The effect of antibiotics on the cell morphology of *Legionella pneumophila*

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Summary. *Legionella pneumophila*, in Buffered Yeast Extract broth, was treated for 5 h at 37°C with rosaramicin, erythromycin, cefotaxime, dibekacin, penicillin, methicillin, cefoxitin, cephalothin, ticarcillin, carbenicillin or polymyxin B at near-MIC levels and above. Electronmicroscopy demonstrated morphological changes to the bacteria in some, but not all, of the antibiotic-treated suspensions. Penicillin, at 1000 µg/ml (40 × MIC) but not less, produced smooth bubble-like structures on cell surfaces; methicillin produced rough bubble-like structures at 100 µg/ml (MIC) but not at 1000 µg/ml. In each case, these structures resembled spheroplasts. Polymyxin B induced small-bleb formation on the bacterial cell surfaces at all concentrations tested (MIC–10 × MIC). The other eight antibiotics did not induce any morphological changes at any concentration tested.

Introduction

Antibiotic susceptibility of *Legionella pneumophila* has been studied extensively by a wide variety of techniques, including agar-dilution and broth-dilution methods, but the effect of antibiotics on the morphology of the organism has not been investigated systematically. *L. pneumophila* has a high proportion of branched-chain fatty acids in its cell wall, a much higher proportion than is found in most other gram-negative bacteria (Moss *et al.*, 1977). This unique composition of its cell wall may allow expression of different cell morphologies when the organism is treated with different concentrations of certain antibiotics. In the present study, the effect of eleven antibiotics on the cell morphology of *L. pneumophila* was observed by electron-microscopy (EM).

Materials and methods

Bacterial strain

Legionella pneumophila (Knoxville strain 1), was kindly provided by Dr Fliermans, Savannah River Laboratory (E. I. DuPont de Nemours and Co., Aiken, SC 29808, USA). One ml of a suspension of *L. pneumophila* containing 10⁵ cfu/ml was injected intraperitoneally into each of four guinea pigs. All animals became moribund within 4 days and were then killed and their spleens and livers removed aseptically, emulsified in sterile saline and frozen (–70°C) for further use.

Antibiotics

The antibiotics employed in this study were first dissolved in distilled water at a concentration of 10 mg/ml, and then further diluted to the desired concentrations. Penicillin G potassium salt (E. R. Squibb and Sons, Inc., Princeton, NJ, USA), methicillin sodium (Beecham Laboratories, Pittsburg, PA, USA), cefoxitin sodium (Merck, Sharp and Dohme, West Point, PA, USA), bisodium ticarcillin (Beecham Laboratories, Pittsburg, PA, USA), cephalothin sodium (Lilly, Indianapolis, IN, USA), rosaramicin (Schering Corporation, Kenilworth, NJ, USA), erythromycin lactobionate I.V. (Abbott Laboratories, Chicago, IL, USA), carbenicillin disodium (Roerig, New York, NY, USA), cefotaxime sodium (Hoechst-Roussel Pharmaceuticals, Somerville, NJ, USA), dibekacin (USV Laboratories, Tuckahoe, NJ, USA) and polymyxin B (Pfizer, Inc., New York, NY, USA), were all tested at minimal inhibitory concentration (MIC) or half-MIC levels and at multiples thereof up to (5–320) × MIC (see table).

Susceptibility testing

MIC values were determined by an agar dilution procedure. Buffered Charcoal Yeast Extract agar (BCYE) (Pasculle *et al.*, 1980) was prepared containing graded (two-fold) dilutions of antibiotics. Inocula were prepared by harvesting *L. pneumophila* from a 48-h plate culture in 1 ml of saline, which resulted in a concentration of c. 10⁹ organisms/ml. Agar dilution plates were inoculated with a Steers replicator. The final test inoculum was approximately 5 × 10⁶ cfu/0.002-ml spot. The large inoculum size was required for optimal compatibility with electron-microscopy procedures. The inoculated plates were incubated at 37°C for 48 h, after which time MIC values

Received 23 Jul. 1985; revised version accepted 11 Jul. 1986.

