Biofilms, homoserine lactones and biocide susceptibility

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Aims: To investigate the susceptibility, to a range of different biocides, of Pseudomonas aeruginosa strains variously deficient in N-acyl homoserine lactone systems, grown either as planktonic or biofilm populations.

Methods and results: Biocide susceptibility data were generated for strains of P. aeruginosa deficient in N-acyl homoserine lactone production, grown planktonically or as biofilm populations using a poloxamer hydrogel construct. Component cells from the biofilm constructs were also tested for their susceptibility. Significant differences in susceptibility were noted between the wild-type strain, a mutant defective in the long chain (C-12) homoserine lactone and a mutant defective in the short chain (C-4) homoserine lactone which could not be related to the biofilm mode of growth. Moreover, differences in susceptibility appeared to be dependent upon the nature of the homoserine lactone deletion and type of biocide rather than the mode of growth.

Conclusions: No general trend exists between homoserine lactone deficiency and biocide susceptibility regardless of mode of growth.

Keywords: biocide susceptibility, N-acyl homoserine lactones, bronopol, chlorhexidine diacetate, cetrimide USP, isothiazolones

Introduction

A notable feature of biofilms is their generalized resistance to a wide range of antibiotics, biocides and disinfectants.1 In this respect, treatment with antimicrobial agents, proven to be effective against suspension cultures in the laboratory, often fails to have any effect in vivo.1 Such resistance has been attributed to a number of biofilm properties which include induction of attachment-specific phenotypes,2,3 reaction-diffusion-limitation through the exopolymeric matrix,4,5 accumulation of hydrolytic enzymes,6 physiological changes associated with growth rate7 and the up-regulation of multidrug efflux pumps.5 However, such mechanisms will only ensure a delay in killing rather than provide a unifying explanation of biofilm resistance.9 The possibility exists that the resistant bacteria could react to the stress of the environment by altering their phenotypic characteristics during this delayed action.1

N-acyl homoserine lactone (HSL)-mediated, quorum sensing has been reported to mediate in biofilm differentiation and the associated changes in susceptibility towards some biocides.8 Quorum-sensing is a cell-density-sensing mechanism that elaborates signal-transduction, thereby facilitating adaptation to the prevailing growth environment.10 Pseudomonas aeruginosa is known to possess at least two quorum-sensing systems. The last-lasR system that uses N3-(oxododecanoyl)-homoserine lactone (C12-HSL)12 and the rhlI-rhlR, that uses N-butanyllhomoserine lactone (C4-HSL) as signal molecules.13 Whilst C4-HSL passively diffuses in and out of the cells, C12-HSL accumulates in the cell by diffusion but is actively pumped out by the MexAB-Oprp pump.14 Davies et al.10 hypothesized that an abnormal, undifferentiated biofilm formed by a lasI mutant, PA0-JP1, might be sensitive to a biocide, sodium dodecyl sulphate (SDS, 0.2%) that would not otherwise disrupt wild-type biofilms (PA01). Their observations demonstrated that whereas there was no detectable effect on the wild-type biofilm or the lasI mutant biofilm grown in the presence of synthetic 3OC12-HSL, most or all of the bacteria in the lasI mutant biofilm detached from the surface. The abnormal biofilm, therefore, appeared to be sensitive to the detergent biocide SDS. Such broader associations between HSL-mediated biofilm phenotypes and biocide susceptibility have been proposed based on biofilms produced by HSL-deficient strains.10

The present study was therefore conducted to determine whether such changes in biofilm susceptibility could be attributed to a general loss of the biofilm phenotype or whether other HSL-associated responses could alter biocide susceptibility.

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Materials and methods

Organisms and media

P. aeruginosa PA01 (HSL wild-type) strain possessing functional lasR and lasI genes and mutants PA0-R1 (lasR; C₅₇-HSL), PA0-JP1 (lasI; C₅₇-HSL), PAN067 (lasB; C₂-HSL) and PA0-JP2 (lasB; C₂-HSL) were used throughout the study. Stock cultures for daily use were maintained on Luria broth (LB) plates at 4°C following overnight incubation (30°C), replaced at weekly intervals.

Luria-Bertani broth (LB broth; Difco) was used for all experiments. Growth curves, used to determine the minimal inhibitory concentrations (MICs) were produced in quarter-strength LB broth to ensure minimal interactions between broth components and biocides. This also allowed for growth to be limited through C/N availability rather than oxygen.

