OXA-17, a Further Extended-Spectrum Variant of OXA-10 β-Lactamase, Isolated from Pseudomonas aeruginosa

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Pseudomonas aeruginosa isolates 871 and 873 were isolated at Hacettepe University Hospital in Ankara and were highly resistant to ceftazidime (MIC, 128 μg/ml). Each produced three β-lactamases, with pIs of 5.3, 6.1, and 7.9. The β-lactamase with a pI of 5.3 was previously shown to be PER-1 enzyme. The antibiograms of the isolates were not entirely explained by production of PER-1 enzyme, insofar as ceftazidime resistance was incompletely reversed by clavulanate. The enzymes with pIs of 6.1 and 7.9 were therefore investigated. The enzyme with a pI of 6.1 proved to be a novel mutant of OXA-10, which we designated OXA-17, and had asparagine changed to serine at position 73 of the protein. When cloned into Ershicheria coli XL1-blue, OXA-17 enzyme conferred greater resistance to cepotaxime, latamoxef, and cefepime than did OXA-10, but it had only a marginal (two- to fourfold) effect on the MIC of ceftazidime. This behavior contrasted with that of previous OXA-10 mutants, specifically OXA-11, -14, and -16, which predominately compromise ceftazidime. Extracted OXA-17 enzyme had relatively greater activity than OXA-10 against oxacillin, cloxacillin, and cefotaxime but, in terms of kM/km, it had lower catalytic efficiency against most β-lactams. The enzyme with a pI of 7.9 was shown by gene sequencing to be OXA-2.

The most frequent mechanisms of resistance to extended-spectrum cephalosporins in Pseudomonas aeruginosa are derepression of the chromosomal AmpC β-lactamase and increased efflux (1). Extended-spectrum β-lactamases (ESBLs) are a greater problem in members of the family Enterobacteriaceae (19). Only one TEM-related ESBL has so far been described from P. aeruginosa (24); nevertheless, several other potent plasmid-mediated β-lactamases have been described from the species. These include the IMP-1 zinc β-lactamase (34), PER-1, which is a class A ESBL (25) that is widespread in Turkey (5, 26, 31); and several extended-spectrum mutants of class D β-lactamases (4, 6, 10). The last group includes the OXA-11, -14, and -16 derivatives of OXA-10 β-lactamase (4, 8, 10), the OXA-15 derivative of OXA-2 enzyme (6), and the OXA-18 enzyme, which is closely related to the OXA-12 and AmpS chromosomal enzymes of Aeromonas sobria (27, 29, 33).

In the present study we describe a further ESBL mutant of the OXA-10 enzyme, obtained from two P. aeruginosa isolates collected in Turkey.

MATERIALS AND METHODS

Bacterial strains and plasmids. P. aeruginosa isolates 871 and 873 were isolated in February and March 1992, respectively, from patients treated for burns at Hacettepe University Hospital, Ankara, Turkey. They were believed to be isolates of the same strain, since they had identical enzyme DNA restriction patterns (5). Previous work showed that they produced PER-1 β-lactamase together with β-lactamases that had pIs of 6.1 and 7.7, and that they carried a gene which hybridized with a probe to blaPER-1 (5).

Both isolates were broadly resistant to β-lactams (Table 1), and ceftazidime resistance was less completely reversed by clavulanate (4 μg/ml) than in strains with PER-1 β-lactamase alone (5). P. aeruginosa PU21 (OXA-10) (12) and a ciprofloxacin-resistant derivative, obtained as described previously (7, 9), were used as recipients in transconjugation. Ershicheria coli XL1-blue MRF7 was used as a recipient in transformation experiments with the pBCKS + cloning vector (Stratagene, La Jolla, Calif.). P. aeruginosa PU21 transconjugants with plasmids pMG40 (encoding OXA-2 β-lactamase), pMLH51 (OXA-10) (10, 20), pMLH52 (OXA-11) (10), pMLH53 (OXA-14) (4), and pMLH75 (PER-1) (7) were used as producers of reference β-lactamases. An E. coli K-12 transconjugant with plasmid R46 was used as a further reference producer of OXA-2 β-lactamase (2).

