolol. “c” represents time points, 1 = T0 (0 min); 2 = T1 (10 min) and 3 = T2 (1 h). Such plots give a comprehensive view of how the concentration levels of the metabolite are changing under each unique combination of the factors (strains, dosage of propranolol and time). Variable 81 (methionine), Variable 88 (glutamine), Variable 95 (phenylalanine), and Variable 103 (fumarate).
Figure S2.12: Box-whisker plots of the detected metabolites of central carbon metabolism in *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18, dashed lines separate different concentration levels of propranolol and solid lines separate different strains. The label is constructed in a format of “Aac”, “A” represents strains, and varies from A to C: A = *P. putida* DOT-T1E; B = *P. putida* DOT-T1E-PS28 and C = *P. putida* DOT-T1E-18. “a” represents 4 different concentration levels, and varies from 0 to 3: 0 = control; 1 = 0.2 mg mL$^{-1}$; 2 = 0.4 mg mL$^{-1}$ and 3 = 0.6 mg mL$^{-1}$ propranolol. “c” represents time points, 1 = T0 (0 min); 2 = T1 (10 min) and 3 = T2 (1 h). Such plots give a comprehensive view of how the concentration levels of the metabolite are changing under each unique combination of the factors (strains, dosage of propranolol and time). Variable 109 (citrate), Variable 119 (lysine), Variable 135 (tyrosine), and Variable 177 (tryptophan).
Table S2.1: Results from the propranolol MIC experiments using *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18. Culture growth was observed after overnight incubation.

<table>
<thead>
<tr>
<th><em>P. putida</em> strains</th>
<th>Propranolol concentration (mg mL$^{-1}$)</th>
<th>Growth (+/-)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOT-T1E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>0.7</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>± or +</td>
<td></td>
</tr>
<tr>
<td>1.4</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>DOT-T1E-PS28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>0.7</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>± or +</td>
<td></td>
</tr>
<tr>
<td>1.4</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>-</td>
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<tr>
<td>DOT-T1E-18</td>
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</tr>
<tr>
<td>0</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>± or +</td>
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<tr>
<td>0.7</td>
<td>±</td>
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<tr>
<td>0.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>-</td>
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</tr>
<tr>
<td>1.4</td>
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</tr>
<tr>
<td>1.5</td>
<td>-</td>
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<tr>
<td>2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*(+) indicates growth, (±) slight growth, and (-) no growth
Table S2.2: Viability of *P. putida* cells 1 h later after exposure to propranolol

<table>
<thead>
<tr>
<th><em>P. putida</em> strains</th>
<th>Propranolol (mg mL$^{-1}$)</th>
<th>Green/Red ratio*</th>
<th>Bacterial viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOT-T1E</td>
<td>0</td>
<td>3.21</td>
<td>96.17</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>2.70</td>
<td>73.07</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>2.57</td>
<td>66.93</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>1.83</td>
<td>33.03</td>
</tr>
<tr>
<td>DOT-T1E-PS28</td>
<td>0</td>
<td>3.19</td>
<td>95.45</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>2.54</td>
<td>65.46</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>2.10</td>
<td>45.19</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>1.67</td>
<td>25.80</td>
</tr>
<tr>
<td>DOT-T1E-18</td>
<td>0</td>
<td>2.52</td>
<td>64.65</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>2.23</td>
<td>51.36</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>1.53</td>
<td>19.09</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>1.26</td>
<td>6.89</td>
</tr>
</tbody>
</table>

\* *BacLight* bacterial viability assay where Green indicates live cells and Red indicates dead cells
Chapter 3: The identification and quantification of toluene in *Pseudomonas putida* DOT-T1E using Raman spectroscopy and liquid chromatography

3.1 Abstract

*Pseudomonas putida* strains have the ability to thrive in the presence of toxic solvents such as toluene. In this study, the level of toluene present in *P. putida* DOT-T1E strains were first investigated using Raman spectroscopy. Using the excitation wavelength of 1064 nm instead of 532 nm resulted in a significant increase in the Raman signal which could be due to the application of higher laser power of the instrument that was accessible at this excitation source, as well as a reduction in fluorescence at longer wavelengths. However, toluene was not successfully quantified in *P. putida* cells since the limit of detection is in the milli-molar range, which indicated the requirement for a technique more specifically suited to the detection of very low levels of toluene in cells, such as HPLC. Results from HPLC demonstrated the technique’s ability to detect and quantify toluene present in *P. putida* strains in concentrations of 33, 71 and 277 µM for DOT-T1E (wild-type), DOT-T1E-PS28 (mutant) and DOT-T1E-18 (mutant), respectively. Finally, the limit of detection was found to be rather high and calculated to be 15 µM which is very close to the levels of toluene present in *P. putida* strains.
3.2 Introduction

Many aromatic hydrocarbons (in particular toluene) are highly toxic solvents that kill most microorganisms, since these solvents are known to accumulate in cell membranes and thereby impair their function, ultimately leading to cell death (Inoue and Horikoshi, 1989; Sikkema et al., 1995; Ramos et al., 1998; De Smet et al., 1978). However, it has been reported that some microorganisms (e.g. *Pseudomonas putida*) have the ability to thrive in the presence of toxic solvents, and that their resistance to these compounds are acquired via several adaptation mechanisms including efflux pumps, as well as changing lipid compositions and up-regulation of chaperones (Garikipati and Peeples, 2015; Ramos et al., 2015; Wijte et al., 2011; Volkers et al., 2009; Molina-Santiago et al., 2014). Solvent tolerant microorganisms play an important and widely recognised role in several applications including bioremediation (Pandey et al., 2009), whole-cell biocatalysis (Neumann et al., 2006; Ramos-Gonzalez et al., 2001) and in the production of biofuels (Ingram et al., 1987; Rude and Schirmer, 2009).

In recent years, Raman-based techniques have become increasingly important and have developed into excellent analytical tools in various scientific areas of research. This is partly due to the versatility of applications, minimal sample preparation, rapid acquisition time, and non-destructive sampling capabilities. This technique offers significant potential for the environmental and industrial monitoring of toxic vapours (Onchoke et al., 2016; Manivel et al., 2013), food analysis (Nicolaou et al., 2011; Ellis et al., 2012; Ellis et al., 2015), clinical diagnostics (Ellis et al., 2013) and biopharmaceutical applications (Wen, 2007; Ashton and Goodacre, 2011), as it provides both qualitative and quantitative information that can be derived from Raman spectra. Moreover, since water has a very weak Raman signal, Raman spectroscopy is attractive for the analysis of bio-fluids. However, a major limitation of Raman spectroscopy is the fact that the Raman effect itself is inherently weak and typically only 1 in $10^6$-$10^8$ incident photons undergo an inelastic light scattering event (Ellis and Goodacre, 2006). Therefore, several techniques have been developed to enhance Raman signals including surface-enhanced Raman scattering (SERS) (Alharbi et al., 2015; Jarvis et al., 2008) and resonance Raman spectroscopy (López-Díez and Goodacre, 2004).
In the present study, we have employed Raman spectroscopy combined with chemometric techniques to investigate whether it is possible to identify and quantify n-toluene and isotopic labelled toluene and their metabolism in *P. putida* cells. As such a method would provide a better understanding of cellular process of microorganisms exposed to abiotic stress which is necessary for the development of new biotechnologies.

### 3.3 Material and methods

#### 3.3.1 Bacterial strains and growth conditions

One isolate of *P. putida* named DOT-T1E (Ramos *et al.*, 1995) and the two mutants DOT-T1E-PS28 (Rojas *et al.*, 2001) and DOT-T1E18 (Ramos *et al.*, 1998) were selected for this study. Cultures were routinely grown in liquid Lysogeny broth (LB) medium and incubated in an orbital shaker for 4 h at 30°C and 200 rpm. Once *P. putida* cultures reached the mid-log phase, toluene (Sigma-Aldrich, U.K.) was added at different levels ranging between 5 to 100 mM. The culture flasks were closed with Suba-Seals to prevent toluene evaporation and then incubated under the same conditions for an additional 12 h.

#### 3.3.2 Sample preparation

##### 3.3.2.1 Bacterial biomass

During the incubation period after the addition of toluene, 2 mL aliquots from culture samples were collected at different time points (1, 2, 6 and 12 h). The collected samples were then centrifuged at 11500 ×g for 5 min at 4°C (ThermoFisher CR3.22, UK). The supernatant was removed, the cell pellets were washed once using 2 mL of sterile saline solution (0.9% NaCl) (Fisher, UK) and then centrifuged under the conditions described above. The biomass pellets were normalised, resuspended in saline solution, and kept on wet ice until further analysis.

##### 3.3.2.2 Direct quenching extraction of intracellular metabolites

After 30 min following exposure to 20 mM toluene, culture samples (45 mL) were collected and transferred to a 50 mL centrifuge tube and centrifuged at 3000 ×g (10
min, 1°C). The supernatant was discarded and the collected biomass pellets were washed once with 10 mL of saline solution following the above centrifugation parameters. Again the supernatant was removed, an aliquot (1.5 mL) of (100%) methanol (-48°C) was added to the biomass, and the resuspended pellets were transferred to new 2 mL microcentrifuge tubes. Freeze-thaw cycles were performed three times on the samples as described previously (Winder et al., 2008) in order to permeabilise the cells. Samples were centrifuged (13500 × g, 5 min) and an aliquot (1200 µL) of supernatant (intracellular extracts) was normalised to OD_{660} and transferred to fresh 2 mL tubes. Finally, all samples were stored on dry ice until further measurements.

3.3.3 Sample analysis

3.3.3.1 Raman spectroscopy

3.3.3.1.1 Biomass analysis

An aliquot (3 µL) of the samples was spotted onto calcium fluoride (CaF₂) disks and dried at room temperature in a desiccator. Raman spectra were obtained using a Renishaw inVia Raman microscope using 3.4 software (Galactic Industries Crop. Salem, NH) equipped with a 532 nm diode laser and an air-cooled CCD detector. A 50× magnifying objective was employed for sample observation. Spectra were acquired by employing a laser power of 10 mW focused on the sample, a 600 l/mm grating, and three accumulations with a 20 s exposure time. The Raman data were imported into Matlab version 2012 (MathWorks, Natick, MA) and then Raman spectra were baseline corrected prior to analysis.

3.3.3.1.2 Liquid extracts

For liquid analysis 300 µL of the pure toluene and cell extracts was transferred onto a quartz 96-well plate. Measurements were again performed on a Renishaw inVia Raman microscope (Galactic Industries Crop. Salem, NH) quipped with an excitation source 532 nm diode laser, delivering approximately 10 mW at the sample level.
Raman spectra were acquired using a grating of 600 l/mm, a 15 s exposure time, one accumulation, and an air-cooled CCD detector. The objective lens used in the study was a 15× magnifying objective. To obtain z-scans of spectra vs. depth into a liquid sample an automated XYZ sample stage was moved axially. Prior to analysis all obtained Raman spectra were imported into Matlab version 2012 (MathWorks, Natick, MA) and followed by baseline corrected.

3.3.3.2 High-performance liquid chromatography (HPLC)

300 µL of each intracellular extract was placed in an LC vial and analysed by HPLC utilising a HPLC system (Agilent Technologies) equipped with an Agilent 1260 Infinity Quaternary Pump, an auto-sampler and a variable wavelength UV Diode Array Detector. The chromatographic separation was achieved by injecting 15 µL, using a C18 column (100 x 4.6 mm) at 20°C and (100%) methanol mobile phase at a flow rate of 1 mL min⁻¹. The output signal was monitored at 218 nm and the total analysis time was 30 min.

3.4 Results and discussion

3.4.1 Raman spectroscopy analysis

*P. putida* strains are well-known microorganisms whose mechanism of solvent tolerance has been studied extensively. It has been reported that these bacteria can reduce the activity of toxic chemicals by the employment of several adaptation mechanisms including energy production to drive efflux pumps and lipid modifications (Ramos et al., 2015; Rojas et al., 2001; Wijte et al., 2011). The ability to detect toxic solvents or drugs rapidly in microorganisms at low levels has obvious benefits and would expand our knowledge in bacterial tolerance mechanisms, which are important in many areas of research such as bio-refinery and biofuels bioprocesses. In this study Raman spectroscopy was used to detect the level of toluene or its metabolites in *P. putida* strains as it has proven very successful in rapidly discriminating and identifying microorganisms.
The carbon-hydrogen stretching vibrational band is usually observed in the region between 2800 and 3100 cm$^{-1}$ and has a high spectral intensity (Socrates, 2004). Therefore, this region is ideal for the identification and quantification of the hydrocarbon moiety of aromatic solvents (Sebek et al., 2013). Figure 3.1 shows the typical Raman spectra from undeuterated and deuterated toluene using the Raman microscope. Comparing spectra of n-toluene, toluene-$d_3$ and toluene-$d_5$ revealed shift in the characteristic regions. It is noted that most of the bands detected in Raman spectra for deuterated toluene are shifted to lower wavenumbers in comparison to unlabelled toluene. The most affected bands were at 3055 and 2922 cm$^{-1}$ which can be attributed to the aromatic C-H stretch and the symmetrical CH$_3$ stretch of the alkyl group respectively. Furthermore, the toluene Raman spectra contain a range of vibrations between 600 and 1650 cm$^{-1}$, which corresponds to benzene C=C bond vibrations. These clear spectral shifts are mainly due to an increased mass of the deuterated atoms (Larkin, 2011) in the labelled toluene compared to the normal toluene, and details of the affected Raman peaks and their corresponding assignments are shown in Table 3.1.

Figure 3.1: Typical Raman spectra of unlabelled toluene (red line) compared to labelled toluene-$d_3$ (blue line) and toluene-$d_5$ (black line). Raman spectra were recorded using Raman spectroscopy with an excitation wavelength of 532 nm.
Chapter Three

Table 3.1: Major Raman spectral shift in wave numbers detected due to deuterated atom

<table>
<thead>
<tr>
<th>Unlabelled toluene (cm(^{-1}))</th>
<th>Toluene-D3 (Δ cm(^{-1}))</th>
<th>Toluene-D5 (Δ cm(^{-1}))</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3055</td>
<td>0</td>
<td>-768</td>
<td>C-H (stretch, aromatic)</td>
</tr>
<tr>
<td>2922</td>
<td>-780</td>
<td>0</td>
<td>C-H (stretch, alkyl)</td>
</tr>
<tr>
<td>1611</td>
<td>0</td>
<td>-41</td>
<td>C=C (stretch in ring)</td>
</tr>
<tr>
<td>1004</td>
<td>0</td>
<td>-34</td>
<td>C=C (stretch in ring)</td>
</tr>
<tr>
<td>790</td>
<td>-24</td>
<td>-46</td>
<td>C=C (stretch in ring)</td>
</tr>
</tbody>
</table>

Since the \textit{P. putida} strains were cultivated under two different conditions (absence or presence of 5 mM toluene), we first investigated the presence of toluene in the cell pellet samples collected at different time points, and the results of Raman spectra are shown in Figure 3.2. To detect the presence of toluene in \textit{P. putida} cells, simple visual inspections of Raman spectra were carried out. It is obvious that similar observations were observed between non-exposed and exposed cells to toluene and the toluene was not detected under the experimental conditions because \textit{P. putida} cultures were challenged with very low concentrations of toluene.
Figure 3.2: Raman spectra of *P. putida* DOT-T1E culture biomass at three different time points following 5 mM toluene stress. (A) 2 h, (B) 6 h, and (C) 12 h after the addition of toluene. Colours represent different conditions. Control-no toluene (blue line) and culture exposed to toluene (red line). Raman spectra were recorded using Raman spectroscopy with an excitation wavelength of 532 nm.
Therefore, cells were challenged with higher concentrations ranging from 5 to 100 mM and the resultant Raman spectra are displayed in Figure 3.3. Unfortunately, we were unable to detect toluene in bacterial cells under these conditions. Toluene is well-known as highly volatile solvent; thus, it is possible that it was lost during the drying process prior to Raman analysis.

Conversely, exposure of *P. putida* cells to toluene resulted in a decrease in band intensity in comparison to non-exposed cells. This could be due to a biological effect resulting from challenging bacterial culture with toluene. Several studies have shown that the presence of many aromatic hydrocarbons (e.g. toluene), resulted in significant alterations in the structure and function of membrane components including disruption and removal of proteins and lipids as well as loss of Mg$^{2+}$ and Ca$^{2+}$ (Sikkema et al., 1995; Sardessai and Bhosle, 2002; Rodriguez-Herva et al., 2007; Pinkart et al., 1996; Clifton et al., 2015).

Figure 3.3: Raman spectra of *P. putida* culture biomass at four different concentrations of toluene. Colours represent different concentrations. Cell exposed to 0 mM toluene (blue line), 5 mM toluene (black line), 30 mM toluene (green line), and 100 mM toluene (red line). Raman spectra were recorded using Raman spectroscopy with an excitation wavelength of 532 nm.
In order to circumvent the drying process prior to Raman analysis, a direct extraction experiment was performed to extract the intracellular metabolites. Figure 3.4 shows the Raman spectra of *P. putida* strains exposed to 20 mM of toluene. Again no bands corresponding to toluene were observed in the exposed cultures to toluene suggesting another factor would prevent the toluene detection in bacterial cells. Although the specificity of Raman spectroscopic technique is very high, the sensitivity is not as high due to the weakness of the Raman effect.

As a consequence, the next stage was to assess the limit of detection of toluene utilising the peak area for 3055 cm\(^{-1}\) corresponding to aromatic C-H stretches. The results of Raman spectra and the calibration curve for serial dilutions of toluene are presented on Figure 3.5A and 5B. As can be seen, the band at 3055 cm\(^{-1}\) is concentration dependant and it starts to disappear at 250 mM. The next stage was to use the equation of the line obtained from the dilution study to establish the limit of detection of toluene using the peak area for 3055 cm\(^{-1}\). The limit of detection was
calculated to be 3 times the standard deviation of the blank, divided by the gradient of the straight line. The calculated limit of detection for the toluene was found to be 536 mM. Our observations strongly suggest that it would be very difficult to detect toluene in *P. putida* strains as the cells were challenged with toluene concentrations below the limit of detection of Raman spectroscopy.

Figure 3.5: Raman spectra of (A) serial dilution of toluene and (B) the calibration curve of 3055 cm\(^{-1}\) peak area which was used to calculate the limit of detection for toluene. Each point represents the median of three replicates of each concentration. Error bars are standard deviations. Raman spectra were recorded using Raman spectroscopy with an excitation wavelength of 532 nm.
In Raman spectroscopy, depending on the type of application, several excitation sources such as a laser of any wavelength from the deep UV (e.g. 244 nm) to visible (405 nm, 532 nm, 633 nm) and into the NIR (785-1064 nm) can be used. Therefore, the next step was to investigate the level of toluene in bacterial cells, and for this a handheld Raman spectrometer with a 1064 nm laser was employed. Typical Raman spectra of serial dilutions of toluene are shown in Figure 3.6A and 6B. It can be seen that the band at 790 cm\(^{-1}\) decrease linearly with decreasing toluene concentration, and the signal was not observed at concentrations equivalent to or lower than 1 mM. Although the applied laser at wavelength of 1064 nm has lower energy than 532 nm, lower levels of toluene were detected. This increase in the Raman signal could be due to the application of higher laser power at 1064 nm, which was 70 mW compared to 10 mW at 532 nm, as well as a reduction in fluorescence at longer wavelengths. The limit of detection of toluene was determined using the peak area for 790 cm\(^{-1}\) from benzene ring vibrations, and the calibration curve was generated (Figure 3.7). The limit of detection of toluene was 3 mM. Although applying the laser at a wavelength of 1064 nm led to an increase in the Raman signal, no bands corresponding to toluene were observed in bacterial samples indicating a very low level of toluene, which is below the limit of detection of Raman spectroscopy.
Figure 3.6: Raman spectra of (A) reference solvents, and (B) serial dilution of toluene. Raman spectra were recorded using a hand-held 1064 nm Raman spectrometer.
As mentioned previously, the Raman effect is very weak and thus Raman spectroscopy suffers from limited sensitivity. Because of this the Raman signal needs to be enhanced and this is typically achieved using either surface-enhanced Raman scattering (SERS) or resonance Raman. In SERS, Raman signal enhancement occurs when molecules are absorbed onto a suitable roughened noble-metal surface (Jarvis and Goodacre, 2004). Since SERS enhancement is mainly attributed to electromagnetic and chemical mechanisms (Sharma et al., 2012), it is particularly suited to polar compounds. Therefore, it is not unreasonable to expect that the SERS spectra may yield valuable data to study the presence of toluene in bacterial cells as toluene is highly non-polar compound. The second important enhancement technique is UV resonance Raman (UVRR) spectroscopy. In this technique, the enhancement of the Raman signal is achieved when the energy of the incident laser is within the molecular absorption band of chromophores, or at 244 nm excitation, aromatics in the molecules. Thus, such a method could be desirable to detect toluene as it is an aromatic compound. Unfortunately, the analysis of toluene using UVRR spectroscopy with an excitation wavelength of 244 nm was not preformed due to instrumental problems resulting in poor signal intensity during the calibration process, which affected the reproducibility of the results. A solution to this problem
is to employ an ideal alternative technique in order to identify and quantify the level of toluene in *P. putida* strains. High performance liquid chromatography (HPLC) is among a number of analytical techniques that are sensitive enough for the determination of trace components in a wide range of samples (Schmidt *et al.*, 2004) and, because of that, we used HPLC for the identification and quantification of toluene in bacterial cells.

### 3.4.2 HPLC analysis

Liquid chromatography (LC) linked to ultraviolet-visible (UV) detection or mass spectrometry (MS) is of great interest as a technique for the analysis of environmental or biological samples (Sun *et al.*, 2014; Hanff *et al.*, 2014). In order to achieve high selectivity and sensitivity in the quantitative analysis of toluene in *P. putida* strains, HPLC-UV was used. The chromatograms of reference toluene and bacterial extracts are shown in Figure 3.8. It is clear that toluene eluted with a retention time of 1.45 min for both reference toluene and exposed cells to toluene indicating much higher selectivity and sensitivity of HPLC in the quantitative analysis of solvent, compared to Raman spectroscopy.
Figure 3.8: Chromatograms for (A) 1 mM toluene, (B) *P. putida* DOT-T1E (no toluene), exposed cells to 20 mM toluene (C) DOT-T1E, (D) DOT-T1E-PS28 and (E) DOT-T1E-18 obtained from HPLC-UV. Toluene is eluted with a retention time of 1.458 ± 0.003 min.
Once it was ascertained that the toluene was observed in *P. putida* cells, a dilution series was carried out to determine the limit of detection and the level of toluene in *P. putida* cultures. The limit of detection of toluene was found to be 15 µM (Figure 3.9). In order to determine the concentration of toluene in bacterial cells, the equation of the calibration line obtained from a serial dilution study was used. The calculations of the amount of toluene present in *P. putida* DOT-T1E cells is presented in Table 3.2. It is obvious that the level of toluene present in bacterial cells is considerably lower than the challenged concentrations suggesting the contribution of efflux pumps and catabolism of solvents in this process.

![Calibration curve for toluene ranging from 0.001 to 1 mM for 3 replicates using HPLC-UV.](image)

**Figure 3.9:** Calibration curve obtained from toluene ranging from 0.001 to 1 mM for 3 replicates using HPLC-UV. Points are means of the 3 replicates and error bars are standard deviations.

**Table 3.2:** Concentrations of toluene in *P. putida* DOT-T1E strains

<table>
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<tr>
<th><em>P. putida</em> strains</th>
<th>Toluene concentration (µM)</th>
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<tr>
<td>DOT-T1E</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>DOT-T1E-PS28</td>
<td>71 ± 11</td>
</tr>
<tr>
<td>DOT-T1E-18</td>
<td>277 ± 18</td>
</tr>
</tbody>
</table>
It should be noted that the level of toluene in the mutant DOT-T1E-PS28 and DOT-T1E-18 were 2 and 7 times higher compared to the wild-type DOT-T1E (Figure 3.10). This result would clearly illustrate the crucial role of efflux pumps in the removal of solvents from bacterial cells to the outer environment as stress responses. A series of early competition assays suggested that efflux pumps are considered the most efficient mechanism of solvent tolerance in Gram-negative bacteria (Isken and DeBont, 1996; Udaondo et al., 2013; Mosqueda and Ramos, 2000). It is also worth noting that *P. putida* DOT-T1E is able to degrade toluene into Krebs cycle intermediates via the toluene dioxygenase (TOD) pathway although there is no correlation between the catabolism of solvent and solvent tolerance (Lau et al., 1997; Mosqueda et al., 1999). Therefore, efflux pumps and the ability of a strain to degrade an aromatic compound would be strong evidence to explain the trace amount of toluene present in *P. putida* DOT-T1E strains.

![Figure 3.10: Box-whisker plot representing the toluene level in *P. putida* strains exposed to 20 mM toluene for 4 replicates. The red lines indicate the median of the peak area. DOT-T1E is the wild type, DOT-T1E-PS28 is the mutant (lacking the TtgGHI pump) and DOT-T1E-18 is the mutant (lacking the TtgABC pump). Error bars are standard deviations of 4 replicates.](image-url)
3.5 Conclusion

This study has shown that the accumulations of organic solvent (e.g. toluene) inside bacterial cells can be measured, and that there may be a connection between the level of accumulation of solvent in bacterial cells and the adaptation responses and/or the catabolism of solvent. Raman spectroscopy is a convenient method for investigating intracellular uptake due to detailed fingerprinting that can be derived from Raman spectra, as well as the ability to detect the shift in the spectra resulted from heavy isotopes. However, the level of toluene in *P. putida* DOT-T1E strains could not be detected using Raman spectroscopy due to poor sensitivity. By contrast, an HPLC assay has been successfully applied to the detection of toluene present within bacterial cells. The assay shows high sensitivity and led to the detection of solvent in bacterial cells. Moreover, *P. putida* cells had far lower toluene concentrations which, might result from the ability of the strains to remove solvent from the cells via efflux pumps and their ability to degrade solvent and use it as source of carbon.
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Chapter 4: Metabolic analysis of the response of Pseudomonas putida DOT-T1E strains to toluene using Fourier transform infrared spectroscopy and gas chromatography mass spectrometry

The work presented in this chapter has been published:

Contributing authors and their roles:
Ali Sayqal is the main author. Yun Xu contributed to GC-MS data analysis and Drupad Trivedi participated in data processing. Najla AlMasoud participated in the quenching process and David Ellis provided assistance in reporting the study. Howbeer Muhamadali helped with preliminary lab work and Nicholas Rattray provided assistance in preliminary analysis. Carole Webb participated in operating the HPLC and Royston Goodacre contributed to this work through supervision and guidance of the study.
Chapter Four

4.1 Abstract

To elucidate any observable metabolic alterations during interactions of several strains of *Pseudomonas putida* (DOT-T1E, and its mutants DOT-T1E-PS28 and DOT-T1E-18) with the aromatic hydrocarbon toluene, metabolomic approaches were employed. Initially, Fourier-transform infrared (FT-IR) spectroscopy, which provided a rapid, high-throughput metabolic fingerprint of *P. putida* strains, was used to investigate any phenotypic changes resulting from exposure to toluene. Principal component discriminant function analysis (PC-DFA) allowed the differentiation between different conditions of toluene on bacterial cells, which indicated phenotypic changes associated with the presence of the solvent within the cell. Examination of PC-DFA loading plots suggested that protein and fatty acids groups were responsible for discrimination of responses by *P. putida* strains to toluene. To identify metabolites of interest, the polar extracts of *P. putida* cells were analysed using gas chromatography-mass spectrometry (GC-MS) and 15 metabolites of *P. putida* central metabolic pathways were detected. Multi-block principal component analysis (MB-PCA) indicated that *P. putida* cultures challenged with toluene were differentially clustered away from the non-challenged cells. Investigation of MB-PCA loading plots and *N*-way ANOVA for condition | strain×time blocking (dosage of toluene) suggested ornithine as the most significant compound that increased upon solvent exposure. Ornithine presents itself as a major feature which may have important functions in toluene stress tolerance mechanisms.
4.2 Introduction

Bacteria can adapt to overcome the activity of toxic substances via the application of several resistant mechanisms. An exceptionally interesting stress response of *Pseudomonas putida* strains to toxic substances is the induction of efflux pumps, which, as their name suggests, remove toxic substances from the bacterial cell out to the external environment (Poole, 2007; Fernandes *et al*., 2003; Ramos *et al*., 1998). This mechanism is probably the most important process that plays an absolutely crucial role in bacterial adaptation mechanisms. The development of solvent-tolerant microorganisms that are able to grow in the presence of toxic organic solvents are useful in many applications, for example in environmental bioremediation (Nicolaou *et al*., 2010) and biocatalysis where organic solvents are often used to dissolve the substrate and product (Ellis and Goodacre, 2012).