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Table. List of antibiotics and their concentrations used in test for effects on *L. pneumophila* cell morphology

Antibiotic	Concentrations ($\mu\text{g/ml}$) used in tests		
Rosaramicin	0.125 ($\frac{1}{2} \times \text{MIC}$)	0.25 (MIC)	25 ($10 \times \text{MIC}$)
Erythromycin	0.5 ($\frac{1}{2} \times \text{MIC}$)	1.0 (MIC)	10 ($10 \times \text{MIC}$)
Dibekacin	1.0 ($\frac{1}{2} \times \text{MIC}$)	2.0 (MIC)	10 ($10 \times \text{MIC}$)
Penicillin	25 (MIC)	100 ($4 \times \text{MIC}$)	1000 ($40 \times \text{MIC}$)
Methicillin	...	100 (MIC)	1000 ($10 \times \text{MIC}$)
Ticarcillin	100 ($\frac{1}{2} \times \text{MIC}$)	200 (MIC)	1000 ($5 \times \text{MIC}$)
Carbenicillin	10 ($\frac{1}{2} \times \text{MIC}$)	20 (MIC)	200 ($10 \times \text{MIC}$)
Cefoxitin	3.12 (MIC)	100 ($32 \times \text{MIC}$)	1000 ($320 \times \text{MIC}$)
Cefotaxime	4.0 ($\frac{1}{2} \times \text{MIC}$)	8.0 (MIC)	80 ($10 \times \text{MIC}$)
Polymyxin B	160 (MIC)	320 ($2 \times \text{MIC}$)	1600 ($10 \times \text{MIC}$)

The concentrations shown in **bold** are those that caused morphological changes.

were determined. The MIC value was the lowest concentration of drug that completely inhibited growth, except that one or two colonies or a faint hazy growth were ignored (see table).

Antibiotic treatment

One ml of the spleen or liver suspension from infected guinea pigs was seeded on BCYE agar and incubated at 37°C for 48 h. *L. pneumophila* organisms from such cultures were washed off with saline, concentrated by centrifugation and resuspended in Buffered Yeast Extract broth (Yeast Extract, Difco, 10 g/L; ACES buffer, Calbiochem-Behring 10 g/L; activated charcoal, Norit A, 2 g/L; L-cysteine $\text{HCl} \cdot \text{H}_2\text{O}$ 0.4 g/L; ferric pyrophosphate 0.25 g/L; pH adjusted to 6.8 with KOH). The resulting bacterial suspension was diluted in the broth to approximately the opacity of a MacFarland No. 3 nephelometer standard (10^9 cfu/ml), and samples (9 ml) were incubated at 37°C for 4 h, before antibiotic treatment. At this time, the organisms were in the logarithmic growth phase as determined by standard plate counts. After pre-incubation, 1 ml of $10 \times$ the desired concentration of each of the respective antibiotics was added to separate samples of the bacterial suspension, which were then incubated for another 5 h at 37°C . At the end of this period, glutaraldehyde 2.5% v/v fixative was added to each of the suspensions, which were then held at 4°C overnight before being prepared for EM examination. Control cultures were prepared and treated in an identical manner, but with the substitution of sterile saline for antibiotic solutions.

Scanning EM

After the overnight fixation with glutaraldehyde, the cell suspensions were each centrifuged and washed three times with deionised water that had previously been filtered through a 0.22- μm "micropore" filter. After resuspension in deionised water, one drop (0.025 ml) of each suspension was added to 1 ml of absolute ethyl

alcohol, which was applied to a standard metal support plate and allowed to dry in air. The metal plates were then coated with gold palladium in a Polaron SEM coating unit, at 7MA for 1 min, after which time they were examined at a 30° angle, in a scanning mode, with a Phillips EM400 electronmicroscope.

Transmission EM

After the overnight fixation with glutaraldehyde, suspensions were centrifuged and washed three times for 10 min in sodium cacodylate buffer (pH 7.2). The cells were then post-fixed for 2 h in 2% buffered osmium tetroxide and again washed three times in the buffer. The cells were centrifuged to a pellet and embedded in 2% Noble agar. The agar blocks were dehydrated in a graded alcohol series, followed by propylene oxide, embedded in Epon and sectioned with a diamond knife. Ultra-thin sections were stained with uranyl acetate and lead citrate and were examined at 20 kV in a Phillips EM400 electronmicroscope.

Results

When examined by scanning EM, the untreated control cells demonstrated a wavy, wrinkled cell surface and had a length of 2–20 μm and a diameter of approximately 0.3–0.9 μm (fig. 1). Transmission EM demonstrated an outer cell wall, an inner membrane and vacuoles within cells (fig. 2), in agreement with previously published descriptions (Chandler *et al.*, 1979). As indicated in the table, antibiotic treatment with rosaramicin (0.125, 0.25, 25 $\mu\text{g/ml}$), erythromycin (0.5, 1.0, 10 $\mu\text{g/ml}$), carbenicillin (10, 20, 200 $\mu\text{g/ml}$), ticarcillin (100, 200, 1000 $\mu\text{g/ml}$), cephalothin (6.25, 100, 1000 $\mu\text{g/ml}$), cefoxitin (3.12, 100, 1000 $\mu\text{g/ml}$), cefotaxime (4.0, 8.0, 80 $\mu\text{g/ml}$) and dibekacin (1.0, 2.0, 20 $\mu\text{g/ml}$) did not result in any morphologic changes observed by either EM method, as compared with control

Fig. 1. Scanning EM of *L. pneumophila* (control). Bar = 0.2 μ m.