P. aeruginosa NCTC 50192, with plasmids of 154, 66, 38, and 7 kb, was used in plasmid sizing (32).

Antibiotics and susceptibility tests. MICs were determined on DST agar (Unipath, Basingstoke, Hampshire, United Kingdom) with inocula of 105 CFU per spot. Antibiotics were supplied by the following manufacturers: aztreonam and ceftazidime, Pfizer, New York; cefotaxime, Glaxo-Wellcome, Stevenage, Hertfordshire, United Kingdom; ciprofloxacin, Bayer, Newbury, Berkshire, United Kingdom; pipera-cillin sodium and tazobactam, Wyeth, Taplow, Berkshire, United Kingdom; cephalothin and moxalactam, Lilly, Basingstoke, Hampshire, United Kingdom; imipenem, Merck Sharp and Dohme, Hoddesdon, Hertfordshire, United Kingdom; cefotaxime and cefepime, Roussel, U. Sarbreg, Middlesex, United Kingdom; benzylpenicillin, cephaloridine, cloxacillin, oxacillin, gentamicin, and rifampin, Sigma, St. Louis, Mo.; ampicillin, sodium chloride, carbenicillin, and clavulante, lithium, SmithKline Beecham, Brentford, Middlesex, United Kingdom; and metronom, Zeneca, Macclesfield, Cheshire, United Kingdom.

Plasmid transfer to P. aeruginosa PU21. Logarithmic-phase cells of P. aeruginosa 871 and 873 were mated overnight with P. aeruginosa PU21cid on DST agar, as described previously (7, 9). Transconjugants were selected on the same medium containing ciprofloxacin (30 μg/ml) plus either ceftazidime (25 or 50 μg/ml) or gentamicin (50 μg/ml).

Detection of β-lactamases and their genes. β-Lactamases were characterized by isoelectric focusing of ultrasonic extracts prepared from overnight nutrient agar cultures described previously (22). For probing, which was undertaken as described previously (4, 10) total DNA was extracted and then digested with BamHI (when probing for blaOXA-10) or HincII (when probing for blaOXA-23) (Promega, Madison, Calif.). The probe for blaOXA-10 was made similarly from plasmid R46, with primers AB1 and AB2 (Fig. 1). The probe for blaOXA-23 was made similarly from plasmid R46, with primers AH1 (GGAAAGATTC ATGCCAATCCGAATCTT) and AH2 (TATCCGCGAGGT-CGAGAT [complementary strand]), corresponding to coordinates 118 to 144 and 955 to 935, respectively, in the sequence published by Danel et al. (6). The amplified fragments were labeled with digoxigenin with a DIG DNA labeling and detection kit (Boehringer, Lewes, East Sussex, United Kingdom).
Cloning β-lactamase genes. Total DNA was extracted from the *E. aeruginosa* isolates 871 and 873 and transconjugant PU21(pMLH51) by a guanidium thiocyanate method (28). Two-microgram amounts of DNA from the test strain and the vector pHBluesK (3) were then digested separately with restriction enzymes as follows: BamHI, HindIII, or PstI (Promega) for DNA from isolates 871 and 873 or *P. aeruginosa* only for DNA from strain PU21(pMLH51). The digestion conditions were as advised by the supplier of the enzymes. After digestion, the DNA was ligated and transformed into *E. coli* XL1-blue by electroporation, as described previously (6). Transformants were selected on Luria-Bertani agar (30) containing ampicillin (100 μg/ml). Plasmids from the transformants were extracted and electrophoresed by the method of Kado and Liu (15) with plasmids of the manufacturer's directions and were digested with the same enzyme used for cloning, and then the fragments were electrophoresed and compared to a 1-kb DNA ladder marker (Gibco BRL, Uxbridge, United Kingdom).

