Cefuroxime resistance in non-β-lactamase *Haemophilus influenzae* is linked to mutations in *ftsI*

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The penicillin binding protein (PBP) genes *dacA*, *dacB* and *ftsI* from 14 cefuroxime-resistant (CXM⁰) isolates and three clinical isolates with low CXM MIC for non-β-lactamase-producing *Haemophilus influenzae* type b were molecularly characterized. One strain, 5788, was used to transform *H. influenzae* Rd to CXM⁰ for direct comparison of the *pbps* in the same genetic background. No obvious mutations in the *dacA* and *dacB* gene products could be associated with CXMR. One amino acid substitution in the *ftsI* gene product in particular, S357N, could give rise to CXM⁰. Sequence analysis from the CXM⁰ transformants also implicated *FtsI*; in this case, the substitutions were V511A and R517H. To verify S357N substitution, the protein sequence of *H. influenzae* FtsI was threaded through the *S. pneumoniae* PBP 2X structure giving an average root mean square deviation of the α-carbon chains of 0.5 Å. The S357N substitution alters both the residue size and charge. One explanation for the contribution of S357N to CXM⁰ is that the asparagine side-chain produces unfavourable steric hindrance with the side chain of Val-362 changing the torsion angles of the asparagine residue, which in turn may influence the position of the loop V362–P366 adjacent to the active site. Whilst other groups have examined the contribution of *H. influenzae* PBPs in ampicillin resistance, this is the first report analysing their role in CXM⁰.

Keywords: penicillin binding proteins, β-lactam resistance

Introduction

*Haemophilus influenzae* non-capsulate strains are usually responsible for non-invasive disease, causing local infections that are often associated with an underlying physiological or anatomical abnormality. The most common involve the respiratory tract: otitis media, sinusitis, and purulent episodes in patients with chronic obstructive pulmonary disease. Invasive infections (most commonly type b) include meningitis, epiglottitis, bacteremia and pneumonia; however, with the advent of the Hib vaccine, cases associated with capsulated strains have diminished appreciably.¹

From its introduction in the early 1960s, ampicillin was regarded as the drug of choice for the treatment of infections in children, and proved to be effective against *H. influenzae*. In more recent times, cefuroxime (CXM) replaced ampicillin, particularly for community-acquired pneumonias due to ampicillin-resistant *H. influenzae*. Resistance to β-lactams is mediated by two mechanisms, namely, production of a constitutively expressed β-lactamase (often TEM but sometimes ROB-1) and mutation of the β-lactam targets, the penicillin-binding proteins (PBPs). Whilst β-lactamase production in *H. influenzae* has been well characterized, there is a paucity of information on alterations of PBPs in ‘non-β-lactamase-producing’ *H. influenzae* (NBHI). The PBPs of *H. influenzae* were first characterized in 1981, when eight major PBPs were detected and the pattern of PBPs was demonstrably different.
from that of *Escherichia coli*. Unfortunately, since this initial characterization, research on *H. influenzae* PBPs has been confused by the use of different naming systems by different research groups. The genome sequence of *H. influenzae* Rd was published in 1995 and studies have utilized this information to study PBP genes. Ubukata et al. molecularly characterized PBPs from ampicillin-resistant *H. influenzae* and reported differences in the *ftsI* gene product. Dabernet et al. also determined the sequence of *ftsI* in almost 200 ampicillin-resistant isolates from different geographical locations and were able to correlate ampicillin resistance with particular mutations in *ftsI*. However, the conclusions of this study were not tested by transformation. In this study, we have analysed PBP genes linked to β-lactam resistance from other studies as possible mediators of cefuroxime resistance (CXM R); namely *dacA*, *dacB* and *ftsI*. This study was facilitated by the published genome sequence of *H. influenzae* Rd and identification of PBP genes based on their homologies to those in *E. coli*. Mutations believed to be responsible for the CXM R phenotype were verified by transformation and gene sequencing. Protein modelling has been used to investigate the molecular significance of the *ftsI* mutations.

