Application of green fluorescent protein to measure antimicrobial efficacy and the kinetics of cell death against *Escherichia coli*

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1 REVISION

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Highlights

- Fluorescent intensity of cytosolic eGFP is strongly correlated with viability.
- Assay enables the kinetics of cell death in response to antimicrobials to be monitored in real time.
- Differentiates between biocidal and biostatic effects.
- Demonstrated differences in antimicrobial efficacy against growing and non-growing cells.

Abstract

Industrial antimicrobials have been extensively used to control unwanted microbial growth by incorporation into a variety of products such as plastics and paints, reducing biodeterioration and biofouling and extending the lifespan of the product. Industrial antimicrobials generally have broad sites of action affecting core cellular functions such as central metabolism, enzyme function, cell wall or DNA synthesis and can either be biocidal or biostatic. In addition, susceptibility can be affected by the metabolic state of the microbe, with metabolically inactive cells generally more resistant than metabolically active cells. Previously it was demonstrated that cytosolically expressed green fluorescent protein could be used as a real-time viability indicator in the yeast *Aureobasidium pullulans* based on the pH dependent fluorescence of GFP and the collapse of the proton gradient across the cell membrane during cell death. In this study we report on the development and validation of an equivalent GFP fluorescence viability assay in *Escherichia coli* and used this assay to study the effect of five antimicrobials commonly used in plastics; 4,5-dichloro-2-octyl-isothiazol-3-one (DCOIT), sodium pyrithione, 1,2-benzisothiazol-3-one (BIT), 2-Octyl-isothiazol-3-one (OIT) and n-butyl-1,2-benzisothiazol-3-one (BBIT). The results demonstrate broad differences amongst the antimicrobials in both relative efficacy, rate of effect and for some antimicrobials, marked differences in sensitivity toward growing and non-growing cells.

Key words

Antimicrobial efficacy, eGFP, *Escherichia coli*, viability assay

1. Introduction

Industrial antimicrobials refer to inorganic and organic compounds with antimicrobial activity that are extensively used commercially by incorporation into a range of commodities including plastics, paints, textiles, wood composites, cosmetics and pharmaceuticals as well as in swimming pools and metal working fluids where they act as protectants against microbes (Rossmoore, 1995; Karsa and Ashworth, 2002; Chapman, 2003; Swedish Chemical Agency, 2012a). In the plastics industry, consumption of antimicrobials was estimated to be ca. 15.4 million tonnes in 2005 with a value of ca. £34 million (Markarian, 2006). While accurate figures are difficult to ascertain, it has been estimated that the true cost of biodeterioration of non-food items globally due to microbial activities could reach \$40 billion annually (Allsopp, 2011).

Industrial antimicrobials can be largely classified depending on their site of action such as the cell wall, membrane or cytoplasm and often have broad physiological and biochemical effects which are still largely uncharacterised in detail (Denyer, 1995; Paulus, 2005; Lambert 2008). Many target essential cellular processes leading to a cessation of growth and/or cell death. For example, the widely used isothiazolone agents have been shown to target the thiol group of proteins including hydroxylases leading to metabolic impairment, whereas cationic agents such as chlorhexidine are thought to act primarily at the cell surface (Denyer, 1995; Maillard, 2002; Paulus, 2005).

Testing the efficacy of single or combinations of antimicrobials has largely relied on direct or indirect measurements of growth retardation against specific test

organisms using disc diffusion or broth dilution assays and many ISO standards for determining antimicrobial efficacy are still based on these methodologies (Bloomfield, 1991; Swedish Chemical Agency, 2012b; Kun and Marossy, 2013). Attempts have been made to introduce alternative methods for evaluating cell viability and antimicrobial efficacy in recent years (Davey, 2011). For example, bioluminescence in engineered bacterial strains including *E.coli* have been shown to be useful *in situ* markers of cell viability as light generation is a metabolically active process relying on ATP production by viable cells (Jones et al., 2002; Alloush et al., 2003; Robinson et al., 2011; Thorn et al., 2013). Other methods studied have included the reduction of tetrazolium salts, live/dead cell staining and measuring ATP content (Petty et al., 1995; Boulos et al., 1999; Zhang and Fang, 2004; Aiken et al., 2011). Intracellular GFP production in GFP transformed bacterial cells has also been shown to be a useful indicator of cellular growth and growth inhibition by antimicrobials (Lehtinen et al., 2006; Deng et al., 2009; Hoogenkamp et al., 2015).

