Effect of temperature on aminoglycoside binding sites in *Stenotrophomonas maltophilia*

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In this study we used strains of *Stenotrophomonas maltophilia* grown at 30°C and 37°C to investigate the role of lipopolysaccharide (LPS) in temperature-dependent variations in sensitivity (TDVS) to gentamicin. TDVS was scored as ‘good’ if a four-fold or greater difference in minimum inhibitory concentration (MIC) was found between the two incubation temperatures (good TDVS strains; \( n = 23 \)), and otherwise as ‘poor’ (poor TDVS strains; \( n = 15 \)). Phosphate content of isolated LPS in the strains exhibiting good TDVS grown at 37°C was significantly (\( P < 0.001 \)) higher than those grown at 30°C. However, the phosphate content from LPS of strains exhibiting poor TDVS did not alter significantly with growth temperature. There was no significant difference in 3-deoxy-\( \alpha \)-manno-octulosonic acid (KDO) content between the strains grown at the different incubation temperatures. Fluorescence-activated cell sorting analysis showed significant differences in binding of fluorescein isothiocyanate conjugated gentamicin to cells grown at 30°C or 37°C. We conclude that the temperature-dependent variation in the aminoglycoside susceptibility of this species was not correlated with any detectable change in KDO content, but correlated well with phosphate content of LPS and that LPS phosphate is the major site of ionic interaction for aminoglycosides in *S. maltophilia*.

Introduction

*Stenotrophomonas maltophilia*, a clinically important microorganism causing a variety of infections, is known to be notably less susceptible to aminoglycoside antibiotics when incubated at 30°C than at 37°C. The mechanisms of this temperature-dependent variation in susceptibility (TDVS) have not been elucidated. Reports suggest that alteration in membrane proteins or lipids may be associated with TDVS in this organism, and more recently we have found temperature-dependent changes in lipopolysaccharide (LPS) structure and outer membrane organization that correlate well with TDVS.

The outer membranes of Gram-negative bacteria are known to be binding sites for cationic agents and antibiotics. The initial action of cationic antibiotics, including aminoglycosides, may be the disturbance of outer membrane integrity through interaction with LPS. These cationic antibiotics bind to and disturb the packing arrangement of isolated LPS suggesting that their interaction site on the outer membrane is LPS. Assuming that cationic antibiotics are electrostatically attracted to the high concentration of negative charge in the LPS core-lipid A region, resistance to these agents might be induced by decreasing the negative charge, either by loss of phosphate moieties or by esterification of the phosphate groups. This hypothesis is supported by studies of cells and isolated LPS from polymyxin-resistant mutants of *Salmonella typhimurium* which were shown to bind less polymyxin B and to have increased levels of arabinosamine, presumably esterified to lipid A phosphate, as well as ethanolamine. A another location for negative charge in LPS is 3-deoxy-\( \alpha \)-manno-octulosonic acid (KDO) present in the linkage between the core-lipid A region.

In this study we examined the phosphate and KDO content of LPS from *S. maltophilia* strains grown at 30°C or 37°C to determine whether these correlated with TDVS.

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MATERIALS AND METHODS

Bacterial strains

Thirty-eight S. maltophilia strains were studied; 33 of these were clinical isolates obtained from the University Hospital of Wales (Cardiff), the Royal Hallamshire Hospital (Sheffield) or the Rigshospitalet Copenhagen (Denmark), and five were reference strains (NCTC 10257, 10258, 10259, 10498 and 10499). Stock cultures were maintained at −70°C in 10% glycerol broth (Pro-Lab, Bromborough, U.K.). Isolates were cultured on blood agar and incubated aerobically at 30°C and 37°C for 24 h. The susceptibility profiles and MICs of these strains to a variety of antibiotics at 30°C and 37°C, and changes in outer and cytoplasmic membranes, whole cell proteins and LPS have been reported previously. Briefly, 23 of the 38 strains showed more than a four-fold difference in MIC to gentamicin and other aminoglycosides being susceptible at 37°C and resistant at 30°C. These strains are referred to as exhibiting ‘good’ temperature-dependent variation in susceptibility (TDVS) to aminoglycosides.