Bioremediation is an incredibly important form of waste management that involves the conversion of harmful substances into non-harmful end products via the use of microorganisms (Bustard *et al*., 2000; Bustard *et al*., 2002; Gupta *et al*., 2006; Pandey *et al*., 2009; Zhao and Poh, 2008). Solvent tolerance is an adaptive process, as it is possible to make the bacteria tolerant to harsh environments through a number of reported methods. One approach to adapt the characteristics of microbial cells to unfavourable culture conditions has included the pre-exposure of bacterial cultures to low concentrations of toxic solvent (Ramos *et al*., 1998; Xin *et al*., 2009). Alternative methods such as genetic engineering can also be used to produce altered strains with superior tolerance characteristics, and this can be achieved through transformation of the microorganism to include a plasmid that confers degradation properties (encodes key enzymes) to specific toxic solvents (Horikoshi *et al*., 2011). This would allow for increased decontamination rates, so an understanding of the mechanisms of solvent toxicity is of great importance in order to explore microorganisms that exhibit sufficient tolerance, thereby enabling them to serve as bioremediation agents for specific chemical contaminants.

Whole-cell biocatalysis in two-phase systems containing an organic phase is an application for the production of specialty or fine chemicals (Heipieper *et al*., 2007; Neumann *et al*., 2006; Sardessai and Bhosle, 2004). In many instances, the initial material and/or the end-product can display some toxicity to the biocatalyst, which of
course leads to limited production yields or may affect the overall performance of
(which could be biotransformation specificity) biocatalysis. Thus, the ability to
exploit these microorganisms to their full potential requires a deeper understanding of
the interactions between the bacteria and organic solvents, which is an important
research goal. Changes identified in the microbial metabolome can be considered to
be hypothesis generating and as such can inform our biochemical knowledge
(Goodacre et al., 2004). Observed metabolite changes can prove to be indicative of
novel adaptation mechanisms, or may support postulated adaptation mechanisms for
which there is little evidence to date.

In this study, the effect of the sudden addition of toluene to *P. putida* DOT-T1E, and
two mutants of this strain – *P. putida* DOT-T1E-PS28 (lacking the TtgGHI pump)
and *P. putida* DOT-T1E-18 (lacking the TtgABC pump) – grown in LB medium, in
the presence/absence of toluene via gas phase has been investigated. Metabolomics
strategies were applied, specifically metabolic fingerprinting (Ellis et al., 2007)
employing FT-IR spectroscopy (Ellis and Goodacre, 2006) in order to identify
general phenotypic alterations in bacterial cultures exposed to toluene, and metabolic
profiling using GC-MS to investigate any metabolome changes in response to solvent
stress. The data sets generated via these approaches were explored further using
multivariate analysis methods in order to model the metabolic effect of organic
solvents on microbial species.
4.3 Material and methods

4.3.1 Bacterial strains and culture medium

Three strains of *P. putida* were chosen for this study to investigate the response of bacteria to toluene stress and these are listed in Table 4.1, and were sourced from the Juan Luis Ramos lab (Consejo Superior de Investigaciones Cientificas, Estacion Experimental del Zaidin, Department of Biochemistry and Molecular and Cellular Biology of Plants, Granada, Spain, http://www.eez.csic.es/?q=en/node/51). Nutrient agar (NA) and lysogeny broth (LB) were used for cultivation of bacteria. NA was prepared as follows: peptone 5 g/L, beef extract 3 g/L, sodium chloride 8 g/L, 12 g/L of agar no. 2. After autoclaving the mixture was allowed to cool prior to pouring into Petri dishes. LB medium contained: tryptone 10 g/L (Formedia, Hunstanton, UK), yeast extract 5 g/L (USP, Cleveland, USA) and NaCl 10 g/L.

<table>
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<tr>
<th>Table 4.1: <em>P. putida</em> strains used in this study</th>
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<tr>
<td><strong>P. putida strains</strong></td>
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<tr>
<td>DOT-T1E</td>
</tr>
<tr>
<td>DOT-T1E-PS28</td>
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<tr>
<td>DOT-T1E-18</td>
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<sup>a</sup> Resistance to Ap<sup>r</sup>: ampicillin, Rif<sup>r</sup>: rifampin, Sm<sup>r</sup>: streptomycin, Km<sup>r</sup>: kanamycin and Tol<sup>r</sup>: toluene

4.3.2 Cultivation of bacteria and culture conditions

All three strains of *P. putida* DOT-T1E were sub-cultured in triplicate on agar plates in order to obtain pure single colonies. Cells were grown in LB and the axenic cultures were incubated overnight with horizontal shaking in an orbital incubator (Infors HT Ltd, UK) at 30°C and 200 rpm.
4.3.3 Growth in response to toluene, sample collection and analysis

*P. putida* cells were normalised to an optical density (OD) of 0.1 and then incubated in an orbital shaker for 1 h at 30°C and 200 rpm. At this point, cultures were divided into two groups: one was kept as a control and for the other toluene was supplied via the gas phase for 30 min. Here an evaporation tube containing 100 µL of toluene was used in order to avoid direct contact with the culture. The culture flasks containing 50 mL of LB medium were sealed with Suba-Seals to prevent toluene leakage and then incubated for an additional 4 h. The concentration of toluene in the flask was approximately 12.5 mM (0.125% (v/v)) under these culture conditions. Once cell cultures reached the mid-log phase, the cultures were split into two halves; to one 0.1% (v/v) toluene was added, while the other was kept as a control. Cell cultures were then incubated for an additional 7 h. The tested concentration of toluene is below the minimum inhibitory concentrations (MICs) which are 5, 0.8 and 0.7% (v/v) for DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18 respectively (Table S4.1).

4.3.3.1 Bacterial growth profiles

During the time-course of 12 h incubation, (100 µL) samples were collected at various time points (0, 1, 3, 5, 7, 9, 11 and 12 h) in triplicate from each flask and from each of the exposure conditions (i.e., positive and negative groups) for OD measurement at 660 nm (OD

4.3.3.2 Analysis of biomass samples by FT-IR spectroscopy

Aliquot (2 mL) samples were collected and centrifuged at 11500 ×g for 5 min at 4°C (ThermoFisher CR3.22, UK). The supernatant was removed, while the cell pellets were washed twice with 2 mL of sterile physiological saline solution (0.9% NaCl) (Fisher, UK) and centrifuged again. The supernatant was discarded prior to storage of the cell pellet at -80°C for further analysis (Muhamadali et al., 2015a). The OD

135
randomised and spotted as 20 µL aliquots in triplicate onto a 96-well silicon FT-IR plate. The silicon plates were then dried in a desiccator at 25°C for 7 h.

The silicon plate was loaded onto a motorised microplate module HTS-XT™ under the control of a computer programmed with OPUS software version 4. Triplicate spectra were obtained from each sample, resulting in a total of nine spectra per biological sample, therefore a total of 324 spectra were collected. Spectra were acquired by employing a Bruker Equinox 55 FT-IR spectrometer (Bruker Optics, Banner Lane, Coventry, UK) as described by Winder and co-workers (Winder et al., 2006). Transmission measurements of the samples were acquired and converted to absorbance spectra, using a deuterated triglycine sulfate (DTGS) detector over the wavenumber range 4000-600 cm⁻¹, with a resolution of 4 cm⁻¹, 64 scans were combined and averaged to improve the signal-to-noise ratio.

The IR data were converted to ASCII format using OPUS reader software and imported into Matlab version 2012 (MathWorks, Natick, MA). Prior to analysis, atmospheric CO₂ vibrations in the 2,400-2,275 cm⁻¹ region were removed and the spectra were scaled using extended multiplicative signal correction (EMSC) (Martens et al., 2003).

Principal component analysis (PCA) was used to generate sets of latent variables (PCs) that retain the most important variance in the data whilst reducing the dimensionality (Wold et al., 1987). In addition, discriminant function analysis (DFA) was then employed, which is a supervised method that discriminates groups by a priori knowledge of sample origin. DFA attempts to maximise the differences between the known groups (classes) whilst minimising the differences within the class (Macfie et al., 1978; Johnson et al., 2003; Gromski et al., 2015). PC-DFA was conducted utilising PCs 1-10, and the class structure for the DFA algorithm was based on the biological replicates of samples from the same conditions.
4.3.3.3 Metabolite profiling

4.3.3.3.1 Sample collection and metabolic quenching

Samples were collected as 15 mL aliquots at several time points (0, 10 and 60 min) in the absence and presence of different toluene conditions (0 min refers to the point immediately before the addition of toluene shock). The metabolic activity of the collected samples were immediately quenched by adding 30 mL of cold (-50°C) 60:40 (v/v) methanol:water followed by centrifugation at 3000 ×g for 10 min at 1°C. After the centrifugation the supernatant was discarded, while the cell pellets were stored at -80°C prior to metabolite extraction (Winder et al., 2008).

4.3.3.3.2 Metabolite extraction

An aliquot (750 µL) of cold (-20°C) 80:20 (v/v) methanol:water was added to the biomass and then transferred into a 2 mL Eppendorf tube, followed by three freeze-thaw cycles to extract the intracellular polar metabolites into the polar phase. The samples were then pelleted by centrifugation (13500 ×g, 3 min, 4°C) and the supernatant stored on dry ice. This procedure was undertaken twice on the cell pellets and both extracts were combined and kept on dry ice.

Aliquots (1400 µL) of intracellular extracts were normalised according to OD$_{660}$, followed by the preparation of a quality control (QC) sample (Fiehn et al., 2008; Dunn et al., 2011). The QC sample was prepared by transferring an equal volume of sample (100 µL) into a 15 mL centrifuge tube. Internal standard solution (0.2 mg mL$^{-1}$ succinic-$d_4$ acid, 0.2 mg mL$^{-1}$ benzoic-$d_5$ acid, 0.2 mg mL$^{-1}$ lysine-$d_4$ and 0.2 mg mL$^{-1}$ glycine-$d_5$) was added (100 µL) to all samples. The samples were then dried for 16 h in speed vacuum concentrator (concentrator 5301; Eppendorf, Cambridge, UK), and stored at -80°C prior to GC-MS analysis.
4.3.3.3 GC-TOF-MS analysis

Metabolite samples were removed from -80°C storage and re-dried for 3 h in a concentrator prior to derivatisation, in order to remove any moisture absorbed by the sample during thawing, which could interfere with derivatisation process. Samples were derivatised for GC-MS following a two stage process as described previously (Wedge et al., 2011). Briefly, an aliquot (50 µL) of O-methylhydroxylamine hydrochloride solution (20 mg mL⁻¹ in pyridine) was added to all samples. The samples were then heated using a heating block at 65°C for 40 min followed by addition of 50 µL of MSTFA (N-methyl-trimethylsilyl trifluoroacetamide) and then heated for 40 min at 65°C. An aliquot (20 µL) of retention index solution (C₁₀/C₁₅/C₁₉/C₂₂ n-alkanes) was added for chromatographic alignment.

Gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) was used to analyse the derivatised samples in a random order. The instrument was operated using an Agilent 6890 GC coupled to a LECO Pegasus III TOF mass spectrometer (Leco, St. Joseph, MI, USA), as described previously (Begley et al., 2009; Dunn et al., 2011) which follows metabolomic standards initiative (MSI) guidelines (Sumner et al., 2007). QC samples were employed prior to statistical analysis as described from a previous report (Wedge et al., 2011), in order to provide quality assurance of the data by the evaluation and removal of mass features that exhibit high deviation within the QC samples.

4.3.3.4 Data analysis

The data were analysed using multi-block PCA (Smilde et al., 2003) with three different types of blockings. Strain | time×condition blocking was the first type of blocking and this partitioned the data into 9 blocks. Each block contained the samples taken under the same toluene condition and at the same time points while the strains were matched across blocks. The second type of blocking was time | strain×condition blocking. This blocking partitioned the data into 12 blocks. Each block had the samples of the same strain and same condition of toluene while the time points were matched across blocks. The last type of blocking was condition | strain×time blocking which partitioned the data into 6 blocks (this type of blocking did not include the samples at 0 min since this time point refers to the point immediately before the addition of toluene shock). Each block contained all the samples from the same strain
with the same time point, while the conditions of toluene were matched across blocks. Such blocking allows for the detection of the effect of each of the factors of interest (the factor which matched across different blocks, e.g. strain | time×condition blocking was used to detect the differences between different strains) separately without the inference from others by MB-PCA (Xu and Goodacre, 2012). A total number of 116 unique GC-MS peaks were detected. The natural logarithm (ln) was used on the peak area of these peaks. Data were then mean-centred, auto-scaled then subjected to MB-PCA. The most significant variables were recognised by choosing the most predominant averaged block loadings and N-way Analysis of Variance (N-way ANOVA). These results were visualised and compared using box-whisker plots. All FT-IR and GC-MS data are freely available at MetaboLights (http://www.ebi.ac.uk/metabolights/); study identifier MTBLS319.

4.4 Results and discussion

4.4.1 The effect of toluene on the Growth of P. putida strains

Growth of P. putida cells was examined in liquid culture medium, once cells were pre-grown on LB medium with and without toluene via the gas phase. After this P. putida cultures were challenged with sudden shock of 0.1% (v/v) toluene which is below the minimum inhibitory concentration (MIC) (see Table S4.1). Growth curves from P. putida cells can be seen in (Figure 4.1A-C). Generally, it can be clearly noted that there is a demonstrable effect caused by the sudden addition of toluene in the flask cultures. Both non-induced and induced cells were sensitive to the sudden shock of toluene, and the final biomass of the samples decreased (as indicated by a decreased final OD reading and the final turbidity measurement being lower than negative control cells) over the 12 h incubation time-course. Our results would suggest that this decrease in the biomass could be due to energy consumption as solvent tolerance is an energy intensive process, and not due to bacterial cell death as the concentration used was below MIC. One study showed the effect of sub-lethal toluene concentrations on the growth yields of a solvent-tolerant Pseudomonas strain (Isken et al., 1999). It was found that cultures exhibited lower yields once grown in the presence of toluene and the biomass was decreased linearly with increasing
toluene concentrations, suggesting that high levels of energy are extremely important for solvent tolerance in order to protect the cells from excessive damage.

Furthermore, the wild type *P. putida* DOT-T1E and the mutant *P. putida* DOT-T1E-PS28 were less sensitive to 0.1% (v/v) toluene, compared to the mutant *P. putida* DOT-T1E-18, when cells were pre-grown in the absence or presence of toluene supplied via the gas phase. In addition, to assess the accumulation of toluene and the role of efflux pumps in *P. putida* DOT-T1E cells, HPLC was used to measure toluene levels in bacterial cells. Figure 3.8 shows the chromatograms obtained for reference toluene and bacterial cultures. To quantify the level of toluene in *P. putida* cells, a calibration curve for toluene was generated (Figure 3.9). As observed in Figure 3.10 and Table 3.2, the level of toluene in the mutant DOT-T1E-PS28 and DOT-T1E-18 were 2-fold and 7-fold higher compared to the wild-type DOT-T1E. Therefore, these results would clearly suggest that the TtgABC pump plays a more important role in toluene efflux than the TtgGHI pump, and these observations are in agreement with previous studies which show that the TtgABC pump is the main extrusion pump for strain tolerance as it has the ability to extrude solvents and antibiotics (Teran et al., 2003; Duque et al., 2007; Roca et al., 2008). The next stage was to assess the bacterial biochemical changes during toluene stress.
Figure 4.1: Growth curves of: (A) the wild-type DOT-T1E, (B) the mutant DOT-T1E-PS28, and (C) the mutant DOT-T1E-18 in LB medium with and without toluene. Symbols represent different growth conditions. Control cultures - no toluene (closed diamonds), exposed cultures to 0.1% (v/v) toluene (closed squares), toluene gas (closed triangles), toluene gas and 0.1% (v/v) toluene (crosses). A 1/10 dilution of 100 µL samples was prepared in order to determine the turbidity at 660 nm. 5 h time point is the point immediately before any toluene addition.
4.4.2 FT-IR spectroscopy of collected biomass samples

FT-IR was employed to assess and compare the metabolic fingerprint of *P. putida* strains under the examined conditions. All FT-IR spectral data were subjected to the supervised method of PC-DFA and the resultant DFA scores plots are displayed in Figure 4.2A-C.

It is evident that cells induced to toluene (vapour) cluster together significantly and separately from the non-induced cells, and also a noticeable shift was observed in the exposed cells to 0.1% (v/v) toluene from the control cultures. This clustering pattern would suggest that toluene stress had an obvious effect on the cell cultures and may cause alterations to the phenotype of cells. In addition, it is clear that non-induced cultures collected from the 0.1% (v/v) toluene exposed cells cluster separately from the positive cultures in the mutant strains compared to the wild-type. These clustering patterns could suggest that the parent strain was less sensitive to 0.1% (v/v) toluene in comparison to the mutants, indicating the important role of the activity of efflux pumps in response to toluene. To ensure that the model quality is of a high standard, and that the obtained subsequent conclusions drawn from the data are valid, these PC-DFA models were validated by test set projection as can be seen in Figure S4.1.

As these results show significant differences and are valid (Figure. S4.1) the FT-IR spectra were investigated further using the loadings plots for PC-DFA and wavenumbers with significant loadings in the PC-DFA were identified. The loadings plots of *P. putida* strains for the first discriminant functions for the three strains are shown in (Figure 4.2D-F). According to these loading plots the largest variances are observed between wavenumbers 1750-1550 cm\(^{-1}\) which is attributed to changes in the protein components of the cells; most notably amide I (C=O stretching at 1690-1620 cm\(^{-1}\)) and amide II (combination of C-N stretching and N-H bending at 1550 cm\(^{-1}\)). However, we also see changes in the spectral regions of 2930-2850 cm\(^{-1}\) in which we would expect C-H stretching from fatty acids to occur. These results would indicate that metabolites within the amide and fatty acid regions contributed to differential responses to toluene challenge. Therefore, the most significant effects of toluene stress on bacterial cultures would be associated with changes in proteinaceous and lipid components of bacteria. Indeed, previous proteomic analysis has revealed that a number of proteins were up-regulated as a result of exposure of *P. putida* DOT-T1E
Chapter Four

or S12 strains to toluene stress (Wijte et al., 2011; van der Werf et al., 2008; Segura et al., 2005). In addition, changes in lipid compositions of DOT-T1E and S12 strains have also been shown to be involved in solvent tolerance in order to adapt membrane fluidity to the presence of toluene (Ramos et al., 1997; Bernal et al., 2007). Unsurprisingly, the interpretation of FT-IR spectra showed the most significant changes in the frequency of the proteins and lipid components. Our observations can deduce that some proteins were up-regulated and also lipid compositions were altered in response to toluene by *P. putida* DOT-T1E strains. As the FT-IR results showed that there was an effect of toluene on the phenotype of *P. putida* strains, GC-MS was employed as a metabolic profiling approach to specifically identify the significant metabolites.
Figure 4.2: PC-DFA scores plots of FT-IR data for three different strains of *P. putida* strains upon toluene stress. Symbols show different strains. (A) *P. putida* DOT-T1E (stars) and the first 10 PCs with a total explained variance (TEV) of 99.94% were used for the DFA, (B) *P. putida* DOT-T1E-PS28 (triangles) and PCs 1-10 with TEV of 99.93% were used for the DFA, (C) *P. putida* DOT-T1E-18 (circles) and first 10 PCs with TEV of 99.90% were used for the DFA. Colour codings represent different conditions: control cultures - no toluene (red), cultures exposed to 0.1% (v/v) toluene (black), toluene gas (brown), toluene gas and sudden 0.1% (v/v) toluene (blue). Arrows indicate the direction of shift because of the presence of toluene. (D) PC-DFA loadings plot for *P. putida* DOT-T1E, (E) DOT-T1E-PS28 and (F) DOT-T1E-18. Significant loadings were assigned to bacterial proteins and lipids.
4.4.3 Metabolic profiling with GC-MS

The aim of metabolite profiling is to measure all, or more realistically a subset of the metabolites present in the sample, and several analytical platforms can be employed for metabolite profiling (Ellis and Goodacre, 2012; Dunn, 2008; Fiehn, 2002). In recent years much attention has been focused on studying the stress responses in microorganisms employing metabolomics-based approaches (Brito-Echeverria et al., 2011; Kol et al., 2010; Allwood et al., 2015; Muhamadali et al., 2015b; Muhamadali et al., 2015a). The knowledge of variations within the metabolome following exposure to a stressor could lead to a more in-depth understanding of strain stress responses within these bacteria, therefore we employed GC-MS for metabolic profiling.

As we have multiple interacting factors (viz. Strain, condition, and time) we used multi-block PCA to allow these factors to be analysed independently, an approach we have used successfully before (Xu and Goodacre, 2012). Therefore to investigate the general metabolic effect of toluene on *P. putida* cells, MB-PCA with condition | strain×time and time | condition×strain were undertaken and the results are presented in Figures 4.3 and 4 respectively. As can be seen in Figure 4.3, a slight separation between the non-exposed and exposed cultures to toluene is observed, indicating that there are metabolic changes caused by toluene. An MB-PCA score plot was also conducted to investigate the metabolome alteration of *P. putida* cells during the time course of the exposure and as can be seen in Figure 4.4, the scores of the 0 min time point are located in the bottom right and as the time of incubation increases the cluster spreads from bottom to top. This clearly indicates that *P. putida* cells have different metabolic responses at different time points.
Figure 4.3: MB-PCA scores plot of GC-MS data showing the effect of different conditions of toluene on *P. putida* strains. Colours represent different conditions of toluene. Control with no toluene (blue), cells exposed to 0.1% (v/v) toluene (T; green), toluene gas (G; pink), and toluene gas and 0.1% (v/v) toluene (GT; red).

Figure 4.4: MB-PCA scores plot of GC-MS data showing the effect of different time points on *P. putida* strains. Colours represent different time points. 0 min is blue which refers to the point immediately before toluene addition (blue), 10 min is (in green) and 1 h (red) refer to the points after 10 min and 60 min of the addition of 0.1% (v/v) toluene.
Following MB-PCA, the next objective was to identify which metabolites were significantly changed between different conditions or time points. The loading plots were inspected for the most significant peaks (Table S4.2) and N-way ANOVA statistical test were conducted (Table S4.3), and the top significant features were selected whose $p$-value computed by N-way ANOVA was below 0.05 and also its corresponding false discovery rate (FDR) was below 0.05. A list of the identified metabolites can be seen in Table S4.4. Interestingly, both statistical methods for condition effect (exposure to solvent) suggested variable 54 as a highly significant feature which was identified as ornithine by our in-house library (Sumner et al., 2007; Brown et al., 2009). Figure 4.5 shows that the levels of ornithine in the non-exposed cultures to toluene are significantly lower than exposed cells. This might indicate a requirement for this metabolite for strain tolerance. It is noteworthy that in the induced cultures to toluene, 60 min following exposure to toluene, the level of ornithine was the highest among the other conditions and this could reveal that under this condition, the cultures exposed longer to low concentrations of toluene may allow for the cells to resist harsh conditions and sudden shock of stress. This could result in increasing the production level of ornithine in order to cope with toluene stress. In contrast, our previous study examined the effect of propranolol on *P. putida* DOT-T1E cells which showed that the ornithine was only produced following the exposure of *P. putida* strains to propranolol but was not found in the control, which also suggests that ornithine could be linked directly to the generalised strain tolerance to toxic assault (Sayqal et al., 2016). Our observations in this present study would infer that the production of ornithine in the control cultures is due to oxidative stress resulting from sealing the flask cultures with Suba-Seal rubber to prevent the toluene leakage from the flask in the exposed cultures.

Subsequently, we tested whether plugging the flask with Suba-Seals reduced the level of oxygen in the flask cultures. To ensure that the cells used for metabolic profiling analysis were exposed to oxidative stress, the flask cultures were plugged with both cotton wool and Suba-Seal rubber and then incubated at 30°C and 200 rpm for 24 h. The resultant growth curves are shown in Figure S4.2. The cultures that were plugged with Suba-Seal rubber exhibited slower growth compared to the control groups over the incubation time, which may indeed be a result of reduced oxygen levels in the cultures promoting slower growth.
Mahendran and colleagues demonstrated the effect of using various oxygen regimes on growth patterns of *Pseudomonas* spp. for the biodegradation of aromatic hydrocarbons, and their results showed that all strains have the ability to grow and degrade aromatic hydrocarbons under varying oxygen levels but in a differing manner (Mahendran *et al.*, 2006). In the DOT-T1E strain, the presence of solvents resulted in the up-regulation of several terminal oxidase genes, suggesting adaptation by *P. putida* DOT-T1E to solvents as well as to variable aerobic and microaerobic conditions, a situation that demands the consumption of energy in order to cope with the stress (Rojo, 2010). In the DOT-T1E (as well as the S12 strains they studied), proteomics analyses revealed that the up-regulation of several proteins of the TCA cycle involved in energy production upon exposure to solvents indicates a requirement for enhanced metabolism and high energy demand in order to power efflux pumps (Segura *et al.*, 2005; Udaondo *et al.*, 2012). As previously reported for *P. putida*, ornithine could be synthesised in several steps from glutamate through the TCA cycle where most of the energy production occurs (Antonia Molina-Henares *et al.*, 2010). Our observation would suggest that the ornithine production in the presence of toluene is interesting, as the *P. putida* strain might activate the metabolic pathways for ornithine to demand energy to power efflux pumps due to the high activity of efflux pumps. Alternatively this may be related to other metabolic pathways that are important in response to toluene in *P. putida*. 
Chapter Four

Ornithine
Variable id. = 54

Figure 4.5: Box-whisker plot showing the alterations in ornithine levels (variable id 54) in control and cells exposed to toluene for 4 biological replicates. The red lines represent the median m/z intensity. Box plot represents the data for 3 *P. putida* strains, 4 conditions of toluene and 3 time points, dashed lines separate different conditions of toluene and solid line separates different strains. Codes: control - no toluene (C), cells exposed to 0.1% (v/v) toluene (T), toluene gas (G), and toluene gas and 0.1% (v/v) toluene (GT).

In this work, we aimed to investigate similarities and differences in the levels of metabolites in the central metabolic pathways between the wild type and the mutants in *P. putida* DOT-T1E when cells had been pre-grown on LB medium in the absence or presence of toluene supplied via the gas phase, and these cells were then challenged with 0.1% (v/v) toluene. Rather than just concentrating on a single metabolite difference we also studied the level of metabolites for each bacterial strain independently. As we used GC-MS for untargeted metabolic profiling we were able to identify the changes in levels of metabolites during toluene stress in central carbon and nitrogen metabolism. Schematic summaries of central metabolic pathways in response to toluene in *P. putida* DOT-T1E, *P. putida* DOT-T1E-PS28, and *P. putida* DOT-T1E-18 are shown in Figures 4.6A-B, S4.3A-B and S4.4A-B, respectively. In
general, the mutants had similar patterns in the levels of metabolites compared to the wild type.

