Susceptibility of compound 48/80-sensitized Pseudomonas aeruginosa to the hydrophobic biocide triclosan

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Abstract

Pseudomonas aeruginosa is intrinsically resistant to the hydrophobic biocide triclosan, and yet it can be sensitized to low concentrations by permeabilization of the outer membrane using compound 48/80. A selective plating assay revealed that compound 48/80-permeabilized YM64, a triclosan-recognizing efflux pump-deficient variant, was unable to initiate growth on a medium containing triclosan. Macrobath dilution assay data revealed that treatment with compound 48/80 synergistically decreased minimal inhibitory concentrations of the hydrophobic antibacterial agents rifamycin SV and chloramphenicol for all cell envelope variant strains examined. A low concentration of triclosan exerted a transient bactericidal effect on permeabilized wild-type strain PAO1, after which exponential growth resumed within 4 h. Permeabilized strain YM64 was unable to overcome the inhibition; yet, both strains remained susceptible to chloramphenicol for as long as 6 h, thereby suggesting that the outer membrane remained permeable to nonpolar compounds. These data support the notion that the transitory nature of compound 48/80 sensitization to triclosan in P. aeruginosa does not involve obviation of the hydrophobic diffusion pathway through the outer membrane. The inability of strain YM64 to overcome the synergistic effect of compound 48/80 and triclosan strongly suggests that triclosan-recognizing efflux pumps are involved in maintaining viability in wild-type cells whose outer membranes are otherwise compromised.

Introduction

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium that commonly causes opportunistic infections in chronically ill and immunocompromised patients. Intrinsic resistance to a wide range of antimicrobial agents often complicates chemotherapy by virtue of multiple cellular and molecular mechanisms, including expression of multidrug efflux pumps (Schweizer, 1998; Li et al., 2000b) and exclusionary properties of the outer cell envelope (Hancock & Wong, 1984; Champlin & Hart, 1990; Li et al., 2000b; Champlin et al., 2005).

The intact P. aeruginosa outer membrane acts as a permeability barrier to hydrophobic compounds, including certain antibiotics and biocides (Angus et al., 1982; Champlin & Hart, 1990; Morris et al., 1995; Champlin et al., 2005). The impermeability of the gram-negative outer membrane to nonpolar compounds has been attributed to the localization of lipopolysaccharide in the outer leaflet and the fact that outer membrane porins allow diffusion of only low-molecular-weight polar compounds, while nonpolar compounds partition through regional areas of phospholipid bilayer exposed to the extracellular environment resulting from such events as rough mutations (Nikaido, 2003). Compromising outer membrane integrity using various chemical permeabilizers renders gram-negative bacteria more susceptible to many antimicrobial compounds, thereby supporting a role for outer membrane impermeability in their respective intrinsic resistance mechanisms (Hancock & Wong, 1984; Morris et al., 1995; Champlin et al., 2005). Triclosan (2,4,4’-trichloro-2’-hydroxydiphenyl ether) is a synthetic hydrophobic biocide commonly incorporated into many household and personal care products. It exhibits a broad spectrum of antibacterial activity that includes many common pathogens, with the notable exception of P. aeruginosa (Jones et al., 2000; Schweizer, 2001). Triclosan has been shown to target FabI, the enoyl-acyl carrier protein reductase involved in type II fatty acid biosynthesis found in bacteria (McMurry et al., 1998). Other workers have suggested that triclosan may nonspecifically disrupt...
cytoplasmic membrane function (Villalain et al., 2001; Guillen et al., 2004). Moreover, while triclosan has been reported to be bacteriostatic at low concentrations (Russell, 2004), the precise nature of its growth-inhibitory ability is yet to be resolved experimentally in P. aeruginosa. Data have been provided by other workers that suggest that triclosan exerts a bactericidal effect on both Escherichia coli (McMurtry et al., 1998; Gomez Escalada et al., 2005) and Porphyromonas gingivalis (McMurtry et al., 1998) at greater than minimal inhibitory concentrations (MICs). A similar bactericidal effect was observed in the gram-positive organisms Staphylococcus aureus (Suller & Russell, 2000) and Enterococcus hirae (Gomez Escalada et al., 2005).