Preparation of LPS

The method of Fomsgaard et al. was used with some modifications. Briefly, strains were cultured on blood agar, incubated at 30°C or 37°C overnight. Colonies were suspended in deionized water to a turbidity of 2.5 at 550 nm. A liquid (0.5 mL) of the suspension were transferred to an Eppendorf tube, and 0.5 mL of 90% phenol was added. The suspensions were vortexed by shaking the tube over a strong flame until the brown colour of iodine had disappeared, then 0.25 mL of thiorbarbituric acid solution (0.6% thiorbarbituric acid, pH 9.0) was added and the mixture was heated at 100°C for 7.5 min. While the reaction mixture was still hot, 1 mL of dimethylsulphoxide was added, the mixture was cooled and the absorption was measured against a reagent blank at 548 nm. This procedure was repeated with 2–20 μg of KDO to produce a standard curve from which the KDO content of test samples was calculated.

Phosphate assay

The phosphate content of LPS was determined by the ashing method of A mes. To 0.01 mL of LPS sample in a Pyrex test tube, 0.03 mL of magnesium nitrate solution was added. The material was taken to dryness and ashed by shaking the tube over a strong flame until the brown fumes disappeared. The tube was then allowed to cool and 0.30 mL of 0.5 M HCl was added. The tube was capped and heated in a boiling water bath for 15 min to hydrolyse to phosphate any pyrophosphate formed in the ashing. After the tube was cooled, 0.7 mL of an ascorbic acid–ammonium molybdate mixture (one part of 10% aqueous ascorbic acid and six parts of 0.42% ammonium molybdate, tetrahydrated in 0.5 M HSO₄) was added to 0.3 mL of the LPS extract in an Eppendorf tube (the same volume of the mixture was added to 0.3 mL of distilled water as a blank) and incubated at 45°C for 20 min. The intensity of the colour reaction was measured at 820 nm after appropriate dilution to bring the absorbance into the 0–1.0 range. The amount of phosphate in each sample was calculated from the relationship that 0.01 μmol of phosphate gives an absorbance at 820 nm of 0.240.

KDO assay

KDO was determined essentially according to Hancock & Poxton. One mL of 0.125 M H₂SO₄ was added to 0.3 mL of LPS sample in an Eppendorf tube and the mixture was heated in a boiling-water bath for 8 min. Periodic acid reagent (25 mmol periodic acid in 62.5 mmol H₂SO₄; 0.25 mL) was mixed with 0.5 mL sample of the acid hydrolysate in a small tube and was incubated at 37°C for 30 min. Sodium arsenite solution (2% w/v sodium arsenite in 0.5 M HCl; 0.25 mL) was added to the cooled tube and was mixed until the brown colour of iodine had disappeared, then 0.25 mL of thiorbarbituric acid solution (0.6% thiorbarbituric acid, pH 9.0) was added and the mixture was heated at 100°C for 7.5 min. While the reaction mixture was still hot, 1 mL of dimethylsulphoxide was added, the mixture was cooled and the absorption was measured against a reagent blank at 548 nm. This procedure was repeated with 2–20 μg of KDO to produce a standard curve from which the KDO content of test samples was calculated.