A direct observation from the metabolomic analysis is that the pool of amino acids (e.g. serine, glycine, alanine, valine, leucine, tryptophan, phenylalanine, tyrosine, lysine, methionine, isoleucine, threonine and ornithine) increased under the exposure of cells to toluene conditions at 10 min (shown in panels A of these figures) followed by an increase or decrease at 60 min (panels B). The levels of the most detected metabolites in the toluene adapted cells followed by toluene shock (GT) were higher than in the non-adapted cells. This observation would indicate that in the presence of toluene vapour, cells might induce gene activated metabolic pathways that are involved in toluene tolerant mechanisms prior to toluene shock. With the result of the production of higher levels of metabolites in comparison to non-induced cells, in order to cope with toluene stress and prevent cell death. A previous study found that exposure of *P. putida* DOT-T1E cultures to toluene supplied via the gas phase resulted in more rigidity of the cell membranes compared to non-exposed cultures (Ramos *et al.*, 1998), and this may also be observed in this study from the FT-IR analyses (Figure 4.2). In addition, under the same conditions it was revealed that the expression level of the *ttgGHI* operon in *P. putida* DOT-T1E was higher in the induced cells in comparison to non-induced cells (Rojas *et al.*, 2001). Therefore, our observation would suggest that an increased pool of amino acids would illustrate the participation of metabolites in response to toluene stress. However, 60 min following the exposure to toluene, the glutamine levels were slightly decreased in exposed cells compared to the control. It is possible that the level of glutamine was decreased as glutamate might be converted into ornithine instead of glutamine, as ornithine would be the key stress-responsive metabolite involved to cope with stresses following perturbation by toluene.
Figure 4.6: Schematic metabolic diagram of central carbon metabolism in *P. putida* DOT-T1E adapted to toluene. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. (A) Represents the level of metabolites at 10 min after toluene exposure, and (B) at 60 min. Box-whisker plots show the changes in metabolite levels in control and cells exposed to toluene for 4 biological replicates. The red lines indicate the median m/z intensity. Codes: control - no toluene (C), cells exposed to 0.1% (v/v) toluene (T), toluene gas (G), and toluene gas and 0.1% (v/v) toluene (GT).
Chapter Four

4.5 Conclusion

Our study shows that metabolic fingerprinting and profiling of *P. putida* cells by FT-IR and GC-MS analyses provides valuable information on the biological changes in these bacterial cultures upon toluene exposure. The growth profiles demonstrated the effect of toluene on bacterial cultures and the mutant *P. putida* DOT-T1E-18 was more sensitive to toluene compared to the other strains. This indicates that efflux pumps play a crucial role in strain tolerance, as also illustrated by the LC analyses of the toluene accumulation in the bacterial cells of three strains when exposed to toluene. The data collected by FT-IR shows that PC-DFA scores plots from metabolic fingerprints reveal excellent separation between non-exposed and exposed cultures to toluene and DF1 loadings vector show that several regions derived from proteins and fatty acids contribute to this separation. An FT-IR approach would be a valuable tool as it can be employed to analyse cellular response rapidly (Correa et al., 2012), thereby allowing more cost effective and high-throughput experiments to be conducted. We have also performed GC-MS analysis to monitor metabolome changes in the cultures and the results revealed that the levels of several amino acids in the central metabolic pathways of *P. putida* DOT-T1E strains were increased in response to toluene stress. The production of ornithine in the presence of toluene could be considered as a major key element and linked directly to solvent tolerance mechanisms. Finally, the combination of metabolic fingerprinting and profiling with suitable multivariate analysis is a valuable method for investigating solvent adaptation mechanisms in these industrially and environmentally significant microorganisms.
4.6 References


Chapter Four


Chapter Four


4.7 Supplementary Information: Experimental

4.7.1 Sampling and analysis of cell extracts by HPLC-UV

To investigate the role of efflux pumps which extrude toluene from *P. putida* cells, all bacterial cells were normalised to an optical density at 660 nm (OD$_{660}$) of 0.2 in 50 mL of LB medium and then incubated in an orbital shaker for 4 h at 30°C and 200 rpm. Once *P. putida* cultures reached the mid-log phase, samples were divided into two groups. One group was challenged with 0.2% (v/v) toluene and the second group was kept as an unexposed control. All flasks were sealed with Suba-Seals and incubated for an additional 30 min.

Cells (45 mL) were pelleted by centrifugation (3000 × g, 10 min, 1°C) and the supernatant was removed, while the cell pellets were washed once with 10 mL of 0.9% saline solution and centrifuged again to ensure the complete removal of LB medium. The pellets were suspended in 1.5 mL of 100% methanol and transferred into a fresh 2 mL Eppendorf tube. To permeabilise the cells, the freeze-thaw cycle liquid nitrogen method was performed three times according to the method of Winder *et al.* (Winder *et al.*, 2008). The samples were then pelleted by centrifugation (13500 × g, 5 min) and an aliquot (1200 µL) of supernatant (intracellular extracts) was normalised according to OD$_{660}$. Finally, an aliquot (300 µL) of intracellular extracts was placed in a LC vial and analysed by high-performance liquid chromatography (HPLC-UV).

All measurements were carried out using HPLC system (Agilent Technologies) equipped with an Agilent 1260 Infinity Quaternary Pump, auto-sampler and programmable UV Diode Array Detector. The output signal was monitored at 218 nm. The chromatographic separation was performed using a C18 column (100 x 4.6 mm) and the column temperature was maintained at 20 °C. HPLC separations were carried out by injecting 15 µL with an isocratic mobile phase methanol (100%) at a flow rate of 1 mL min$^{-1}$. The total analysis time was 30 min.
4.8 Supplementary Information: Results

Figure S4.1: Validated PC-DFA models of (A) \textit{P. putida} DOT-T1E, (B) DOT-T1E-PS28, (C) DOT-T1E-18 upon toluene stress. Symbol coding: control with no toluene (circles), cells exposed to 0.1\% (v/v) toluene (squares), toluene via gas phase (triangles), and toluene via gas phase and 0.1\% (v/v) toluene (upside down triangles). Closed symbols represent the training set while open symbols represent the test set that was projected into the PC-DFA scores space constructed from the training set.
Figure S4.2: Effect of oxidative stress on *P. putida* strains growth. Symbols and colours represent different strains. (Closed black diamonds) represents the wild-type DOT-T1E, (Closed red circle) the mutant DOT-T1E-PS28, and (Closed green triangles) the mutant DOT-T1E-18. (Solid lines) represent the growth curves of the control cells, while (dotted lines) cells exposed to oxidative stress.
Figure S4.3: Schematic metabolic diagram of central carbon metabolism in P. putida DOT-T1E-PS28 adapted to toluene. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. (A) Represents the level of metabolites at 10 min after toluene exposure, and (B) at 60 min. Box-whisker plots show the changes in metabolite levels in control and cells exposed to toluene for 4 biological replicates. The red lines indicate the median m/z intensity. Codes: control - no toluene (C), cells exposed to 0.1% (v/v) toluene (T), toluene gas (G), and toluene gas and 0.1% (v/v) toluene (GT).
Figure S4.4: Schematic metabolic diagram of central carbon metabolism in *P. putida* DOT-T1E-18 adapted to toluene. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. (A) Represents the level of metabolites at 10 min after toluene exposure, and (B) at 60 min. Box-whisker plots show the changes in metabolite levels in control and cells exposed to toluene for 4 biological replicates. The red lines indicate the median m/z intensity. Codes: control - no toluene (C), cells exposed to 0.1% (v/v) toluene (T), toluene gas (G), and toluene gas and 0.1% (v/v) toluene (GT).
Table S4.1: Results from the toluene MIC experiments using *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18. Culture growth was recorded after overnight incubation.

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* indicates growth, (±) slight growth, and (-) no growth
Table S4.2: List of the top 30 significant variables from MB-PCA loading

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Table S4.3: List of the top 30 significant variables from N-way ANOVA test

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Table S4.4: A list of detected metabolites by GC-MS. All identifications are based on minimum metabolite reporting standards (Sumner et al., 2007).

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<th>RT</th>
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<th>Metabolite</th>
<th>MSI ID level</th>
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Code: ID, identifier on plots; RT, retention time; RI, retention index; MSI, Metabolomics Standards Initiative
Chapter 5: Metabolic fingerprinting of *Pseudomonas putida* DOT-T1E strains: understanding the influence of divalent cations in adaptation mechanisms following exposure to toluene

The work presented in this chapter has been published:


Contributing authors and their roles:

This chapter apart from GC-MS analysis is a manuscript (metabolites-120907) of an article has been submitted for publication in *Metabolites*. Ali Sayqal is the main author. Yun Xu contributed to GC-MS data analysis and Drupad Trivedi participated in data processing. Najla AlMasoud participated in the quenching process and David Ellis provided assistance in reporting the study. Royston Goodacre contributed to this work through supervision and guidance of the study.
5.1 Abstract

*Pseudomonas putida* strains can adapt and overcome the activity of toxic organic solvents by the employment of several resistance mechanisms including efflux pumps and modification to lipopolysaccharides (LPS) in their membranes. Divalent cations such as magnesium and calcium play a crucial role in the development of solvent tolerance in bacterial cells. Here, we have used Fourier transform infrared (FT-IR) spectroscopy directly on cells (metabolic fingerprinting) to monitor bacterial response to the absence and presence of toluene, along with the influence of divalent cations present in the growth media. Multivariate analysis of the data using principal components discriminant function analysis (PC-DFA) showed trends in scores plots, illustrating phenotypic alterations related to the effect of Mg\(^{2+}\), Ca\(^{2+}\) and toluene on cultures. Inspection of PC-DFA loadings plots revealed that several IR spectral regions including lipids, proteins and polysaccharides contribute to the separation in PC-DFA space, thereby indicating a large phenotypic response to toluene and these cations. Finally, the saturated fatty acid ratio from the FT-IR spectra showed that upon toluene exposure, the saturated fatty acids ratio was reduced, while it increased in the presence of divalent cations. This study clearly demonstrates that the combination of metabolic fingerprinting with appropriate chemometric analysis can result in practicable knowledge on the responses of important environmental bacteria to external stress from pollutants such as highly toxic organic solvents, and indicates that these changes are manifest in the bacterial cell membrane. Finally, we demonstrate that divalent cations improve solvent tolerance in *P. putida* DOT-T1E strains.
Chapter Five

5.2 Introduction

Organic solvents such as benzene, toluene, styrene and xylenes are known to be highly toxic to microorganisms, as these aromatic solvents are known to partition and preferentially accumulate in the bacterial cell membrane, thereby disorganising its structure and impairing cell membrane integrity and function, ultimately leading to cell death (Inoue and Horikoshi, 1989; Sikkema et al., 1995; Isken and de Bont, 1998; De Smet et al., 1978). Nevertheless, it has been reported that some microorganisms have the ability to assimilate these toxic organic solvents even when the solvent concentration is very high. In 1989, the first report of an organic solvent resistant bacterium, resistant to high toxic levels of solvent was observed (Inoue and Horikoshi, 1989). Inoue and Horikoshi isolated a strain of *Pseudomonas putida* (strain HI-2000) which was able to grow in the presence of 50 % (v/v) toluene. This surprising finding has since been confirmed by others (Cruden et al., 1992; Kim et al., 1998; Li et al., 1998; Ramos et al., 1995; Weber et al., 1993), and the search has begun in earnest to discover the mechanisms behind this solvent tolerance.

Bacteria can defend themselves from the action of organic solvents by various adaptation mechanisms. Several studies have suggested that efflux pumps (Garikipati and Peeples, 2015; Rojas et al., 2001), divalent ions, such as magnesium ions (Inoue et al., 1991; Ramos et al., 1995), and the order organisation of cell surface lipopolysaccharides (Pinkart et al., 1996) contribute to solvent tolerance. In *P. putida* DOT-T1E, although high solvent tolerance is acquired mainly by the presence of efflux pumps (Rojas et al., 2001; Rodriguez-Herva et al., 2007), various other mechanisms contribute to organic solvent tolerance as well (Ramos et al., 2002).

Solvent-tolerant microorganisms play an important role in several biotechnological applications and areas such as bioremediation, agriculture and biocatalysis (Rojas et al., 2004; Nicolaou et al., 2010; Garcia et al., 2010; Pandey et al., 2009). Bioremediation involves the employment of microorganisms to convert toxic chemicals found in the environment into benign or less toxic species of chemicals (Yong et al., 2015; Bustard et al., 2002; Zhao and Poh, 2008). Whole-cell biocatalysis involves the production of specialty or fine chemicals, and often employs two-phase systems in order to extract and reduce the concentration of toxic products (or indeed substrates) from the aqueous phase (Heipieper et al., 2007; Neumann et al., 2005). This would decrease the deleterious effects of any toxic products and
hence the biocatalyst remains active, making product recovery easier and less costly (Bruce and Daugulis, 1991; Leon et al., 1998). Solvent tolerant microorganisms are a growing field of study in biotechnological applications, and more in-depth knowledge to aid in the understanding of the mechanisms of solvent tolerance is required. Researchers have suggested that genetic engineering, pre-exposure of bacterial cultures to low concentrations of toxic solvent, and magnesium ions contribute to the enhancement of solvent tolerance (Ramos et al., 1998; Horikoshi et al., 2011; De Smet et al., 1978; Ramos et al., 1995). One study investigated the effect of various metal ions such as Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, Pb\textsuperscript{2+} and W\textsuperscript{6+} on the stabilization of toluene tolerance of \textit{P. putida} IH-2000, and it was found that among the ions examined, Mg\textsuperscript{2+} and Ca\textsuperscript{2+} were the most effective in stabilisation of toluene tolerance, thereby suggesting that metal ions may enhance solvent tolerance in living cells (Inoue et al., 1991).

Metabolomics covers the identification and quantification of the metabolome (small molecules involved in cellular metabolic processes) employing different analytical techniques (Goodacre et al., 2004; Oliver et al., 1998; Dunn et al., 2005; Winder et al., 2011). One of the core high throughput approaches within the expanding field of metabolomics is metabolic fingerprinting (Ellis et al., 2007). With this approach, a rapid biochemical snapshot is obtained from cells, tissue, or biofluids that have been perturbed and any changes detected and correlated with fingerprints from ‘normal’ or typical control samples. Therefore, metabolic fingerprinting can be considered as a rapid, global, high-throughput approach to provide sample provenance (classification), which can also be utilised as a screening tool to differentiate and classify samples quickly from different biological status or origin (Ellis et al., 2007). Metabolic fingerprinting also normally entails minimal sample preparation and can be undertaken via one of a number of technologies, here, we used FT-IR spectroscopy.

FT-IR spectroscopy allows for a very rapid, high-throughput and non-destructive analysis of a broad range of sample types, and has been shown to be a valuable tool for the characterisation of cultured bacteria (Ellis and Goodacre, 2006; Goodacre et al., 1998; Lang and Sang, 1998; Naumann et al., 1991). Indeed, its application to the analysis of bacteria by Dieter Naumann and co-workers in the 1980s led to an
explosion of activity in this area, and has subsequently continued to be applied to many others areas of research (Naumann, 2006). The technique involves the observation of vibrations of molecules following the interrogation of a sample with an infrared beam, and the resultant infrared absorbance spectrum represents a so-called “fingerprint” which is characteristic of any (bio)chemical substance (Gillie et al., 2000; Ellis and Goodacre, 2006; Ellis et al., 2002). The aim of this study was to elucidate whether divalent cations interact with efflux pumps or other resistant mechanisms to improve solvent tolerance in P. putida DOT-T1E strains, and to highlight the role of the analytical techniques to measure these microbial responses. We employed FT-IR spectroscopy to generate rapid and robust biochemical fingerprints of P. putida DOT-T1E strains: wild type and mutants with impaired efflux pump activity. Gas chromatography mass spectrometry (GC-MS) was investigated to identify variation of the metabolome of the biological samples due to strain stress. These analytical techniques in combination with chemometrics can be used to observe metabolite changes that could be indicative of novel adaptation mechanisms, or support postulated adaptation mechanisms, and add to our knowledge in this important area of environmental microbiology.

5.3 Material and methods

5.3.1 Bacterial strains and growth conditions
The bacterial strains used in this study were P. putida DOT-T1E (Ramos et al., 1995), P. putida DOT-T1E-PS28 (lacking the TtgGHI pump) (Rojas et al., 2001) and P. putida DOT-T1E-18 (lacking the TtgABC pump) (Ramos et al., 1998). P. putida strains were routinely grown in nutrient agar plates to obtain fresh axenic cultures, which were then inoculated onto LB liquid medium and grown for 24 h at 30°C with shaking (200 rpm) in an orbital incubator (Infors HT Ltd, UK).
5.3.2 Growth in response to toluene in the absence and presence of divalent cations, sample collection and analysis

The overnight cultures were diluted to an optical density at 660 nm (OD$_{660}$) of 0.2 in 50 mL of fresh LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% $(v/v)$ toluene and grown overnight at 30°C with shaking (200 rpm). All flasks cultures were sealed with Suba-Seal to prevent toluene leakage.

5.3.2.1 Growth curve monitoring

Bacterial cultures were monitored at various time points (1, 5, 10, 22, 30, 48 h) using a Biomate 5 (CarePlanTM, UK) at 660 nm (100 µL samples were measured) and the growth recorded as an increase or decrease in the turbidity of cultures during incubation.

5.3.2.2 Analysis of bacterial cells by FT-IR spectroscopy

5.3.2.2.1 Sample preparation

After 8 h incubation, cells had reached the mid-exponential phase, and 2 mL aliquots of $P$. putida cultures were harvested by centrifugation at 11500 ×g for 5 min at 4°C (ThermoFisher CR3.22, UK). Culture supernatant was discarded, and cells washed twice with 2 mL of physiological saline solution (0.9% NaCl). Cell pellets were stored at -80°C until further required, and the procedure was conducted in triplicate.

Samples were defrosted on wet ice, suspended in saline solution and normalised according to OD at 660 nm. 20 µL aliquots of the suspension were pipetted in triplicate onto a 96-well silicon FT-IR sampling plate (Bruker Optics, Banner Lane, Coventry, UK). Moisture was evaporated from the samples by drying the prepared plates in a desiccator at ambient temperature for 7 h. This step was applied to avoid strong water absorption in the mid-IR region.
Chapter Five

5.3.2.2 Instrument set up

Prepared sample plates were then loaded onto a motorised microplate module (HTS-XT\textsuperscript{TM}) (Winder et al., 2006), attached to an Equinox 55 infrared spectrometer (Bruker Optics, Banner Lane, Coventry, UK), equipped with a deuterated triglycine sulfate (DTGS) detector for transmission measurements of the sample to be acquired. Spectra were collected over the wavenumber range 4000-600 cm\textsuperscript{-1}, with a resolution of 4 cm\textsuperscript{-1}, and 64 scans were combined and averaged to improve the signal-to-noise ratio. The resulting spectra were displayed as absorbance spectra.

5.3.2.2.3 Data analysis

For spectral pre-processing, infrared data were converted to ASCII format by OPUS reader software prior to statistical analysis, and imported into Matlab version 2012 (MathWorks, Natick, MA). To minimise problems arising from baseline shifts, the initial step was to remove atmospheric CO\textsubscript{2} vibrations in the area of 2,400-2,275 cm\textsuperscript{-1} and replace this with a trend, and the spectra were then normalised using extended multiplicative signal correction (EMSC) (Martens et al., 2003).

For cluster analysis, the unsupervised dimension reduction method of principal components analysis (PCA) (Joliffe, 1986) was conducted on the spectra in order to reduce the dimensionality of the multivariate data whilst preserving the variance, prior to the supervised clustering method discriminant function analysis (DFA). In PCA, the inputs are clustered without a priori knowledge (Goodacre et al., 2004). By contrast, DFA was performed to create a set of discriminant functions (DFs) on the basis of the retained principal components (PCs) which minimise within class differences whilst maximising the differences between the known groups (classes) (Johnson et al., 2003; Macfie et al., 1978). PC-DFA was performed using PCs 1-30 and the first 3 DFs were extracted. The class structure for the DFA algorithm was on the basis of the biological replicates from each sample of the same conditions.
5.3.2.3 GC-MS analysis

5.3.2.3.1 Metabolic quenching and extraction

To quench metabolism, culture samples (15 mL) were transferred rapidly into 50 mL tubes filled with 30 mL of mixture of methanol/water (60:40, v/v) (-50°C). Subsequently the quenched culture was centrifuged (3000 × g, 10 min, 1°C). The supernatant was discarded rapidly, while the pellets were stored at -80°C until required for further analysis (Winder et al., 2008).

In order to extract the intracellular metabolites, the biomass pellets were suspended in 750 µL of 80% methanol (-20°C) and then transferred into 2 mL tubes. The freeze-thaw cycle in liquid nitrogen was conducted three times to permeabilize the cells. The samples were then centrifuged for 3 min at 13500 × g and 4°C. The supernatants were transferred to fresh 2 mL tubes and stored on dry ice, while the cell pellets were resuspended in 750 µL of 80% methanol to perform the extraction again. Both extracts were combined together and kept on dry ice. Final aliquots (1400 µL) of intracellular extracts from each sample were normalised according to respective OD at 660 nm, followed by transferring an equal amount of each extract (100 µL) into a fresh 15 mL centrifuge tube in order to prepare the quality control (QC) sample (Dunn et al., 2011). Aliquots (1300 µL) of each sample extract were spiked with 100 µL of internal standard solution (0.2 mg mL⁻¹ succinic-d₄, 0.2 mg mL⁻¹ benzoic-d₅ acid, 0.2 mg mL⁻¹ lysine-d₄, and acid 0.2 mg mL⁻¹ glycine-d₅) and dried for 16 h employing speed vacuum concentrator (concentrator 5301; Eppendorf, Cambridge, UK), and stored at -80°C for further analysis.

5.3.2.3.2 Derivatisation

Prior to GC-MS analysis two-stage chemical derivatisations were performed. First, oximation was performed by adding 50 µL of O-methylhydroxylamine (20 mg mL⁻¹ in pyridine) to each sample followed by heating at 65°C for 40 min using a heating block. Second, the samples were trimethylsilylated by the addition of 50 µL of MSTFA (N-acetyl-N-(trimethylsilyl)-trifluoroacetamide) and heating at 65°C for 40 min. For chromatographic alignment, 20 µL of retention index solution (C₁₀/CH₁₂/C₁₅/C₁₀/C₂₂ n-alkanes) was then added.
5.3.2.3 GC-MS setup

Samples were analysed in a random order and a LECO Pegasus III time-of-flight mass spectrometer (TOF/MS) was employed in order to conduct gas chromatography electron ionisation time-of-flight mass spectrometry (GC-TOF/MS). The mode of operation is provided as described previously by (Begley et al., 2009; Dunn et al., 2011). Raw data were exported as netCDF files and then processed via deconvolution procedure for further analysis as described from the previous report (Begley et al., 2009). Prior to statistical analysis, QC samples were used as described previously (Wedge et al., 2011) to provide quality assurance of data.

5.3.2.3.4 Data analysis

Multivariate analysis was conducted using Matlab 2014a (Mathworks, Natick, MA). All peaks that had more than 20% missing values were eliminated from the analysis. All data were normalised and mean centred then subsequently subjected to PC-DFA (40 PCs were retained) and Two-way Analysis of Variance (ANOVA). Metabolites with significant p-values (<0.05) after false discovery rate correction for groups were identified as significant. Box-whisker plots were used to visualise the level of metabolites.

5.4 Results and discussion

5.4.1 Effect of toluene on the growth of P. putida DOT-T1E cells

The ability of P. putida DOT-T1E strains to grow on LB medium in the presence of different levels of toluene was examined, and the resultant growth curves of the three strains of DOT-T1E are displayed in Figure S5.1). It is evident that the growth of P. putida DOT-T1E strains were inhibited by the addition of different concentrations of toluene to the growth medium, and that the yield of bacterial cells decreased monotonically with increasing toluene concentrations. We note that in control medium the wild type DOT-T1E and DOT-T1E-PS28 had similar growth profiles, but that the DOT-T1E-18 mutant grew a little slower. Similar findings were obtained in previous reports which investigated the effects of solvents on bacterial biomass yield and it was deduced that the yields were reduced in the presence of toluene in the
culture (Abe et al., 1995; Aono et al., 1992; Ramos et al., 1997). Solvent tolerance is an energy intensive process, thus, a possible suggestion is that the decrease in the yield could be caused by energy-consuming adaptation mechanism such as efflux pump systems in *P. putida* (Isken and DeBont, 1996; Kieboom et al., 1998; Rojo, 2010) being used to protect the cells from further damage. It was reported that the biomass yield of *P. putida* S12 and DOT-T1E were reduced when grown in the presence of solvents, suggesting that solvent tolerance demand high levels of energy to cope with the solvent stress (Ramos et al., 1995; Isken et al., 1999).

In Gram-negative bacteria, efflux pumps are considered to be the most important adaptation mechanism for solvent tolerance (Ramos et al., 2002). Several studies have demonstrated that an energy-dependent efflux system is responsible for the resistance to toluene in *P. putida* DOT-T1, DOT-T1E and S12 (Rojas et al., 2001; Ramos et al., 1997; Isken and DeBont, 1996). To evaluate the role of efflux pump systems in toluene tolerance, growth of the parent strain was directly compared with that of the mutant strains, both in the presence and absence of toluene. The growth of the wild-type was inhibited at all tested concentrations (0.1, 0.5, 1.0, 5.0% (v/v) toluene), while the mutants could not grow in the media containing equal to or greater than 1% (v/v) toluene. Obviously, the mutants were found to be more sensitive to toluene than DOT-T1E, suggesting that TtgABC and TtgGHI pumps play an important role in toluene extrusion.

### 5.4.2 The effect of divalent cations on the growth of *P. putida* DOT-T1E cultures in the absence and presence of toluene

Many Gram-negative bacteria are less sensitive to organic solvents upon the addition of cations (most notably Mg$^{2+}$, Ca$^{2+}$) (Stanlotter et al., 1979). Therefore, the influence of Mg$^{2+}$ and Ca$^{2+}$ on the stabilisation of the toluene tolerance of *P. putida* DOT-T1E cultures was investigated and the resultant growth curves are shown in (Figure 5.1). Growth was observed to have increased when Mg$^{2+}$ was added to the LB medium containing toluene, compared with the control with the absence of the metal ions. In the presence of the magnesium ions the lag period was found to be shorter and higher cell biomass yields were obtained. The mutants were unable to grow in the presence of toluene at 1% (v/v) without the metal ion. However, it was observed that the addition of Mg$^{2+}$ improved toluene solvent tolerance and that cultures grew after 20 h
incubation time. The effect of the addition of various concentrations of magnesium ions was also tested. Growth increased in the presence of metal ions and 3.5 mM Mg$^{2+}$ was as effective for solvent tolerance as 30 mM (Figure S5.2). One study showed the influence of various combined concentrations of Ca$^{2+}$ and Mg$^{2+}$ ions on the growth of *P. putida* IH-2000, where growth was improved by the addition of more than 0.5 mM Ca$^{2+}$ and in the presence of more than 2 mM Mg$^{2+}$ (Inoue *et al.*, 1991).

To test the role of metallic cations and anions on the stability of the toluene tolerance, growth was determined after incubation for 24 h at 30°C in LB containing MgSO$_4$, MgCl$_2$, Ca(NO$_3$)$_2$, and CaCl$_2$ at 7 mM for Mg$^{2+}$ and 3 mM for Ca$^{2+}$ (other concentrations of these two cations were tested and these found to be the most optimal (data not shown)) supplemented without or with toluene at 5% (v/v). The reason that 5% (v/v) was chosen rather than the lower toluene concentrations (0.5%, 1%) was that in preliminary experiments the most striking effect on bacterial growth/toluene tolerance was seen at 5% beyond 10 h of solvent exposure (data not shown). The *P. putida* DOT-T1E strain has a higher tolerance for toluene in the presence of metal ions and the lag phase period was also significantly shorter (Figure S5.3). These divalent cations certainly exert beneficial effects as determined by higher cell yield in the presence and absence of toluene. Addition of Mg$^{2+}$ was found to be slightly more effective than Ca$^{2+}$ in improving solvent tolerance in *P. putida* DOT-T1E cells at 22 h time point. With different anions, such as Cl$^-$, SO$_4^{2-}$ and NO$_3^-$, we found that similar growth patterns were obtained under the same culture conditions although the cultures were supplemented with different anions. These observations suggest that the cations were more effective than anions, or that anions may not play a crucial role for stability of solvent tolerance in *P. putida* strains. These results were in agreement with previous observations that Mg$^{2+}$ and Ca$^{2+}$ ions are important for bacterial solvent tolerance (Inoue *et al.*, 1991; Stanlotter *et al.*, 1979; Ramos *et al.*, 1995).
Figure 5.1: Influence of 7 mM MgSO$_4$ on growth of *P. putida* DOT-T1E strains in the presence of toluene. Growth curves of: (A) the wild-type DOT-T1E, (B) the mutant DOT-T1E-PS28, and (C) the mutant DOT-T1E-18. Symbols and colours represent different growth conditions. Control cultures with no toluene (blue closed diamonds), exposed cultures to 0.1% (v/v) toluene (red closed circles), 0.5% (v/v) toluene (yellow closed triangles), 1% (v/v) toluene (green closed square). Solid and dotted lines represent the absence and presence of metal ions in the culture respectively. A 1/10 dilution of 100 µL samples was prepared to determine the turbidity at 660 nm.
5.4.3 FT-IR fingerprinting of *P. putida* DOT-T1E cultures

In recent years, much attention in the literature has been paid to investigating stress responses in bacteria via the application of metabolomics-based methods (Brito-Echeverria *et al.*, 2011; Kol *et al.*, 2010; Allwood *et al.*, 2015), an area which has been applied within a broad range of disciplines including medical sciences, metabolic engineering and drug discovery (Anton *et al.*, 2013; Kim *et al.*, 2014; Dunn *et al.*, 2015; Ellis and Goodacre, 2012).

