Cytological changes in chlorhexidine-resistant isolates of *Pseudomonas stutzeri*

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Transmission electron microscopy (TEM), scanning electron microscopy (SEM) and energy-dispersive analysis of X-ray (EDAX) have been used to examine chlorhexidine diacetate (CHA)-sensitive and -resistant isolates of *Pseudomonas stutzeri* and to determine the effects of CHA on the cells. Significant differences were observed in the structure, size and elemental composition of CHA-sensitive and -resistant cells. Treatment with CHA produced considerably greater changes in CHA-sensitive cells, with widespread peeling of the outer membrane, a substantial loss of cytoplasmic electron-dense material and extensive lysis. Cells from the resistant isolates showed no blebbing of the outer membrane and no structural damage. X-ray mapping confirmed the difference in CHA uptake between CHA-sensitive and CHA-resistant cells. It is proposed that changes in the outer membrane form a major mechanism of resistance to CHA in *P. stutzeri*.

**Materials and methods**

**Bacterial isolates**

Bacterial test isolates (in parentheses, MICs of chlorhexidine in mg/L) were *P. stutzeri* JM 302 (2.5), JM 302R (100), NCIMB 10783 (2.5) and 10783R (50). Aqueous cell suspensions were prepared and adjusted to a cell density of $c. 2 \text{mg dry wt/mL}$ as described previously.\textsuperscript{8} In EDAX studies, Elgastat water was used in place of Water for Injections, B.P.

**Negative staining, TEM and SEM**

Techniques were carried out as described in previous studies.\textsuperscript{9,10}

**Effects of chlorhexidine diacetate on *P. stutzeri* ultrastructure**

Chlorhexidine diacetate (CHA) was purchased from Sigma Ltd (Poole, UK). Equal volumes of bacterial cell suspensions were prepared and adjusted to a cell density of $c. 2 \text{mg dry wt/mL}$ as described previously. Microliter drops of the suspensions were placed on a glow-discharged carbon-coated copper grid and negatively stained with 2% phosphotungstic acid (pH 7.0). TEM, SEM and EDAX were carried out as described previously.\textsuperscript{9,10}

**Introduction**

Electron microscopy (EM) is a useful tool for detailed ultrastructural analysis of microorganisms. Both scanning electron microscopy (SEM) and transmission electron microscopy (TEM), which provide information about surface and intracellular changes, can be used for studying the effects of antimicrobial agents on microorganisms. EM has previously been used to study the ultrastructural basis of the resistance of *Pseudomonas aeruginosa* to antiseptics, disinfectants and antibiotics,\textsuperscript{2} and has been widely used in this laboratory for investigating the effects of antimicrobial agents on a variety of different types of organism.

X-ray microanalysis is a method of elemental analysis at the ultrastructural level and can correlate morphological appearance with chemical composition. The technique exploits the fact that different elements give off X-rays having characteristic energies when a sample is irradiated with an electron beam. The elemental composition of a sample can be determined by analysing the X-ray spectrum.\textsuperscript{3} An energy-dispersive X-ray analyser offers the advantage that all elements of interest are analysed simultaneously. Energy-dispersive analysis of X-ray (EDAX) is a useful technique for the localization of elements in microbial cells and has previously been used in this laboratory for studying the effects of chlorhexidine on *Saccharomyces cerevisiae*, *P. aeruginosa* bacteriophage F116 and *Acanthamoeba castellanii*.\textsuperscript{4–6}

Stable resistance to chlorhexidine has been demonstrated in some isolates of *Pseudomonas stutzeri*,\textsuperscript{7,8} and in this study we have used SEM, TEM and EDAX to better understand the underlying mechanisms involved.

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suspension and a CHA solution were mixed at 20°C to give a final CHA concentration of 100 mg/L. After exposure for 5, 15, 30 and 60 min the suspensions were fixed and prepared for TEM and SEM according to the methods described above.