Previously, an *in vitro* assay based on the dependence of the fluorescence intensity of green fluorescent protein (eGFP) on intracellular pH (Kneen et al, 1998) has been used as a real time indicator of cellular viability in the biodeteriogenic fungus *Aureobasidium pullulans* (Webb et al., 2001; Sabev et al. 2004). In this assay, constitutively expressed cytosolic eGFP was brightly fluorescent in viable cells due to the energy dependent maintenance of an intracellular cytosolic pH of ca. 7.5-8.0. However, in an acidic buffer or medium, cell death leads to the subsequent loss of intracellular pH regulation, the acidification of the cytoplasm and consequent pH dependent reduction in eGFP fluorescence which can be quantified in real time and correlates with cell viability.

In this study, we extended this approach by developing and validating an analogous eGFP fluorescence viability assay in *E. coli* and monitored the response of both log phase (growing) and stationary phase (non-growing) cells in real time following antimicrobial challenge with agents commonly incorporated into plastics (D'Arcy, 2001).

2. Materials and Methods

2.1. Antimicrobials and source

With the exception of 4,5-dichloro-2-octyl-isothiazol-3-one (DCOIT) (Sanitized, Switzerland) which was in powder form, all antimicrobials were supplied as liquids as follows: sodium pyrithione (40%), Arch, UK; 1,2-benzisothiazol-3-one (BIT, 20%), Thor, UK; 2-Octyl-isothiazol-3-one (OIT, 100%), Sanitized, Switzerland; n-butyl-1,2-benzisothiazol-3-one (BBIT, 100%) Sanitized, Switzerland. Antimicrobials were dissolved in 10% (v/v) ethanol in PBS (pH 6.0) or LB (pH 6.0) to give stock solutions of 4% and 8% and used to challenge stationary or log phase cells respectively.

2.2. Strains and culture maintenance

Escherichia coli (BL21-DE3), which constitutively expresses cytosolic eGFP under the t7 promotor (Chew et al, 20112), was revived from glycerol stock cultures stored at -80°C on LB agar (Sigma-Aldrich, UK) at 30°C for 2 days.

2.3. Cultures, growth and viability determination

E. coli (BL21-DE3) was cultured in 250 ml conical flasks containing 50 ml LB (adjusted to pH 6.0 before inoculation) and incubated at 25°C on a rotary shaker at 200 rpm. Cultures were incubated for 18 h (stationary phase as determined by no additional increase in OD₅₂₀ over 4 consecutive hours). For stationary phase tests,

cells were harvested by centrifugation at 3000 X g for 10 min, the supernatant discarded and the cells washed three times by resuspending in 50 mM phosphate buffered saline (PBS, adjusted to pH 6.0) and centrifuging at 3000 X g for 10 min. After the final wash, the resulting pellets were re-suspended in 50 ml PBS (pH 6.0) and incubated at 25°C for at least 6 h prior to use. For log phase tests, 250 µl of the 18 h overnight culture was used to inoculate 50 ml of LB broth (adjusted to pH 6.0) and used immediately.

2.4. Correlation between culture absorbance, fluorescence and cell viability

In order to determine if eGFP fluorescence intensity could be used as an indicator of cell viability for *E. coli* (BL21-DE3), 300 µl of LB broth (adjusted to pH 6.0) inoculated with *E. coli* (BL21-DE3) was aliquoted into wells of a flat bottomed 96 well plate (Corning, UK) and incubated at 25°C in a microplate reader (Synergy HTMulti-Mode Microplate Reader, Biotek, UK) and fluorescence (excitation 488 nm, emission 520 nm) and absorbance (OD₅₂₀) measured at 20 min intervals for up to 24 h from 8 wells with 15 sec shaking immediately prior to the reading. Periodically, 5 µl of cell suspension was removed from triplicate wells, serially diluted in PBS, plated in triplicate onto LB agar plates and incubated at 25°C for 48 h after which CFU per ml of the original culture was determined.