In this study, a metabolic fingerprinting approach (Ellis *et al.*, 2007) based on FT-IR spectroscopy (Ellis *et al.*, 2012) was employed to study the influence of metal ions on the whole organism phenotype of *P. putida* DOT-T1E strains in the presence and absence of toluene. To ensure that there was sufficient biomass for metabolomics analysis, cultures were grown in LB medium supplemented without or with 7 mM magnesium and 3 mM calcium in the absence/presence of 0.05% (v/v) toluene and incubated for 8 h (see Figure 5.2). The results showed that all strains have the ability to grow in the absence/presence of toluene; however, all *P. putida* strains had higher tolerance to toluene in the presence of divalent cations. By contrast, similar growth profiles were observed in the culture medium supplemented with or without divalent cations in the absence of toluene.

![Figure 5.2: Turbidity at OD 660 nm of *P. putida* strains after 8 h incubation in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. Colours represent different strains: the wild-type DOT-TIE (red), the mutant DOT-TIE-PS28 (yellow), and the mutant DOT-TIE-18 (green). Bars of the means of 4 replicates and error bars are standard deviations.](image-url)
To generate robust biochemical fingerprints of *P. putida* DOT-T1E strains FT-IR spectroscopy was employed. Since subtle and important variations in FT-IR spectra are not easy to interpret visually (Figure S5.4), chemometric methods were conducted in order to analyse these data in far more detail. Initially, a PCA scores plot was produced (data not shown) and no obvious clusters were observed in this analysis. PCA failed to discriminate data as in many previous studies (Johnson *et al.*, 2003; Goodacre, 2003). Therefore, it would seem sensible to employ a supervised clustering approach such as DFA in order to visualise the distribution of samples based on their IR metabolic fingerprint (Ellis and Goodacre, 2006). The first and second discriminant function (DF) scores were generated to identify variation or relationships between the samples, and the resultant PC-DFA scores plot of DF1 vs. DF2 is displayed in (Figure 5.3). As can be seen in Figure 5.3, a clear separation between the wild type DOT-T1E and the mutants DOT-T1E-PS28 and DOT-T1E-18 is observed in the first discriminant function which explains the majority of the total group variance (here the groups relate to the biological replicates and are not biased based on either the level of toluene or the addition of cations). This observation could be due to the lack of efflux pump in the mutants compared to the parent strain or an indirect effect on growth of mutant DOT-T1E-18, indicating the ability of FT-IR to discriminate between bacterial cells within the same strain. Figure 5.3 also clearly shows that a similar trend (through DF2) was observed between the wild type and the mutants under the same conditions, indicating clear metabolic changes caused by metal ions in the absence and presence of toluene. The parent and the mutant strains have the same genetic background and the only difference between the three cell types is the absence of one of the efflux pump protein in the mutants compared with the parent strain. Therefore, the results from DFA would suggest that the influence of Mg$^{2+}$ and Ca$^{2+}$ on the stabilisation of the toluene tolerance of *P. putida* DOT-T1E may be due to the contribution of metal ions in other bacterial tolerance mechanisms rather than only the efflux pump(s).

In addition, cells exposed to 0.05% (v/v) toluene in the absence of metal ions in the wild type *P. putida* DOT-T1E and the mutant *P. putida* DOT-T1E-PS28 (a mutant in the TtgGHI pump) are clustered more closely to the control cultures compared with the mutant *P. putida* DOT-T1E-18 (which lacks the TtgABC pump), indicating that DOT-T1E-18 cells were more sensitive to 0.05% (v/v) toluene compared to DOT-
T1E and DOT-T1E-PS28 cells. This clustering pattern would suggest that the TtgABC pump might play a more crucial role in toluene efflux than the TtgGHI pump. This observation was in agreement with previous investigations which conclude that the TtgABC pump is the main extrusion pump, and is able to extrude solvents and antibiotics (Teran et al., 2003; Duque et al., 2007; Roca et al., 2008). Therefore, the results from DFA clearly illustrate that the metabolic fingerprinting approach has the ability to detect a clear effect upon the cell cultures caused by metal ions and toluene which may cause changes to the phenotype of cells.

Figure 5.3: PC-DFA scores plots of FT-IR data of P. putida DOT-T1E strains in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. Symbols represent different strains: P. putida DOT-T1E wild type (stars), P. putida DOT-T1E-PS28 (closed circles), and P. putida DOT-T1E-18 (closed triangles) PCs 1-30 with a total explained variance (TEV) of 99.92% were used for the DFA. Colour coding: control with no toluene (red), cells without toluene in the presence of 7mM Mg$^{2+}$ (brown), 3mM Ca$^{2+}$ (black), mixed 7mM Mg$^{2+}$ and 3mM Ca$^{2+}$ (green), cells challenged with 0.05% (v/v) toluene in the presence of 7mM Mg$^{2+}$ (dark blue), 3mM Ca$^{2+}$ (light blue), mixed 7mM Mg$^{2+}$ and 3mM Ca$^{2+}$ (grey), and cells with 0.05% (v/v) toluene in the absence of divalent cations (pink).
To investigate which spectral regions discriminated between different conditions within strains, DFA loadings vectors were calculated and plotted for DF2 (Figure 5.4) which largely discriminated between different conditions (Figure 5.3). Several changes occur within these loadings plot with the greatest variances being observed between 2950-2850 cm\(^{-1}\), 1700-1600 cm\(^{-1}\) and 1110-945 cm\(^{-1}\) contributing to the DFA scores plot clustering. Vibrational assignments are provided in Table 5.1; in this region of mid-infrared the bands at 2918 cm\(^{-1}\) and 2853 cm\(^{-1}\) can be attributed to C-H stretching vibrations from membrane lipids and the peaks at 1630 cm\(^{-1}\) and 1550 cm\(^{-1}\) would be attributed to C=O stretching (amide I) and a combination of C-N stretching and N-H bending (amide II) vibrations, respectively, from protein components. In addition, the bands at 1105 cm\(^{-1}\) and 952 cm\(^{-1}\) could arise from a range of vibrations from the carbohydrate family including complex polysaccharides within the cells. These large variations in lipids, proteins and carbohydrates between different conditions within the *P. putida* DOT-T1E cells are due to the biological effects caused by the metal ions and toluene.

Table 5.1: Wavenumber regions of biological interest and assignment for *P. putida* DOT-T1E cells (Kim *et al.*, 2010)

<table>
<thead>
<tr>
<th>Wavenumbers (cm(^{-1}))</th>
<th>Assignment</th>
<th>FT-IR vibrational modes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2958-2873)</td>
<td>Membrane lipid</td>
<td>Asymmetric CH(_3) stretches mode of CH(_3) end groups from membrane lipid</td>
</tr>
<tr>
<td>(2924-2850)</td>
<td>Membrane lipid</td>
<td>Symmetric CH(_2) stretches mode of CH(_2) chain from membrane lipid</td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3400-3300)</td>
<td>Amide A</td>
<td>N-H stretching</td>
</tr>
<tr>
<td>(1690-1620)</td>
<td>Amide I</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>(1590-1530)</td>
<td>Amide II</td>
<td>C-N stretching and N-H bending</td>
</tr>
<tr>
<td>(1450-1200)</td>
<td></td>
<td>COOH of proteins, free amino acids, polysaccharides</td>
</tr>
<tr>
<td><strong>Carbohydrate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1200-900)</td>
<td>Polysaccharides</td>
<td>C-O or O-H stretching from polysaccharides</td>
</tr>
</tbody>
</table>
Chapter Five

Figure 5.4: PC-DFA loadings plot from DF 2 of *P. putida* DOT-T1E strains in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. Significant loadings were assigned to bacterial lipids (highlighted in the yellow box), proteins (blue box) and polysaccharides (red box).

The outer membrane of Gram-negative bacteria is an effective barrier for many toxic agents, and divalent cations (in particular, Mg$^{2+}$ and Ca$^{2+}$) are important in the organisation of the outer membrane (Nikaido and Vaara, 1985) as lipopolysaccharide (LPS) molecules are linked to each other electrostatically via divalent cations (Schneck *et al.*, 2010; Labischinski *et al.*, 1985). In several cases it has been observed that when the structure of the outer membrane of certain organisms (which are able to acquire resistance against toxic solvents (e.g. toluene)), are modified by chemical or enzymatic removal of parts of the LPS molecule or mutation, the resistance of these bacteria to these solvents is decreased (Sheu and Freese, 1973; Hancock, 1984; Vaara, 1993). On the other hand, Junker *et al.* (2001) observed that in WbpL mutant of *P. putida* DOT-T1E, LPS may not be important for aromatic hydrocarbon tolerance (Junker *et al.*, 2001). If Mg$^{2+}$ and Ca$^{2+}$ are essential for the integrity of the outer membrane and LPS layer, the presence of many aromatic hydrocarbons (e.g. toluene), ethylenediaminetetraacetic acid (EDTA) and antimicrobial peptides (AMPs), lead to significant changes in the structure and function of membrane components, such as disruption and removal of lipids and proteins as well as loss of
Mg$^{2+}$ and Ca$^{2+}$ (Sikkema et al., 1995; Zhang et al., 2000; Brogden, 2005; Nikaido, 2003).

In Gram-negative bacteria, Clifton et al. (Clifton et al., 2015) reported that the removal of calcium ions from the LPS bilayer led to the destabilisation of the bilayer and mixing of LPS molecules between the inner and outer leaflets; indicating the important role of salt bridges which are formed by divalent cations (e.g. Mg$^{2+}$ and Ca$^{2+}$) with negatively charged sugar in LPS core oligosaccharide to strengthen the integrity of the outer membrane. It has been found that calcium has the ability to block the binding of a cationic antimicrobial peptide to LPS and thus decrease its antimicrobial activity (Pink et al., 2003). The effect of AMPs, EDTA and Mg$^{2+}$ on the LPS layer was examined in Gram-negative bacteria (Clifton et al., 2015), showing that cationic AMPs or anionic EDTA effectively modify the LPS layer electrostatically by displacing Mg$^{2+}$ ions from the LPS layer competitively, while Mg$^{2+}$ tightens and stabilises the LPS layer (Lam et al., 2014).

Therefore, it is perhaps not surprising that a similar trend in the DFA scores plot between the wild type and the mutants were observed suggesting the contribution of Mg$^{2+}$ and Ca$^{2+}$ in LPS stabilisation but not efflux pumps. This observation would suggest that the efflux pumps system in *P. putida* might not require a magnesium or calcium gradient to export substrates such as toluene. In addition, the most significant changes observed from the interpretation of FT-IR spectra were in the vibration frequency of the polysaccharide, proteins and lipid components, and we can infer from this that the important role of divalent cations in *P. putida* DOT-T1E strain is related to the LPS mechanism to cope with the presence of toluene.

Finally, the ratio of saturated fatty acid composition was calculated from the raw (Figure 5.5) and scaled infrared spectra (Figure S5.5) to investigate the effect of divalent cations and toluene on *P. putida* DOT-T1E strains. It is clear that upon toluene exposure, the saturated fatty acid ratio (CH$_3$:CH$_2$) was lower compared to the control cultures in the absence of these divalent cations. This result is in agreement with previous observations showed that the fluidity of *P. putida* S12 outer membrane increased in the presence of toluene, as toluene may displace divalent cations from the LPS layer, causing increased membrane permeability (Wijte et al., 2011). By contrast, the saturated fatty acids ratio of *P. putida* cells was increased with the
addition of Mg$^{2+}$ and Ca$^{2+}$ to medium with and without toluene. However, under the same conditions there was a slight decrease in the saturated fatty acids ratio for *P. putida* DOT-T1E-PS28 in the presence of Mg$^{2+}$. In *Pseudomonas aeruginosa*, Schneck *et al.* (Schneck *et al.*, 2009) were able to show that the conformation of the O-antigen was a shorter and denser layer in the presence of Ca$^{2+}$ compared to the absence of calcium ions. Our results would suggest that divalent cations are essential for the integrity of the LPS layer and the outer membrane and therefore they may play an important role to improve solvent tolerance in *P. putida* cells.

![Box-whisker plot for FT-IR scaled spectra showing the ratio of saturated fatty acids (CH$_3$/CH$_2$) of *P. putida* DOT-T1E strains grown in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. Red lines indicate the median of peak area of saturated fatty acids ratio of infrared spectra. The median was used to compare the level of saturated fatty acids ratio. Red plus signs represent the outliers.](image-url)
5.4.4 GC-MS metabolic profiling of *P. putida* DOT-T1E cultures

Historically, GC-MS has proven to be one of the most valuable bio-analytical tools in metabolome analysis due to its high separation efficiency, which can resolve very complex mixtures, assisted by identification of compounds with mass detection using MS (Villas-Boas *et al.*, 2005). Therefore, we used GC-MS to create metabolic profiles of bacterial stress to toluene and investigate the effect of divalent cations on the stability of *P. putida* DOT-T1E strains at the metabolome levels.

As a first step towards the assessment of the general metabolic effect of bivalent cations on *P. putida* cells in the absence and presence of toluene, PC-DFA was applied and a scores plot for (DF1 vs. DF2) were generated (Figure 5.6). As can be seen in Figure 5.6A, a clear separation between the three *P. putida* DOT-T1E cells is observed resulting from the absence of efflux pump in the mutants compared to the parent strain. However, a weak separation and no obvious trends under different conditions within each strain are observed. As a result, it would be sensible to generate PC-DFA scores plot for its discriminant function DF1 and DF3 scores to visualise the distribution of samples from another angle see (Figure 5.6B). It can be noted that no clear trends under different conditions within each strain are observed. In addition, to investigate which metabolites were significantly altered between different conditions, two-way ANOVA statistical test was conducted (see Table S5.1). This showed that four metabolites were statistically significant because of the presence of metal ions and toluene in cell cultures. Unfortunately, it was found that three of these metabolites are currently unable to be identified, but the other metabolite is identified by in house database matching to leucine. This can be due to mis-matched fragmentation patterns between samples and library compounds or due to drift in retention times. These results from metabolic profiling analysis would suggest that due to the complexity of metabolic profiling results under these different metal ions conditions, it would be difficult to interpret these results and illustrate the influence of divalent cations on the stability of the toluene tolerance.
Figure 5.6: DFA scores plots of GC-MS data of *P. putida* DOT-T1E strains in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. (A) represents scores plot for (DF1 vs. DF2), while (B) for (DF1 vs. DF3). Colours represent different strains. *P. putida* DOT-T1E is the wild type (red), *P. putida* DOT-T1E-PS28 is the mutant (green), and *P. putida* DOT-T1E-18 is the mutant (blue). Symbols represent different conditions. Control with no toluene (triangles), cells without toluene in the presence of 7mM Mg$^{2+}$ (squares), 3mM Ca$^{2+}$ (circles), 7mM Mg$^{2+}$ and 3mM Ca$^{2+}$ (diamonds), cells with 0.05% (v/v) toluene in the presence of 7mM Mg$^{2+}$ (pluses), 3mM Ca$^{2+}$ (upside down triangles), 7mM Mg$^{2+}$ and 3mM Ca$^{2+}$ (stars), and cells with 0.05% (v/v) toluene in the absence of divalent cations (crosses).
5.5 Conclusion

In this study we have shown that different levels of toluene inhibit the growth and reduce the biomass yields of *P. putida* DOT-T1E strains, suggesting that solvent tolerance demands high levels of energy to cope with toluene stress. In addition, our results clearly show how divalent cations improve toluene tolerance in *P. putida* cells, indicating that Mg$^{2+}$ and Ca$^{2+}$ ions are important for bacterial solvent tolerance. We report that results of PC-DFA from metabolic fingerprinting show obvious separation between different culture conditions and the DFA loadings vectors reveal that several mid-infrared regions derived from lipids, proteins and polysaccharides contribute to this separation. Since results from PC-DFA obtained from the wild-type strain shows a very similar trend to that from the mutant cells, it is clearly demonstrated that the influence of divalent cations to improve toluene tolerance in *P. putida* cells may be correlated to other bacterial tolerance mechanisms including LPS, but they do not contribute to efflux pumps. Furthermore, divalent cations increase the saturated fatty acids ratio of *P. putida* cells, indicating that Mg$^{2+}$ and Ca$^{2+}$ would be essential for the integrity of the LPS layer and the outer membrane and therefore improve solvent tolerance in bacterial cells. Unfortunately this metabolic profiling approach using GC-MS failed to illustrate the effect of the divalent cations on the stability of the toluene tolerance at the metabolome level due to the complexity of metabolic profiling results.

In conclusion, we have demonstrated that metabolic fingerprinting with appropriate chemometric analysis is a valuable approach for studying the influence of divalent cations on the stabilisation of the toluene tolerance of *P. putida* DOT-T1E cultures, advancing our understanding of the role of metal ions in these environmentally and industrially important bacterial cells.
5.6 References


Chapter Five


5.7 Supplementary Information:

Figure S5.1: Growth of *P. putida* DOT-T1E strains on toluene at four different concentrations. Growth curves of: (A) the wild-type DOT-T1E, (B) the mutant DOT-T1E-PS28, and (C) the mutant DOT-T1E-18. Symbols and colours represent different concentrations of toluene. Control cultures - no toluene (blue closed squares), exposed cultures to 0.1% (v/v) toluene (green closed triangles), 0.5% (v/v) toluene (red closed circles), 1% (v/v) toluene (yellow closed diamonds) and 5% (v/v) toluene (purple stars). A 1/10 dilution of 100 µL samples was prepared to determine the turbidity at 660 nm.
Figure S5.2: Influence of three different concentrations of magnesium ions on growth of *P. putida* DOT-T1E strains in the presence of 0.1% (v/v) toluene. Growth curves of: (A) DOT-T1E, (B) DOT-T1E-PS28 and (C) DOT-T1E-18. Symbols and colours represent different concentrations of metal ion. Control cultures - no toluene and metal ion (blue closed diamonds), exposed cells to 0.1% (v/v) in the presence of 3.5 mM Mg\(^{2+}\) (red closed squares), 14 mM Mg\(^{2+}\) (green closed triangles), 30 mM Mg\(^{2+}\) (purple crosses). A 1/10 dilution of 100 µL samples was prepared to determine the turbidity at 660 nm.
Figure S5.3: The influence of cations and anions of metal ions on the growth of *P. putida* DOT-T1E strain in the absence and presence of 5% (v/v) toluene. Symbols and colours represent different growth conditions. Solid lines represent the absence of toluene in the culture, while dotted lines represent the presence of toluene in the culture. A 1/10 dilution of 100 µL samples was prepared to determine the turbidity at 660 nm.
Figure S5.4: FT-IR spectra collected for *P. putida* DOT-T1E cultures in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. (A) FT-IR raw spectra, while (B) scaled spectra using extended multiplicative signal correction (EMSC).
Figure S5.5: Box-whisker plot for FT-IR raw spectra showing the ratio of saturated fatty acids (CH$_3$/CH$_2$) of \textit{P. putida} DOT-T1E strains grown in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. The red lines indicate the median of peak area of saturated fatty acids ratio of infrared spectra. The median was used to compare the level of saturated fatty acids ratio. Red plus signs represent outliers.

<table>
<thead>
<tr>
<th>Metal ions effect</th>
<th>Metal ions</th>
<th>p-value</th>
<th>FDR</th>
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</tr>
<tr>
<td>38</td>
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<td>4.7E-05</td>
<td>1.1E-03</td>
</tr>
<tr>
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<td>3.7E-04</td>
<td>5.6E-03</td>
</tr>
<tr>
<td>76</td>
<td>Unknown</td>
<td>7.1E-04</td>
<td>8.0E-03</td>
</tr>
</tbody>
</table>
Chapter 6: Conclusion and future works

The ability of a number of microorganisms to thrive and adapt in the presence of a range of toxic chemicals such as pharmaceuticals and organic solvents is becoming an increasingly important field of research. This is due to the fact that microorganisms that can grow in toxic solvents are needed in several biotechnological applications. Therefore, the elucidation of the biological mechanisms of solvent tolerance is very important to enhance the resistant systems in non-pathogenic microorganisms and create altered strains with superior tolerance characteristics for bioprocessing. The mechanisms of adaptation and tolerance toward solvent toxicity have been extensively studied and reported in the literature (Ramos et al., 2015; Ramos et al., 2001; Ramos et al., 1998; Ramos et al., 1997; Sikkema et al., 1995; Wijte et al., 2011; Volkers et al., 2009; Nicolaou et al., 2010; Zgurskaya and Nikaido, 1999; Mosqueda and Ramos, 2000). In general, the toxicity of solvent and the intrinsic resistance of the microorganism are the main two factors that illustrate the process and overall impact of a solvent on a microorganism. Toxic solvents will partition and accumulate in the cytoplasmic membrane of bacteria and disrupt the cell membrane structure, impairing its function and ultimately leading to cell death.

By contrast, most microorganisms have evolved mechanisms in order to adapt and overcome the damage imparted by these chemicals. These mechanisms include induction of efflux pumps (Aono et al., 1995), induction of Krebs cycle enzymes related to energy production (Wijte et al., 2011), and changes in cell membranes composition (Junker and Ramos, 1999; Heipieper and Debont, 1994). The expression of these mechanisms of solvent tolerance can be reflected as phenotypic changes to the cell. Therefore, it is desirable to have an approach to monitor such changes in order to establish whether the phenotype of an organism has changed by exposing it to different levels of toxic solvents.

Metabolomics is the study of the whole metabolome in a certain organism under a defined set of conditions. The holistic qualitative and quantitative measurements of the metabolome of an organism can be used to determine the function of genes (Fiehn, 2001; Fiehn, 2002). In recent years, attention has been paid to understanding the stress responses in bacteria by employing metabolomics-based approaches (Kol et
al., 2010; Brito-Echeverria et al., 2011; Allwood et al., 2015), which has involved a broad range of disciplines such as drug discovery, metabolic engineering and medical sciences (Mashego et al., 2007; Anton et al., 2013; Kim et al., 2014; Dunn et al., 2015; Ellis and Goodacre, 2012). Since it is not currently possible to determine the entire metabolome in an organism by using an individual extraction method or analytical instrument, close attention is needed at all stages of workflow in terms of sample numbers, sample preparation, appropriate choice of analytical instruments and strategies for data processing and analysis.

In this thesis metabolomics strategies were employed to investigate the adaptation response in *P. putida* DOT-T1E which is mainly achieved by a number of energy dependent active efflux pumps belonging to the RND family (Daniels and Ramos, 2009). In this strain, three active efflux pumps, named TtgABC, TtgDEF and TtgGHI, has been identified. The TtgABC and TtgGHI efflux systems are involved in the resistance to a wide range of organic solvents and pharmaceuticals (Ramos et al., 1998; Rojas et al., 2001), while the TtgDEF extrusion system appears to be involved in excreting organic solvents (Mosqueda and Ramos, 2000). Metabolomics can provide insight into the adaptation mechanisms of microorganisms, which was applied to *P. putida* DOT-T1E, DOT-T1E-PS28 (lacking TtgGHI pump) and DOT-T1E-18 (lacking the TtgABC pump) exposed to abiotic stresses such as propranolol and toluene.

In Chapter 2, the detected growth profiles in combination with FT-IR spectroscopy, GC-MS and chemometrics has been employed in order to monitor the phenotypic alterations between different culture conditions to illustrate the adaptation responses of these strains to abiotic stresses. Although previous genomic, transcriptomics and proteomics studies have focused on the adaptation mechanisms of *P. putida* strain to a wide range of organic solvents (e.g. toluene, styrene and xylenes) or pharmaceutical such as ampicillin, tetracycline and flavonoids (Udaondo et al., 2012; Ramos et al., 2015), our study was novel as it included the effect of propranolol and analysis with complementary metabolomics techniques. We have generated a global snapshot of bacterial phenotypic and untargeted metabolic profiles utilising FT-IR spectroscopy and GC-MS respectively.
In this instance exposure of the three strains of *P. putida*, DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18 to propranolol has been monitored at three different concentrations (below the minimum inhibitory concentrations (MIC)). Initial results from growth curves demonstrated that the mutant *P. putida* DOT-T1E-18 was more sensitive to propranolol than the other strains due to the lack of TtgABC pump, indicating the important role of efflux pumps in this adaptation processes. FT-IR spectroscopy was used to generate a biochemical fingerprint to monitor the general phenotypic effects of propranolol through the whole cell. FT-IR data revealed global metabolic changes with high reproducibility, while demonstrating the effect of propranolol exposure on protein components of the examined *P. putida* strains. Subsequently, GC-MS was used to investigate the alterations in the metabolome of *P. putida* DOT-T1E strains upon exposure to propranolol. Generally, in the central metabolism of *P. putida* DOT-T1E large effects were seen in amino acid levels between control and propranolol challenged cells. Untargeted analysis revealed that the levels of propranolol in the mutants were higher than the wild-type indicating the importance of efflux pumps in solvent tolerance. Moreover, ornithine can be considered as major key element for adaptation mechanisms since it was only produced in the presence of propranolol within the bacterial cultures. By contrast, glutamine was down-regulated after challenging with propranolol which could be consumed by the cells in order to generate the higher energy demands due to propranolol exposure.

This chapter provides a wider view of adaptation responses of bacterial cells to propranolol and shows that there are some areas in the adaptation mechanisms of *P. putida* that are necessary to be studied in more details. Future work should aim to provide further investigations employing other approaches including targeted analysis, fluxomics and lipidomics. Accurate quantitative measurement of toxic compounds and their metabolites is very important for many applications such as bioremediation, biocatalysis and production of biofuels, as some of the starting materials or end products can be toxic to cells which may affect the performance of microorganisms in biotechnological applications. In Chapter 3, the identification and quantification of toluene in *P. putida* DOT-T1E strains was investigated using Raman spectroscopy and liquid chromatography techniques. In this study toluene was not detected in bacterial cells using Raman spectroscopy as the levels of toluene present in the cells were below the
limit of detection of this technique. Therefore, several other methods (e.g. resonance Raman scattering) were investigated to enhance the Raman signal. Unfortunately this was not successful for the detection of toluene in bacterial cells. In recent years, graphene oxide (GO) and reduced graphene oxide (RGO) have become a promising candidate in biotechnology and have found a broad range of applications such as pollutant management (Zhao et al., 2011) and surface-enhanced Raman scattering (SERS) (Yu et al., 2011). The oxygen-containing functional groups and the interconnected \( \text{sp}^2 \) network of graphene gives it the capability to undergo \( \pi-\pi \) stacking with aromatic molecules (Wojcik and Kamat, 2010; Emery et al., 2011; Park et al., 2009; Goncalves et al., 2009). Future work may investigate the potential of the graphene-SERS technique for the identification and quantification of toluene in \( P. \ putida \) cells. In addition, the levels of toluene in \( P. \ putida \) strains were successfully detected and quantified using HPLC. The HPLC results revealed that the levels of toluene present in the mutants were considerably higher in comparison to the wild-type suggesting the contribution of efflux pumps in toluene tolerance.