Meincke et al. (1980) found that P. aeruginosa accumulated considerably more cell envelope-associated triclosan than did two other organisms, and yet was more resistant to its growth-inhibitory action. Because P. aeruginosa possessed greater cell envelope lipid content, they reasoned that it was better able to retard triclosan diffusion into the cell. Recent work from this laboratory (Champlin et al., 2005) revealed that P. aeruginosa can be sensitized to a low concentration (2.0 µg mL⁻¹) of triclosan using compounds such as polymyxin B-nonapeptide, EDTA, and compound 48/80, which selectively permeabilize the gram-negative outer membrane (Hancock & Wong, 1984; Vaara, 1992). Compound 48/80 is a mixture of polymers formed from the condensation of p-methoxyphenethylamine with formaldehyde (Katsu et al., 1985a), which effectively permeabilizes the gram-negative outer membrane to nonpolar compounds (Katsu et al., 1985b).

The expression of five active multidrug efflux pumps, which are members of the resistance nodule division family, has also been shown to be involved in intrinsic triclosan resistance in P. aeruginosa (Schweizer, 2001). Chuanchuen et al. (2003) more recently reported that P. aeruginosa strains that lack the ability to express any one of the triclosan-recognizing efflux pumps were more susceptible to the biocide. However, other reports have revealed that strain YM64, which expresses a nontriclosan-recognizing efflux pump (Morita et al., 2001), exhibits triclosan resistance comparable to that of its parental strain PAO1 (Sekiya et al., 2003; Champlin et al., 2005), a wild-type strain that constitutively expresses the triclosan-recognizing MexAB-OprM efflux system (Schweizer, 2001; Sekiya et al., 2003).

Despite the fact that the growth of P. aeruginosa was transiently inhibited by a synergistic combination of triclosan and compound 48/80, all strains examined except the efflux pump-deficient YM64 appeared to be able to initiate exponential growth within 4 h of treatment (Champlin et al., 2005; unpublished data). While these data support the notion that intrinsic triclosan resistance in P. aeruginosa is influenced by outer membrane exclusion, it is also apparent that triclosan-recognizing efflux pumps may be necessary to restore resistance in the event the outer membrane is rendered permeable to triclosan. The present study was undertaken to obtain a better understanding of the relationship between outer cell envelope impermeability and active efflux in intrinsic triclosan resistance by using P. aeruginosa cell envelope variant strains and the outer membrane permeabilizer compound 48/80.

### Materials and methods

#### Bacterial strains and culture conditions

The model system strains used for this study are described in Table 1. Pseudomonas aeruginosa strains PAO1 and Z61 (Zimmermann, 1980; ATCC 35151) are maintained as stock cultures in this laboratory. Pseudomonas aeruginosa YM64 was provided by Dr Tomofusa Tsuchiya (Okayama University, Okayama, Japan), and P. aeruginosa K799 was provided by Dr Robert E.W. Hancock (University of British Columbia, Vancouver, Canada). Pseudomonas aeruginosa ATCC strains 27853 and 10145 were obtained from the American Type Culture Collection (Manassas, VA).

All strains were maintained as cryopreserved stock cultures at −80 °C (Darnell et al., 1987) and cultivated on Mueller Hinton medium (MHA; Difco Laboratories, Detroit, MI) plates for 18 h at 37 °C to obtain working cultures. Starter cultures were prepared by inoculating 125-mL screw-capped flasks, each containing 20 mL of sterile Mueller Hinton broth (MHB; Difco Laboratories), with cells from working cultures and incubating for 12–15 h at 37 °C with rotary aeration (180 r.p.m.) in a G24 Environmental