**Materials and methods**

### Haemophilus isolates

Seventeen clinical isolates of *H. influenzae* type b were collected from patients at Southmead Hospital, Bristol, and the Royal United Hospital, Bath (Table 1). Of these, three were cefuroxime sensitive (CXM<sup>+</sup>) and 14 were CXM<sup>−</sup>. The isolates were confirmed as *H. influenzae* by API-NH testing. All isolates were tested for β-lactamase activity using the chromogenic substrate nitrocefin. Strains were stored at −70°C in horse blood with 15% glycerol.

### Media and antibiotics

The medium used for growth and determination of MICs was brain–heart infusion agar or broth supplemented with 10 µg of histidine, 10 µg of haemin and 15 µg of β-NAD per mL. Chocolate agar was used for routine culturing. Plate cultures were incubated at 37°C in an atmosphere of 5% CO₂. The medium used for transformation was M-IV stage II medium. Selective agar was brain–heart infusion agar supplemented with 10 µg of histidine, 10 µg of haemin and 15 µg of β-NAD per mL, plus an appropriate concentration of CXM. The antibiotics used in the study were CXM, ampicillin, kanamycin (Sigma, Poole, Dorset, UK) and imipenem (Merck, Sharpe & Dohme, Hoddesdon, Herts, UK).

### Determination of the MIC

The MICs of CXM and other β-lactams for clinical strains and transformants were determined by both Etest (AB Biodisk, Solna, Sweden) and agar dilution according to NCCLS guidelines. MICs were determined in triplicate. The MIC was defined as the lowest concentration of antibiotic that totally inhibited growth of the inoculum.

**Table 1.** Cefuroxime (CXM) MICs and sequence variation for *ftsI* in clinical strains of *H. influenzae* used in this study and compared with the grouping of Dabernet *et al.*

<table>
<thead>
<tr>
<th>Strain</th>
<th>CXM MIC</th>
<th>Sequence variations from genomic sequence of <em>H. influenzae</em> Rd</th>
<th>Group classification of Dabernet <em>et al.</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>HIBLN</td>
<td>0.25</td>
<td>D350N</td>
<td>–</td>
</tr>
<tr>
<td>4731</td>
<td>0.5</td>
<td>NONE</td>
<td>–</td>
</tr>
<tr>
<td>4722</td>
<td>0.75</td>
<td>D350N</td>
<td>–</td>
</tr>
<tr>
<td>4717</td>
<td>2</td>
<td>A502T, N526K</td>
<td>IIb</td>
</tr>
<tr>
<td>4718</td>
<td>8</td>
<td>D350N, M377I, A502V, N526K</td>
<td>IIb</td>
</tr>
<tr>
<td>5782</td>
<td>8</td>
<td>R501H, N526K</td>
<td>–</td>
</tr>
<tr>
<td>5783</td>
<td>8</td>
<td>R501H, N526K</td>
<td>–</td>
</tr>
<tr>
<td>5794</td>
<td>8</td>
<td>S357N, N526K, Y528H</td>
<td>–</td>
</tr>
<tr>
<td>5801</td>
<td>8</td>
<td>A502V, N526K</td>
<td>IIb</td>
</tr>
<tr>
<td>5804</td>
<td>8</td>
<td>A337V, S357N, N526K, Y528H</td>
<td>–</td>
</tr>
<tr>
<td>4831</td>
<td>8</td>
<td>A368T, A502T, N526K</td>
<td>IIc</td>
</tr>
<tr>
<td>5788</td>
<td>16</td>
<td>V511A, R517H</td>
<td>–</td>
</tr>
<tr>
<td>5785</td>
<td>&gt;32</td>
<td>R501H</td>
<td>–</td>
</tr>
<tr>
<td>5793</td>
<td>&gt;32</td>
<td>S357N, T443A, R501L, N526K</td>
<td>–</td>
</tr>
<tr>
<td>4719</td>
<td>8</td>
<td>A502T, N526K</td>
<td>IIb</td>
</tr>
<tr>
<td>5795</td>
<td>8</td>
<td>S357N, A502T</td>
<td>–</td>
</tr>
<tr>
<td>5800</td>
<td>8</td>
<td>A502V, N526K</td>
<td>IIb</td>
</tr>
<tr>
<td>4797</td>
<td>&gt;32</td>
<td>S357N, N526K, T532S</td>
<td>–</td>
</tr>
</tbody>
</table>
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Table 2. Nucleotide sequences for primers used for PCR and sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference</th>
<th>Oligonucleotide sequence (5’ → 3’)</th>
<th>Size of PCR product (base pairs)</th>
</tr>
</thead>
</table>
| *ftsI* | GenBank accession number AB035738 | FP: TAGCTATGGCGACTGCGCCC
RP: GGGCAGAAACCGCACCACCA | 776 |
| *dacB* | GenBank accession number AB035865 | FP: CCACCTCGCCCCACCTGATCGT
RP: GCCTGAAGGTCCTAAACGCAG | 1063 |
| *dacB* | GenBank accession number AB035864 | FP: GCCAGCTAGGTATAAACGCAG
RP: CTGCCTGAAGGTCCTAAACGT | 1147 |
| *dacA* | TIGR locus HI0029 | FP: CCGTATCTGAGAGATATGC
RP: CCCGCTTCTCCCCACATATT | 1022 |