We next studied the effect of toluene on \( P. \ putida \) DOT-T1E strains employing metabolomics strategies (Chapter 4). The growth profiles revealed that the wild type strain had higher resistance to toluene than the mutant strains, suggesting that the TtgABC and TtgGHI pumps play a role in toluene extrusion. Previous reports have demonstrated the role of efflux pumps in solvent extrusion (Rojas et al., 2001; Rodriguez-Herva et al., 2007; Wijte et al., 2011). FT-IR spectroscopy results show that toluene had an effect on protein and lipid components of the bacterial cells. In addition GC-MS was employed to monitor metabolome alterations in these cultures. Such a technique gave greater details with regard to the changes in the levels of various metabolites. Obvious differences were observed in the level of metabolites of the toluene exposed cells. Moreover, the level of ornithine was increased in the presence of toluene which presents itself as a major feature that may have important functions in toluene tolerance. As the FT-IR pointed towards the alteration of lipids in the cells exposed to toluene, future work would concentrate on lipidomics analysis employing powerful techniques such as GC-MS (employing FAME), reversed-phase LC-MS and MALDI-MS in order to expand our knowledge and provides a wider view of adaptation mechanisms of \( P. \ putida \) cultures to toxic solvents. In addition, it
is perhaps worth investigating the adaptation of *P. putida* strains to other aliphatic and aromatic organic solvents.

The effects of both propranolol and toluene on the phenotype of *P. putida* strains were successfully investigated in Chapters 2 and 4, and it was therefore important to explore the factors that would improve bacterial tolerance to toxic solvents. Previous research had investigated the impact of various metal ions on the stabilisation of the toluene tolerance in *P. putida*, and it was found that magnesium and calcium were the most effective in stabilising toluene tolerance (Inoue *et al.*, 1991). The effect of Mg$^{2+}$ and Ca$^{2+}$ on the stabilisation of the toluene tolerance in *P. putida* DOT-T1E cells was therefore investigated in Chapter 5. Growth curves results revealed that *P. putida* cultures showed higher tolerance to toluene in the presence of these divalent cations, irrespective of the anion used in the salt. Inspection of the FT-IR data revealed that the mutants had the same profile as the wild-type suggesting that the influence of Mg$^{2+}$ and Ca$^{2+}$ to enhance toluene tolerance in *P. putida* cells might be correlated to other toluene tolerance mechanisms such as lipopolysaccharides (LPS) instead of efflux pumps. In addition GC-MS was investigated in order to identify variation of the metabolome to elucidate the influence of divalent cations in greater details; however, GC-MS did not successfully illustrate the effect of divalent cations in stabilising toluene tolerance due to the complexity of the metabolic profiling results.

Future work in this area should aim to use alternative techniques such LC-MS to monitor phenotypic alterations at the metabolome level. Magnesium is an essential co-factor for many cellular processes including cellular protein synthesis and activator of many enzyme systems (Kung *et al.*, 1976). In addition to this, further investigation can be conducted by employing an atomic absorption spectrophotometer (AAS) for measuring intracellular free magnesium and extracellular magnesium buffering during cells growth in the absence and presence of toxic solvents to produce new insights into the regulation of cellular magnesium under harsh conditions.

In conclusion, the work undertaken in this thesis has provided further insights into the adaptation responses of microorganisms subjected to abiotic stress at the metabolite level, and demonstrated that both rapid physicochemical screening tools and metabolic profiling, in combination with chemometrics, seem ideally suited for
monitoring the phenotypic responses occurring within microbial cultures under different growth conditions.
6.1 References


Appendix

Published works


Appendix

Collaboration in other published works

RESEARCH ARTICLE

Metabolomics Analysis Reveals the Participation of Efflux Pumps and Ornithine in the Response of Pseudomonas putida DOT-T1E Cells to Challenge with Propranolol

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Abstract

Efflux pumps are critically important membrane components that play a crucial role in strain tolerance in Pseudomonas putida to antibiotics and aromatic hydrocarbons that result in these toxicants being expelled from the bacteria. Here, the effect of propranolol on P. putida was examined by sudden addition of 0.2, 0.4 and 0.6 mg mL\(^{-1}\) of this \(\beta\)-blocker to several strains of P. putida, including the wild type DOT-T1E and the efflux pump knockout mutants DOT-T1E-PS28 and DOT-T1E-18. Bacterial viability measurements reveal that the efflux pump TtgABC plays a more important role than the TtgGHI pump in strain tolerance to propranolol. Mid-infrared (MIR) spectroscopy was then used as a rapid, high-throughput screening tool to investigate any phenotypic changes resulting from exposure to varying levels of propranolol. Multivariate statistical analysis of these MIR data revealed gradient trends in resultant ordination scores plots, which were related to the concentration of propranolol. MIR illustrated phenotypic changes associated with the presence of this drug within the cell that could be assigned to significant changes that occurred within the bacterial protein components. To complement this phenotypic fingerprinting approach metabolic profiling was performed using gas chromatography mass spectrometry (GC-MS) to identify metabolites of interest during the growth of bacteria following toxic perturbation with the same concentration levels of propranolol. Metabolic profiling revealed that ornithine, which was only produced by P. putida cells in the presence of propranolol, presents itself as a major metabolic feature that has important functions in propranolol stress tolerance mechanisms within this highly significant and environmentally relevant species of bacteria.

Introduction

Active pharmaceutical compounds (APCs), in their original states or their metabolites, are ubiquitous in the environment [1], and the levels of APCs in the aquatic ecosystems (e.g., lakes,
rivers, seawater and estuaries) are a growing concern [2]. Pharmaceuticals are not only being introduced into the environment after consumption, but also via the disposal of unused or expired pharmaceuticals [3]. The levels of many pharmaceuticals in sewage treatment plants (STPs) have been detected at low concentrations in the range of ng L\(^{-1}\) to μgL\(^{-1}\) [1, 4–6]. A study in the United Kingdom revealed that the β-blocker propranolol is widely used, and for instance, around 12 tonnes of propranolol are consumed each year [4, 6, 7]. In addition, Ashton and co-workers (2004) showed that the presence of the β-blocker propranolol in STP effluents was highly likely at 76 ng L\(^{-1}\) (median level) [4].

Despite the fact that APCs are designed to have specific modes of action in the organism they were designed for, similar targets might control different metabolic processes in different species for which the original APC was not designed for [8]. In addition, the modes of action of the drugs within microbial systems are not fully understood. Thus, we and others believe it is necessary to increase our knowledge of the biological effects and fate of pharmaceuticals on microorganisms in the environment to appreciate the risks [9–11].

Indeed, bacterial communities inhabiting the benthic environment of riverbeds can be exposed to higher levels of APCs than expected, as it is known that these compounds can become concentrated in these areas [12–14]. Additionally, pharmaceuticals tend to bioaccumulate and induce impacts in aquatic and terrestrial environments due to their intrinsic pharmacokinetic properties [12]. A major adverse side effect of the presence of APCs in the environment is an increase in antimicrobial resistance that poses huge potential risk for the future, making the treatment of infections very difficult to cure, and there are several studies that have eloquently described the link between exposure to effluent and antimicrobial resistance [15–18].

Bacteria can adapt the activity of toxic substances by the employment of several resistant mechanisms including altering lipid composition, energy production, efflux pumps as well as other processes [19–22]. Efflux pumps, which transport toxic chemicals (usually waste products from normal metabolism) from the bacterial cell into the extra-cellular environment, are probably the most highly significant process which plays an important role in bacterial tolerance. One of these mechanisms is controlled by the ATP-binding cassette (ABC) transporters via the hydrolysis of ATP, whereas the transmembrane electrochemical gradient, particularly the proton motive force, is used by secondary transporters in order to drive drug efflux [23, 24]. In Pseudomonas putida DOT-T1E cells, three efflux pumps, which are genome-encoded, have been identified, and are termed TtgABC, TtgDEF, and TtgGHI. The TtgABC and TtgGHI pumps remove both organic solvents and some antibiotics, whereas the TtgDEF pump has been shown to be induced only by aromatic hydrocarbons [25–27].

Many studies have found that an enormous number of multidrug resistance (MDR) transport proteins are involved in the export of a wide range of antimicrobial compounds [23, 24, 28]. In Pseudomonas species, various studies linked solvent and antibiotic tolerance to the action of several efflux pumps [22, 25, 29, 30]. Moreover, solvent-tolerant microorganisms (e.g. P. putida DOT-T1E) play a crucial role in several biotechnological applications such as bioremediation, biocatalysis and agriculture [31–34]. Thus, an understanding of bacterial tolerant mechanisms is very important, in order to enhance the resistant systems for non-pathogenic strains and create altered strains with superior tolerance characteristics for industrial bioprocessing.

The qualitative and quantitative measurements of the metabolome of an organism can reveal its biochemical status and these data can be used to monitor and determine the function of genes [35, 36]. Metabolomics enables the identification and quantification of endogenous biochemical reaction products of cellular regulatory pathways and metabolite levels can be regarded as the ultimate response of biological system to environmental alterations and/or
genetic factors. Metabolome analysis provides relevant information about specific cell types under different conditions that is important for a more holistic understanding of cell functions and properties [35]. A comprehensive assessment of the alteration in the metabolite levels in P. putida strains can be acquired using a combination of metabolic profiling and multivariate data analysis approaches. The interpretation of metabolic data is complicated, thus a wide range of different analytical strategies have been employed to measure the metabolome [37, 38]. By understanding metabolomics data the effect of stress on lowest molecular levels is revealed. This enables better understanding of altering metabolic pathways that are directly affected by change in bacterial genome.

In order to investigate the effects of propranolol on biological system, we have employed Fourier-transform infrared (FT-IR) spectroscopy to acquire metabolic fingerprints [39–41]. FT-IR spectroscopy involves the observation of bond vibrations from within molecules when a sample is excited by a beam from the mid-infrared region of the electromagnetic spectrum. Briefly, the infrared beam is transmitted through or reflected from a sample, with some of the infrared radiation being absorbed at particular wavelengths within the sample, and the remainder continuing on to a detector, before being Fourier transformed and analysed via a computer. This results in an infrared absorbance spectrum which can be referred to as a metabolic “fingerprint” as it is characteristic of any chemical or biochemical substance. The fundamentals of FT-IR have been described in far greater detail elsewhere [40, 42] but its main advantages are that it is very rapid (taking seconds per sample), high-throughput, with 96 and 384 well sampling plates, reagentless, and non-destructive. FT-IR has been applied to a very wide-range of biological studies including clinical [41, 43] and microbiological [44] analyses since the very early 1990s when Dieter Naumann and co-workers demonstrated its potential use for bacterial characterization [45]. Metabolic profiling approaches are powerful in that in contrast to FT-IR spectroscopy they can be used to identify, quantify and detect the metabolites within the biological system, and gas chromatography mass spectrometry (GC-MS) is currently a very popular method for analyzing central carbon and nitrogen metabolism [46–48]. Changes identified in the metabolome can be considered to be hypothesis generating and as such can inform our biochemical knowledge [49, 50]. With respect to bacterial strain tolerance we believe that the observed metabolite changes can prove to be indicative of novel adaption mechanisms or may support postulated adaption mechanisms for which there is little evidence up to date.

The aim of this study was to investigate the changes in metabolite levels within P. putida DOT-T1E strains in the presence and absence of propranolol and determine if these changes were associated to efflux pumps or other adaptation mechanisms within these bacteria. To enable this, FT-IR spectroscopy was utilised as a rapid, high-throughput screening tool in order to identify phenotypic alterations in bacterial cultures exposed to propranolol, and metabolic profiling using GC-MS was employed to examine the change in metabolites at specific time points before and after challenge with propranolol.

Material and Methods
Bacterial Strains and Cultivation of Bacteria
Three bacterial strains of P. putida DOT-T1E were used in this study, their relevant characteristics, and references for further information on each strain are listed in Table 1. All strains were sub-cultured in triplicate to obtain axenic cultures. Individual colonies were then picked and transferred from plates into 250 mL flasks containing 50 mL of autoclaved Lysogeny broth (LB) medium and incubated at 24 h at 30°C in an orbital incubator (Infors HT Ltd, UK) shaking at 200 rpm.
Growth Curve Monitoring

Bacterial growth curves were monitored manually using an orbital incubator and UV instrument at 660 nm (Biomate 5, CarePlanTM, UK). All samples were normalised to an optical density (OD) of 0.02 in 250 mL flasks containing 50 mL LB medium. *P. putida* DOT-T1E cultures were incubated at 30°C and 200 rpm. During the 24 h time course of, 100 μL samples were taken at various time points (0, 2, 4, 6, 8, 10, 12 and 24 h) for OD measurement.

Growth in Response to Propranolol Shock, Sample Collection and Analysis

Cells were grown in 50 mL of LB medium for 5 h at 30°C and 200 rpm. Once cell cultures reached the mid-exponential phase, samples were divided into two groups. One group was kept as a control, and to the second group propranolol was added at three different concentrations (0.2, 0.4 and 0.6 mg mL\(^{-1}\)). These cultures were then incubated for an additional 8 h.

**Growth curve measurement.** At various time points (0, 1, 3, 5, 7, 9, 11 and 13 h) before and after the addition of propranolol, a 100 μL sample was taken for OD measurement. Growth was recorded as an increase or decrease in turbidity at 660 nm. This work was undertaken in biological triplicates.

**FT-IR sample collection.** After 60 min of the addition of propranolol, an aliquot (2 mL) sample was transferred to 2 mL tube, and the ODs of the samples were recorded for normalisation. All measurements were performed in triplicate.

**Sample preparation for FT-IR spectroscopy.** An aliquot (2 mL) sample from each flask was transferred to 2 mL tube and centrifuged at 11500 × g for 5 min at 4°C. The supernatant was removed and discarded, and the remaining pellet was washed twice with 2 mL of physiological saline solution (0.9% NaCl) and centrifuged (11500 × g, 5 min, 4°C) and the supernatant discarded. The remaining cell pellets were stored at -80°C until required.

A 96-well silicon FT-IR plate (Bruker Optics, Banner Lane, Coventry, UK) was cleaned with 5% sodium dodecyl sulfate (SDS) and rinsed with deionised water and allowed to dry at room temperature. Cell pellets were then removed from -80°C and allowed to thaw on ice. Samples were normalised according to OD at 660 nm and resuspended in physiological saline and gently vortexed. Aliquots (20 μL) of each sample were randomized and spotted in triplicate onto a silicon FT-IR plate. The prepared plates were then dried on a desiccator at ambient temperature for 7 h. This step was applied to minimise any signal arising from water absorbance in the mid-IR region.

**FT-IR setup.** The prepared silicon sample plate was loaded onto a motorised microplate module HTS-XT™ under the control of a PC programmed with OPUS software version 4. Spectra were acquired using a Bruker Equinox 55 FT-IR spectrometer (Bruker Optics, Banner Lane, Coventry, UK) in transmission mode as described previously [51], with a deuterated triglycerine sulfate (DTGS) detector over the wavenumber range 4000–600 cm\(^{-1}\), with a resolution of 4 cm\(^{-1}\), 64 scans were co-added and averaged in order to improve the signal-to-noise ratio. Three technical replicates were obtained from each sample, and a total of 324 spectra were collected.
**FT-IR data analysis.** FT-IR data were converted to ASCII format using OPUS reader software and analysed using Matlab version 2012 (MathWorks, Natick, MA). All FT-IR spectra were CO₂ corrected by replacing the region from 2400 to 2275 cm⁻¹ with a linear trend and then scaled using extended multiplicative signal correction (EMSC) [52].

Statistical analysis of the preprocessed data was performed using principal component analysis (PCA) [53] and discriminant function analysis (DFA). PCA was used to generate set of latent variables (PCs) which retain the major variance of the data whilst decreasing the dimensionality; DFA was then used to create a set of discriminant functions (DFs) based on PCs which maximize the differences between the known groups (classes) [54, 55]. PC-DFA was performed using 10 PCs and 3 DFs, and the class structure for the DFA algorithm was based on the biological replicates of samples of the same conditions.

**GC-MS sample collection.** 15 mL samples were quenched at three time points 0, 10 and 60 min before and after the addition of propranolol (0 min refers to the point immediately before the addition of propranolol). This procedure was performed with four biological replicates.

**Metabolic quenching and metabolite extraction.** Generally, a rapid inactivation of metabolism is achieved by alteration in pH or temperature [56]. Thus, in order to halt metabolism culture samples (15 mL) were plunged into a double volume of 60% cold methanol (-50°C) in a 50 mL tube. The quenched culture mixture was centrifuged (3000 x g, 10 min, 1°C), and then the supernatant was discarded, while the cell pellets were stored at -80°C until required for metabolite extraction [57].

The biomass pellets were resuspended in 750 μL of freshly prepared cold methanol (80%). The solution was then transferred to a 2 mL Eppendorf tube. This was followed by a freeze-thaw cycle in order to extract the intracellular polar metabolites from the cells. Samples were centrifuged at (13500 x g, 3 min, 4°C) and the supernatant was transferred to new tubes and stored on dry ice [57]. The extraction was performed again on the remaining pellet and both supernatants were combined and again stored on dry ice. A final aliquot (1400 μL) of metabolite extracts were normalised using 80% methanol according to OD at 660 nm. A quality control (QC) sample [58] was prepared by transferring 100 μL from each of the sample to a new (15 mL) centrifuge tube. This was followed by the addition of (100 μL) of internal standard solution (0.2 mg mL⁻¹ glycine-d₅, 0.2 mg mL⁻¹ benzoic-d₅ acid, 0.2 mg mL⁻¹ lysine-d₄, and 0.2 mg mL⁻¹ succinic-d₄ acid) to all samples. The samples were lyophilized for 16 h by speed vacuum concentrator (concentrator 5301; Eppendorf, Cambridge, UK), and then the pellet was stored at -80°C for further analysis.

**GC-MS derivatization process.** Samples were derivatized prior to GC-MS analysis in two stages as described previously by Wedge and co-workers [59]. The first step, (50 μL) of O-methoxylamine hydrochloride diluted in pyridine (20 mg mL⁻¹) was added to the samples and then samples were heated on a heating block at 65°C for 40 min. The second step, (50 μL) of MSTFA (N-methyl-trimethylsilyl trifluoroacetamide) was added to the samples followed by heating for 40 min. At the end of second step, 20 μL of retention index was added. After each addition in all three steps described above samples were vortexed for 10 s and centrifuged at 13500 x g for 15 min.

**GC-MS instrument setup.** Samples were randomised and analysed by gas chromatography electron ionisation time-of-flight mass spectrometry (GC-TOF-MS) using an Agilent 6890 GC instrument coupled to a LECO Pegasus III TOF mass spectrometer (Leco, St. Joseph, MI, USA), as described previously [59–61]. GC column (VF-17MS column, 0.25 mm ID × 30 m × 0.25 μm film thickness, Varian, cat. no. CP8982) was employed at a constant helium carrier gas flow of 1 mL min⁻¹, with a temperature program starts at 70°C and end at 300°C. The mass spectrometer source is operated at a temperature of 250°C in electron ionization (EI).
mode, with an electron energy of 70 eV and the detector is operated in the range 1400–1800 V. Raw data processing was undertaken using LECO ChromaTOF v3.26 in order to construct a data matrix of metabolite peak vs. sample and infilled with peak areas for metabolites that were detected. A reference database was prepared that contained retention times, quant mass, peak area, retention index value and peak number related to each peak by analysing QC samples. The identification of analytes was based on both spectral similarity and matched with retention indices. In-house library as well as NIST library was used for identification, and we followed MSI guidelines for metabolite identification [62].

**GC-MS data analysis.** For statistical analysis multi-block PCA [63] was used with three different types of blockings. The first type of blocking is strain | time×dosage blocking. This blocking partitioned the data into 9 blocks. Each block contained all the samples from the same time point with the same dosage of propranolol, e.g. all the samples with 0.2 mg mL\(^{-1}\) propranolol, collected at 0 min were assigned to one block, those with 0.4 mg mL\(^{-1}\) propranolol, collected at 10 min were assigned to another block and so on. Across different blocks, the strains were matched so that in every block the first 4 samples were \(P.\) putida DOT-T1E, the next 4 samples were \(P.\) putida DOT-T1E-18 and the last 4 samples were \(P.\) putida DOT-T1E-PS28. Based on the same principle, dosage | strain×time blocking partitioned the data into 6 blocks (samples at 0 min were not included for this type of blocking as this time point refers to the point immediately before the addition of propranolol), each block had the samples of the same strain and same time points, the dosage of propranolol were matched. Such blocking allowed MB-PCA to detect the effect of each of the factor of interest (i.e., strain, time and dosage of propranolol) separately without the inference from others [64].

A total of 200 features were detected by GC-MS. The natural logarithm (ln) was first applied on the peak area of the detected peaks. Data were mean centred and then auto-scaled then subjected to MB-PCA. The potentially most significant variables were identified by selecting the most predominant averaged block loadings. Finally, box-whisker plots were used to visualise the data. These analyses were conducted using in-house scripts under the Matlab 2014a (Mathworks, Natick, MA) environment. The data are available at MetaboLights (http://www.ebi.ac.uk/metabolights/): study identifier MTBLS320.

**Results and Discussion**

**Characterization of \(P.\) putida DOT-T1E Strains**

Growth curve experiments were undertaken for \(P.\) putida strains to determine the optimum points to induce abiotic stress using propranolol. The resultant growth curves are displayed in (Fig 1A) and these show that there were no significant differences in the pattern of growth between the wild type DOT-T1E and the mutant DOT-T1E-PS28 (lacking the TtgGHI pump) over the 24 h incubation period. Whilst under the same conditions, the mutant DOT-T1E-18 (lacking the TtgABC pump) grew slightly poorly in comparison to the other strains. This result was in agreement with previous observations which show that \(P.\) putida DOT-T1E-PS28 grew on LB medium and had similar growth generation time to the wild type [27]. However, the mutant DOT-T1E-18 showed less growth compared to the wild-type and this could be a result of the waste products made during cellular metabolic processes accumulating to toxic levels due to the lack of TtgABC pump, resulting in slower growth. To be able to investigate the metabolome changes between the wild type and the two mutants, cells were cultured in the absence of propranolol, GC-MS analysis was performed and this was followed by chemometrics.

MB-PCA of all \(P.\) putida strains was carried out and the result showed an obvious clustering pattern as can be seen in (S1 Fig). It was clear from this analysis that \(P.\) putida DOT-T1E-18 was very different to the other two strains although weak separation can also be observed.
between \textit{Pseudomonas putida} DOT-T1E and \textit{Pseudomonas putida} DOT-T1E-PS28. In addition, MB-PCA loading plots were plotted in order to investigate the significant metabolites associated with the different growth behaviour. It can be seen that many metabolites were most abundant in \textit{Pseudomonas putida} DOT-T1E-18 and least abundant in \textit{Pseudomonas putida} DOT-T1E. Box-whisker plots were generated and these generally supported the increased metabolite levels in DOT-T1E-18 (S2 Fig). During the growth of the three \textit{Pseudomonas putida} strains, a number of metabolites detected by GC-MS were compared (e.g. carbon and nitrogen metabolism; \textit{viz.}, sugars, sugar phosphates, amino acids, organic acids).

A schematic summary of the detected metabolites by GC-MS of central metabolic pathways in \textit{Pseudomonas putida} DOT-T1E strains is shown in S3 Fig and S1 Table. It can be seen that the level of a total of 9 metabolites were similar in the mutant DOT-T1E-PS28 compare to the wild-type DOT-T1E, while only 3 metabolites had similar levels in the mutant \textit{Pseudomonas putida} DOT-T1E-18 in comparison to the wild type. These results would suggest that the TtgABC pump is involved in

Fig 1. Growth curves of \textit{Pseudomonas putida} strains. (A) all three \textit{Pseudomonas putida} DOT-T1E strains in LB medium without propranolol; (B) \textit{Pseudomonas putida} DOT-T1E, (C) \textit{Pseudomonas putida} DOT-T1E-PS28, and (D) \textit{Pseudomonas putida} DOT-T1E-18 in the presence of propranolol. A 1/10 dilution of 100 μL samples were prepared for OD measurement at 660 nm.

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the removal of toxic metabolites produced during the log phase. In addition, the accumulation of toxic products might result in changes in the level of amino acids due to the activation of other metabolic pathways to deal with waste products.

**Characterization of P. putida DOT-T1E Strains to Propranolol Shocks**

**Minimal inhibitory concentration (MIC).** In order to study the effect of propranolol on P. putida DOT-T1E cultures, it was necessary to establish the MIC of each bacterial strain when cultured in LB media and challenged with different levels of propranolol and the results are recorded in S2 Table. The visible growth of the wild-type DOT-T1E, mutant DOT-T1E-PS28 and mutant DOT-T1E-18 were inhibited at 1.5, 1.5 and 0.8 mg mL\(^{-1}\) of propranolol respectively. The resistance of DOT-T1E-PS28 to propranolol was the same as the wild-type. However, it was reduced for the mutant DOT-T1E-18, suggesting that the extrusion of propranolol by the TtgABC pump could play a more crucial role than TtgGHI pump. Observations similar to these findings have been reported by Rojas and co-workers [27] testing MIC of several antibiotics for P. putida DOT-T1E strains, in which the DOT-T1E-18 mutant was more sensitive to those antibiotics than DOT-T1E. Nevertheless, the DOT-T1E-PS28 mutant showed similar sensitivity to the wild-type.

**Bacterial growth in the presence of propranolol.** From interpretation of the growth curves (Fig 1A), it was decided to induce propranolol stress after 5 h (once the cultures reached their mid-exponential phase) at three different concentrations of propranolol (0.2, 0.4 and 0.6 mg mL\(^{-1}\)) below the MIC. The effect of propranolol on P. putida cells was then studied in liquid culture medium after cells had been pre-grown on LB liquid medium, and following challenge with propranolol. Growth curve results from P. putida cultures are shown in (Fig 1B–1D). In general, slight variations were noted in the growth patterns between P. putida DOT-T1E and DOT-T1E-PS28 species exposed to 0.2 and 0.4 mg mL\(^{-1}\) propranolol, though considerable effects on the same cultures were observed when cultures were exposed to 0.6 mg mL\(^{-1}\) propranolol across a 13 h growth period.

By contrast, a marked effect was observed in P. putida DOT-T1E-18 when exposed to 0.4 and 0.6 mg mL\(^{-1}\) concentrations of propranolol. Strain tolerance is an energy intensive process, and it was noted that the growth yields of P. putida DOT-T1E cultures in the presence of 0.6 mg mL\(^{-1}\) were reduced by five-fold compared to the control cultures. This decrease in the growth yield might result in consumption of energy by various mechanisms in order to protect the cells from further damage. One study examined the growth yields of *Pseudomonas* upon sub-lethal toluene dosages and it was found that the presence of toluene led to lower yields and that the growth yield reduced linearly with increasing toluene concentrations [65]. This report deduced that the decrease in yield associated with the presence of toluene could be due to energy-consuming adaptation mechanisms initiated to protect cells from excessive damage.

To assess bacterial membrane integrity during the growth of bacteria following propranolol perturbation a LIVE/DEAD BacLight bacterial viability assay was used, and the green and red fluorescence emissions were measured using a Flexstation 3 Microplate Reader (Molecular Devices, USA). The ratio of green to red fluorescence and the percentage of live cells from TVC plates estimations in the *P. putida* suspension are shown (S3 Table). It was clear from these measurements that cell viability decreased linearly with increasing propranolol indicating the toxic effect of propranolol on *P. putida* DOT-T1E strains.