Plasmids from the transformants were extracted and electrophoresed by the method of Kado and Liu (15) with plasmids of *E. coli* 50192 as size markers. To obtain better estimates of their sizes, plasmids were extracted and purified with Quiagen (Hybaid, Teddington, United Kingdom) kits used in accordance with the manufacturer's directions and were digested with the same enzyme used for cloning, and then the fragments were electrophoresed and compared to a 1-kb DNA ladder marker (Gibco BRL, Uxbridge, United Kingdom).

Sequencing the β-lactamase gene. The OXA-10-related genes from isolates 871 and 873 were amplified by PCR with ABD1 and 5′-biotin-labeled ABD4 (Fig. 1) as primers. The products were sequenced by chain termination with ABD1, ABD2, and ABD3 primers, exactly as described by Hall et al. (10). The OXA-2-related gene was amplified with AH1 and 5′-biotin-labeled AH2 and then sequenced with primers AH1 (see above); AH3, GCCTGCATCGACATTCAAGA (coordinates 434 to 453 in the sequence published by Danel et al. [6]); AH4, GCGCGGCTTTAAGAAG (536 to 553); and AH5, TCGCAACTGGATACGTGTCGTT (833 to 851).

β-Lactamase purification. An *E. coli* XL1-blue transformant with the cloned OXA-17-β-lactamase was grown overnight, with shaking, at 37°C in 0.6 liters of Luria-Bertani broth and then diluted into 12 liters of fresh, warm, identical medium. After 5 h of incubation at 37°C, the bacteria were harvested by centrifugation at 5,000 × g for 15 min at 37°C and washed once in 20 mM triethanolamine buffer, pH 7.6. The pellet was resuspended in the same buffer and frozen and thawed six times. Further purification was undertaken as described in the OXA-16 enzyme, with one anion- and one cation-exchange chromatographic fractionation (8). OXA-10-β-lactamase was purified from *P. aeruginosa* PU21(pMLH51) as described previously (7, 8). β-Lactamase purity was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on the gel system of Lugtenberg et al. (21). Protein concentrations were determined by the micro-bicinchoninic acid method (Pierce, Rockford, Ill.).

**TABLE 1. MICs of β-lactams for *P. aeruginosa* isolates 871 and 873**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pip</td>
</tr>
<tr>
<td>871</td>
<td>32</td>
</tr>
<tr>
<td>873</td>
<td>64</td>
</tr>
<tr>
<td>PU21(pMLH57)/PER-1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>8</td>
</tr>
<tr>
<td>PU21</td>
<td>2</td>
</tr>
</tbody>
</table>

* Axt, aztreonam; Cb, carbencillin; Clav, clavulanate at 4 μg/ml; Csp, ceftizoxime; Ctx, cefotaxime; Imp, imipenem; Mex, moxalactam (latamoxef); Pip, piperacillin; Taz, tazobactam at 4 μg/ml.

<sup>1</sup> *P. aeruginosa* PU21 with pMLH57 plasmid coding PER-1-β-lactamase.

**RESULTS**

Susceptibilities and β-lactamases of *P. aeruginosa* isolates 871 and 873. Extracts of isolates 871 and 873 yielded β-lactamases that focused at pIs 5.3, 6.1, and 7.9. The enzyme with a pI of 5.3 corresponded to PER-1, as shown previously (5); the other two enzymes were characterized in this study.

Restriction patterns of DNA digests and hybridization with gene probes for OXA-10 and 2-β-lactamases. DNA from isolates 871 and 873 hybridized with both the bla<sub>OXA-2</sub> and bla<sub>OXA-10</sub> probes, whereas DNA from PU21(pMG40) hybridized with only the bla<sub>OXA-2</sub> probe and DNA from PU21 (pMLH51) bound only the bla<sub>OXA-10</sub> probe.