Binding of FITC-labelled gentamicin by fluorescence-activated cell sorting

Organisms grown at 30°C or 37°C overnight in tryptone soya broth (TSB) were diluted to 10⁸ cfu/mL (OD = 0.15 at 460 nm). A liquid of suspensions (0.5 mL) were added to 0.5 mL of TSB containing 2 mM potassium cyanide (KCN) and 4 mM dinitrophenol (DNP) (final concentrations; 1 mM KCN and 2 mM DNP) and incubated at 4°C for 10 min. Fluorescein isothiocyanate (FITC) labelled gentamicin (100 μL) (Abbott Laboratories, Maidenhead, U.K.) was added and incubated at 4°C for 30 min. The suspension was washed three times with TSB, resuspended in 0.5 mL of TSB and kept in an ice-bath. Samples were analysed on a FACScan (Becton–Dickinson, San Jose, CA, USA) using 15 mW argon-ion laser for excitation at 488 nm. Filters for fluorescence detection were FL1: 530/30 nm bandpass (optimized for FITC) and FL2: 585/42 bandpass (optimized for phycoerythrin). The sheath fluid was phosphate-buffered saline (PBS) and data sets were collected on 10,000 events (S. maltophilia) using Lysys II software (Becton–Dickinson). A fter transferring data to an IBM PC compatible format using HPD1sk (A pplied Cytometry Systems, Sheffield, U.K.) analysis was performed with WinMDI (J. Trotter, Salk Institute, La Jolla, CA, USA) using mean fluorescence intensity (FL1-height) from histogram statistics.
Variations in binding sites in S. maltophilia

Results

LPS isolated from S. maltophilia grown at 37°C had a significantly greater content of phosphate than their counterparts grown at 30°C (P < 0.001; Table I). However, there was no significant difference in KDO content of LPS for strains grown at the two incubation temperatures (P > 0.05). This indicates that the number of negatively charged phosphate groups present in the LPS in isolates grown at 37°C is greater than that in isolates grown at 30°C. Differences in TDVS to aminoglycosides in these organisms may therefore relate to variations in phosphate content, and the resulting change in the number of binding sites available to the cationic aminoglycoside antibiotics. This theory is supported by the fact that the temperature-induced change in phosphate content was significant for good TDVS strains but not significant for poor TDVS strains (Table II). The KDO content of strains exhibiting good TDVS was not significantly different at growth temperatures of 30°C or 37°C (not shown).

To determine if growth temperature could affect the binding of gentamicin to S. maltophilia, FITC-labelled gentamicin binding was determined by FACS analysis. Typical three-dimensional density plots of fluorescence intensity versus forward scatter light and number of events for fluorescent gentamicin bound to S. maltophilia cells at 30°C and 37°C for a clinical isolate are shown in the Figure. The mean values of FL1-height (corresponding to fluorescence) for all the strains grown at 30°C and 37°C were: 11.38 ± 2.084 and 15.34 ± 4.620 respectively.

The amount of gentamicin bound to cells grown at 37°C was greater than at 30°C and this difference was significant (P < 0.01) for strains exhibiting good TDVS. This suggests that the number of gentamicin binding sites on the cell membranes was altered by incubation temperature and could account for differences in gentamicin susceptibility between strains grown at 30°C and 37°C.

Discussion

Many clinical isolates of S. maltophilia exhibit marked differences in susceptibility to aminoglycosides with growth temperature, being resistant at 30°C but susceptible at 37°C. This can have important consequences for both the laboratory susceptibility testing and the treatment of patients for this organism; low core body temperatures may exist in patients with sepsis and lower temperatures are often found in peripheral regions of the body.10 In this study, we have analysed anionic sites in the LPS of S. maltophilia exhibiting temperature-dependent variations in susceptibility (TDVS) to aminoglycosides and correlated the expression of such ionic groups with gentamicin binding at 30°C and 37°C.

It has been demonstrated that phosphate and KDO residues in the core region of LPS are the main cell surface groups25 possessing negative charge.15 It has been shown that the core region of S. maltophilia contains no heptose but significant amounts of mannose residues, unlike most other Gram-negative organisms. Mannose would therefore be the major site of phosphorylation in

Table I. Phosphate and KDO content of LPS in all S. maltophilia strains grown at 30°C and 37°C

<table>
<thead>
<tr>
<th>Growth temperature</th>
<th>Phosphate (µmol/mL) mean ± s.d.</th>
<th>KDO (µmol/mL) mean ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>30°C (n = 38)</td>
<td>2.98 ± 0.588</td>
<td>0.0926 ± 0.0339</td>
</tr>
<tr>
<td>37°C (n = 38)</td>
<td>3.58 ± 0.635&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0963 ± 0.0279&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.001 versus 30°C.
<sup>b</sup> Not significant versus 30°C.