**DNA isolation**

Strain 5788 was grown overnight on chocolate agar. After 24 h, the confluent bacterial lawn was suspended in 9.5 mL of TE buffer (10 mM Tris, 1 mM EDTA) to which were added 1 mg of proteinase K and 0.5 mL of 10% SDS. The cell suspension was incubated at 37°C until no longer cloudy (usually after 1 h); 0.5 mL of 5 M NaCl was added and mixed thoroughly; 1.5 mL of CTAB/NaCl solution [0.3 M CTAB (hexadecyltrimethyl ammonium bromide), 0.7 M NaCl] was added and the solutions mixed then incubated at 65°C for 20 min. An equal volume of chloroform/isooamyl alcohol (24:1) was added, the mixture shaken and centrifuged at 13 000 rpm for 15 min. The liquid was removed and the DNA pellet was suspended in 1 mL of 70% ethanol. The suspension was transferred to a 1.5 mL Eppendorf tube and spun in a microcentrifuge (Eppendorf 5415D) at 13 000 rpm for 15 min. The supernatant was poured off and the DNA was centrifuged at 13 000 rpm for 30 min at 4°C. The supernatant was poured off and the DNA pellet was suspended in 1 mL of 70% ethanol. The suspension was transferred to a 1.5 mL Eppendorf tube and spun in a microcentrifuge (Eppendorf 5415D) at 13 000 rpm for 15 min. The liquid was removed and the DNA pellet was washed with absolute alcohol and then dried at 30°C. The pellet was finally dissolved in 50 µL of diethyl pyrocarbonate (DEPC)-treated H₂O. DNA was stored at −20°C until needed.

**Transformation**

Transformation was based on the method of Herriott et al. using M-IV stage II medium. The recipient *H. influenzae* Rd strain RM 132b was grown overnight on chocolate agar. Five colonies were suspended in 1 mL of M-IV medium and incubated at 37°C for 100 min. The suspension was centrifuged at 13 000 rpm for 2 min, the supernatant removed and the cell pellet resuspended in 1 mL of M-IV medium; 10 µL of the DNA solution prepared as described was added to the cell suspension and the mixture incubated at 37°C for 30 min. DEPC-H₂O was used as a negative control, and a kanamycin resistance plasmid (*H. influenzae* 132b P5 construct) was used as the positive control; in 10 and 1 in 100 dilutions of the DNA-treated cells (and controls) were plated on to selective agar containing 8 mg/L CXM. The positive control cells were plated on agar containing 20 mg/L kanamycin. Plates were incubated at 37°C for 24 h.