Both of the isolates carried the OXA-10-related gene on a 5-kb BamHI fragment. Banding patterns with the bla<sub>OXA-2</sub>

![FIG. 1. Nucleotide and protein sequences of the coding region of OXA-17-β-lactamase in comparison to those of OXA-10, -11, -14, and -16 enzymes. The nucleotide sequence shown in full corresponds to that determined in this study and was allocated the GenBank accession no. AF060206. Nucleotide differences in other OXA-10 family genes are marked above the bla<sub>OXA-17</sub> sequence, and the deduced amino acid changes are indicated below. The signal peptide extends from amino acid residues 1 to 20, and the proposed cleavage site is shown by a vertical line in the protein sequence depicted in italics. The three conserved elements previously described in class D β-lactamases are in boldface (14, 17).](http://aac.asm.org/DownloadedFrom/90752245)
probe were complex because HinIII cuts twice inside the OXA-2 gene, giving a constant 0.5-kb internal fragment and two further fragments with sizes that varied according to the restriction sites in the flanking genes. The 3' fragment had only 21 nucleotides homologous to the probe and was not detected, whereas that corresponding to the 5' end of blaOXA-2 was detected. Isolates 871 and 873 yielded 0.5- and 1.4-kb bands with the OXA-2 gene probe, and PU21(pMG40) yielded 0.5- and 0.45-kb bands.

**Sequencing blaOXA-10 and blaOXA-2 genes from isolates 871 and 873.** The blaOXA-10-related genes were amplified from both isolates by PCR, and base sequences corresponding to positions 179 to 912 of blaOXA-2 (11) were determined (see Fig. 1). This allowed prediction of the protein sequence except for the first 16 amino acids, which are part of the signal peptide, and the last 5 residues. Both isolates had the same base substitution with blaOXA-10, with guanine replacing adenine at position 192, predicting a change of asparagine to adenine at position 899 of the protein. This new β-lactamase was named OXA-17.

The blaOXA-2-related genes were sequenced between bases 145 and 935, corresponding to the coding sequence, except for the six amino acids at the N terminus (which are part of the signal peptide) and the last five amino acids at the C terminus (6). Both sequences corresponded exactly to that of blaOXA-2 (3).

**Cloning of blaOXA-17 into E. coli XL1-blue.** The presence of three β-lactamases in isolates 871 and 873 complicated purification of the OXA-17 enzyme and determination of its contribution to resistance. Transconjugation experiments did not achieve transfer of any of the β-lactamase genes, and cloning into *E. coli* XL1-blue was adopted to separate OXA-17 from the other β-lactamases. Transformants resistant to ampicillin at 10 µg/ml were obtained from both isolates, and crude β-lactamase extracts were prepared from representatives containing recombinant plasmids with inserts of different sizes. These extracts were subjected to isoelectric focusing (Table 2). Transformants with only the β-lactamase that focused at pH 6.1 hybridized with the probe to blaOXA-10 but not with that to blaOXA-2; those with the β-lactamase that focused at pH 7.9 showed the reverse hybridization pattern (Table 2). Transformants with each of these patterns were observed from each of the isolates 871 and 873. No producer of PER-1 β-lactamase (pI 5.3) was detected by isoelectric focusing from any of the libraries.

As a control, the OXA-10 β-lactamase gene from *P. aeruginosa* PU21(pMLH51) was cloned and transformed into *E. coli* XL1-blue. Two transformants were obtained by selection on ampicillin (10 µg/ml), and each contained a 16-kb plasmid which encoded the β-lactamase.

**Susceptibilities of the E. coli transformants with OXA-17 and -2 β-lactamases.** Susceptibility tests were performed on representative *E. coli* XL1-blue transformants with different inserts encoding OXA-17 and -2 β-lactamases (Table 3). Transformants with OXA-17 β-lactamase (pI 6.1) from the BamHI, PstI, and HindIII libraries possessed similar resistance profiles. Both the cloned OXA-17 and OXA-10 β-lactamases increased the MICs of amino and carboxy penicillins and cephalosporins for *E. coli* XL1-blue by 64- to 128-fold and raised those of aztreonam and ceftiraxone by 8- to 16-fold. In addition, production of OXA-17 enzyme, but not OXA-10, increased the MICs of cefotaxime, cefsulodin, ceftazidime, latamoxef, and cepimef by at least fourfold. Neither the OXA-10 nor the OXA-17 enzyme protected against carbenapens.