| Table 1. Pseudomonas aeruginosa model system strains used in this study |
|-----------|-----------------|----------------|
| Strain    | Description                                             | Reference         |
| PAO1      | Wild-type strain possessing a highly refractory outer membrane for nonpolar compounds. | Champlin et al. (2005) |
| YM64      | PAO1 Δ MexAB, Δ MexCD, Δ MexEF, Δ MexXY.                  | Morita et al. (2001) |
| K799      | Wild-type strain possessing a highly refractory outer membrane for nonpolar compounds. | Angus et al. (1982) |
| Z61       | K799 Δ OprM, expresses MexCD-OprM and MexEF-OprN; possesses a highly permeable outer membrane for nonpolar compounds. | Li et al. (2000a), Angus et al. (1982) |
| 10145     | American Type Culture Collection type strain.             | Editorial Secretary (1970) |
| 27853     | American Type Culture Collection strain recommended for use in antibiotic susceptibility testing in P. aeruginosa. | National Committee for Clinical Laboratory Standards (2000) |
Incubator Shaker (New Brunswick Scientific Co. Inc., Edison, NJ). Test cultures were prepared by inoculating MHB with stationary-phase cells from starter cultures to an OD at 620 nm (OD_{620\,\text{nm}}) of 0.05 using a Spectronic 20 optical spectrophotometer (Milton Roy Co., Rochester, NY), and then transferring 5.0-mL aliquots into individual culture tubes (18 × 150 mm; Kimax) and incubating as described above after the aseptic application of experimental treatments as low-volume transfers.

**Selective plating**

The effect of compound 48/80 (Sigma-Aldrich Chemical Co., St Louis, MO) permeabilization of the outer membrane on the ability of strains PAO1 and YM64 to initiate growth on media containing hydrophobic molecules was determined using MHA, MHA plus triclosan (2.0\,\mu\text{g}\cdot\text{mL}^{-1}; Irgasan DP 300; Ciba Specialty Chemicals Corp, High Point, NC), and MHA plus Bile Salts No. 3 (0.5%; Difco Laboratories) as described by Hart & Champlin (1988).

**Antimicrobial agent susceptibility**

The susceptibilities of all the test strains to triclosan and selected hydrophobic antibiotics were determined using a standard macrobroth two-fold serial dilution bioassay (Darnell et al., 1987) with either MHB or MHB plus compound 48/80 (10.0\,\mu\text{g}\cdot\text{mL}^{-1}) as a diluent. Stock solutions of triclosan were prepared by first solubilizing in ethanol (95%), and then diluting to the desired final concentration in sterile MHB. Novobiocin sodium (Sigma-Aldrich Chemical Co.), rifamycin SV (Sigma-Aldrich Chemical Co.), and chloramphenicol (also solubilized in 95% ethanol; Sigma-Aldrich Chemical Co.) were prepared to desired concentrations in MHB, filter sterilized (0.22-\mu\text{m} Fisherbrand syringe filter assemblies; Fisher Scientific, Houston, TX), and stored at 4°C until needed. The final ethanol concentrations never exceeded 0.26%. Each dilution tube (13 × 100 mm; Pyrex) was inoculated with 1.0 mL of a mid-exponential-phase MHB batch culture (5.0 × 10^{9}\text{CFU}\cdot\text{mL}^{-1} viable cell density). The MIC was defined as the lowest concentration of the antimicrobial agent that completely inhibited initiation of growth after incubation as described above for 18 h.

**Viable cell density growth kinetics**

In order to determine the effect of a synergistic combination of compound 48/80 and triclosan on culture viability, viable cell density assessments of batch cultural growth kinetics were performed using a method similar to that of Cappelletty & Rybak (1996). A 125-mL flask containing 20 mL of MHB was inoculated with stationary-phase starter culture cells to an initial OD_{620\,\text{nm}} of 0.05. Test cultures were prepared by transferring 5.0-mL aliquots of the cell suspension to each of four sterile culture tubes (18 × 150 mm; Kimax). Each culture was treated by aseptically adding compound 48/80 and triclosan alone or in combination to final concentrations of 10.0 and 2.0\,\mu\text{g}\cdot\text{mL}^{-1}, respectively. An untreated culture was included for control purposes. A triclosan stock solution having a concentration of 500\,\mu\text{g}\cdot\text{mL}^{-1} was prepared in 95% ethanol, such that 20 \mu L additions to each designated 5.0-mL test culture yielded the desired final triclosan concentration and a final ethanol concentration of 0.4%. Ethanol, at this concentration, was shown not to affect control growth (data not shown). Cultures were incubated as described above and aliquots of 0.1 mL were removed at 0, 2, 4, and 6 h, subjected to 10-fold serial dilutions in sterile physiological saline (0.85% NaCl), and 0.1 mL of appropriate dilutions was aseptically spread inoculated onto MHA plates using alcohol flame sterilization. Plates were incubated at 37°C for 24 h, after which CFU were counted visually with the aid of a Quebec Darkfield Colony Counter (Leica, Buffalo, NY).