**PCR amplification of PBP genes**

PCR was carried out using a Hybaid MBS system (Hybaid, Middlesex, UK). *H. influenzae* strains were grown overnight on chocolate agar. Two colonies from each plate were suspended in 50 µL of DEPC-H₂O and incubated at 95°C for 10 min. The suspension was centrifuged at 13 000 rpm for 10 min to give a crude cell lysate containing template DNA. To 10 µL of this lysate was added 5 µL of 10× buffer, 1 mM MgCl₂, 0.1 mM each dNTP, 2 µL each primer and 2.5 U Taq DNA polymerase (MBI Fermentas) and water to give a total volume of 50 µL.

Primer pairs for the genes *ftsI* and *dacB* were designed from sequences in the GenBank nucleotide sequence database, and those for *dacA* from the Institute for Genomic Research nucleotide database (Table 2). Two different sequences were available for *dacB*; therefore, two primer sets were designed to amplify both alleles. All primers were from Sigma-Genosys Ltd, Pampisford, Cambs, UK and are listed in Table 2.

Amplification conditions were 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C, followed by 5 min at 72°C. PCR products were analysed on 1% agarose gels to visualize them and were then purified using the Qiagen QIAquick PCR purification kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer’s instructions.

**Sequencing**

Purified PCR products were sequenced at the Advanced Biotechnology Centre (Imperial College of Science, Technology...
Protein modelling

A homology model of *H. influenzae* Rd PBP3 was produced. The primary amino acid sequence was threaded through the published crystal structure of *S. pneumoniae* PBP 2X (PDB entry code 1QME) using SWISS-MODEL. The resulting structure was inspected manually for unusual torsion angles and steric clashes. Torsion angles were changed manually to correct such conflicts. In particular, the torsion angles of Asn-357 were altered from their threaded values of $\phi = 179^\circ$, $\psi = -162^\circ$ and $\omega = -169^\circ$ to $\phi = -168^\circ$, $\psi = -167^\circ$ and $\omega = 173^\circ$ to relieve steric hindrance between this residues side-chain and Val-362. The resulting structure was energy minimized with steepest descent and≤100 of conjugate gradient minimization using the GROMOS96 program with parameter set 43B1, until the energy differential between steps was <0.01 kJ/mol.

Protein docking

A solvent-accessible Connolly surface of the resultant homology model was created with Quantum Chemistry Program Exchange program 429 (University of Indiana, IN, USA) using a 1.4 Å solvent probe. The CXM structure was docked into this surface using the flexX program (Tripos Inc., Cambridge, UK) with a 60 × 60 × 60 grid centred on the $\alpha$-carbon atom of the active site residue Thr-328 and a grid spacing of 0.375 Å.

Results

$\beta$-Lactam MICs for *H. influenzae* clinical isolates and transformants

All CXMR isolates were also ampicillin resistant (>32 mg/L). Clinical isolate 4717, with a CXM MIC of 2 mg/L had an ampicillin MIC of 16 mg/L. CXM$^R$ transformants of *H. influenzae* strain Rd gave comparable CXM MIC results (32 mg/L) to that of the donor strain 5788 (16 mg/L). CXM MICs for clinical strains are shown in Table 1.

PCR amplification and sequencing

PCR products were obtained using all primer sets, with the exception of the $\beta$-lactamase-negative control strain HIBLN, for which no products were obtained using primer sets targeted to dasB and dasA. The reason for this is unclear. Nucleotide sequences produced by PCR amplification were translated into predicted amino acid sequences and compared with the published *H. influenzae* Rd genome sequence.

Changes in the ftsI gene product in clinical isolates

Three amino acid substitutions in the ftsI gene product are common among the clinical CXMR isolates: A502T/V in 6/14 isolates; N526K in 11/14 and S357N (Table 1). As A502T/V and N526K are also found in one of the isolates with a low MIC (2 mg/L) they are unlikely to be solely or primarily responsible for the CXMR phenotype. D350N substitution, found in one strain with low CXM MIC (4722) and one CXMR strain, as well as the HIBLN control strain, can also be discounted as being responsible for the CXMR phenotype. The substitution S357N was only found in CXMR strains. Of the CXMR isolates, only one, 5785, has the single amino acid substitution R501H. Seven have two substitutions, three have three substitutions and three have four substitutions (Table 1). This variability was only apparent in strains with a CXMR phenotype where four substitutions were found in more than one CXMR isolate: S357N was seen in five isolates, R501H in three isolates and A502V and T528H in two each.