Transformants with OXA-2 β-lactamase showed resistance to ampicillin and carbenicillin and had reduced susceptibilities to piperacillin, ceftazidime, and cephaloridine, whereas susceptibilities to cefotaxime, ceftiraxone, cefsulodin, cefepime, cephrone (not shown), aztreonam, and imipenem were not significantly altered.

**Purification of OXA-17 β-lactamase.** An *E. coli* XL1-blue MRF*" isoform from the HindIII library of *P. aeruginosa* 871 was used as a source of enzyme for purification to preclude contamination by any of the other β-lactamases produced by the original *P. aeruginosa* isolates. OXA-10 β-lactamase was purified from *P. aeruginosa* PU21(pMLH51). For both enzymes, the final purity exceeded 99%; 12 liters of culture yielded 4.4 mg of pure OXA-17 β-lactamase and 3.75 mg of OXA-10 protein.

**Kinetic parameters of the OXA-17 β-lactamase.** Unlike other OXA-10 derivatives (4, 8, 10), OXA-17 β-lactamase gave linear kinetics for all of the substrates tested. The lowest $K_m$
was for penicillin G (34 μM), whereas values between 153 and 300 μM were seen for oxacillin, ampicillin, carbenicillin, and cephalothin and values of 500 to 600 μM were seen for cloxacin and cefoxitin. The highest $K_s$ (≥2000 μM) were for cefotaxime and cephaloridine (Table 4). The $k_{cat}$ exceeded 100 s$^{-1}$ only for oxacillin; the values for all of the other β-lactams were less than 30 s$^{-1}$. In term of $K_m/K_{cat}$, the best substrate was oxacillin, followed by penicillin G and ampicillin, whereas $k_{cat}/K_m$ values for other substrates were at least 20-fold lower than for oxacillin. Hydrolysis of ceftazidime and carbapenems was not detected. For comparison, Table 4 also shows kinetic data for OXA-10 β-lactamase, which had biphasic kinetics for many substrates. OXA-17 generally had higher $K_m$ values than OXA-10 for cephalosporins and, except for cefotaxime, had lower $k_{cat}$ rates for both penicillins and cephalosporins.

**DISCUSSION**

Isolates 871 and 873 were previously found to have PER-1 β-lactamase together with two further β-lactamases, one with a pI of 6.1 and the other with a pI of 7.9 (5). DNA from each isolate hybridized with a probe to blaOXA-10 and the enzyme with a pI of 6.1 was surmised to be related to OXA-10. The β-lactamase with a pI of 7.9 type was wrongly proposed to be an AmpC type (5). It appeared likely that one or both of these enzymes contributed to the resistance phenotype of the isolates, since their ceftazidime resistance was only partly reversed by clavulanate (Table 1) whereas strains with PER-1 β-lactamase alone became highly sensitive to ceftazidime in the presence of this inhibitor.

The present study confirmed that the two isolates had an OXA-10-related enzyme, and sequencing revealed this to be a new variant, OXA-17, with serine replacing asparagine at position 157. This might also explain why cloned OXA-10 showed no resistance to cefotaxime or ceftazidime in P. aeruginosa (unpublished observations). Alternatively, OXA-17 enzyme may have been partially denatured during purification or may genuinely be less efficient than the other members of the family in hydrolyzing β-lactams, though with equally good substrate binding.