**Outer membrane permeability**

A chloramphenicol susceptibility bioassay was used to determine whether P. aeruginosa wild-type strains were able to overcome compound 48/80-sensitization to triclosan by obviating an induced hydrophobic pathway for diffusion of nonpolar solutes through the outer membrane. The macrobroth dilution bioassay described above was used to determine MICs for test culture cells grown in the absence and presence of compound 48/80 at 0 and 6 h.

**Results and discussion**

Previous work in this laboratory revealed that treatment of P. aeruginosa cell envelope variant strains with compound 48/80 and other outer membrane permeabilizers rendered cells susceptible to low concentrations of the hydrophobic antimicrobial agents novobiocin and triclosan (Champlin et al., 2005; unpublished data). All but one of six strains tested were able to overcome the synergic growth-inhibitory effect within 4 h when compound 48/80 was used as the permeabilizer. The exception was strain YM64, a genetic derivative of wild-type strain PAO1 that does not express triclosan-recognizing efflux pumps (Morita et al., 2001). In order to examine more fully the relationship between outer cell envelope exclusion and active efflux of triclosan, a model system comprised of P. aeruginosa strains exhibiting disparate phenotypes with regard to both properties was constructed for the present study (Table 1).

The apparent inability of compound 48/80-sensitized strain YM64 to overcome its susceptibility to triclosan (Champlin et al., 2005) suggests that active efflux is involved in the transitory nature of such a synergy in parental strain PAO1. To further test this hypothesis, the ability of
compound 48/80-permeabilized YM64 to initiate growth on MHA containing either triclosan or nonpolar bile salts was compared with that of parental strain PAO1 (data not shown). Strain YM64 was able to grow in the presence of triclosan, despite the absence of triclosan-recognizing efflux pumps. In contrast, compound 48/80-sensitized strain YM64 was unable to initiate growth in the presence of a low concentration of triclosan (2.0 μg mL⁻¹) and grew only poorly on media containing bile salts. Strain PAO1 was able to establish good growth by 18 h on all media in both the absence and presence of compound 48/80. These data clearly support the idea that outer membrane integrity is vital in order for P. aeruginosa to undergo population growth in the presence of low concentrations of triclosan. However, efflux pump expression appears to be involved in the ability to overcome the deleterious nature of compound 48/80 sensitization to triclosan. These findings are in contrast with a previous report wherein triclosan resistance in P. aeruginosa was thought to be a function only of triclosan-recognizing efflux pump expression (Chuanchuen et al., 2003).

Should compound 48/80 permeabilization of the outer cell envelope for triclosan function by establishing a hydrophobic pathway for diffusion of nonpolar molecules through the outer membrane, a generalized increased susceptibility to hydrophobic antibacterial agents should occur concomitantly. This relationship was investigated by determining the MICs of triclosan and disparate hydrophobic antibiotics for model system strains in both the presence and absence of compound 48/80 (Table 2). Compound 48/80 sensitization decreased the triclosan MIC for wild-type strain PAO1 to levels such that a precise value could be obtained. Strain YM64 was more susceptible to the nonpolar compounds than four wild-type P. aeruginosa reference strains in a manner comparable to cell envelope variant strain Z61, thereby suggesting that both the outer membrane exclusion and efflux pump expression underlie intrinsic resistance to nonpolar compounds. The inability of compound 48/80 to mitigate the high triclosan MIC for wild-type strains is likely due to a compensatory induction of normally silent triclosan-recognizing pumps. All strains examined were rendered significantly more susceptible to the hydrophobic antibiotics rifamycin SV and chloramphenicol in a manner consistent with the notion that compound 48/80 confers a nonspecific hydrophobic diffusion pathway through which they are able to dissolve through the outer membrane.