Biocides

Stock solutions of the following biocides were prepared in sterile distilled water, filter sterilized and stored either at 4°C or frozen: chlorhexidine diacetate hydrate 98% (Aldrich Chemical Co. Gillingham, UK), cetrimide USP, bronopol (both Sigma Chemical Co.), MIT (N-methyl isothiazolone; Rohm and Haas, Philadelphia, USA), CMIT (4-chloro, N-methyl isothiazolone; Rohm and Haas), and BIT (benzisothiazolone; ICI plc Organics Division, Manchester UK).

MIC determination

Mid-log phase cultures were prepared by inoculating 1 mL of an overnight culture into fresh LB broth (50 mL) in a 250 mL Erlenmeyer flask. The flask was incubated (30°C, 200 osc/min) in a shaking incubator until the culture reached an optical density (470 nm) of 0.6. After a one in two dilution, aliquots (100 µL) were added to equal volumes of biocide solution in 98-well microtitre plates. This gave a concentration of 5×10⁶ cfu/mL in quarter strength LB broth. Biocide concentrations were chosen on an incremental scale, between the endpoints of a doubling dilution MIC. Growth was monitored over 24 h at 30°C using an Anthos Microtiter Plate Reader (Model HTIII, Labtec Instruments, Austria). Optical density readings (492 nm) were taken every 30 min following 60 s of high shaking. Appropriate controls were used and four replicate wells selected for each combination of biocide and microorganism.

MIC values were determined by relating mid-log phase growth rate to biocide concentration and extrapolating to give the concentration required for 100% growth inhibition. In this manner, a more precise MIC value was determined than is possible by the more traditional tube-dilution method.

Biocide susceptibility of planktonic cultures

Aliquots (200 µL) of 24 h liquid culture, grown in quarter strength LB broth, were transferred to sterile Eppendorf tubes and centrifuged in a benchtop Microfuge (13 000 r.p.m., 10 min). The supernatants were discarded and cell pellets resuspended in pre-warmed biocide (200 µL, 37°C, 10 min) before transfer to chilled sterile neutralizer (900 µL, 20 min). Most-probable-number (MPN) counts were carried out using the wells (98) of sterile microtitre plates. Accordingly, flakes of poloxamer (40%) were added to quarter strength LB broth and refrigerated (4°C) overnight for hydration to occur. The dissolved poloxamer solutions were then autoclaved and returned to the refrigerator until required. Before use, sterile, chilled poloxamer (3 mL) was inoculated with 1:100 dilution of an overnight culture (300 µL) in fresh quarter strength LB broth to give ~10⁷-10⁸ cfu/mL. This was mixed well and drops (200 µL) of the poloxamer gel carefully placed onto sterile glass supports and incubated for 24 h (static incubator, 30°C) in a fully hydrated chamber. Following incubation, the glass supports (in triplicate) were transferred to pre-warmed biocide (37°C, 10 min) before removing to an appropriate chilled neutralizer solution (1800 µL, 20 min).

The poloxamer hydrogel was dispersed in the chilled solution, thereby releasing the cells, after which an MPN count was carried out.

Biocide susceptibility of component biofilm cells

Following incubation (24 h), the poloxamer hydrogel constructs were transferred together with their glass supports to chilled sterile saline (5 min). The dispersed hydrogel solution was then transferred to a sterile Eppendorf tube and treated as per the planktonic cells above.

Neutralization of biocides

Chlorhexidine diacetate and cetrimide USP were neutralized using a lecithin (1% w/v; Sigma)-Tween 80 (2% w/v; BDH, Poole, UK) solution, whereas bronopol was neutralized using sodium thioglycolate (Sigma) at an equimolar concentration to the highest bronopol concentration used. The antimicrobial effects of the other biocides were arrested by dilution (›1:100).