Changes in the dasA and dasB gene products in clinical isolates

The control strain HIBLN has 15 amino acid substitutions in the dasB gene product compared with that of the dasB gene product predicted from the RD genome sequence: S67P; N84G; Q88K; S95T; G102V; D105G; N107H; R120S; S125T; K189G; G192R; I194T; V213I; Y244S; N255D; Q262A. Fourteen of these (excluding R120S) are also present in the CXMS strain 4722. Four were also found in CXMR isolates: S67P; D84G in all bar one (5794, which does have a substitution here but to serine); N107H; and Q262A.

Amongst the CXMR isolates, four amino acid substitutions in DasB were each found once: V143I (5794); A172Ser (5804); A261V (5804); M306L (5783). Two substitutions (other than those previously mentioned that were common to both CXMR strains and those with low CXM MICs) were found in more than one isolate: S250N in two CXMR strains and R111S in four CXMR strains.

No changes in the amino acid sequences of the dasA gene products were detected among any of the isolates studied.

Analysis of CXMR transformants

CXM$^R$ transformants of *H. influenzae* Rd, designated TX1–TX6, were obtained using donor DNA from the CXMR isolate 5788 at a transformation frequency of $\sim 2 \times 10^5$ per µg of DNA. The cefuroxime MIC for the transformants increased.
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from 0.25 to 32 mg/L, similar to the donor organism 5788. The amino acid changes found in FtsI in the CXMR transformants were compared with the FtsI sequence in H. influenzae Rd. The substitutions V511A and R517H found in FtsI in the donor strain 5788 were also found in FtsI in all six transformants. The dacA and dacB gene products in the CXM R transformants were identical to those of the CXM S recipient H. influenzae Rd.

Homology modelling

The identity between the amino acid sequences of S. pneumoniae PBP 2X and H. influenzae FtsI was 29%. This was sufficient to allow threading of the ftsI gene product between amino acids Q312 and S446. The initial threading produced an excellent fit within this region with an average root mean square deviation of the α-carbon chains of 0.5 Å. For the S357 structure, the α-carbon of Ser-357 was 11.7 Å from the active site region. On changing this from serine to asparagine, reducing steric hindrance with V362 and energy minimization the energy of this residue changed from −13 kJ/mol (serine) to −143 kJ/mol (asparagine). The distance of the α-carbon to the active site in this case was 11.4 Å. The values of the torsion angles for this residue were found to influence the position of the loop V362–P366 (sequence VDVAP), which lies adjacent to the active site. The structures of the homology model for both the Ser-357 and Asn-357 forms are shown in Figure 1.

Cefuroxime docking

Cefuroxime docked into the active site region of the ftsI gene product in a conformation consistent with the binding of β-lactams to both PBPs and serine proteases. The lowest energy conformation is shown in Figure 2.
Discussion

Ubukata et al.\(^6\) showed that ampicillin-resistant non-\(\beta\)-lactamase-producing isolates of \(H.\) influenzae found in Japan carried mutations in their \(ftsI\) genes. The mutants were categorized into three distinct groups (I–III) according to which amino acid substitutions had occurred in the transpeptidase domain of the \(ftsI\) gene product. Group I mutants were those with an R517H change. Most of these also carried substitutions at D350N and S357N. Mutations in isolates assigned to Groups II and III generated an N526K change, as well as the D350N substitution. Most also have the S357N alteration. Group III isolates are distinguished by having three additional substitutions, M377I, S385T and L389F. All the isolates retained susceptibility to most cephalosporins, except cefaclor, to which they were resistant. In a more recent study, Dabernat et al.\(^7\) investigated 109 clinical isolates of \(H.\) influenzae that were resistant to ampicillin and had reduced susceptibilities to cephalosporins. The isolates were basically grouped according to Ubukata et al.\(^6\) but the majority (101) fell within class II. A single isolate was assigned to Group III, whereas the remainder (seven) were in class I. Class II was subdivided into four divisions, according to which amino acid substitutions, other than S357N, were found in the PBP encoded by \(ftsI\). Hence, IIA isolates only have the N526K change. IIB also have an A502V alteration, with or without these other changes: G490Q, A373S, M377L, D350N. Group IIc isolates are distinguished by the N526K change coupled with an A502T substitution. Approximately half the isolates (12/25) in this subdivision were also found to have substitutions at position A368T or D350N. Group IIid isolates comprised a single type with an I449V substitution, in addition to the D526N change. In both studies, quantitative changes in susceptibilities to \(\beta\)-lactams were not easily correlated with the alterations found in the PBP3 sequence.