Fig. 2. Transmission EM of *L. pneumophila* (control). Bar = 0.4 μ m.

Fig. 3. Scanning EM of *L. pneumophila* treated with methicillin 100 μ g/ml. Note the swelling at the middle of the cell (arrow). Bar = 0.4 μ m.

cells. Some morphological changes were seen with methicillin, penicillin and polymyxin B.

Methicillin at a concentration of 100 μ g/ml (MIC), but not at 1000 μ g/ml (10 $\times$ MIC), produced a sequence of events in the *L. pneumophila* cell that was demonstrated by both transmission and scanning EM. Initially, methicillin caused the cell to swell at the centre (fig. 3). The centre portion continued to enlarge and a spheroplast-like structure developed (fig. 4). The cell wall remained sufficiently intact to contain the contents of the cell. Finally, the spheroplast-like structure collapsed (fig. 5). The collapse of the cell as observed by scanning EM could be correlated with the transmission EM findings of outer cell wall and inner membrane break down (fig. 6). Throughout the methicillin treatment the cell surface remained rough and wavy, regardless of spheroplast development and enlargement as observed by scanning EM (figs. 3 and 5).

Penicillin at a concentration of 1000 μ g/ml (40 $\times$ MIC), but not 25 or 100 μ g/ml, caused bubble-like structures and openings to appear on the cell surface of *L. pneumophila* (fig. 7). Unlike those previously noted after methicillin treatment, these penicillin-induced structures demonstrated a smooth surface and appeared to originate from the interior of the cell. One or more such structures could be found on cells treated with this concentration of penicillin, although the remainder of the cell surface appeared normal.

Polymyxin B caused formation of small blebs on the bacterial surfaces at all concentrations tested (160, 320, 1600 μ g/ml); 1 $\times$, 2 $\times$, 10 $\times$ MIC). These blebs were not readily detected by scanning EM after the low-concentration treatment (160 μ g/ml), but were clearly observed at the highest (1600 μ g/ml) (fig. 8). With transmission EM however, blebs were easily observable with all three concentrations of polymyxin B (fig. 9). The blebs appeared to arise due to selective folding of the outer cell wall, while the inner membrane remained unchanged. At the highest antibiotic concentration, nuclear material was also observed, resembling a patchwork pattern within the cytoplasm (fig. 9). This patchwork of nuclear material was not seen with the lower concentrations (160 and 320 μ g/ml), nor with other antibiotic-treated cell suspensions.

Discussion

Legionella pneumophila is an organism that possesses a unique cellular lipid composition, with a high proportion (> 68%) of branched-chain fatty acids (Moss *et al.*, 1977; Moss and Dees, 1979).

Fig. 4. Transmission EM of *L. pneumophila* treated with methicillin 100 µg/ml. The spheroplast is about four times the original size, with intact cell walls (arrow). Bar = 0.2 µm.

Fig. 6. Transmission EM of *L. pneumophila* treated with methicillin 100 µg/ml demonstrating loose attachment of the cell membrane (arrow). Bar = 0.2 µm.

Fig. 5. Scanning EM of *L. pneumophila* treated with methicillin 100 µg/ml. Collapse of the spheroplast-like structure (arrow). Bar = 0.4 µm.

This unusual chemical make-up may affect the organism's morphological response to certain antibiotics. Its known lag phase of 3–4 h and generation time of 4–6 h required the bacterial culture to be pre-incubated for 4 h to ensure that the logarithmic growth phase had been attained (Warren and Miller, 1979). This was done because most of the antibiotics used in this study were most effective during cell division. In the present study, the response of *L. pneumophila* was similar to that expected of a gram-positive organism with regard to penicillin, but similar to that of a gram-negative bacillus with regard to methicillin. In its observed effects upon *L. pneumophila*, penicillin appears to