2.5. Antimicrobial efficacy testing

2.5.1. Stationary phase antimicrobial efficacy determination

For stationary phase tests, 150 µl aliquots of the cells suspended in PBS (pH 6.0) and shaken for 6 h at 25°C, were aliquoted into wells of a flat bottomed 96 well plate (Corning, UK) and 150 µl of PBS containing 8% or 4% antimicrobial agent added to give final concentrations of 4% and 2% respectively and a final ethanol

concentration of 2.5% (v/v). To determine background fluorescence due to the antimicrobial agents, 150 µl of PBS replaced the stationary phase cells. Wells containing 150 µl of the cell suspension and 150 µl of PBS were used as antimicrobial free controls and wells containing 300 µl of PBS were used as negative PBS controls. Plates were incubated in a microplate reader (Synergy HTMulti-Mode Microplate Reader, Biotek, UK) at 25°C. Fluorescence (excitation 488 nm, emission 520 nm) was measured at hourly intervals up to 24 h with 15 s shaking immediately prior to each measurement. To calculate the impact of the antimicrobials, the background fluorescence from antimicrobial-containing wells in the absence of cells was subtracted from the wells containing the antimicrobial agent and cells. Data was plotted as the mean fluorescence of triplicate wells following background subtraction.

2.5.2. Log phase antimicrobial efficacy determination

For log phase tests, 150 µl aliquots of stationary phase cells suspended in 50 ml of LB broth (pH 6.0) were aliquoted into wells of a flat bottomed 96 well plate (Corning, UK) and incubated for 15 h in a microplate reader (Synergy HTMulti-Mode Microplate Reader, Biotek, UK) at 25°C and fluorescence (excitation 488 nm, emission 520 nm) measured at hourly intervals with 15 s shaking immediately prior to each measurement. After 15 h, the 96 well plate was removed and 150 µl of LB broth (pH 6.0) containing 8% or 4% antimicrobial agent added to give final concentrations of 4% and 2% respectively and a final ethanol concentration of 2.5% (v/v) and incubated for a further 9 h. To determine background fluorescence due to the biocides, 150 µl of PD broth (pH 6.0) replaced the cell suspension. Antimicrobial free controls in which there was no antimicrobial challenge were prepared by adding 150 µl of LB broth (pH 6.0) lacking antimicrobial agent to the wells and wells containing 300 µl of media (no cells) were used as negative controls. To calculate the impact of

the biocides, the background fluorescence from wells containing LB medium in the absence of cells into which antimicrobial agent was added was subtracted from the wells containing biocide and cells. Data was plotted as the mean fluorescence of triplicate wells following background subtraction.

3. Results and discussion

Conventional antimicrobial efficacy testing requires time consuming viable colony counts or indirect measures of growth and growth inhibition. Here we used a constitutively expressed cytosolic eGFP in *E.coli* (BL21-DE3) (Chew et al., 2012) that can readily distinguish between bacteriocidal and bacteriostatic effects by measuring changes in eGFP fluorescence in real-time.

3.1. Correlation between eGFP fluorescence and cell viability in *E. coli* (BL21-DE3).

E.coli (BL21-DE3) was grown in LB broth in a 96 well microtitre plate format and GFP fluorescence, absorbance and viable CFU determined periodically. The correlation between fluorescence intensity and viable CFU is shown in Figure 1. Fluorescence was shown to be significantly ($P < 0.05$) correlated with viable CFU up to ca. 5×10^8 CFU ml⁻¹ with an R^2 of 0.9955 indicating fluorescence is a reliable indicator of cell viability. Recently, Alvalos Vizcarra et al (2013) also demonstrated a strong positive correlation between intracellular eGFP fluorescence and cell viability in *E. coli* (BL21-DE3) expressing eGFP under the control of the *tac* promoter, although in this study, eGFP was used to measure the percentage fluorescing cells microscopically rather than total fluorescence emission on a plate reader.