MPN count

MPNs were carried out using the wells (98) of sterile microtiter plates. Five replicates were used for each sample with 1:10 serial dilutions in quarter strength LB broth. Growth of the microorganism in the broth caused a visible change in turbidity after incubation (48 h) permitting a well to be scored either positive or negative for growth. The first serial dilution of the test sample was into the neutralizer solution and from this the serial dilutions were continued in the LB broth solution. Since the neutralizer was not a specific growth substrate for the microorganism, the presence or absence of growth could not be monitored with a turbidity change. Instead, five replicate droplets (30 µL) were placed onto a nutrient agar plate divided into five sections, with any colony formation in each section scored as positive growth. On the basis of probability theory it is possible to calculate, from the numbers of positive and negative samples taken from each microtiter plate well receiving a certain amount of inoculum, the most probable number of microorganisms in that quantity of inoculum. The MPN is then obtained by multiplying the result by the appropriate dilution factor. A table of MPN values using five tubes per dilution at a dilution ratio of 1:10 is provided by Alexander. Each experiment was carried out in duplicate and repeated on a separate occasion to give quadruplicate test samples for statistical analysis.

Statistical analysis

A Student’s t-test and one-way ANOVA were carried out using Micro-soft Excel to evaluate statistical significance.

Results and discussion

Homoserine lactones are known to have a role in biofilm formation, the consequence of which may affect biofilm susceptibility to antimicrobial agents. Apart from one early study, it is not however known to what extent HSLs affect antimicrobial susceptibility.


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Table 1. MIC values for P. aeruginosa strains PA01 (HSL wild-type), PA0-R1 (lasR; C12-HSL; ) and PAN067 (lasB; C4-HSL; ).

<table>
<thead>
<tr>
<th>Biocide</th>
<th>MIC values (mg/L) with standard error</th>
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<tr>
<td></td>
<td>PA01</td>
</tr>
<tr>
<td>Chlorhexidine diacetate</td>
<td>75.5 ± 1.65</td>
</tr>
<tr>
<td>Cetrimide USP</td>
<td>8.0 ± 0.471</td>
</tr>
<tr>
<td>Bronopol</td>
<td>6.3 ± 0.236</td>
</tr>
<tr>
<td>MIT</td>
<td>25 ± 0</td>
</tr>
<tr>
<td>CMIT</td>
<td>0.85 ± 0.24</td>
</tr>
<tr>
<td>BIT</td>
<td>70 ± 3.3</td>
</tr>
</tbody>
</table>

Figure 1. Susceptibility of P. aeruginosa PA0-R1 (lasR; C12-HSL; filled) and PAN067 (lasB; C4-HSL; hatched) to a range of biocides presented as a percentage of the MIC of the HSL wild-type strain, PA01 (no pattern).

Assumptions have been made that when cells deficient in cell-cell signalling are grown as a biofilm, the physiology of the biofilm is modified such that it then has an increased susceptibility to biocides. In no instance has the innate susceptibility of HSL-deficient strains been compared when grown as planktonic cultures. Hence, the studies described in this article were carried out using strains deficient in their ability to produce key HSLs, where the mutant strains were regarded to possess an antimicrobial susceptibility similar to that of the parent wild-type. Comparisons of susceptibility were made between the wild-type organism and the HSL-mutant strains, grown both planktonically (positive control) and as biofilm populations.

Initially, MIC values were determined for P. aeruginosa PA01, PA0-R1 and PAN067 to a range of biocides when grown as planktonic cultures (Table 1). Unexpectedly, the MICs of the HSL-deficient strains were not comparable to those of the wild-type for all biocides. The MICs were similar for chlorhexidine diacetate and bronopol but markedly different for the isothiazolones. Interestingly, no significant difference was demonstrated between the wild-type and P. aeruginosa PAN067 for cetrimide USP, but was observed with strain PA0-R1. P. aeruginosa PA0-R1 was generally the least susceptible strain. As shown in Figure 1 it possessed a similar susceptibility to the wild-type strain for the membrane interactive biocides, chlorhexidine diacetate and cetrimide USP, but was significantly decreased for the isothiazolones. This was particularly apparent for CMIT, where PA0-R1 was over 150% less sensitive to the biocide compared to the wild-type, P. aeruginosa PA01. P. aeruginosa PAN067 was significantly more susceptible to all biocides except chlorhexidine diacetate and bronopol.