**FT-IR fingerprinting of cell cultures.** FT-IR spectroscopy was employed to investigate whether the phenotype of an organism had changed by exposing it to gradient levels of propranolol. PC-DFA scores plots were produced in order to visualise the distribution of samples based on their IR metabolic fingerprints (Fig 2A–2C). From inspection of the PC-DFA scores
Fig 2. PC-DFA scores plots of FT-IR data for three different strains of *P. putida* strains upon propranolol shock. Symbols represent different strains. (A) *P. putida* DOT-T1E is the wild type (stars) and ten PCs with a total explained variance (TEV) of 99.43% were used for the DFA. (B) *P. putida* DOT-T1E-PS28 is the mutant (closed triangles) and ten PCs with a TEV of 99.65% were used for the DFA. (C) *P. putida* DOT-T1E-18 is the mutant (closed circles) and ten PCs with a TEV of 99.03% were used for the DFA. Colour coding: control with no propranolol (red), cells exposed to 0.2 mg mL⁻¹ propranolol (black), 0.4 mg mL⁻¹ propranolol (black).
plots of the biomass samples, it was possible to determine that there was an obvious separation between the different culture conditions. There was also a clear trajectory based on concentration (annotated with arrows) with samples from control cultures following a trend from right to left across the plot space due to the increase of propranolol concentrations. This clustering pattern was anticipated and suggests that propranolol stress has had a clear additive effect on the bacterial cells and this is reflected in the FT-IR results. In other analyses these PC-DFA models were validated by test set projection (S4 Fig) and these ensure that the model quality is of a high standard, and that the obtained subsequent conclusions drawn from the data are valid and robust.

To assess the relevant metabolites causing these separations in PC-DFA scores plots, the loadings plots for the first discriminant functions were plotted (Fig 2D–2F). Multiple changes occur within these loadings plot with the largest variances being observed between wavenumbers 1700–1600 cm\(^{-1}\). In this region of the mid-infrared the majority of vibrational bands are associated with protein components of the sample; most notably amide I (C = O stretching at 1690–1620 cm\(^{-1}\)) and amide II (combination of C-N stretching and N-H bending). These results suggest that the most significant effect over the duration of the 1 h incubation period following drug shock is associated with alterations to proteinaceous components of bacteria. The profile of proteins in different \(P.\) \(putida\) strains—T1E and S12—upon exposure to toluene has been investigated previously, and it was revealed that almost 90 proteins were up-regulated as a result of an exposure of strains to toluene in which some of these proteins relate to efflux pump systems \[22, 66, 67\]. Therefore, it is perhaps not surprising that the most significant changes observed from the interpretation of infrared spectra were in the vibrational frequency of the proteins components, and we can infer from this that some proteins were up-regulated to cope with the presence of propranolol.

**GC-MS metabolic profiling of cell cultures.** Recently, attention has been focused on studying the stress responses in bacteria employing metabolomics-based approaches \[68–70\], and this has involved a wide range of disciplines such as drug discovery, metabolic engineering and medical sciences \[71–75\]. In this study, we employed GC-MS to create metabolic profiles of bacterial stress to propranolol, as the knowledge of variations within the metabolome following chemical perturbation could lead to a more in-depth understanding of strain specific stress responses within these bacteria.

As there are multiple potentially interacting factors that we have in our experiment with respect to propranolol dose, bacterial strain, as well as time, MB-PCA was used for analysis. MB-PCA with dosage | strain×time blocking (see materials and methods) was undertaken and a gradient effect corresponding to differing dosages of propranolol can be seen on the resultant scores plot (S5 and S6 Figs). We observed nine metabolites that were differentially expressed between control and different dosages of propranolol and these were statistically significant. However, four metabolites (cystathionine, glutamine and two unknowns) decreased with increase in dosage of propranolol, four metabolites (ornithine, propranolol and two unknowns) increased with dosage whereas no clear pattern was seen for one metabolite (unknown).

Interestingly, it was found that two of these metabolites (variables 180 and 100) were only detected following the exposure of \(P.\) \(putida\) strains to all three concentrations of propranolol groups but not in the control. Variable 180 was identified by an in-house database as propranolol itself, and Fig 3A shows that exposure of cells to propranolol resulted in the accumulation

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Propranolol
Variable id. = 180

Fig 3. Box-whisker plots showing the changes in metabolite levels in control and cells exposed to propranolol for 4 biological replicates. Variable 180 was identified as propranolol. (Red line) indicates the median m/z intensity. (A) Represent the data for 3 P. putida strains, 4 concentrations of propranolol and 3 time points, dashed lines separate different concentration levels of propranolol and solid line separates different strains. (B) Represent the data for 3 P. putida strains, 3 concentrations of propranolol and 1 time point at 60 min, dashed lines separates different strains.

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of propranolol in comparison to non-exposed cells. These data also show that the level of propranolol in *P. putida* stains were detected at both time points at 10 and 60 min, and it was noticed that the accumulation of propranolol in the exposed cells increased as the concentration of the propranolol increased.

In addition, comparing the level of propranolol between the wild-type and the mutants only at 60 min (Fig 3B), it was observed that *P. putida* DOT-T1E (wild-type) and *P. putida* DOT-T1E-PS28 (lacking TtgGHI pump) showed high similarities in the level of propranolol at all tested concentrations. By contrast, the amount of propranolol accumulating in the *P. putida* DOT-T1E-18 (lacking TtgABC pump) was higher than the other strains. This could be further evidence for the activity of efflux pump system in *P. putida* cells due to the presence of propranolol at different levels. In addition, these results would suggest that the TtgABC efflux pump is the main extrusion pump for propranolol and that it plays a more important role than the TtgGHI pump. These findings, which agree well with other studies, show that the TtgABC pump in *P. putida* DOT-T1E is the main antibiotic extrusion pump, and it has the ability to extrude flavonoids, tetracycline, chloramphenicol and ampicillin in addition to other solvents such as toluene [76–78].

Interestingly, the other significant variable, 100, was identified as ornithine (ChEBI ID 15729) again from an in-house library generated on the same instrument [62]. Ornithine production was detected within 10 min after exposure to propranolol, and the level of ornithine in the wild-type DOT-T1E and mutant DOT-T1E-PS28 shows an increase at 0.2 mg mL⁻¹ propranolol and a further almost linear increase in the presence of 0.4 and 0.6 mg mL⁻¹ propranolol (Fig 4). By contrast, the production of this metabolite in the mutant DOT-T1E-18 exhibits an increase at 0.2 mg mL⁻¹ propranolol followed by a further increase at 0.4 mg mL⁻¹ followed by a decrease toward 0.6 mg mL⁻¹ propranolol. Furthermore, the level of ornithine was further decreased, from 10 to 60 min at 0.6 mg mL⁻¹ propranolol for both *P. putida* DOT-T1E and DOT-T1E-PS28, while it was increased for *P. putida* DOT-T1E-18 under the same conditions. This metabolite is very important, as it is only produced by the *P. putida* cells in the presence of propranolol and our data suggest that this is linked to bacterial tolerance mechanisms, further studies are needed in order to understand this role and comprehend whether this is a cause or effect relationship.

In addition, in *P. putida* T1E and S12 proteomic analysis found that several proteins of the TCA cycle involved in energy production were up-regulated upon toluene exposure, indicating a requirement for enhanced metabolism and high energy demands because of toluene exposure in order to power efflux pumps that extrude solvent from the cells [22, 66], which is in agreement with several proteomics, and transcriptomics studies [66, 79]. The up-regulation of several terminal oxidase genes upon solvent stress in *P. putida* T1E suggests that demands on energy consumption are necessary to cope with the presence of solvents, in particular due to high activity of efflux pumps [80]. Ornithine can be synthesised via the TCA cycle in which glutamate is converted into ornithine, as previously reported for *P. putida* [81]. The production of ornithine in the presence of propranolol is interesting, as this observation would suggest that *P. putida* DOT-T1E may use this amino acid for energy production to power efflux pumps, or in order to activate other metabolic pathways that are important in bacterial tolerance to propranolol.

In addition, the primary building block of biological membranes mainly consists of glycerophospholipids such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and cardiolipin (CL); however, other lipids classes (e.g. ornithine lipids) have been described as well, which contain a 3-hydroxy fatty acyl group attached in amide linkage to the α-amino group of ornithine. This lipid can be formed only by specific groups of bacteria or under certain stress conditions [82]; although these have not yet been reported in *P. putida*. It is possible that the
ability to produce ornithine under propranolol stress in *P. putida* strains is linked to lipid production, however we have no direct evidence for this yet.

It is clear from the above that there are changes in central metabolism in response to propranolol exposure. Therefore, we investigated whether the levels of metabolites in the central metabolic pathways of *P. putida* strains were significantly altered or not between control and propranolol challenged samples for each bacterial strain independently. Metabolic pathways that were changed during propranolol stress were identified utilising untargeted GC-MS analysis. A comparative summary of central metabolic pathways between control and propranolol challenged cells for 10 or 60 min in *P. putida* DOT-T1E (Fig 5), *P. putida* DOT-T1E-18 (Fig 6) and *P. putida* DOT-T1E-PS28 (S7 Fig) were generated and large effects were seen in amino acid biosynthesis. In total, 17 metabolites were differentially produced or consumed in the presence of 3 different concentrations of propranolol, compared to the control sample at two time points. Major metabolites that were changed significantly during propranolol stress were serine, glycine, tryptophan, phenylalanine, tyrosine, alanine, valine, leucine, citrate, fumarate, glutamine, ornithine, aspartic acid, lysine, methionine, threonine and isoleucine, and box-whisker plots of these metabolites show the changes in these metabolite levels (S8–S11 Figs).
Fig 5. Schematic metabolic diagram of central carbon metabolism in *P. putida* DOT-T1E adapted to propranolol showing the level of metabolites for cells exposed to propranolol compared to the control. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. The median m/z intensity (red line) in the box-whisker plots was used to compare the level of metabolites. (A) Represent the level of metabolites at 10 min, while (B) the level of
In *P. putida* DOT-T1E, 10 metabolites were found to be consumed, 4 metabolites produced and 3 metabolites did not change at 10 min, while 4 metabolites were down-regulated and 13 metabolites up-regulated at 60 min. In the mutant DOT-T1E-18, 10 min following exposure to propranolol the levels of 2 metabolites increased, 8 metabolites were consumed and 7 metabolites remained constant. After 60 min following exposure to propranolol, the levels of 11 metabolites were increased, 3 metabolites were consumed and 3 remained constant. In *P. putida* DOT-T1E-PS28, although similar patterns in the level of metabolites was observed compared to the wild-type in the absence of propranolol, different patterns were observed in the presence of propranolol. Both mutants showed different metabolic profiles compared to the wild type and this could be due to the lack of the pump leading to over-expression of certain amino acids that are important to activate specific pumps or other metabolic pathways to cope with the stress.

Pathway analysis also revealed that glutamine and ornithine, which shows similar metabolic changes in all *P. putida* DOT-T1E strains, as major pathways impacted by propranolol stress. It is possible that glutamine could be being consumed by the cells in order to respond to high energy demands due to propranolol exposure. Another possible suggestion is that the decrease in the level of glutamine may be due to the biosynthesis of ornithine which could be the key stress-responsive metabolite involved to cope with stress following perturbation by propranolol. Therefore, cells may convert glutamate into ornithine instead of glutamine, resulting in a decrease in the level of glutamine. In contrast, in comparison to the wild type both mutants undergo different metabolic changes in other detected metabolites, mainly aliphatic amino acids, aromatic amino acids, and the aspartate family. This might be explained by the lack of the efflux pump in each mutant leading to the induction of certain metabolic pathways resulting in the production or consumption of certain amino acids associated with specific pumps.

**Conclusion**

Here we have shown that propranolol had a measurable biological effect on all three strains of bacteria studied. The results demonstrated that the mutant *P. putida* DOT-T1E-18 was more sensitive to propranolol than the other strains analysed due to the lack of TtgABC pump. With respect to exposure to propranolol, data from FT-IR revealed that propranolol had an effect on protein components of the bacterial cells. The investigation of the characterization of the metabolome of *P. putida* DOT-T1E strains upon exposure to propranolol revealed the important role of efflux pump activity and the production of ornithine as major key elements for adaptation mechanisms. This information can be useful in bioengineering to create engineered *P. putida* strains or even other bacteria with superior tolerance characteristic for bioprocesses, which in turn can help to remediate simple or complex mixtures of pollutants from environment. Similar to the case where lactate tolerance was improved in an engineered strain producing ascorbic acid, a well-known reactive-oxygen species scavenger [83]. Furthermore, both screening tools and metabolic profiling in combination with multivariate statistical methods, seem ideally suited to monitoring the phenotypic responses occurring within microbial cultures under different growth conditions and subjected to abiotic stress.
Metabolomics Analysis of *Pseudomonas putida* Challenged with Propranolol

Fig 6. Schematic diagram of central carbon metabolism in *P. putida* DOT-T1E-18 adapted to propranolol showing the level of metabolites for cells exposed to propranolol compared to the control ones. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. The median m/z intensity (red line) in the box-whisker plots was used to compare the level of metabolites. (A) Represent the level of metabolites at 10 min, while (B) the
level of metabolite at 60 min. Traffic light system represents different concentration of propranolol. Red, yellow and green represent exposed cells to 0.2, 0.4 and 0.6 mg/mL of propranolol respectively. Up-arrow, down-arrow and steady arrow indicate an increase, a decrease and no change in the level of metabolite respectively. The number of arrows represents the level of metabolites. Slight change (single arrow), medium change (double arrows) and high change (triple arrows).

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Supporting Information

S1 Fig. MB-PCA scores plot of GC-MS data for the wild type and the mutants in the absence of propranolol. Colours represent different strains. (A) *P. putida* DOT-T1E is the wild type (red), (B) *P. putida* DOT-T1E-PS28 (green), and (C) *P. putida* DOT-T1E-18 (blue).

S2 Fig. Box-whisker plots of a few selected most significant metabolites between the wild type and the mutants in the absence of propranolol. (A) *P. putida* DOT-T1E is the wild type, (B) *P. putida* DOT-T1E-PS28, and (C) *P. putida* DOT-T1E-18. Variables 9 (unknown), Variables 29 (leucine), Variables 37 (leucine\(^\wedge\)), Variables 70 (unknown), Variables 134 (\(\alpha\)-D-glucopyranoside\(^-\)), Variables 163 (D-ribonic acid/ D-glucose\(^-\)), Variables 185 (\(\alpha\)-N-acetylneuraminic acid/ D-Glucose\(^-\)), Variables 188 (sucrose), and Variables 198 (\(\alpha\)-N-acetylneuraminic acid\(^-\)). ^ multiple derivatives of same compound. * multiple assignments as identification is putative only.

S3 Fig. Schematic metabolic pathway diagram of central carbon metabolism in *P. putida* DOT-T1E showing the level of metabolites for both mutants compared to the wild type. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. The median m/z intensity (red line) in the box-whisker plots was used to compare the level of metabolites. Blue and brown represent the mutant DOT-T1E-PS28 and DOT-T1E-18 respectively. Up-arrow, down-arrow and steady arrow indicate an increase, a decrease and no change in the level of metabolite respectively. The number of arrows represents the level of metabolites. Slight change (single arrow) and medium change (double arrows).

S4 Fig. Validated PC-DFA models of (A) *P. putida* DOT-T1E, (B) *P. putida* DOT-T1E-PS28, (C) *P. putida* DOT-T1E-18 upon 0.2, 0.4 and 0.6 mg mL\(^{-1}\) Propranolol shock. Symbols coding: control with no propranolol (circles), cells exposed to 0.2 mg mL\(^{-1}\) propranolol (squares), 0.4 mg mL\(^{-1}\) propranolol (triangles), and 0.6 mg mL\(^{-1}\) propranolol (upside down triangles). Opened symbols represent the test set while closed symbols represent the training set.

S5 Fig. MB-PCA score plot of GC-MS data showing the effect of different concentrations on *P. putida* strains. Colours represent different dosage of propranolol. (D0) exposed to 0 mg/mL propranolol (blue), (D1) exposed to 0.2 mg mL\(^{-1}\) propranolol (green), and (D2) exposed to 0.4 mg mL\(^{-1}\) propranolol (pink). (D3) exposed to 0.6 mg mL\(^{-1}\) propranolol (red).

S6 Fig. MB-PCA loading plot of GC-MS data showing the most significant metabolites in the presence of different concentrations of propranolol. Significant loadings were observed in the positive and negative sides of the plot.
S7 Fig. Schematic metabolic diagram of central carbon metabolism in *P. putida* DOT--T1E-PS28 adapted to propranolol showing the level of metabolites for cells exposed to propranolol compared to the control. Metabolites were detected and identified by GC-MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. The median m/z intensity (red line) in the box-whisker plots was used to compare the level of metabolites. (A) Represent the level of metabolites at 10 min, while (B) the level of metabolite at 60 min. Traffic light system represents different concentration of propranolol. Red, yellow and green represent exposed cells to 0.2, 0.4 and 0.6 mg mL⁻¹ of propranolol respectively. Up-arrow, down-arrow and steady arrow indicate an increase, a decrease and no change in the level of metabolite respectively. The number of arrows represents the level of metabolites. Slight change (single arrow), medium change (double arrows) and high change (triple arrows).

PDF

S8 Fig. Box-whisker plots of the detected metabolites of central carbon metabolism in *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18. Dashed lines separate different concentration levels of propranolol and solid line separates different strains. The label is constructed in a format of “Aac”, “A” represents strains, varies from A to C: A = *P. putida* DOT-T1E; B = *P. putida* DOT-T1E-PS28 and C = *P. putida* DOT-T1E-18. “a” represents 4 different concentration levels, varies from 0 to 3: 0 = control; 1 = 0.2 mg mL⁻¹; 2 = 0.4 mg mL⁻¹ and 3 = 0.6 mg mL⁻¹ propranolol. “c” represents time points, 1 = T0 (0 min); 2 = T1 (10 min) and 3 = T2 (1 h). Such plots give a comprehensive view of how the concentration levels of the metabolite changing under each unique combination of the factors (strains, dosage of propranolol and time). Variables 14 (alanine), Variables 20 (valine), Variables 29 (leucine), and Variables 34 (isoleucine).

PDF

S9 Fig. Box-whisker plots of the detected metabolites of central carbon metabolism in *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18. Dashed lines separate different concentration levels of propranolol and solid line separates different strains. The label is constructed in a format of “Aac”, “A” represents strains, varies from A to C: A = *P. putida* DOT-T1E; B = *P. putida* DOT-T1E-PS28 and C = *P. putida* DOT-T1E-18. “a” represents 4 different concentration levels, varies from 0 to 3: 0 = control; 1 = 0.2 mg mL⁻¹; 2 = 0.4 mg mL⁻¹ and 3 = 0.6 mg mL⁻¹ propranolol. “c” represents time points, 1 = T0 (0 min); 2 = T1 (10 min) and 3 = T2 (1 h). Such plots give a comprehensive view of how the concentration levels of the metabolite changing under each unique combination of the factors (strains, dosage of propranolol and time). Variables 40 (glycine), Variables 53 (threonine), Variables 54 (serine), and Variables 78 (aspartic acid).

PDF

S10 Fig. Box-whisker plots of the detected metabolites of central carbon metabolism in *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18. Dashed lines separate different concentration levels of propranolol and solid line separates different strains. The label is constructed in a format of “Aac”, “A” represents strains, varies from A to C: A = *P. putida* DOT-T1E; B = *P. putida* DOT-T1E-PS28 and C = *P. putida* DOT-T1E-18. “a” represents 4 different concentration levels, varies from 0 to 3: 0 = control; 1 = 0.2 mg mL⁻¹; 2 = 0.4 mg mL⁻¹ and 3 = 0.6 mg mL⁻¹ propranolol. “c” represents time points, 1 = T0 (0 min); 2 = T1 (10 min) and 3 = T2 (1 h). Such plots give a comprehensive view of how the concentration levels of the metabolite changing under each unique combination of the factors (strains, dosage of propranolol and time). Variables 81 (methionine), Variables 88 (glutamine), Variables 95
S11 Fig. Box-whisker plots of the detected metabolites of central carbon metabolism in *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18. Dashed lines separate different concentration levels of propranolol and solid line separates different strains. The label is constructed in a format of “Aac”, “A” represents strains, varies from A to C: A = *P. putida* DOT-T1E; B = *P. putida* DOT-T1E-PS28 and C = *P. putida* DOT-T1E-18. “a” represents 4 different concentration levels, varies from 0 to 3: 0 = control; 1 = 0.2 mg mL⁻¹; 2 = 0.4 mg mL⁻¹ and 3 = 0.6 mg mL⁻¹ propranolol. “c” represents time points, 1 = T0 (0 min); 2 = T1 (10 min) and 3 = T2 (1 h). Such plots give a comprehensive view of how the concentration levels of the metabolite changing under each unique combination of the factors (strains, dosage of propranolol and time). Variables 109 (citrate), Variables 119 (lysine), Variables 135 (tyrosine), and Variables 177 (tryptophan).

S1 Table. The level of metabolites for both mutants compared to the wild type in the central carbon metabolism in *P. putida* DOT-T1E. Metabolites were detected and identified by GC-MS.

S2 Table. Results from the propranolol MIC experiments using *P. putida* DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18. Culture growth was observed after overnight incubation.

S3 Table. Viability of *P. putida* cells 1 h later after exposure to propranolol.

Author Contributions

Conceived and designed the experiments: AS RG. Performed the experiments: AS DKT NA DIE NJWR. Analyzed the data: YX. Contributed reagents/materials/analysis tools: RG. Wrote the paper: AS YX DTK NA DIE RG NJWR.

References


Metabolic analysis of the response of *Pseudomonas putida* DOT-T1E strains to toluene using Fourier transform infrared spectroscopy and gas chromatography mass spectrometry

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Abstract

Introduction An exceptionally interesting stress response of *Pseudomonas putida* strains to toxic substances is the induction of efflux pumps that remove toxic chemical substances from the bacterial cell out to the external environment. To exploit these microorganisms to their full potential a deeper understanding of the interactions between the bacteria and organic solvents is required. Thus, this study focuses on investigation of metabolic changes in *P. putida* upon exposure to toluene.

Objective Investigate observable metabolic alterations during interactions of three strains of *P. putida* (DOT-T1E, and its mutants DOT-T1E-PS28 and DOT-T1E-18) with the aromatic hydrocarbon toluene.

Methods The growth profiles were measured by taking optical density (OD) measurement at 660 nm (OD\(_{660}\)) at various time points during incubation. For fingerprinting analysis, Fourier-transform infrared (FT-IR) spectroscopy was used to investigate any phenotypic changes resulting from exposure to toluene. Metabolic profiling analysis was performed using gas chromatography-mass spectrometry (GC–MS). Principal component—discriminant function analysis (PC-DFA) was applied to the FT-IR data while multiblock principal component analysis (MB-PCA) and N-way analysis of variance (N-way ANOVA) were applied to the GC–MS data.

Results The growth profiles demonstrated the effect of toluene on bacterial cultures and the results suggest that the mutant *P. putida* DOT-T1E–18 was more sensitive (significantly affected) to toluene compared to the other two strains. PC-DFA on FT-IR data demonstrated the differentiation between different conditions of toluene on bacterial cells, which indicated phenotypic changes associated with the presence of the solvent within the cell. Fifteen metabolites associated with this phenotypic change, in *P. putida* due to exposure to solvent, were from central metabolic pathways. Investigation of MB-PCA loading plots and N-way ANOVA for condition | strain × time blocking (dosage of toluene) suggested ornithine as the most significant compound that increased upon solvent exposure.

Conclusion The combination of metabolic fingerprinting and profiling with suitable multivariate analysis revealed some interesting leads for understanding the mechanism of *Pseudomonas* strains response to organic solvent exposure.

Keywords Metabolomics • Efflux pumps • *P. putida* DOT-T1E • Toluene • Tolerance • Ornithine • FT-IR • GC–MS

1 Introduction

Bacteria can adapt to overcome the activity of toxic substances via the application of several resistant mechanisms. An exceptionally interesting stress response of *Pseudomonas putida* strains to toxic substances is the induction of efflux pumps, which, as their name suggests, remove toxic substances from the bacterial cell out to the external environment (Fernandes et al. 2003; Poole 2007; Ramos et al. 1998). This mechanism is probably the most important process that plays an absolutely crucial role in
bacterial adaptation mechanisms. The development of solvent–tolerant microorganisms that are able to grow in the presence of toxic organic solvents are useful in many applications, for example in environmental bioremediation (Nicolau et al. 2010) and biocatalysis where organic solvents are often used to dissolve the substrate and product (Ellis and Goodacre 2012).

Bioremediation is an incredibly important form of waste management that involves the conversion of harmful substances into non-harmful end products via the use of microorganisms (Bustard et al. 2000, 2002; Gupta et al. 2006; Pandey et al. 2009; Zhao and Poh 2008). Solvent tolerance is an adaptive process, as it is possible to make the bacteria tolerant to harsh environments through a number of reported methods. One approach to adapt the characteristics of microbial cells to unfavourable culture conditions has included the pre-exposure of bacterial cultures to low concentrations of toxic solvent (Ramos et al. 1998; Xin et al. 2009). Alternative methods such as genetic engineering can also be used to produce altered strains with superior tolerance characteristics, and this can be achieved through transformation of the microorganism to include a plasmid that confers degradation properties (encodes key enzymes) to specific toxic solvents (Horikoshi et al. 2011). This would allow for increased decontamination rates, so an understanding of the mechanisms of solvent toxicity is of great importance in order to explore microorganisms that exhibit sufficient tolerance, thereby enabling them to serve as bioremediation agents for specific chemical contaminants.

Whole-cell biocatalysis in two-phase systems containing an organic phase is an application for the production of specialty or fine chemicals (Heipieper et al. 2007; Neumann et al. 2006; Sardessai and Bhosle 2004). In many instances, the initial material and/or the end-product can display some toxicity to the biocatalyst, which of course leads to limited production yields or affect the overall performance (which could be biotransformation specificity) of biocatalysis. Thus, the ability to exploit these microorganisms to their full potential requires a deeper understanding of the interactions between the bacteria and organic solvents, which is an important research goal. Changes identified in the microbial metabolome can be considered to be hypothesis generating and as such can inform our biochemical knowledge (Goodacre et al. 2004; Kell and Oliver 2004). Observed metabolite changes can prove to be indicative of novel adaptation mechanisms, or may support postulated adaptation mechanisms for which there are little evidence to date.

In this study, the effect of the sudden addition of toluene to P. putida DOT-T1E, and two mutants of this strain—P. putida DOT-T1E-PS28 (lacking the TtgGHI pump) and P. putida DOT-T1E-18 (lacking the TtgABC pump)—grown in LB medium, in the presence/absence of toluene via gas phase has been investigated. Metabolomics strategies were applied, specifically metabolic fingerprinting (Ellis et al. 2007) employing FT-IR spectroscopy (Ellis and Goodacre 2006) in order to identify general phenotypic alterations in bacterial cultures exposed to toluene, and metabolic profiling using GC–MS to investigate any metabolome changes in response to solvent stress. The data sets generated via these approaches were explored further using multivariate analysis methods in order to model the metabolic effect of organic solvents on microbial species.

2 Materials and methods

2.1 Bacterial strains and culture medium

Three strains of P. putida were chosen for this study to investigate the response of bacteria to toluene stress and these are listed in Table 1, and were sourced from the Juan Luis Ramos lab (Consejo Superior de Investigaciones Cientificas, Estacion Experimental del Zaidin, Department of Biochemistry and Molecular and Cellular Biology of Plants, Granada, Spain, http://www.eez.csic.es/?q=en/node/51). Nutrient agar (NA) and lysogeny broth (LB) were used for cultivation of bacteria. NA was prepared as follows: peptone 5 g/L, beef extract 3 g/L, sodium chloride 8 g/L, 12 g/L of agar. LB medium contained: tryptone 10 g/L (Formedia, Hunstanton, UK), yeast extract 5 g/L (USP, Cleveland, USA) and NaCl 10 g/L.

2.2 Cultivation of bacteria and culture conditions

All three strains of P. putida DOT-T1E were sub-cultured in triplicate on agar plates in order to obtain pure single colonies. Cells were grown in LB and the axenic cultures were incubated overnight with horizontal shaking in an orbital incubator (Infors HT Ltd, UK) at 30 °C and 200 rpm.