3.2. Efficacy of antimicrobial agents against stationary phase and log phase cells of *E. coli* (BL21-DE3).

Numerous studies have indicated that the sensitivity of microbes to antimicrobial agents can be influenced by the metabolic state of the organisms and that non-growing cells are often more resistant compared to growing cells (Prax and Bertram, 2014). In order to study if antimicrobial efficacy was influenced by the metabolic state of *E. coli* (BL21-DE3), each biocide was used to challenge cells from both the stationary phase (non-growing) and log phase (growing).

3.2.1. Efficacy of antimicrobial agents against stationary phase cells

To study the effect of the antimicrobials on stationary phase cells, cells were grown for 18 h (stationary phase), washed and resuspended in PBS (pH 6) and shaken for 6 h before challenging with the antimicrobial agent and fluorescence monitored over 24 h (Fig 2A, C, E, G and I).

BBIT at both 2% and 4% was highly effective at reducing fluorescence (Fig 2A). After only 4 h, both concentrations of BBIT showed a ca. 50% reduction in fluorescence when compared to the antimicrobial free control. For 2% BBIT, fluorescence remained approximately constant after 4 h whereas at 4% BBIT, fluorescence decreased further up to 24 h indicating a further loss in viability. Similarly, BIT also reduced fluorescence in *E. coli* (BL21-DE3) at both 2 % and 4 % but in this case there was a gradual decrease over 24 h by ca 30% (Fig 2C).

Addition of 2% sodium pyrrhione to *E. coli* (BL21-DE3) caused a ca. 50% reduction in fluorescence within the first hour after addition and then remained approximately constant up to 24 h whereas 4% sodium pyrrhione rapidly reduced fluorescence to almost zero within the first hour (Fig 2E).

OIT caused a rapid reduction in fluorescence of ca. 35% against *E. coli* (BL21-DE3) within 1 h (Fig 2G) with little further affect thereafter at both 2% and 4 % and

DCOIT, a dichlorinated analogue of OIT, was also effective against *E. coli* (BL21-DE3) causing a slow decrease in fluorescence over the first 8 h to ca. 50% and 20% at 2% and 4% respectively and changed little thereafter (Fig 2I).

3.2.2. Efficacy of antimicrobial agents against log phase cells

In order to study the efficacy of antimicrobial agents against growing (log phase) cells, *E. coli* (BL21-DE3) was cultured in LB broth for 15 h in a microtitre plate before challenging with the antimicrobial agent at 2% or 4% and the effect on eGFP fluorescence determined over the subsequent 9 h.

2% BBIT had little effect on *E. coli* (BL21-DE3) log phase cells while 4% caused a gradual reduction in fluorescence to ca. 40% respectively 9 h after antimicrobial addition (Fig 2B). When BIT was added to log phase cells of *E. coli* (BL21-DE3), cells reacted rapidly with a reduction in fluorescence to ca. 6% within 1 h of addition at both concentrations compared to the antimicrobial free control and remained low thereafter (Fig 2D).

Sodium pyrrhione caused a reduction in fluorescence in *E. coli* (BL21-DE3) log phase cells to ca. 20% and ca. 5% within 1 h when challenged with concentrations of 2% and 4% respectively and then remained approximately constant thereafter (Fig 2F).

OIT displayed little efficacy against log phase cells of *E. coli* (BL21-DE3) at a concentration of 2% while at 4% fluorescence fell slowly to ca. 75% following antimicrobial addition (Fig 2H). DCOIT showed a similar effect against *E. coli* (BL21-DE3) with only 4% causing a reduction to ca. 75% after 24 h (Fig 2J).

A summary showing the effects of each antimicrobial agent on fluorescence of stationary phase cells after 24 h and 1 h or 9 h after antimicrobial addition to growing log phase cells compared to the antimicrobial-free control is shown in Table 1.