Viable cell density growth kinetic experiments were performed using strains PAO1 and YM64 in order to determine the precise nature of the growth inhibition exerted by triclosan subsequent to outer membrane disruption (Fig. 1). As can be seen in Fig. 1a, neither compound 48/80 nor triclosan significantly affected strain PAO1 cultural viability at the concentrations used. However, the combination of both exerted an initial bactericidal effect that was overcome with the initiation of exponential growth within 4 h. Data presented in Fig. 1b similarly reveal that neither compound 48/80 nor triclosan were able to inhibit growth of strain YM64 significantly. However, the combination of both compounds exerted a bactericidal effect from which it was unable to recover by 6 h. That both strains exhibited control growth in the presence of triclosan alone underscores the importance of the outer membrane impermeability barrier function to the cellular mechanism forming the basis for intrinsic triclosan resistance. Moreover, the inability of strain YM64 to surmount the initially bactericidal effect of triclosan in the absence of an intact outer membrane suggests a fundamentally important function for triclosan-recognizing efflux pumps in wild-type strains. So far, this is the first report of a relatively low concentration of triclosan exerting a lethal effect in P. aeruginosa.

While triclosan efflux appears to underlie the mechanism by which parental strain PAO1 is able to overcome compound 48/80 sensitization to triclosan, the possibility remains that the disruption of the outer membrane may itself

### Table 2. Susceptibility of Pseudomonas aeruginosa model system strains to hydrophobic antibacterial agents as a function of compound 48/80 permeabilization of the outer membrane

<table>
<thead>
<tr>
<th>Strain</th>
<th>TCS</th>
<th>TCS+Cpd 48/80</th>
<th>NOV</th>
<th>NOV+Cpd 48/80</th>
<th>Rif</th>
<th>Rif+Cpd 48/80</th>
<th>Chl</th>
<th>Chl+Cpd 48/80</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>&gt; 512.0</td>
<td>512.0</td>
<td>512.0</td>
<td>ND</td>
<td>128.0</td>
<td>8.0</td>
<td>64.0</td>
<td>2.0</td>
</tr>
<tr>
<td>YM64</td>
<td>256.0</td>
<td>8.0</td>
<td>16.0</td>
<td>ND</td>
<td>64.0</td>
<td>16.0</td>
<td>4.0</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>K799</td>
<td>&gt; 512.0</td>
<td>&gt; 512.0</td>
<td>&gt; 512.0</td>
<td>ND</td>
<td>128.0</td>
<td>8.0</td>
<td>256.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Z61</td>
<td>256.0</td>
<td>&lt; 1.0</td>
<td>16.0</td>
<td>ND</td>
<td>2.0</td>
<td>2.0</td>
<td>8.0</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>10145</td>
<td>&gt; 512.0</td>
<td>&gt; 512.0</td>
<td>256.0</td>
<td>ND</td>
<td>128.0</td>
<td>4.0</td>
<td>256.0</td>
<td>4.0</td>
</tr>
<tr>
<td>27853</td>
<td>&gt; 512.0</td>
<td>&gt; 512.0</td>
<td>256.0</td>
<td>ND</td>
<td>128.0</td>
<td>8.0</td>
<td>256.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*Each value was determined on the basis of three to five replicate two-fold serial dilutions. ND, not determined; TCS, triclosan; NOV, novobiocin; Rif, rifamycin SV; Chl, chloramphenicol; Cpd 48/80, compound 48/80.

1Precipitation was observed at triclosan concentrations of 512.0 μg mL⁻¹.

Not determined owing to the insolubility of novobiocin in the presence of compound 48/80.
These data confirm the notion that intrinsic resistance to low triclosan concentrations in \textit{P. aeruginosa} is due at least in part to the impermeability properties of the intact outer membrane for nonpolar molecules. In addition, the transitory nature of triclosan-induced decreased viability in multidrug efflux-sufficient strains does not appear to involve obviation of the hydrophobic diffusion pathway through the outer membrane. The inability of strain YM64 to overcome the synergistic effect of compound 48/80 and triclosan strongly suggests that triclosan-recognizing efflux pumps are involved in maintaining viability in cells whose outer membranes are otherwise compromised. Moreover, triclosan is bactericidal at relatively low concentrations in \textit{P. aeruginosa} when able to permeate the outer cell envelope in the absence of sufficient efflux capacity.

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**References**