precipitate a classic antibacterial sequence which we interpret as follows: peptidoglycan cross-linkage formation is inhibited by high concentrations of penicillin, which consequently produce a cell-membrane lesion, or opening, in the cellular envelope. This could allow extra-cellular fluid to enter the cell osmotically, resulting in cellular swelling. It then follows that the expanded inner cell membrane would protrude through resultant antibiotic-induced lesions causing the bubble-like structures to appear. Eventually these bubble-like structures would burst, leaving behind an opening in the cell surface. The documented presence of a peptidoglycan layer in the cell envelope of *L. pneumophila* (Flesher *et al.*, 1979) and the ability of this species to produce β -lactamase (Fu and Neu, 1979) may explain both the observed effects on the cell wall and why only a high concentration (1000 µg/ml) of penicillin is capable of producing these bubble-like structures, whereas lower concentrations (25 and 100 µg/ml) do not. This would be due to the inactivation of penicillin at lower concentrations by the β -lactamase produced by *L. pneumophila*.

The effect of methicillin on the morphology of *L. pneumophila* was different from that of penicillin but comparable to the response of a gram-negative bacillus to β -lactam antibiotics. Thus the morphological changes that we noted can be set in a sequence from bulge formation at mid-cell, to spheroplast-like formation and, finally, to cell lysis due to the dissolution of the outer membrane as observed with transmission EM. The effect of methicillin on *L. pneumophila* can also be compared

with that of cephalothin on gram-negative bacteria, in that spheroplast-like formation occurs with high frequency at drug levels near the MIC, but less frequently as drug concentrations are increased (Nishino and Nakazawa, 1976). Methicillin could exert an influence upon a unique penicillin-binding protein in a manner analogous to that of β -lactam antibiotics binding to the penicillin-binding protein-1 of *Escherichia coli* (Kitano and Tomasz, 1979), which effect is known to be associated with

Fig. 7. Scanning EM of *L. pneumophila* treated with penicillin 1000 $\mu\text{g/ml}$. Note smooth bubble-like structures on the cell surface. Arrow shows opening after the inner membrane has burst. Bar = 0.4 μm .

Fig. 8. Scanning EM of *L. pneumophila* treated with polymyxin B 1600 $\mu\text{g/ml}$, demonstrating bleb formation on the cell surface. Bar = 0.2 μm .

Fig. 9. Transmission EM of *L. pneumophila* treated with polymyxin B 1600 $\mu\text{g/ml}$, demonstrating bleb formation from the outer cell membrane (arrow) and patchwork nuclear material. Bar = 0.1 μm .

spheroplast-like formation and triggers of autolytic wall degradation. Further investigation is needed to determine the actual mechanism(s) responsible for the events induced by methicillin.

With regard to the surface blebs caused by treatment with polymyxin B, the induction of bleb formation by this antibiotic on gram-negative bacteria is well documented (Koike *et al.*, 1969; Lounatmaa *et al.*, 1976). The chemical nature of the binding site on the cell wall is reported to be phospholipid (Newton, 1956), and the binding of polymyxin B to the cell membrane of *L. pneumophila* is therefore understandable because of the reportedly high phospholipid content of this organism (Keel *et al.*, 1979). According to the finding of Hsueh and Feingold (1973), bleb-formation probably results from the intercalated polymyxin B in the cell wall, in close association with negatively charged phospholipids. Lounatmaa *et al.* (1976) demonstrated that the intercalation of a large amount of polymyxin B in the cell membrane resulted in increased surface area and bleb formation in *Salmonella* strains. Because of the tight binding of the outer membrane to the rigid peptidoglycan layer in *L. pneumophila* (Flesher *et al.*, 1979), the outer membrane is probably not free to expand. The increase of the surface area under these conditions thus forces the cell wall into folds and bleb formation, as explained by Lounatmaa *et al.* (1976). This bleb formation induced by polymyxin B alters the virulence of the organism (Chan E L, Harris R C, Weeks B S and Dalton H P; unpublished observation). The patch-distribution

pattern of the nuclear material has not been previously reported as an effect of polymyxin B on *L. pneumophila*.

With regard to the failure of the other antibiotics tested to induce morphological changes in *L. pneumophila*, this may be due to its high susceptibility to the cephalosporin antibiotics. The organism is weakly β -lactamase positive. This β -lactamase is more active against the penicillins and less active against cephalosporins, as evidenced by the lower MICs of cephalosporins than of the penicillins for *L. pneumophila*. Carbenicillin and

ticarcillin probably bind to a different penicillin-binding protein from that for penicillin or methicillin and were therefore unable to induce lesions or other morphological changes. Rosaramicin, erythromycin and dibekacin were highly active against *L. pneumophila*. All organisms in the suspensions were killed within 1 h, before any morphologic changes could have taken place. However, such changes would not be expected as these antibiotics are known to act on protein synthesis rather than on cell wall synthesis.

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