**CHA-treated cells and EDAX analysis**

Equal volumes of the cell suspension and CHA solution (final concentration 100 mg/L) were mixed at room temperature. Samples (1 mL) were removed at the required time intervals [0 (control), 0.5, 15, 30 and 60 min] and immediately washed twice with 25% w/v polyvinyl pyrrolidone. The cell pellets were harvested by centrifugation at 1500g for 10 min, and used as described previously.4-6

The transmission electron microscope (Philips EM 400) with its associated energy-dispersive X-ray detector and multichannel analyser (EDAX 9100/60) were used to analyse the elements in the specimens.3,4-6,11

**Results**

**CHA-resistant P. stutzeri: TEM**

Cells of CHA-resistant isolates of P. stutzeri differed from those of parent sensitive isolates when negatively stained and examined by TEM (Figure 1a–d). Cells of both showed polar flagella; however, the resistant isolates stained with 1% phosphotungstic acid also carried a fibril-like structure on their cell surfaces (Figure 1b and d, arrow). This surface structure was found in the form of the electron-dense material around the cells when stained with 1% amine tungstate (data not shown). No surface structures were observed in their parent isolates prepared in the same manner (Figure 1a and c).

Cells of CHA-resistant isolates were larger than those of parent isolates, e.g. cells of JM 302 were 1.65 ± 0.21 μm (s.d.) in length and 0.43 ± 0.05 μm in width, with corresponding values of 2.17 ± 0.21 μm and 0.83 ± 0.05 μm for JM 302R. These results were statistically different (Student’s t test, P = 0.95).

TEM of thin sections of P. stutzeri revealed that the flagella and fibril-like structure usually found on the surface by negative staining (Figure 1) had been lost. However, the extracellular structure in the form of granular materials was present on some cells of isolate 10783R (data not shown). There was a marked difference in cell structure between sensitive and resistant isolates. The cell envelope of JM 302R was wavy, thicker and more densely stained than its parent sensitive isolate. The cell envelope of NCIMB 10783R did not show any difference from its parent isolate; however, amorphous material, which did not appear in the sensitive isolate, was observed outside the cell.

**CHA-resistant P. stutzeri: SEM**

The results of SEM studies (Figure 2a–d) demonstrated the rough surface of cells of resistant isolates compared with those of sensitive isolates. A morphous material was observed outside the resistant cells (Figure 2d, arrow).

**Effect of CHA on cell structures**

Exposure of the CHA-sensitive isolate JM 302 to CHA (100 mg/L) produced progressive cellular damage, the extent depending on the period of treatment (Figure 3a–d). A fter exposure to CHA for 5 min (Figure 3a), numerous small blebs of the outer membrane were seen, with an occasional larger ballooning showing evidence of inner membrane breakage, which resulted in cytoplasmic leakage (Figure 3a, arrow). There was no apparent cytological lysis and the cytoplasm still contained electron-dense material.

A fter exposure for 15 min (Figure 3b), cells had lost their electron-dense material and large blebs on the outer membrane were seen. Cytoplasmic swelling was observed at the area where the outer membrane appeared to be ruptured. A lthough the cytoplasmic outgrowth was lightly stained and contained filamentous materials, cytological lysis was not apparent.

F ollowing exposure for 30 and 60 min (Figure 3c and d), the cells showed widespread peeling of the outer membrane. Most cells underwent extensive lysis and, as a result, lost a substantial amount of cytoplasmic electron-dense material. Many cells exposed for 60 min became ghost cells with complete extraction of cytoplasmic contents (Figure 3d, arrow).

The results obtained from a parallel experiment using the resistant isolate (JM 302R) showed no blebbing of the outer membrane and no structural damage when the cells were exposed to CHA at 100 mg/L (Figure 4a–d). However, in comparison with untreated JM 302R, a decreased outer membrane thickness of treated cells was observed. A fter exposure for 60 min, the outer membrane of some treated cells showed blisters (Figure 4d).