2.3 Growth in response to toluene, sample collection and analysis

P. putida cells were normalised to an optical density (OD) of 0.1 and then incubated in an orbital shaker for 1 h at

<table>
<thead>
<tr>
<th>P. putida strains</th>
<th>Relevant characteristics</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>DOT-T1E</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Rif&lt;sup&gt;+&lt;/sup&gt; Tol&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Ramos et al. (1995)</td>
</tr>
<tr>
<td>DOT-T1E-PS28</td>
<td>Rif&lt;sup&gt;-&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; ttgH::VSm</td>
<td>Rojas et al. (2001)</td>
</tr>
<tr>
<td>DOT-T1E-18</td>
<td>Rif&lt;sup&gt;-&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; ttgB::'phoA-Km</td>
<td>Ramos et al. (1998)</td>
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<sup>a</sup> Resistance to Ap<sup>+</sup> ampicillin, Rif<sup>-</sup> rifampin, Sm<sup>+</sup> streptomycin, Km<sup>+</sup> kanamycin, Tol<sup>-</sup> toluene; note that the mutants have different deletions in the two genes involved in toluene tolerance (ttg)
30 °C and 200 rpm. At this point, cultures were divided into two groups: one was kept as a control and for the other toluene was supplied via gas phase for only 30 min in which an evaporation tube containing 100 μL of toluene was used in order to avoid direct contact with the culture. The culture flasks containing 50 mL of LB medium were sealed with Suba-Seal to prevent toluene leakage and then incubated for an additional 4 h. The concentration of toluene in the flask was approximately 12.5 mM (0.125 % (v/v)) under these culture conditions. Once cell cultures reached the mid-log phase, the cultures were split into two halves; to one 0.1 % (v/v) toluene was added and the other was kept as a control, and then cell cultures were incubated for additional 7 h. The tested concentration of toluene is below the minimum inhibitory concentrations (MICs) which are 5, 0.8 and 0.7 % (v/v) for DOT-T1E, DOT-T1E-PS28 and DOT-T1E-18 respectively (Table S1).

2.3.1 Bacterial growth profiles

During the time-course of 12 h incubation, (100 μL) samples were collected at various time points (0, 1, 3, 5, 7, 9, 11 and 12 h) in triplicate from each flask and from each of the exposure conditions (i.e., positive and negative samples were collected at various time points (0, 1, 3, 5, 7, 9, 11 and 12 h) in triplicate from each flask and from each of the exposure conditions (i.e., positive and negative groups) for OD measurement at 660 nm (OD$_{660}$).

2.3.2 Analysis of biomass samples by FT-IR spectroscopy

Aliquot (2 mL) samples were collected and centrifuged at 11,500×g for 5 min at 4 °C (ThermoFisher CR3.22, UK). The supernatant was removed, while the cell pellets were washed twice with 2 mL of sterile physiological saline solution (0.9 % NaCl) (Fisher, UK) and centrifuged again. The supernatant was discarded prior to storage of the cell pellet at −80 °C for further analysis (Muhamedali et al. 2015a). The OD$_{660}$ of samples were recorded for normalisation and the procedure was conducted in three replicates. Samples were defrosted on wet ice and then normalised and resuspended in saline solution and vortexed briefly. Normalised samples were randomised and spotted as 20 μL aliquots in triplicate onto a 96-well silicon FT-IR plate. The silicon plates were then dried in a desiccator at 25 °C for 7 h.

The silicon plate was loaded onto a motorised microplate module HTS-XT™ under the control of a computer programmed with OPUS software version 4. Triplicate spectra were obtained from each sample, resulting in a total of nine spectra per biological sample, therefore a total of 324 spectra were collected. Spectra were acquired by employing a Bruker Equinox 55 FT-IR spectrometer (Bruker Optics, Banner Lane, Coventry, UK) as described by Winder and co-workers (Winder et al. 2006). Transmission measurements of the samples were acquired and converted to absorbance spectra, using a deuterated triglycerine sulfate (DTGS) detector over the wavenumber range 4000–600 cm$^{-1}$, with a resolution of 4 cm$^{-1}$, 64 scans were co-added and averaged to improve the signal-to-noise ratio.

The IR data were converted to ASCII format using OPUS reader software and imported into Matlab version. 2012 (MathWorks, Natick, MA). Prior to analysis, atmospheric CO$_2$ vibrations in the 2400–2275 cm$^{-1}$ region were removed and the spectra were scaled using extended multiplicative signal correction (EMSC) (Martens et al. 2003).

Principal component analysis (PCA) was used to generate sets of latent variables (PCs) that retain the most important variance in the data whilst reducing the dimensionality (Wold et al. 1987). In addition, discriminant function analysis (DFA) was then employed, which is a supervised method that discriminates groups by a priori knowledge of sample origin. DFA attempts to maximise the differences between the known groups (classes) whilst minimising the differences within the class (Gromski et al. 2015; Johnson et al. 2003; Macfie et al. 1978). PC-DFA was conducted utilising PCs 1–10, and the class structure for the DFA algorithm was based on the biological replicates of samples from the same conditions.

2.3.3 Metabolite profiling

2.3.3.1 Sample collection and metabolic quenching

Samples were collected as 15 mL aliquots at several time points (0, 10 and 60 min) in the absence and presence of different toluene conditions (0 min refer to the point immediately before the addition of toluene shock). The metabolic activity of the collected samples were immediately quenched by adding 30 mL of cold (−50 °C) 60:40 (v/v) methanol:water followed by centrifugation at 3000×g for 10 min at 1 °C. After the centrifugation the supernatant was discarded, while the cell pellets were stored at −80 °C prior to metabolite extraction (Winder et al. 2008).

2.3.3.2 Metabolite extraction

An aliquot (750 μL) of cold (−20 °C) 80:20 (v/v) methanol:water was added to the biomass and then transferred into a 2 mL Eppendorf tube, followed by three freeze–thaw cycles to extract the intracellular polar metabolites into the polar phase. The samples were then pelleted by centrifugation (13,500×g, 3 min, 4 °C) and the supernatant stored on dry ice. This procedure was undertaken twice on the cell pellets and both extracts were combined and kept on dry ice.

Aliquots (1400 μL) of intracellular extracts were normalised according to OD$_{660}$, followed by the preparation of a quality control (QC) sample (Dunn et al. 2011; Fiehn et al. 2008). The QC sample was prepared by transferring
an equal volume of sample (100 µL) into a 15 mL centrifuge tube. Internal standard solution (0.2 mg mL\(^{-1}\) succinic-\(d_4\) acid, 0.2 mg mL\(^{-1}\) benzoic-\(d_5\) acid, 0.2 mg mL\(^{-1}\) lysine-\(d_6\) and 0.2 mg mL\(^{-1}\) glycine-\(d_5\)) was added (100 µL) to all samples. The samples were then dried for 16 h in a speed vacuum concentrator (centrifuge 5301; Eppendorf, Cambridge, UK), and stored at -80 °C prior to GC–MS analysis.

2.3.3.3 GC–TOF–MS analysis Metabolite samples were removed from -80 °C storage and re-dried for 3 h in a concentrator prior to derivatisation, in order to remove any moisture absorbed by the sample during thawing, which could interfere with derivatisation process. Samples were derivatised for GC–MS following a two stage process as described previously (Wedge et al. 2011). Briefly, an aliquot (50 µL) of O-methylhydroxylamine hydrochloride solution (20 mg mL\(^{-1}\) in pyridine) was added to all samples. The samples were then heated using a heating block at 65 °C for 40 min followed by addition of 50 µL of MSTFA (N-methyl-trimethylsilyltrifluoroacetamide) and then heated for 40 min at 65 °C. An aliquot (20 µL) of retention index solution (\(C_{10}/C_{12}/C_{15}/C_{18}/C_{22}\) n-alkanes) was added for chromatographic alignment.

The gas chromatography time-of-flight mass spectrometry (GC–TOF–MS) method was used to analyse the derivatised samples in a random order. The instrument was operated using an Agilent 6890 GC coupled to a LECO Pegasus III TOF mass spectrometer (Leco, St. Joseph, MI, USA), as described previously (Begley et al. 2009; Dunn et al. 2011) which follows metabolomic standards initiative (MSI) guidelines (Sumner et al. 2007). QC samples were employed prior to statistical analysis as described from a previous report (Wedge et al. 2011), in order to provide quality assurance of the data by the evaluation and removal of mass features that exhibit high deviation within the QC samples.

2.3.3.4 Data analysis All data collected in this study were analysed on Matlab version 2014a (Mathworks, Natick, MA). The data were analysed using multi-block PCA (Smilde et al. 2003) with three different types of blockings. Strain \(\times\) time \(\times\) condition blocking was the first type of blocking and it partitioned the data into nine blocks, each block had the samples taken under the same toluene condition and at the time points while the strains were matched across blocks. The second type of blocking was time \(\times\) strain \(\times\) condition blocking. This blocking partitioned the data into 12 blocks, each block had the samples of the same strain, same condition of toluene while the time points were matched across blocks. The last type of blocking was condition \(\times\) strain \(\times\) time blocking which partitioned the data into six blocks (this type of blocking did not include the samples at 0 min since this time point refers to the point immediately before the addition of toluene shock), each block contained all the samples from the same strain with the same time point, while the conditions of toluene were matched across blocks. Such blocking allows for the detection of the effect of each of the factors of interest (the factor which matched across different blocks, e.g. strain \(\times\) time \(\times\) condition blocking was used to detect the differences between different strains) separately without the inference from others by MB-PCA (Xu and Goodacre 2012). A total number of 116 unique GC–MS peaks were detected. The natural logarithm (ln) was used on the peak area of these peaks. Data were then mean-centred, auto-scaled then subjected to MB-PCA. The most significant variables were recognised by choosing the most predominant averaged block loadings and N-way Analysis of Variance (N-way ANOVA). These results were visualised and compared using box-whisker plots.

All FT-IR and GC–MS data are freely available at MetaboLights (http://www.ebi.ac.uk/metabolights/): study identifier MTBLS319.

3 Results and discussion

3.1 The effect of toluene on the growth of \(P.\) putida strains

Growth of \(P.\) putida cells was examined in liquid culture medium, once cells were pre-grown on LB medium with and without toluene via the gas phase. After this \(P.\) putida cultures were challenged with sudden shock of 0.1 % (v/v) toluene which is below the minimum inhibitory concentration (MIC) (see Table S1). Growth curves from \(P.\) putida cells can be seen in (Fig. 1a–c). Generally, it can be clearly noted that there is a demonstrable effect caused by the sudden addition of toluene in the flask cultures. Both non-induced and induced cells were sensitive to the sudden shock of toluene, and the final biomass of the samples decreased (as indicated by a decreased final OD reading and the final turbidity measurement being lower than negative control cells) over the 12 h incubation time-course. Our results would suggest that this decrease in the biomass could be due to energy consumption as solvent tolerance is an energy intensive process, and not due to bacterial cell death as the concentration used was below MIC. One study showed the effect of sub-lethal toluene concentrations on the growth yields of a solvent-tolerant \(Pseudomonas\) strain (Isken et al. 1999). It was found that cultures exhibited lower yields once grown in the presence of toluene and the biomass was decreased linearly with increasing toluene concentrations, suggesting that high levels of energy are extremely important for solvent tolerance in order to protect the cells from excessive damage.
Furthermore, the wild type \textit{P. putida} DOT-T1E and the mutant \textit{P. putida} DOT-T1E-PS28 were less sensitive to 0.1 % (v/v) toluene, compared to the mutant \textit{P. putida} DOT-T1E-18, when cells were pre-grown in the absence or presence of toluene supplied via the gas phase. In addition, to assess the accumulation of toluene and the role of efflux pumps in \textit{P. putida} DOT-T1E cells, HPLC was used to measure toluene levels in bacterial cells. Figure S1 shows the chromatograms obtained for reference toluene and bacterial cultures. To quantify the level of toluene in \textit{P. putida} cells, a calibration curve for toluene was generated (Fig. S2). As observed in Fig. S3 and Table S2, the level of toluene in the mutant DOT-T1E-PS28 and DOT-T1E-18 were twofold and sevenfold higher compared to the wild-type DOT-T1E. Therefore, these results would clearly suggest that the TtgABC pump plays a more important role in toluene efflux than the TtgGHI pump, and these observations are in agreement with previous studies which show that the TtgABC pump is the main extrusion pump for strain tolerance as it has the ability to extrude solvents and antibiotics (Duque et al. 2007; Roca et al. 2008; Teran et al. 2003). The next stage was to assess the bacterial biochemical changes during toluene stress.

3.2 FT-IR spectroscopy of collected biomass samples

FT-IR was employed to assess and compare the metabolic fingerprint of \textit{P. putida} strains under the examined conditions. All FT-IR spectral data were subjected to the supervised method of PC-DFA and the resultant DFA scores plots are displayed in Fig. 2a–c. It is evident that cells induced to toluene (vapour) cluster together significantly and separately from the non-induced cells, and also a noticeable shift was observed in the exposed cells to 0.1 % (v/v) toluene from the control cultures. This clustering pattern would suggest that toluene stress had an obvious effect on the cell cultures and may cause alterations to the phenotype of cells. In addition, it is clear to be seen that non-induced cultures collected from the 0.1 % (v/v) toluene exposed cells cluster separately from the positive cultures in the mutant strains compared to the wild-type. These clustering patterns could suggest that the parent strain was less sensitive to 0.1 % (v/v) toluene in comparison to the mutants, indicating the important role of the activity of efflux pumps in response to toluene. To ensure that the model quality is of a high standard, and that the obtained subsequent conclusions drawn from the data are valid, these PC-DFA models were validated by test set projection as can be seen in Fig. S4.

As these results show significant differences and are valid (Fig. S4), the FT-IR spectra were investigated further using the loadings plots for PC-DFA and wavenumbers with significant loadings in the PC-DFA were identified. The loadings plots of \textit{P. putida} strains for the first discriminant functions for the three strains are shown in (Fig. 2d–f). According to these loading plots the largest variances are observed between wavenumbers...
1750–1550 cm$^{-1}$ which is attributed to changes in the protein components of the cells; most notably amide I (C=O stretching at 1690–1620 cm$^{-1}$) and amide II (combination of C–N stretching and N–H bending at 1550 cm$^{-1}$). However, we also see changes in the spectral regions of 2930–2850 cm$^{-1}$ in which we would expect C–

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Fig. 2  PC-DFA scores plots of FT-IR data for three different strains of $P$. putida strains upon toluene stress. Symbols show different strains. a $P$. putida DOT-T1E (stars) and the first 10 PCs with a total explained variance (TEV) of 99.94 % were used for the DFA, b $P$. putida DOT-T1E-PS28 (triangles) and PCs 1–10 with TEV of 99.93 % were used for the DFA, c $P$. putida DOT-T1E-18 (circles) and first 10 PCs with TEV of 99.90 % were used for the DFA. Colours coding represent different conditions: control cultures—no toluene (red), cultures exposed to 0.1 % (v/v) toluene (black), toluene gas (brown), toluene gas and sudden 0.1 % (v/v) toluene (blue). Arrows indicate the direction of shift because of the presence of toluene. d PC-DFA loadings plot for $P$. putida DOT-T1E, e DOT-T1E-PS28 and f DOT-T1E-18. Significant loadings were assigned to bacterial proteins and lipids.
H stretching from fatty acids to occur. These results would indicate that metabolites within the amide and fatty acid regions contributed to differential responses to toluene challenge. Therefore, the most significant effects of toluene stress on bacterial cultures would be associated with changes in proteinaceous and lipid components of bacteria. Indeed, previous proteomic analysis has revealed that a number of proteins were up-regulated as a result of exposure of \textit{P. putida} DOT-T1E or S12 strains to toluene stress (Segura et al. 2005; van der Werf et al. 2008; Wijte et al. 2011). In addition, changes in lipid compositions of DOT-T1E and S12 strains have also been shown to be involved in solvent tolerance in order to adapt membrane fluidity to the presence of toluene (Bernal et al. 2007; Ramos et al. 1997). Unsurprisingly, the interpretation of FT-IR spectra showed the most significant changes in the frequency of the proteins and lipid components. Our observations can deduce that some proteins were up-regulated and also lipid compositions were altered in response to toluene by \textit{P. putida} DOT-T1E strains. As the FT-IR results showed that there was an effect of toluene on the phenotype of \textit{P. putida} strains, GC–MS was employed as a metabolic profiling approach to specifically identify the significant metabolites.

3.3 Metabolic profiling with GC–MS

The aim of metabolite profiling is to measure all, or more realistically a subset of the metabolites present in the sample, and several analytical platforms can be employed for metabolic profiling (Dunn 2008; Ellis and Goodacre 2012; Fiehn 2002). In recent years much attention has been focused on studying the stress responses in microorganisms employing metabolomics-based approaches (Allwood et al. 2015; Brito-Echeverria et al. 2011; Kol et al. 2010; Muhamadali et al. 2015a, b). The knowledge of variations within the metabolome following exposure to a stressor could lead to a more in-depth understanding of strain stress responses within these bacteria, therefore we employed GC–MS for metabolic profiling.

As we have multiple interacting factors (viz. strain, condition, and time) we used multi-block PCA to allow these factors to be analysed independently, an approach we have used successfully before (Xu and Goodacre 2012). Therefore to investigate the general metabolic effect of toluene on \textit{P. putida} cells, MB-PCA with condition | strain × time and time | condition × strain were undertaken and the results are presented in Figs. 3 and 4 respectively. As can be seen in Fig. 3, a slight separation between the non-exposed and exposed cultures to toluene is observed, indicating that there are metabolic changes caused by toluene. An MB-PCA score plot was also conducted to investigate the metabolome alteration of \textit{P. putida} cells during the time course of the exposure and as can be seen in Fig. 4, the scores of the 0 min time point are located in the bottom right and as time of incubation increases the cluster spreads from bottom to top. This clearly indicates that \textit{P. putida} cells have different metabolic responses at different time points. Following MB-PCA, the next objective was to identify which metabolites were significantly changed between different conditions or time points.

The loading plots were inspected for the most significant peaks (Table S3) and \textit{N}-way ANOVA statistical test were conducted (Table S4), and the top significant features were selected whose \textit{p} value computed by \textit{N}-way ANOVA was below 0.05 and also its corresponding false discovery rate (FDR) was below 0.05. A list of the identified metabolites can be seen in Table S5. Interestingly, both statistical methods for condition effect (exposure to solvent) suggested variable 54 as a highly significant feature which was identified as ornithine by our in-house library (Sumner et al. 2007; Brown et al. 2009). Figure 5 shows that the levels of ornithine in the non-exposed cultures to toluene are significantly lower than exposed cells. This might indicate a requirement for this metabolite for strain tolerance. It is noteworthy that in the induced cultures to toluene, 60 min following exposure to toluene, the level of ornithine was the highest among the other conditions and this could reveal that under this condition, the cultures exposed longer to low concentrations of toluene may allow for the cells to resist harsh conditions and sudden shock of stress. This could result in increasing the production level of ornithine in order to cope with toluene stress. In contrast, our previous study examined the effect of propranolol on \textit{P. putida} DOT-T1E cells which showed that the ornithine was only produced following the exposure of \textit{P. putida} strains.
to propranolol but was not found in the control, which also suggests that ornithine could be linked directly to the generalised strain tolerance to toxic assault (Sayqal et al. 2016). Our observations in this present study would infer that the production of ornithine in the control cultures is due to oxidative stress resulting from sealing the flask cultures with Suba-Seal rubber to prevent the toluene leakage from the flask in the exposed cultures.

Subsequently, we tested whether plugging the flask with Suba-Seal reduced the level of oxygen in the flask cultures. To ensure that the cells used for metabolic profiling analysis were exposed to oxidative stress, the flask cultures were plugged with both cotton wool and Suba-Seal rubber and then incubated at 30 °C and 200 rpm for 24 h. The resultant growth curves are shown in Fig. S5. The cultures that were plugged with Suba-Seal rubber exhibited slower growth compared to the control groups over the incubation time, which may indeed be a result of reduced oxygen level in the cultures promoting slower growth.

Mahendran and colleagues demonstrated the effect of using various oxygen regimes on growth patterns of Pseudomonas spp. for the biodegradation of aromatic hydrocarbons, and their results showed that all strains have the ability to grow and degrade the aromatic hydrocarbons under varying oxygen levels but in a differing manner (Mahendran et al. 2006). In the DOT-T1E strain, the presence of solvents resulted in the up-regulation of several terminal oxidase genes, suggesting adaptation by P. putida DOT-T1E to solvents as well as to variable aerobic and microaerobic conditions, a situation that demands the consumption of energy in order to cope with the stress (Rojo 2010). In the DOT-T1E (as well as the S12 strain they studied), proteomics analyses revealed that the up-regulation of several proteins of the TCA cycle involved in energy production upon exposure to solvents indicates a requirement for enhanced metabolism and high energy demand in order to power efflux pumps (Segura et al. 2005; Udaondo et al. 2012). As previously reported for P. putida, ornithine could be synthesised in several steps from glutamate through the TCA cycle where most of the energy production occurs (Antonia Molina-Henares et al. 2010). Our observation would suggest that the ornithine production in the presence of toluene is interesting, as the

**Ornithine**

- **Variable id. = 54**

![Box-whisker plot showing the alterations in ornithine levels (variable id 54) in control and cells exposed to toluene for four biological replicates. The red lines represent the median m/z intensity. Box plot represents the data for three P. putida strains, four conditions of toluene and three time points, dashed lines separate different conditions of toluene and solid line separates different strains. Code: control—no toluene (C), cells exposed to 0.1 % (v/v) toluene (T), toluene gas (G), and toluene gas and 0.1 % (v/v) toluene (GT).](image-url)
Fig. 6  Schematic metabolic diagram of central carbon metabolism in P. putida DOT-T1E adapted to toluene. Metabolites were detected and identified by GC–MS. Metabolites indicated in black were observed, while metabolites indicated in grey were not detected. a Represent the level of metabolites at 10 min after toluene exposure, and b at 60 min. Box-whisker plot showing the changes in metabolite levels in control and cells exposed to toluene for four biological replicates. The red lines indicate the median m/z intensity. Code: control—no toluene (C), cells exposed to 0.1 % (v/v) toluene (T), toluene gas (G), and toluene gas and 0.1 % (v/v) toluene (GT)
P. putida strain might activate the metabolic pathways for ornithine to demand energy to power efflux pumps due to the high activity of efflux pumps. Alternatively this may be related to other metabolic pathways that are important in response to toluene in P. putida.

In this work, we aimed to investigate similarities and differences in the levels of metabolites in the central metabolic pathways between the wild type and the mutants in P. putida DOT-T1E when cells had been pre-grown on LB medium in the absence or presence of toluene supplied via the gas phase, and these cells were then challenged with 0.1 % (v/v) toluene.

Rather than just concentrating on a single metabolite difference we also studied the level of metabolites for each bacterial strain independently. As we used GC–MS for untargeted metabolic profiling we were able to identify the changes in the levels of metabolites during toluene stress in central carbon and nitrogen metabolism. Schematic summaries of central metabolic pathways in response to toluene in P. putida DOT-T1E, P. putida DOT-T1E-PS28, and P. putida DOT-T1E-18 are shown in Figs. 6a, b, S6A, B and S7A, B, respectively. In general, the mutants had similar patterns in the levels of metabolites compared to the wild type.

A direct observation from the metabolomic analysis is that the pool of amino acids (e.g. serine, glycine, alanine, valine, leucine, tryptophan, phenylalanine, tyrosine, lysine, methionine, isoleucine, threonine and ornithine) increased under the exposure of cells to toluene conditions at 10 min (shown in panels A of these figures) followed by an increase or decrease at 60 min (panel B). The levels of the most detected metabolites in the toluene adapted cells followed by toluene shock (GT) were higher than in the non-adapted cells. This observation would indicate that in the presence of vapour toluene, cells might activate metabolic pathways that are involved in toluene tolerant mechanisms prior to toluene shock. With the result of the production of higher levels of metabolites in comparison to non-induced cells, in order to cope with toluene stress and prevent cell death. A previous study found that exposure of P. putida DOT-T1E cultures to toluene supplied via the gas phase resulted in more rigidity of the cell membranes compared to non-exposed cultures (Ramos et al. 1998), and this may also be observed in this study from the FT-IR analyses (Fig. 2). In addition, under the same conditions it was revealed that the expression level of the ttgGHI operon in P. putida DOT-T1E was higher in the induced cells in comparison to non-induced cells (Rojas et al. 2001). Therefore, our observation would suggest that an increased pool of amino acids would illustrate the participation of metabolites in response to toluene stress. However, 60 min following the exposure to toluene, the glutamine levels were slightly deceased in exposed cells compared to the control. It is possible that the level of glutamine was decreased as glutamate might be converted into ornithine instead of glutamine, as ornithine would be the key stress-responsive metabolite involved to cope with stresses following perturbation by toluene.

4 Conclusion

Our study shows that metabolic fingerprinting and profiling of P. putida cells by FT-IR and GC–MS analyses provides valuable information on the biological changes in these bacterial cultures upon toluene exposure. The growth profiles demonstrated the effect of toluene on bacterial cultures and the mutant P. putida DOT-T1E-18 was more sensitive to toluene compared to the other strains. This indicates that efflux pumps play a crucial role in strain tolerance, as also illustrated by the LC analyses of the toluene accumulation in the bacterial cells of three strains when exposed to toluene. The data collected by FT-IR shows that PC-DFA scores plots from metabolic fingerprints reveal excellent separation between non-exposed and exposed cultures to toluene and DF1 loadings vector show that several regions derived from proteins and fatty acids contribute to this separation. An FT-IR approach would be a valuable tool as it can be employed to analyse cellular response rapidly (Correa et al. 2012), thereby allowing more cost effective and high-throughput experiments to be conducted. We have also performed GC–MS analysis to monitor metabolome changes in the cultures and the results revealed that the levels of several amino acids in the central metabolic pathways of P. putida DOT-T1E strains were increased in response to toluene stress. The production of ornithine in the presence of toluene could be considered as a major key element and linked directly to solvent tolerance mechanisms. Finally, the combination of metabolic fingerprinting and profiling with suitable multivariate analysis is a valuable method for investigating solvent adaptation mechanisms in these industrially and environmentally significant microorganisms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.
Ethical approval This article does not contain any studies with human or animal subjects.

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Metabolic Fingerprinting of *Pseudomonas putida* DOT-T1E Strains: Understanding the Influence of Divalent Cations in Adaptation Mechanisms Following Exposure to Toluene

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Abstract: *Pseudomonas putida* strains can adapt and overcome the activity of toxic organic solvents by the employment of several resistant mechanisms including efflux pumps and modification to lipopolysaccharides (LPS) in their membranes. Divalent cations such as magnesium and calcium play a crucial role in the development of solvent tolerance in bacterial cells. Here, we have used Fourier transform infrared (FT-IR) spectroscopy directly on cells (metabolic fingerprinting) to monitor bacterial response to the absence and presence of toluene, along with the influence of divalent cations present in the growth media. Multivariate analysis of the data using principal component-discriminant function analysis (PC-DFA) showed trends in scores plots, illustrating phenotypic alterations related to the effect of Mg$^{2+}$, Ca$^{2+}$ and toluene on cultures. Inspection of PC-DFA loadings plots revealed that several IR spectral regions including lipids, proteins and polysaccharides contribute to the separation in PC-DFA space, thereby indicating large phenotypic response to toluene and these cations. Finally, the saturated fatty acid ratio from the FT-IR spectra showed that upon toluene exposure, the saturated fatty acid ratio was reduced, while it increased in the presence of divalent cations. This study clearly demonstrates that the combination of metabolic fingerprinting with appropriate chemometric analysis can result in practicable knowledge on the responses of important environmental bacteria to external stress from pollutants such as highly toxic organic solvents, and indicates that these changes are manifest in the bacterial cell membrane. Finally, we demonstrate that divalent cations improve solvent tolerance in *P. putida* DOT-T1E strains.