3.3. Comparison of antimicrobial agent efficacy and the effect of growth phase

The antimicrobial agents used in this study have broad industrial applications including preservation of paints, adhesives and plastics as well for controlling microbial growth in cosmetics and industrial coolants such as metal working fluids and are used in this study at concentrations typically used in these applications (Denyer, 2005; Paulus, 2005; Rossmoore, 2005).

Comparing the effects of the antimicrobial agents, distinct differences could be seen both in terms of relative efficacy and in the rate of loss of viability. While some antimicrobials affected cells over the course of several hours and were relatively slow acting, for example BIT and BBIT on stationary phase *E. coli* (BL21-DE3) cells (Fig 2A and 2C, Table 1), others had a rapid effect with their maximal effect within 1 h of exposure, for example 4% sodium pyrithione on stationary phase cells (Fig 2E, Table 1). Sodium and zinc pyrithiones are thought to affect microbial cell membranes of both bacteria and fungi (Hyde and Nelson, 1984; Khattar et al, 1988) and pyrithiones have been shown to effect substrate uptake, ATP synthesis and cause membrane depolarization leading to cell death (Dinning et al, 1998a,b; Khattar et al, 1988; Khattar and Salt, 1993; Ermolayeva and Sanders, 1995; Yasokawa et al., 2010) and would therefore be predicted to collapse the proton gradient leading to rapid cytoplasmic acidification and loss of eGFP fluorescence and viability. Log phase cells were more sensitive than stationary phase cells to some biocides (e.g. BIT, Table 1), while others appeared to effect stationary phase cells

more than log phase cells (e.g. OIT, Table 1). As the RFU for stationary phase cells and log phase cells at the point of biocide addition differed, an experiment using 2% and 4% BIT was conducted on stationary phase cells with the initial RFU adjusted to 2,000, 3,000, 4,000 and 5,000 RFU. There was no significant difference in the effect of either 2% or 4% BIT at the different cell densities demonstrating that differences in cell densities was not a factor in biocide efficacy (Results not shown).

DCOIT, OIT, BIT and BBIT are members of the Isothiazolone antimicrobials which are thought to have multiple modes of action but all appear to effect thiol rings within proteins leading to ring opening and deactivation thereby inhibiting key metabolic processes including energy generation through the Krebs cycle as well as causing free radical accumulation (Williams 2007) and may explain the generally slower effect seen in this study. Interestingly, while BIT and BBIT, which differ only in an additional butyl group, were both active against stationary phase cells of *E. coli* (BL21-DE3) (Fig 2A and 2B), both 2% and 4% BIT reduced fluorescence to almost zero against log phase cells of *E. coli* (BL21-DE3) (Fig 2D) whereas BBIT was only active at 4% and showed less efficacy compared to BIT (Fig 2D) although both antimicrobial agents have been reported to have bactericidal activity (Nichols, 2005, Table 1). Similarly DCOIT and OIT, which differ only in DCOIT containing two chlorinated groups, were both active against *E. coli* (BL21-DE3) stationary phase cells (Fig 2G and 2I) but less effective against *E.coli* log phase cells (Fig 2H and 2J). Both antimicrobial agents have also been reported to have a broad antimicrobial activity (Paulus, 2005).

For several of the antimicrobials, an initial decrease in fluorescence against log phase cells was followed by growth (for example, DCOIT against log phase cells; Fig 2J, Table 1) suggesting the concentrations used temporarily inhibited growth but

that it later recovered. Likewise, several agents were able to reduce the viability of a proportion of stationary phase cells while the remainder were still viable (for example BBIT and OIT against *E. coli* (BL21-DE3), Fig 2A and 2G respectively). This is not unexpected since antimicrobial agents show differing efficacies due to phenotypic variability within the same population (Turnidge and Paterson, 2007; Jorgensen and Ferraro, 2009).

4. Conclusion

Overall, this study has demonstrated the value of using eGFP as an *in situ* viability indicator in assessing the efficacy of industrial antimicrobial agents against *E. coli* (BL21-DE3) compared to conventional culture based counting and broth MIC determinations and has revealed that the impact of these antimicrobials on cell viability is a complex one, dependent on both the metabolic state of the cells as well as the agent itself. This technique enables both the rate and extent of the impact of antimicrobial agents on cell viability to be measured in real-time and as well as examining differential effects on growing and non-growing cells and could be adapted to study the kinetics of cell death in response to other antimicrobial agents including antibiotics.