**Elemental distribution in P. stutzeri**

The EDAX spectra of P. stutzeri isolates demonstrated the presence of Mg, P, Cl, K and Ca. A silicon (Si) peak may originate from the absorption of X-rays in the inactive region of the detector or from Si-containing contamination products in the microscope. Elemental concentrations are provided in Tables I and II. Changes in Cl concentrations after exposure of JM 302 and JM 302R to CHA (100 mg/L) are depicted in Table I. A s untreated P. stutzeri cells contained Cl, the Cl concentration cannot represent the amount of CHA taken up by the cells. However, after cell
Chlorhexidine-resistant Pseudomonas stutzeri

Treatment with CHA the Cl concentration increased, indicating uptake of the antibacterial agent. The results of X-ray mapping confirmed the difference in CHA uptake between CHA-sensitive and -resistant isolates. X-ray dots of Cl in CHA-treated JM 302 increased with time and were evenly distributed over the entire cell (data not shown). By contrast, these dots in CHA-exposed JM 302R were less dense and difficult to distinguish from the background signal at 0.5 and 15 min exposure, but can be seen to increase after 30 and 60 min (Figure 5).

Concentrations of various other elements in CHA-treated cells are detailed in Table II. As the period of contact increased so the concentrations of P, Mg and Ca decreased, followed by an increase after 60 min.

Discussion

Both TEM and SEM provide cytological evidence to help explain both the mechanism of action of, and cellular resistance to, CHA. Ultrastructural differences were observed in cells of CHA-sensitive isolates of P. stutzeri and those trained to high-level resistance to the biguanide (Figures 1 and 2). Cell envelopes of resistant

Figure 1. Transmission electron micrographs of negatively stained cells of CHA-resistant isolates and their parent sensitive isolates of P. stutzeri stained with 1% phosphotungstic acid. (a) JM 302; (b) JM 302R, showing densely stained cell ‘wall’ and fibril-like structure around the cell; (c) NCIMB 10783; (d) NCIMB 10783R. Magnification in each case ×12,500. Bars represent 1 μm.
cells stained more intensely than sensitive cells, implying greater absorption of staining materials. The resistant cells had rough surfaces, with external amorphous material (Figure 2d). This may be exopolysaccharide in nature, having reacted with chemicals during EM preparation. The conventional method of producing thin-sectioned preparations caused a slight loss of surface structure. It is noteworthy, however, that the subcellular structure of P. stutzeri cells has been well preserved using the preparation method described here. There was no evidence of cytoplasmic extraction or of cell envelope disruption in any of the samples examined.

The results presented here suggest that CHA affects outer membrane alterations. The acquisition by stepwise training of CHA resistance is associated with alterations in the architecture of the outer membrane, as no ultrastructural damage was observed in CHA-resistant cells exposed to concentrations of CHA that produce extensive damage in sensitive cells (Figures 3 and 4). Polymyxin resistance in P. aeruginosa has been linked to both ultrastructural and chemical alterations in the outer membrane so that uptake of the antibiotic to its target site, the underlying cytoplasmic membrane, is reduced.12-14 Ultrastructural changes in benzalkonium-treated P. aeruginosa have also been described.15

EDAX is a useful tool for studying the distribution of elements within bacterial cells (Figure 5, Tables I and II), yeast cells4 and bacteriophages,5 and of the effects of antimicrobial agents thereon. Elements of interest in this investigation were Mg and Ca (both having a role in maintaining the structural organization of the outer membrane16-18), P (primarily associated with structural elements such as lipids, proteins and polysaccharides19) and Cl, used previously as a marker for CHA.4,5 Concentrations of Mg, Ca and P were significantly lower in the CHA-resistant isolate, JM302R, than in the sensitive isolate JM302; whereas the Cl concentrations were not significantly different.

P was located entirely within the bacterial cell (data not shown). Because the concentrations of Mg, Ca and Cl were

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>JM 302 (n = 10)</th>
<th>JM 302R (n = 10)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>22.58 ± 3.61a</td>
<td>22.83 ± 5.21</td>
</tr>
<tr>
<td>0.5</td>
<td>33.86 ± 6.88</td>
<td>20.76 ± 7.64</td>
</tr>
<tr>
<td>15</td>
<td>85.66 ± 25.01</td>
<td>21.18 ± 3.33</td>
</tr>
<tr>
<td>30</td>
<td>61.67 ± 12.21</td>
<td>51.98 ± 6.55</td>
</tr>
<tr>
<td>60</td>
<td>102.46 ± 12.74</td>
<td>66.53 ± 9.16</td>
</tr>
</tbody>
</table>