Keywords: fingerprinting; efflux pumps; *P. putida*DOT-T1E; toluene; stress tolerance; LPS; Mg$^{2+}$; Ca$^{2+}$; FT-IR

1. Introduction

Organic solvents such as benzene, toluene, styrene and xylenes are known to be highly toxic to microorganisms, as these aromatic solvents are known to partition and preferentially accumulate in the bacterial cell membrane, thereby disorganising its structure and impairing cell membrane integrity and function, ultimately leading to cell death [1–4]. Nevertheless, it has been reported that some microorganisms have the ability to assimilate these toxic organic solvents even when the solvent concentration is very high. In 1989, the first report of an organic solvent-resistant bacterium, resistant to high toxic levels of solvent, was observed [1]. Inoue and Horikoshi isolated a strain of *Pseudomonas putida* (strain HI-2000) which was able to grow in the presence of 50% (v/v) toluene. This surprising...
finding has since been confirmed by others [5–9], and the search has begun in earnest to discover the mechanisms behind this solvent tolerance.

Bacteria can defend themselves from the action of organic solvents by various adaptation mechanisms. Several studies have suggested that efflux pumps [10,11], divalent ions, such as magnesium ions [8,12], and the order organisation of cell surface lipopolysaccharides [13] contribute to solvent tolerance. In P. putida DOT-T1E, although high solvent tolerance is acquired mainly by the presence of efflux pumps [11,14], various other mechanisms contribute to organic solvent tolerance as well [15].

Solvent-tolerant microorganisms play an important role in several biotechnological applications and areas such as bioremediation, agriculture and biocatalysis [16–19]. Bioremediation involves the employment of microorganisms to convert toxic chemicals found in the environment into benign or less toxic species of chemicals [20–22]. Whole-cell biocatalysis involves the production of specialty or fine chemicals, and often employs two-phase systems in order to extract and reduce the concentration of toxic products (or indeed substrates) from the aqueous phase [23,24]. This would decrease the deleterious effects of any toxic products and hence the biocatalyst remains active, making product recovery easier and less costly [25,26]. Solvent tolerant microorganisms are a growing field of study in biotechnological applications, and more in-depth knowledge to aid in the understanding of the mechanisms of solvent tolerance is required. Researchers have suggested that genetic engineering, pre-exposure of bacterial cultures to low concentrations of toxic solvent, and magnesium ions contribute to the enhancement of solvent tolerance [4,8,27,28]. One study investigated the effect of various metal ions such as Mg\(^{2+}\), Ca\(^{2+}\), Pb\(^{2+}\) and W\(^{6+}\) on the stabilization of toluene tolerance of P. putida IH-2000, and it was found that among the ions examined, Mg\(^{2+}\) and Ca\(^{2+}\) were the most effective in stabilization of toluene tolerance, thereby suggesting that metal ions may enhance solvent tolerance in living cells [12].

Metabolomics covers the identification and quantification of the metabolome (small molecules involved in cellular metabolic processes) employing different analytical techniques [29–32]. One of the core high-throughput approaches within the expanding field of metabolomics is metabolic fingerprinting [33]. With this approach, a rapid biochemical snapshot is obtained from cells, tissue, or biofluids that have been perturbed and any changes detected and correlated with fingerprints from “normal” or typical control samples. Therefore, metabolic fingerprinting can be considered as a rapid, global, high-throughput approach to provide sample provenance (classification), which can also be utilized as a screening tool to differentiate and classify samples quickly from different biological status or origin [33]. Metabolic fingerprinting also normally entails minimal sample preparation and can be undertaken via one of a number of technologies, here, we used FT-IR spectroscopy.

FT-IR spectroscopy allows for a very rapid, high-throughput and non-destructive analysis of a broad range of sample types, and has been shown to be a valuable tool for the characterization of cultured bacteria [34–37]. Indeed, its application to the analysis of bacteria by Dieter Naumann and co-workers in the 1980s led to an explosion of activity in this area, and has subsequently continued to be applied to many others areas of research [38]. The technique involves the observation of vibrations of molecules following the interrogation of a sample with an infrared beam, and the resultant infrared absorbance spectrum represents a so-called “fingerprint” which is characteristic of any (bio)chemical substance [34,39,40].

The aim of this study was to elucidate whether divalent cations interact with efflux pumps or other resistant mechanisms to improve solvent tolerance in P. putida DOT-T1E strains, and to highlight the role of the analytical techniques to measure these microbial responses. We employed FT-IR spectroscopy to generate rapid and robust biochemical fingerprints of P. putida DOT-T1E strains: wild-type and mutants with impaired efflux pump activity. This analytical technique in combination with chemometrics can be used to observe metabolite changes that could be indicative of novel adaptation mechanisms, or support postulated adaptation mechanisms, and add to our knowledge in this important area of environmental microbiology.
2. Material and Methods

2.1. Bacterial Strains and Growth Conditions

The bacterial strains used in this study were \textit{P. putida} DOT-T1E [8], \textit{P. putida} DOT-T1E-PS28 (lacking the TtgGHI pump) [11] and \textit{P. putida} DOT-T1E-18 (lacking the TtgABC pump) [27]. \textit{P. putida} strains were routinely grown in nutrient agar plates to obtain fresh axenic cultures, which were then inoculated onto LB liquid medium and grown for 24 h at 30 °C with shaking (200 rpm) in an orbital incubator (Infors HT Ltd., Surrey, UK).

2.2. Growth in Response to Toluene in the Absence and Presence of Divalent Cations, Sample Collection and Analysis

The overnight cultures were diluted to an optical density at 660 nm (OD$_{660}$) of 0.2 in 50 mL of fresh LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene and grown overnight at 30 °C with shaking (200 rpm). All flask cultures were sealed with Suba-Seal to prevent toluene leakage.

2.2.1. Growth Curve Monitoring

Bacterial cultures were monitored at various time points (1, 5, 10, 22, 30, 48 h) using a Biomate 5 (CarePlanTM, UK) at 660 nm (100 µL samples were measured) and the growth recorded as an increase or decrease in the turbidity of cultures during incubation.

2.2.2. Analysis of Bacterial Cells by Fourier Transform Infrared (FT-IR) Spectroscopy

Sample Preparation

After 8 h incubation, cells had reached the mid-exponential phase, and 2 mL aliquots of \textit{P. putida} cultures were harvested by centrifugation at 11,500 ×g for 5 min at 4 °C (ThermoFisher CR3.22, UK). Culture supernatant was discarded, and cells washed twice with 2 mL of physiological saline solution (0.9% NaCl). Cell pellets were stored at −80 °C until further required, and the procedure was conducted in triplicate.

Samples were defrosted on wet ice, suspended in saline solution and normalised according to OD at 660 nm. From the suspension, 20 µL aliquots were pipetted in triplicate onto a 96-well silicon FT-IR sampling plate (Bruker Optics, Banner Lane, Coventry, UK). Moisture was evaporated from the samples by drying the prepared plates in a desiccator at ambient temperature for 7 h. This step was applied to avoid strong water absorption in the mid-IR region.

Instrument Setup

Prepared sample plates were then loaded onto a motorised microplate module (HTS-XT™) [41], attached to an Equinox 55 infrared spectrometer (Bruker Optics, Banner Lane, Coventry, UK), equipped with a deuterated triglycine sulfate (DTGS) detector for transmission measurements of the sample to be acquired. Spectra were collected over the wavenumber range 4000–600 cm$^{-1}$, with a resolution of 4 cm$^{-1}$, and 64 scans were co-added and averaged to improve the signal-to-noise ratio. The resulting spectra were displayed as absorbance spectra.

Data Analysis

For spectral pre-processing, infrared data were converted to ASCII format by OPUS reader software prior to statistical analysis, and imported into Matlab version 2012 (MathWorks, Natick, MA, USA). To minimize problems arising from baseline shifts, the initial step was to remove atmospheric CO$_2$ vibrations in the area of 2400–2275 cm$^{-1}$ and replace this with a trend, and the spectra were then normalised using extended multiplicative signal correction (EMSC) [42].
For cluster analysis, the unsupervised dimension reduction method of principal component analysis (PCA) [43] was conducted on the spectra in order to reduce the dimensionality of the multivariate data whilst preserving the variance, prior to the supervised clustering method discriminant function analysis (DFA). In PCA, the inputs are clustered without a priori knowledge [29]. By contrast, DFA was performed to create a set of discriminant functions (DFs) on the basis of the retained principal components (PCs) which minimize within class differences whilst maximizing the differences between the known groups (classes) [44,45]. PC-DFA was performed using PCs 1-30 and the first three DFs were extracted. The class structure for the DFA algorithm was on the basis of the biological replicates from each sample of the same conditions.

3. Results and Discussion

3.1. Effect of Toluene on the Growth of P. putida DOT-T1E Cells

The ability of P. putida DOT-T1E strains to grow on LB medium in the presence of different levels of toluene was examined, and the resultant growth curves of the three strains of DOT-T1E are displayed in Figure S1. It is evident that the growth of P. putida DOT-T1E strains were inhibited by the addition of different concentrations of toluene to the growth medium, and that the yield of bacterial cells decreased monotonically with increasing toluene concentrations. We note that in control medium, the wild-type DOT-T1E and DOT-T1E-PS28 had similar growth profiles, but that the DOT-T1E-18 mutant grew a little slower, possibly due to the fact that this strain contained higher levels of toluene compared to either DOT-T1E or DOT-T1E-PS28. Indeed, similar findings were obtained in previous reports which investigated the effects of solvents on bacterial biomass yield and it was deduced that the yields were reduced in the presence of toluene in the culture [46–48]. Solvent tolerance is an energy intensive process, thus, a possible suggestion is that the decrease in the yield could be caused by an energy-consuming adaptation mechanism such as efflux pump systems in P. putida [49–51] being used to protect the cells from further damage. It was reported that the biomass yield of P. putida S12 and DOT-T1E were reduced when grown in the presence of solvents, suggesting that solvent tolerance demand high levels of energy to cope with the solvent stress [8,52].

In Gram-negative bacteria, efflux pumps are considered to be the most important adaptation mechanism for solvent tolerance [15]. Several studies have demonstrated that an energy-dependent efflux system is responsible for the resistance to toluene in P. putida DOT-T1, DOT-T1E and S12 [11,48,49]. To evaluate the role of efflux pump systems in toluene tolerance, growth of the parent strain was directly compared with that of the mutant strains, both in the presence and absence of toluene. The growth of the wild-type was inhibited at all tested concentrations (0.1%, 0.5%, 1.0%, 5.0% (v/v) toluene), while the mutants could not grow in the media containing equal to or greater than 1% (v/v) toluene. Obviously, the mutants were found to be more sensitive to toluene than DOT-T1E, suggesting that TtgABC and TtgGHI pumps play an important role in toluene extrusion.

3.2. The Effect of Divalent Cations on the Growth of P. putida DOT-T1E Cultures in the Absence and Presence of Toluene

Many Gram-negative bacteria are less sensitive to organic solvents upon the addition of cations (most notably Mg$^{2+}$, Ca$^{2+}$) [53]. Therefore, the influence of Mg$^{2+}$ and Ca$^{2+}$ on the stabilization of the toluene tolerance of P. putida DOT-T1E cultures was investigated and the resultant growth curves are shown in Figure 1. Growth was observed when Mg$^{2+}$ was added to the LB medium containing toluene, compared with the control with the absence of metal ions. In the presence of the magnesium ions, the lag period was found to be shorter, and higher cell biomass yields were obtained. The mutants were unable to grow in the presence of toluene at 1% (v/v) without the metal ion. However, it was observed that the addition of Mg$^{2+}$ improved toluene solvent tolerance and that cultures grew after 20 h incubation time. The effect of the addition of various concentrations of magnesium ions was also tested. Growth increased in the presence of metal ions and 3.5 mM Mg$^{2+}$ was as effective for solvent
tolerance as 30 mM (Figure S2). One study showed the influence of various combined concentrations of Ca$^{2+}$ and Mg$^{2+}$ ions on the growth of P. putida IH-2000, where growth was improved by the addition of more than 0.5 mM Ca$^{2+}$ and in the presence of more than 2 mM Mg$^{2+}$ [12].

![Figure 1](image-url)

**Figure 1.** Influence of 7 mM MgSO$_4$ on growth of P. putida DOT-T1E strains in the presence of toluene. Growth curves of: (A) the wild-type DOT-T1E; (B) the mutant DOT-T1E-PS28; and (C) the mutant DOT-T1E-18. Symbols and colours represent different growth conditions. Control cultures with no toluene (blue closed diamonds), exposed cultures to 0.1% (v/v) toluene (red closed circles), 0.5% (v/v) toluene (yellow closed triangles), 1% (v/v) toluene (green closed square). Solid and dotted lines represent the absence and presence of metal ion in the culture respectively. A 1/10 dilution of 100 µL samples was prepared to determine the turbidity at 660 nm.

To test the role of metallic cations and anions on the stability of the toluene tolerance, growth was determined after incubation for 24 h at 30 °C in LB containing MgSO$_4$, MgCl$_2$, Ca(NO$_3$)$_2$, and CaCl$_2$ at 7 mM for Mg$^{2+}$ and 3 mM for Ca$^{2+}$ (other concentrations of these two cations were tested and these were found to be the most optimal (data not shown)) supplemented without or with toluene at 5% (v/v). The reason that 5% (v/v) was chosen rather than the lower toluene concentrations (0.5%, 1%) was that in preliminary experiments the most striking effect on bacterial growth/toluene tolerance was seen at 5% beyond 10 h of solvent exposure (data not shown). The P. putida DOT-T1E strain has a higher tolerance for toluene in the presence of metal ions and the lag phase period was also significantly shorter (Figure S3). These divalent cations certainly exert beneficial effects as determined by higher cell yield in the presence and absence of toluene. Addition of Mg$^{2+}$ was found to be slightly more effective than Ca$^{2+}$ in improving solvent tolerance in P. putida DOT-T1E cells at 22 h time point. With different anions, such as Cl$^-$, SO$_4^{2-}$ and NO$_3^-$, we found that similar growth patterns were obtained under the same culture conditions although the cultures were supplemented with different anions. These observations suggest that the cations were more effective than anions, or that anions may not play a crucial role for stability of solvent tolerance in P. putida strains. These results were in agreement with previous observations that Mg$^{2+}$ and Ca$^{2+}$ ions are important for bacterial solvent tolerance [8,12,53].

### 3.3. FT-IR Fingerprinting of P. putida DOT-T1E Cultures

In recent years, much attention in the literature has been paid to investigating stress responses in bacteria via the application of metabolomics-based methods [54–56], an area which has been applied to a broad range of disciplines including medical sciences, metabolic engineering and drug discovery [57–60].

In this study, a metabolic fingerprinting approach [33] based on FT-IR spectroscopy [61] was employed to study the influence of metal ions on the whole-organism phenotype of P. putida DOT-T1E strains in the presence and absence of toluene. To ensure that there was sufficient biomass for metabolomics analysis, cultures were grown in LB medium supplemented without or with 7 mM magnesium and 3 mM calcium in the absence/presence of 0.05% (v/v) toluene and incubated for 8 h (see Figure 2). The results showed that all strains have the ability to grow in the absence/presence of toluene; however, all P. putida strains had higher tolerance to toluene in the presence of divalent
cations. By contrast, similar growth profiles were observed in the culture medium supplemented with or without divalent cations in the absence of toluene.

Figure 2. Turbidity at OD 660 nm of *P. putida* strains after 8 h incubation in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. Colours represent different strains: the wild-type DOT-T1E (red), the mutant DOT-T1E-PS28 (yellow), and the mutant DOT-T1E-18 (green). Bars of the means of four replicates and error bars are standard deviations.

To generate robust biochemical fingerprints of *P. putida* DOT-T1E strains, FT-IR spectroscopy was employed. Since subtle and important variations in FT-IR spectra are not easy to interpret visually (Figure S4), chemometric methods were conducted in order to analyse these data in far more detail. Initially, a PCA scores plot was produced (data not shown) and no obvious clusters were observed in this analysis. PCA failed to discriminate data as in many previous studies [44,62]. Therefore, it would seem sensible to employ a supervised clustering approach such as DFA in order to visualise the distribution of samples based on their IR metabolic fingerprint [34]. The first and second discriminant function (DF) scores were generated to identify variation or relationships between the samples, and the resultant PC-DFA scores plot of DF1 vs. DF2 is displayed in (Figure 3). As can be seen in Figure 3, a clear separation between the wild-type DOT-T1E and the mutants DOT-T1E-PS28 and DOT-T1E-18 is observed in the first discriminant function which explains the majority of the total group variance (here the groups relate to the biological replicates and are not biased based on either the level of toluene or the addition of cations). This observation could be due to the lack of efflux pump in the mutants compared to the parent strain or an indirect effect on growth of mutant DOT-T1E-18, indicating the ability of FT-IR to discriminate between bacterial cells within the same strain. Figure 3 also clearly shows that a similar trend (through DF2) was observed between the wild-type and the mutants under the same conditions, indicating clear metabolic changes caused by metal ions in the absence and presence of toluene. The parent and the mutant strains have the same genetic background and the only difference between the three cell types is the absence of one of the efflux pump proteins in the mutants compared with the parent strain. Therefore, the results from DFA would suggest that the influence of Mg$^{2+}$ and Ca$^{2+}$ on the stabilization of the toluene tolerance of *P. putida* DOT-T1E may be due to the contribution of metal ions in other bacterial-tolerance mechanisms rather than only the efflux pump(s).
TtgABC pump, indicating that DOT-T1E-18 cells were more sensitive to 0.05\% (v/v) toluene, and is able to extrude solvents and antibiotics [63–65]. Therefore, the results from DFA clearly illustrate that the metabolic fingerprinting approach has the ability to detect agreement with previous investigations which conclude that the TtgABC pump is the main extrusion phenotype of cells.

To investigate which spectral regions discriminated between different conditions within strains, DFA loadings vectors were calculated and plotted for DF2 (Figure 4) which largely discriminated between different conditions (Figure 3). Several changes occur within these loading plots with the greatest variances being observed between 2950–2850 cm$^{-1}$, 1700–1600 cm$^{-1}$ and 1110–945 cm$^{-1}$ contributed to the DFA score plot clustering. Vibrational assignments are provided in Table 1; in this region of mid-infrared, the bands at 2918 cm$^{-1}$ and 2853 cm$^{-1}$ can be attributed to C-H stretching vibrations from membrane lipids and the peaks at 1630 cm$^{-1}$ and 1550 cm$^{-1}$ would be attributed to C=O stretching (amide I) and a combination of C-N stretching and N-H bending (amide II) vibrations, respectively, from protein components. In addition, the bands at 1105 cm$^{-1}$ and 952 cm$^{-1}$ could arise from a range of vibrations from the carbohydrates family including complex polysaccharide within the cells. These large variations in lipids, proteins and carbohydrates between different conditions within the P. putida DOT-T1E cells are due to the biological effects caused by the metal ions and toluene.

**Figure 3.** PC-DFA scores plots of FT-IR data of P. putida DOT-T1E strains in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05\% (v/v) toluene. Symbols represent different strains: P. putida DOT-T1E wild-type (stars), P. putida DOT-T1E-PS28 (closed circles), and P. putida DOT-T1E-18 (closed triangles). PCs 1-30 with a total explained variance (TEV) of 99.92\% were used for the DFA. Colour coding: control with no toluene (red), cells without toluene in the presence of 7 mM Mg$^{2+}$ (brown), 3 mM Ca$^{2+}$ (black), mixed 7 mM Mg$^{2+}$ and 3 mM Ca$^{2+}$ (green), cells challenged with 0.05\% (v/v) toluene in the presence of 7 mM Mg$^{2+}$ (dark blue), 3 mM Ca$^{2+}$ (light blue), mixed 7 mM Mg$^{2+}$ and 3 mM Ca$^{2+}$ (grey), and cells with 0.05\% (v/v) toluene in the absence of divalent cations (pink).

In addition, cells exposed to 0.05\% (v/v) toluene in the absence of metal ions in the wild-type P. putida DOT-T1E and the mutant P. putida DOT-T1E-PS28 (a mutant in the TtgGHI pump) are clustered more closely to the control cultures compared with the mutant P. putida DOT-T1E-18 (which lacks the TtgABC pump), indicating that DOT-T1E-18 cells were more sensitive to 0.05\% (v/v) toluene compared to DOT-T1E and DOT-T1E-PS28 cells. This clustering pattern would suggest that the TtgABC pump might play a more crucial role in toluene efflux than the TtgGHI pump. This observation was in agreement with previous investigations which conclude that the TtgABC pump is the main extrusion pump, and is able to extrude solvents and antibiotics [63–65]. Therefore, the results from DFA clearly illustrate that the metabolic fingerprinting approach has the ability to detect a clear effect upon the cell cultures caused by metal ions and toluene which may cause changes to the phenotype of cells.
In Gram-negative bacteria, Clifton et al. [77] reported that the removal of calcium ions from the LPS bilayer led to the destabilisation of the bilayer and mixing of LPS molecules between the inner and outer leaflets; indicating the important role of salt bridges which are formed by divalent cations (e.g., Mg$^{2+}$ and Ca$^{2+}$) with negatively charged sugar in LPS core oligosaccharide to strengthen the integrity of the outer membrane. It has been found that calcium has the ability to block the binding of a cationic antimicrobial peptide to LPS and thus decrease its antimicrobial activity [78]. The effect of

Table 1. Wavenumber regions of biological interest and assignment for *P. putida* DOT-T1E cells [66].

<table>
<thead>
<tr>
<th>Wavenumbers (cm$^{-1}$)</th>
<th>Assignment</th>
<th>FT-IR Vibrational Modes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
<td>Membrane lipid</td>
<td>Asymmetric CH$_3$ stretches mode of CH$_3$ end groups from membrane lipid</td>
</tr>
<tr>
<td>(2958–2873)</td>
<td>Membrane lipid</td>
<td>Symmetric CH$_2$ stretches mode of CH$_2$ chain from membrane lipid</td>
</tr>
<tr>
<td>Protein</td>
<td>Amide A</td>
<td>N-H stretching</td>
</tr>
<tr>
<td>(3400–3300)</td>
<td>Amide I</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>(1690–1620)</td>
<td>Amide II</td>
<td>C-N stretching and N-H bending</td>
</tr>
<tr>
<td>(1590–1530)</td>
<td></td>
<td>COOH of proteins, free amino acids, polysaccharides</td>
</tr>
<tr>
<td>(1450–1200)</td>
<td>Polysaccharides</td>
<td>C-O or O-H stretching from polysaccharides</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td></td>
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<td>(1200–900)</td>
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The outer membrane of Gram-negative bacteria is an effective barrier for many toxic agents, and divalent cations (in particular, Mg$^{2+}$ and Ca$^{2+}$) are important in the organisation of the outer membrane [67] as lipopolysaccharide (LPS) molecules are linked to each other electrostatically via divalent cations [68,69]. In several cases it has been observed that when the structure of the outer membrane of certain organisms (which are able to acquire resistance against toxic solvents (e.g., toluene)), are modified by chemical or enzymatic removal of parts of the LPS molecule or mutation, the resistance of these bacteria to these solvents is decreased [70–72]. On the other hand, Junker et al. (2001) observed that in a WbpL mutant of *P. putida* DOT-T1E, LPS may not be important for aromatic hydrocarbon tolerance [73]. If Mg$^{2+}$ and Ca$^{2+}$ are essential for the integrity of the outer membrane and LPS layer, the presence of many aromatic hydrocarbons (e.g., toluene), ethylenediaminetetraacetic acid (EDTA) and antimicrobial peptides (AMPs), lead to significant changes in the structure and function of membrane components, such as disruption and removal of lipids and proteins as well as loss of Mg$^{2+}$ and Ca$^{2+}$ [2,74–76].
AMPs, EDTA and Mg\textsuperscript{2+} on the LPS layer was examined in Gram-negative bacteria [77], showing that cationic AMPs or anionic EDTA effectively modify the LPS layer electrostatically by displacing Mg\textsuperscript{2+} ions from the LPS layer competitively, while Mg\textsuperscript{2+} tightens and stabilises the LPS layer [79].

Therefore, it is perhaps not surprising that a similar trend in the DFA scores plot between the wild-type and the mutants were observed, suggesting the contribution of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} in LPS stabilisation but not efflux pumps. This observation would suggest that the efflux pumps system in \textit{P. putida} might not require a magnesium or calcium gradient to export substrates such as toluene. In addition, the most significant changes observed from the interpretation of FT-IR spectra were in the vibration frequency of the polysaccharide, protein and lipid components, and we can infer from this that the important role of divalent cations in \textit{P. putida} DOT-T1E strain is related to LPS mechanism to cope with the presence of toluene.

Finally, the ratio of saturated fatty acid composition was calculated from the raw (Figure 5) and scaled infrared spectra (Figure S5) to investigate the effect of divalent cations and toluene on \textit{P. putida} DOT-T1E strains. It is clear that upon toluene exposure, the saturated fatty acid ratio (CH\textsubscript{3}:CH\textsubscript{2}) was lower compared to the control cultures in the absence of these divalent cations. This result is in agreement with previous observations showing that the fluidity of \textit{P. putida} S12 outer membrane increased in the presence of toluene, as toluene may displace divalent cations from the LPS layer, causing increased membrane permeability [80]. By contrast, the saturated fatty acid ratio of \textit{P. putida} cells was increased with the addition of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} to medium with and without toluene. However, under the same conditions there was a slight decrease in the saturated fatty acid ratio for \textit{P. putida} DOT-T1E-PS28 in the presence of Mg\textsuperscript{2+}. In \textit{Pseudomonas aeruginosa}, Schneck \textit{et al.} [81] were able to show that the conformation of the O-antigen was shorter and had a denser layer in the presence of Ca\textsuperscript{2+} compared to the absence of calcium ions. Our results would suggest that divalent cations are essential for the integrity of the LPS layer and the outer membrane and therefore they may play an important role to improve solvent tolerance in \textit{P. putida} cells.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{box-whisker.png}
\caption{Box-whisker plot for FT-IR scaled spectra showing the ratio of saturated fatty acids (CH\textsubscript{3}:CH\textsubscript{2}) of \textit{P. putida} DOT-T1E strains grown strains in LB medium supplemented with or without 7 mM magnesium and 3 mM calcium in the absence and presence of 0.05% (v/v) toluene. Red lines indicate the median of peak area of saturated fatty acid ratio of infrared spectra. The median was used to compare the level of saturated fatty acid ratio. Red plus signs represent the outliers.}
\end{figure}
4. Conclusions

In this study we have shown that different levels of toluene inhibit the growth and reduce the biomass yields of *P. putida* DOT-T1E strains, suggesting that solvent tolerance demands high levels of energy to cope with toluene stress. In addition, our results clearly show how divalent cations improve toluene tolerance in *P. putida* cells, indicating that Mg\(^{2+}\) and Ca\(^{2+}\) ions are important for bacterial solvent tolerance. We report that results of PC-DFA from metabolic fingerprinting show obvious separation between different culture conditions and the DFA loadings vectors reveal that several mid-infrared regions derived from lipids, proteins and polysaccharides contribute to this separation. Since results from PC-DFA obtained from the wild-type strain show a very similar trend to that of the mutant cells, it is clearly demonstrated that the influence of divalent cations to improve toluene tolerance in *P. putida* cells may be correlated to other bacterial-tolerance mechanisms including lipopolysaccharides (LPS), but they do not contribute to efflux pumps. Furthermore, divalent cations increase the saturated fatty acid ratio of *P. putida* cells, indicating that Mg\(^{2+}\) and Ca\(^{2+}\) would be essential for the integrity of the LPS layer and the outer membrane and therefore improve solvent tolerance in bacterial cells.

In conclusion, we have demonstrated that metabolic fingerprinting with appropriate chemometric analysis is a valuable approach for studying the influence of divalent cations on the stabilization of the toluene tolerance of *P. putida* DOT-T1E cultures, advancing our understanding of the role of metal ions in these environmentally and industrially important bacterial cells.

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**Author Contributions:** Ali Sayqal conducted the biological experiments. Yun Xu contributed to the data analysis and Drupad K. Trivedi participated in data processing. Najla AlMasoud participated in the laboratory work and David I. Ellis provided assistance in reporting the study. Royston Goodacre contributed to this work through supervision and guidance of the study.

**Conflicts of Interest:** The authors declare that they have no conflict of interest. Compliance with ethical requirements: This article does not contain any studies with human or animal subjects.

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