In essence, no definite trend could be drawn between HSL deficiency and susceptibility towards the different groups of biocides for planktonic cultures. There was, however, much variation in susceptibility between the three strains to the isothiazolones. These differences may provide information on the mode-of-action of the biocide and upon additional role(s) of the HSL. Curiously the C12-HSL deletion mutant (PA0-R1) produced cells that were significantly less susceptible to the isothiazolones, but which differed to the wild-type, for chlorhexidine diacetate and cetrimide USP. This situation was reversed for the C4-HSL deletion mutant (PAN067), suggesting the susceptibility differences were not the result of changes in the outer or cytoplasmic membrane of the mutant strain. Given that PAN067 has been described as a pleiotropic mutant, other factors such as changes on outer-membrane proteins may affect the relative susceptibility of this strain. Moreover, the exact way the chemical mutation has affected the phenotype of the cell additional to modification of HSL production is unclear.

The susceptibilities of total biofilms, component cells dispersed from biofilms and planktonic equivalents to chlorhexidine diacetate (>5 MIC), cetrimide USP (>1.5 MIC) and bronopol (>250 MIC) were assessed. The biocide concentrations used were based on their ability to give sufficient kill in 24 h cultures of the wild-type strain. Results (Figure 2) are expressed as the log percentage survival relative to controls that had been exposed to distilled water.

For chlorhexidine diacetate, complete biofilms were inherently more resistant than either the planktonically grown cells or the component biofilm cells (Figure 2a). The latter were significantly more susceptible than the planktonic cells. There was no difference in resistance pattern between each strain and mode of growth, suggesting that HSLs do not contribute in resistance to chlorhexidine diacetate.

Similar observations were made with cetrimide USP (Figure 2b). Biofilms were more resistant to cetrimide than either the planktonic cells or the component biofilm cells. There was, however, no significant difference in response amongst the four strains tested. The component biofilm cells of PA0-JP1 and PA0-JP2 were very susceptible to cetrimide exposure compared to either the wild-type or PA0-R1. There was also variation between the strains in response when grown as planktonic cells that were unrelated to the response of the biofilm-grown cells. For both chlorhexidine diacetate and cetrimide USP, the response of the biofilms was similar, providing almost total protection with no significant difference in susceptibility between the wild-type and the mutant strains. This was in contrast to the planktonic cells and component biofilm cells that were significantly more susceptible to the biocides than the complete biofilm. This could either suggest that HSL deletion does not affect susceptibility to these two biocides or that the biofilm matrix acted as a reaction-diffusion limitation of penetration of these agents. It is unlikely, therefore, that HSLs play a role in the response of P. aeruginosa biofilms to these membrane active biocides.

With bronopol, significant differences in response were observed between the planktonic cells and the component biofilm cells that were not reflected in the complete biofilm (Figure 2c). For biofilm populations, there was no significant difference in susceptibility...
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between the wild-type or the C_{12}-HSL mutants, whereas PA0-JP2 was most susceptible. Strain PA0-JP1 was generally the least susceptible organism for each mode of cell preparation. These differences may suggest a role for HSLs in resistance to bronopol.

Bronopol possesses two different action mechanisms. Under aerobic conditions, bronopol catalytically oxidizes thiol-containing materials to produce reactive free oxygen radicals which are directly responsible for the bactericidal activity. Catalytic oxidation of thiols in the presence of excess thiols leads to the creation of an anoxic state. Under such conditions, a slower anoxic reaction with thiols consumes the bronopol and slows growth. Consumption of bronopol by the anoxic route leads to the eventual removal of bronopol from the electrophilic biocide, bronopol, through possibly increasing the amount of reductive thiols available for interaction.

Other antimicrobial agents are known to act in this manner, i.e. through a reactive consumption route, notably the organomercurials and isothiazolones. We are not aware, however, of any antibiotics that are primarily thiol-interactive.

The studies reported here have demonstrated that whilst intact biofilms were generally less susceptible to the biocides tested than their planktonic counterparts, this was not necessarily related to HSL deficiency. Furthermore, biofilm component cells were generally more susceptible than the intact biofilm, a feature observed previously and suggestive of a community-specific resistance phenomenon. Significant differences were however observed between the relative susceptibilities of the HSL mutant strains and the wild-type P. aeruginosa when grown planktonically. Such differences were not reflected in the biofilm.

In Gram-negative bacteria, HSLs are known to be associated with antibiotic susceptibility. Antibiotics act primarily thiol-interactive. We are not aware, however, of any antibiotics that are primarily thiol-interactive. Regardless, caution should be exerted when attempting to relate planktonic susceptibility to that of a biofilm. Moreover, our study indicates that there is no definite trend between HSL deficiency and biocide susceptibility.

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References


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