The amino acid substitutions found in the present study (Table 1) do not fit neatly into the classification scheme described, although several of the substitutions reported by Ubukata et al.\(^6\) and Dabernat et al.\(^7\) (D350N, S357N, A368T, M377I, A502V/T, R517H and N526K) were found in our isolates. However, we also found substitutions at A373V, T443A, R501L, V511A, Y528H and T532S (Table 1), which are previously unreported. Of the amino acid changes to the \(ftsI\) gene product previously reported to correlate with conversion to ampicillin resistance, three were detected in two of the lower CXM MIC isolates used in this study (Table 1), specifically D350N, A502T and N526K. Accordingly, these alterations are not responsible for the CXM\(^8\) phenotype, although they may act in concert with other substitutions that do confer high-level CMX\(^8\).

The substitutions that define group III, which confer significant resistance to cepham \(\beta\)-lactams, would be expected to be those most likely to be found in CXM\(^8\) strains; however, this is not the case. Only one amino acid substitution in the PBP encoded by \(ftsI\) that was reported by Ubukata et al.\(^6\) was found in more than one of the CXM\(^8\) isolates and not in the strains with lower CXM MICs investigated in the present study, namely S357N. To understand the possible effect of this substitution on the enzyme, a molecular model of part of the transpeptidase encoded by \(H.\) influenzae \(ftsI\) was constructed, based on the structure of \(S.\) pneumoniae PBP2X.\(^8\) The two amino acid sequences have 29% homology, which is sufficient to allow the region of the \(ftsI\) gene product to be modelled. PBP2X is a high molecular weight PBP with three domains: an N-terminal domain (amino acid residues 1–265), a central domain (amino acids 266–616) and a small C-terminal domain (amino acids 635–750). The active site serine, S337, is located in the central domain with a 3D structure comprising eight \(\alpha\)-helices and five strands, which is similar to that of class A \(\beta\)-lactamases. The majority of penicillin-binding enzymes (high and low molecular weight PBPs and serine active site \(\beta\)-lactamases) have three amino acid motifs, S-X-X-L, S-X-N and K-S/T-G, which are highly conserved in sequence and spacing and are essential for function.\(^7\) In PBP2X, these motifs are at S337-K340, S395-N397 and K547-G549; in the \(H.\) influenzae \(ftsI\) gene product the motifs are S326-T-V-K, S378-S-N and K512-T-G. When the appropriate section of the \(H.\) influenzae PBP was matched to that of PBP2X, a similar structure emerged in which the first two motifs aligned perfectly. Unfortunately, it was not possible to thread the \(H.\) influenzae PBP sequence beyond S446 because the level of amino acid identity with PBP2X falls below that required for modelling, so the third motif could not be incorporated into the hypothetical structure.