The emergence in P. aeruginosa of an OXA-10 variant that gives greater resistance to cefotaxime than to other oxyimino- 

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**TABLE 4. Kinetic parameters for OXA-17 β-lactamase compared with OXA-10**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$</th>
<th>$V_{cat}$ (μM)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>34 ± 0.6</td>
<td>5 ± 0.1</td>
<td>147</td>
<td>63 ± 6</td>
<td>89 ± 10</td>
<td>1,412</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>245 ± 37</td>
<td>26 ± 0.2</td>
<td>106</td>
<td>235 ± 30</td>
<td>587 ± 30</td>
<td>2,500</td>
<td>77 ± 9</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>296 ± 31</td>
<td>2 ± 0.4</td>
<td>21</td>
<td>195 ± 13</td>
<td>31 ± 1</td>
<td>159</td>
<td>370 ± 44</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>153 ± 13</td>
<td>120 ± 2.0</td>
<td>784</td>
<td>222 ± 16</td>
<td>608 ± 10</td>
<td>2,739</td>
<td>96 ± 8</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>573 ± 79</td>
<td>20 ± 1.0</td>
<td>35</td>
<td>2,640 ± 323</td>
<td>520 ± 36</td>
<td>196</td>
<td>1,110 ± 100</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>2,940 ± 182</td>
<td>23 ± 1.0</td>
<td>8</td>
<td>2,340 ± 300</td>
<td>79 ± 8</td>
<td>33</td>
<td>395 ± 83</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>286 ± 60</td>
<td>5 ± 0.1</td>
<td>17</td>
<td>38 ± 2</td>
<td>6 ± 0.1</td>
<td>158</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>2,240 ± 427</td>
<td>22 ± 3.0</td>
<td>10</td>
<td>346 ± 19</td>
<td>9 ± 0.2</td>
<td>26</td>
<td>54 ± 3</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>544 ± 52</td>
<td>1 ± 0.05</td>
<td>2</td>
<td>55 ± 2</td>
<td>3 ± 0.3</td>
<td>54</td>
<td>ND</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* $a$ OXA-10 β-lactamase was shown previously (8) to give biphasic kinetics, so two sets of kinetics parameters could be determined for the initial ($V_{cat}$) and steady-state ($V_{cat}$) phases of hydrolysis.
* $b$ $(k_{cat}/K_m) = 1.000$ in micromolar$^{-1}$ second$^{-1}$.
* $c$ Values are means ± standard error.
* $d$ –, results not available.
* $\times ND$, hydrolysis not detected.

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by aspartate at position 157, and this may be critical to ceftazidime resistance. The only previous OXA-10 relative to have serine at position 73 was OXA-13, which gave no resistance to cefotaxime or ceftazidime in P. aeruginosa although it did reduce the susceptibility of E. coli, especially to cefotaxime (23). However, comparison is complicated by the fact that OXA-13 has nine other amino acid differences from OXA-10, although two of these lie in the signal peptide. However, seven of the nine differences in the mature OXA-13 protein, but not the serine at position 73, are present in OXA-7, which is not an ESBL.

The mutation in OXA-17 lies close to the first conserved element of class D enzymes (15, 17), which contains the active serine. This is in contrast to the mutations in previous OXA-10 ESBLs, which lie at positions 124, 143, and 157, and to the mutations that give ESBL activity in TEM and SHV enzymes, which affect critical residues that fold towards the active serine but are remote from it in the primary sequence (16).

OXA-17 was more active than OXA-10 against oxacillin, cloxacillin, and cefoxacin and had a higher $k_{cat}$ for cephalosporins and, except for cefotaxime, had lower $k_{cat}$ rates for both penicillins and cephalosporins.
is not therapeutically useful against *P. aeruginosa* infections. It may be that the mutation emerged by chance, or it may have been selected by low-level ceftazidime exposure or the enzyme may have transferred from another species. The absence of transmissibility from isolates 871 and 873 does not preclude the last possibility: plasmids from other species often transfer into *P. aeruginosa* but then cannot replicate and become chromosomally associated (13). The contributions of PER-1, OXA-2, and OXA-17 enzymes to the resistance of the original *P. aeruginosa* isolates is unclear, but each enzyme appeared able to contribute to give protection against ceftazidime (Table 4). Finally, it should be added that the evolution of *P. aeruginosa* strains with such complex arrays of potent β-lactamases is striking: the species has not, historically, been a major host for single secondary β-lactamases, which have remained much rarer than in members of the family Enterobacteriaceae (19).

**REFERENCES**