Competing interests

The authors declare no competing interests.

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Figure 1. Correlation between eGFP fluorescence and viable counts in a growing culture of *E. coli* (BL21-DE3). *E.coli* (BL21-DE3) was grown in a 96 well microtitre plate in LB medium at 25°C and fluorescence (excitation 488 nm, emission 540 nm) and colony forming units (CFU) determined over 24 h after inoculation. CFU's were determined from serial dilutions on LB agar. Data represents the mean of at least 3 replicates $\pm$ SEM. The line represents the best line of fit and R^2 the correlation coefficient.

Figure 2. Effect of the biocides BBIT, BIT, sodium pyrithione, OIT and DCOIT on the eGFP fluorescence of stationary phase and log phase cells of *E.coli* (BL21-DE3). For stationary phase cells, an overnight culture of *E.coli* (BL21-DE3) was washed and resuspended in PBS (pH 6) with shaking for 6 h before dispensing into 96 well plates. Mean fluorescence of the cells was determined over 24 h following the addition of PBS (biocide free control, circles), 2% (squares) or 4% (triangles) BBIT (A), BIT (C), sodium pyrithione (E), OIT (G) or DCOIT (I). For log phase cells, overnight stationary phase cultures cells were diluted in LB broth and grown in 96 well plates for 15 h (log phase, circles) before the addition (indicated by the vertical dotted line) of PBS (biocide free, circles), 2% (squares) or 4% (triangles) BBIT (B), BIT (D), sodium pyrithione (F), OIT (H) or DCOIT (J). Mean fluorescence of the cells was determined over 24 h (15 h prior to and 9 h after biocide addition). Data represents the means of three replicate wells $\pm$ SEM. RFU = Relative Fluorescence Units.

Table 1. Summary of biocide effects on stationary and log phase cells of *E. coli* (BL21-DE3).

Biocide	Concentration	Percentage Biocide Efficacy (%E)*			
		Stationary phase		Log phase	
		Within 1 h	After 24 h	Within 1 h	After 9 h
BBIT	2%	81.7±7.3	65.5±5.9 ^a	83.6±24.7	85.4±22.6
	4%	67.4±8.4 ^a	54.1±7.0 ^a	72.6±26.4 ^a	39.9±31.5 ^a
BIT	2%	87.6±4.7	73.0±3.9 ^a	7.1±8.1 ^a	6.2±9.1 ^a
	4%	99.6±3.2	65.2±2.6 ^a	2.1±4.1 ^a	3.8±17.4 ^a
Pyrithione	2%	52.4±20.8 ^a	47.6±8.6 ^a	19.0±21.3 ^a	16.9±30.6 ^a
	4%	2.0±4.7 ^a	0 ^a	7.9±6.7 ^a	7.0±8.9 ^a
OIT	2%	33.9±7.9 ^a	41.4±8.3 ^a	92.0±9.9	94.4±12.5
	4%	36.0±5.4 ^a	29.9±7.3 ^a	96.2±25.1	78.2±30.6
DCOIT	2%	83.6±4.9	30.0±2.0 ^a	82.2±6.7	94.1±14.1
	4%	90.5±21.6	29.4±20.9 ^a	92.9±8.3	78.6±24.2

*The percentage biocide efficacy (%E) was calculated at 1 and 9 h after the addition of biocide using the formula $\%E = F_{\text{biocide}}/F_{\text{control}} \times 100$ where F_{biocide} is the fluorescence of the biocide treated sample and F_{control} the fluorescence of the untreated sample. Means represent the average of three replicates ± compounded %SEM. ^a Signifies a significant difference $P < 0.05$) compared to the biocide untreated control (paired t-test).

Figure 1

Figure 1

Figure 2

Figure 2

Figure 2 (continued)

Figure 2 (continued)