Table II. Phosphate content of LPS from isolates exhibiting poor and good TDVS<sup>a</sup>

<table>
<thead>
<tr>
<th>Growth temperature</th>
<th>Number of strains</th>
<th>Phosphate (µmol/mL) mean ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor TDVS strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td>15</td>
<td>3.08 ± 0.678</td>
</tr>
<tr>
<td>37°C</td>
<td>15</td>
<td>3.41 ± 0.628&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Good TDVS strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td>23</td>
<td>2.92 ± 0.598</td>
</tr>
<tr>
<td>37°C</td>
<td>23</td>
<td>3.57 ± 0.647&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> KDO (mean value 0.0942 ± 0.0351 µmol/mL) was not significantly different in good and poor TDVS strains at each temperature.
<sup>b</sup> Not significant versus 30°C.
<sup>c</sup> P < 0.001 versus 30°C.
S. maltophilia. Phosphate residues were found to be esterified to a greater extent on the LPS from resistant strains of E. coli than the phosphate of LPS from susceptible strains. Esterification of phosphate would, therefore, decrease the overall negative charge of the core-lipid A region and may alter polycation binding sites on the LPS.

The results of this study showed that the amount of phosphate in the LPS of the isolates exhibiting good TDVS grown at 30°C was significantly lower (P < 0.001) than that of the isolates grown at 37°C, indicating that fewer anionic binding sites would be available for aminoglycosides at 30°C. This is in agreement with the results of McConnell & Wright, who showed that LPS molecules from Salmonella anatum grown at low temperature were deficient in phosphate and primary amine. Taken together with the fact that increasing the incubation temperature shifts the LPS structure to a lower molecular weight with smaller polysaccharide side chains, these results are in agreement with the results of Vanhoof et al. who stated that rough LPS of S. maltophilia had a relatively high phosphorus content. It seems likely that the phosphate molecules in the LPS of S. maltophilia as in other organisms undergo esterification in the strains grown at 30°C, decreasing the number of binding sites available to aminoglycosides. This is in agreement with studies on Burkholderia cepacia (formerly Pseudomonas cepacia) which suggested that resistance to cationic antibiotics stemmed from ineffective binding to the outer membrane as a consequence of the low content of phosphate groups. However, there was no significant difference in the amount of KDO relative to LPS in the organisms grown at the two incubation temperatures, indicating no changes in KDO content with alteration in incubation temperature. This was supported by McGroarty who showed that the KDO content of LPS in mutant and parental strains of E. coli and P. aeruginosa was identical, indicating no major changes in KDO content due to variation in physical factors, such as temperature.

The results of FACS analysis of aminoglycoside binding were as predicted from the phosphate results. The amount of fluorescently labelled gentamicin bound to the cell membrane of the strains exhibiting TDVS was significantly greater at 37°C than at 30°C (P < 0.01). Furthermore, when gentamicin or other agents known to disrupt the outer membrane organization (polymyxin B and EDTA) were added to the S. maltophilia strains in the presence of lysozyme, greater lysis of the organisms resulted at 37°C than at 30°C compared with organisms not incubated with lysozyme (our unpublished data). This suggests that these outer membrane disrupting agents were more active in permeabilizing the outer membrane to lysozyme at the higher growth temperature. This in turn reflects alterations in the outer membrane packing or structural conformation resulting in increased interaction with these agents at 37°C, or increased anionic binding sites (phosphate). The results of this study, and our previous study suggest that the combined alteration in size of the O-polysaccharide and changes in phosphate content of the LPS from S. maltophilia induced by different growth temperatures are responsible for the temperature-dependent susceptibility to aminoglycosides seen in this organism. The mechanism by which growth temperature can induce these changes in LPS composition and structure should now be investigated.
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References


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