Table I. Chlorine concentration in P. stutzeri after treatment with chlorhexidine 100 mg/L at 20°C

4Mean ± s.d. Statistical significance of the results was determined by Student’s t test on the means, at the 95% confidence level.
Chlorhexidine-resistant *Pseudomonas stutzeri*

Figure 3. Transmission electron micrographs of *P. stutzeri* isolate JM302 treated with CHA 100 mg/L: (a) 5 min, blebbing of the outer membrane; arrow shows breakage of the inner membrane resulting in cytoplasmic leakage; (b) 15 min, cytoplasmic swelling; (c) 30 min, cell lysis; (d) 60 min, ghost cells. Magnification in all cases ×40,000. Bars represent 0.1 μm.

Table II. Concentrations of phosphorus, magnesium and calcium in *P. stutzeri* after treatment with chlorhexidine 100 mg/L at 20°C

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>JM302</th>
<th>JM302R</th>
<th>JM302</th>
<th>JM302R</th>
<th>JM302</th>
<th>JM302R</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>815.73 ± 133.77</td>
<td>648.95 ± 78.16</td>
<td>160.78 ± 14.38</td>
<td>116.34 ± 7.85</td>
<td>87.28 ± 6.50</td>
<td>56.98 ± 10.56</td>
</tr>
<tr>
<td>15</td>
<td>596.26 ± 112.17</td>
<td>600.62 ± 126.71</td>
<td>108.04 ± 29.99</td>
<td>112.60 ± 26.20</td>
<td>52.32 ± 9.57</td>
<td>48.61 ± 7.16</td>
</tr>
<tr>
<td>30</td>
<td>353.57 ± 87.01</td>
<td>514.47 ± 96.71</td>
<td>64.64 ± 8.28</td>
<td>75.12 ± 11.07</td>
<td>24.81 ± 3.14</td>
<td>29.19 ± 7.72</td>
</tr>
<tr>
<td>60</td>
<td>553.71 ± 39.86</td>
<td>575.60 ± 58.22</td>
<td>85.96 ± 12.06</td>
<td>114.63 ± 23.23</td>
<td>34.63 ± 6.78</td>
<td>38.92 ± 4.56</td>
</tr>
</tbody>
</table>

*a* Mean ± s.d.; *n* = 10.
much lower (Table I), the X-ray maps of these elements could not distinguish their localization from the background signal. Using X-ray microanalysis, Chang et al.\textsuperscript{19} reported that P and Mg were located in the cytoplasm and the cell envelope whereas Ca was found predominantly in the cell envelope of \textit{Escherichia coli} B cells.

The Cl concentration in CHA-treated cells of JM302 decreased over a 30 min contact period and then rose at 60 min (Table I). This may equate with the well-known biphasic leakage phenomenon that occurs with this chemical agent.\textsuperscript{20} By contrast, the Cl content of CHA-treated JM302R remained constant for the first 15 min and then increased (Table I). This suggests that the biphasic phenomenon does not apply with the resistant isolate; this is not borne out when the P, Mg and Ca contents are considered (Table II), although the effects of CHA on the resistant isolate are, as expected, much less. P release from CHA-treated \textit{E. coli} has been described by Rye & Wiseman.\textsuperscript{21}

In conclusion, the reduced uptake of CHA by the resistant isolate coincided with smaller amounts of Mg, Ca and P in the cell. A major mechanism of CHA resistance in \textit{P. stutzeri} is likely to be linked to changes in the binding site(s) available at the outer membrane.

\textbf{References}


Chlorhexidine-resistant *Pseudomonas stutzeri*


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**Figure 5.** Image and X-ray maps of Cl in *P. stutzeri* JM302R treated with CHA 100 mg/L at 20°C, showing localization of chlorine in the cell. (a) Control (untreated) (x18,750); (b) 15 min (x18,750); (c) 30 min (x18,750); (d) 60 min (x31,250). Bar represents 0.1 µm.


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