The fit between PBP2X and the \(ftsI\) gene product of \(H.\) influenzae in the region of S357 is very good, and this hypothetical structure allows us to predict the likely effect of an S357N substitution. This substitution alters both the residue size and charge at that point in the structure, whereas the distance between the new amino acid and the active site is predicted to decrease by only 0.3 Å. Such a distance precludes a direct interference with substrate binding by this residue. However, asparagine has a higher molecular volume than serine. One explanation for the contribution of this substitution to the CMX\(^8\) phenotype is that the asparagine side-chain produces unfavourable steric hindrance with the side-chain of Val-362 (to which it is adjacent and in close proximity in our model). This hindrance can result in a change in the torsion angles of the asparagine residue, which in turn influences the position of the loop V362–P366, which is adjacent to the active site and may orient the loop so as to deny access of certain \(\beta\)-lactams to the transpeptidase active site of the enzyme (Figure 1), so reducing the affinities of the PBPs for these \(\beta\)-lactams (Figure 2). Given that transpeptidase encoded by \(ftsI\) is essential to the cell, the amino acid alteration would not be expected to prevent interaction of the enzyme with its...
natural substrate, as such inhibition would be lethal. Mutations that change enzymes essential to cell survival so that they are less susceptible to a particular antibiotic(s) but retain essential function often impose a fitness cost on the bacterium; however, the CXM® isolates grew as well on laboratory medium as strains with low CXM MICs.

With H. influenzae, mutation to CXM® appears generally to involve multiple amino acid substitutions in the ftsI gene product. Only one of the 14 CXM® isolates examined, strain 5785, showed a single change (R501H), and although the S357N substitution was that most commonly found (in five CXM® isolates), it was not detected in the majority of CXM® isolates. Two other substitutions in the ftsI gene product are likely to be important regarding CXM®; namely, R501H (found in three isolates) and Y528H (found in two isolates). The first of these substitutions involves a major change of structure, from a dibasic amino acid to an aromatic one. Given that neither substitution was detected in previous studies that investigated mutations responsible for acquisition of resistance to ampicillin, it is not unreasonable to suggest that the R501H and Y528H changes specifically alter the interaction of the ftsI gene product with some cephalosporins.

There appears to be considerable allelic variation among the dacB genes in low and high CXM MIC clinical isolates of H. influenzae. Several differences in the amino acid sequences of the gene products, compared with the comparator gene product from H. influenzae Rd, were noted, as was the case for the Japanese ampicillin-resistant isolates analysed by Ubukata et al. [S67P was found in 12 of 20 clinical isolates and in the β-lactamase-negative control strain; D84G was found in all clinical isolates except one (5794), which has a serine substitution instead, and also in the [β-lactamase-negative control strain]. However, these substitutions were found in isolates with marked MICs, so are very unlikely to account for the resistant phenotype. Interestingly, the β-lactamase-negative control strain and the isolates with low CXM MICs show the greatest dacB allelic variation, indicating that the PBP encoded by dacB is not so tightly conserved as that encoded by ftsI. Interestingly, the Q270K substitution was found in seven of 10 CXM® isolates, including strain 5788, which was used as the DNA donor in transformation experiments; however, this change was not found in the CXM® transformants, i.e. strains TX1–TX6, and so is not likely to contribute to the resistant phenotype in the clinical isolates, a conclusion that agrees with that of Ubukata et al.

It was important to establish whether mutations in ftsI in a particular CXM® H. influenzae isolate alone are responsible for the resistance. Hence, the CXM® laboratory strain H. influenzae Rd was transformed to CXM® using DNA from the CXM® clinical isolate, 5788 (Table 1). We reasoned that if the mutations in ftsI in the clinical isolate confer the CXM® phenotype, then all transformants should possess the same mutations, and the dacB gene should be unaltered. Isolate 5788 was chosen as the DNA donor because the two amino acid substitutions found in its ftsI gene product (V511A, R517H) are unique to this isolate. All six CXM® transformants possess ftsI alleles encoding gene products with the two amino acid substitutions, consistent with attribution of CXM® to these changes. In no case was dacB altered. Unfortunately, both substitutions are beyond the region of the PBP that can be modelled on PBP2X, so how these substitutions affect enzyme structure cannot, at present, be predicted. Attempts to transfer resistance to CXM from other clinical isolates to H. influenzae Rd have, so far, been unsuccessful. Why is not known.

References

K. Straker et al.


