TEM-1 β-lactamase as a source of resistance to sulbactam in clinical strains of Acinetobacter baumannii

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Objectives: Sulbactam is well known to have clinically relevant intrinsic activity against Acinetobacter baumannii. Although secondary resistance to this drug has long been reported in acinetobacters, virtually nothing is known about its molecular basis. The aim of this study was to test the hypothesis that β-lactamase TEM-1 is responsible for sulbactam resistance in A. baumannii.

Methods: Seventeen clinical strains of A. baumannii were selected to represent different combinations of quantitative susceptibilities to sulbactam and molecular typing characteristics. The strains were screened by PCR for the presence of the blaTEM-1 gene and its variants. Amplicons encompassing the blaTEM genes, including their promoters, were sequenced. The expression and copy number of the blaTEM genes were assessed using semi-quantitative real-time PCR. Transfer of the blaTEM-1 gene into a susceptible A. baumannii strain was achieved by electroporation.

Results: Six strains were negative for the blaTEM gene and had sulbactam MICs of 0.5–1.0 mg/L, 10 strains harboured blaTEM-1 and showed MICs ≥8.0 mg/L, except for one strain with an MIC of 2 mg/L, while the remaining strain carried blaTEM-19 and had an MIC of 1 mg/L. The level of blaTEM-1 expression positively correlated with the MICs of sulbactam (r = 0.92). Promoter P4 was linked to the blaTEM gene in all strains except for a P3-carrying strain (an MIC of 2 mg/L). Transformation of the susceptible A. baumannii strain with blaTEM-1 resulted in a 64-fold increase in sulbactam MIC and in resistance to ticarcillin and piperacillin, but no change in susceptibility to broad-spectrum generation cephalosporins, aztreonam or carbapenems.

Conclusions: The results presented suggest that TEM-1 represents a clinically relevant mechanism of sulbactam resistance in A. baumannii.

Keywords: multidrug resistance, transformation, gene expression, mechanisms of resistance

Introduction

Acinetobacter baumannii is an important nosocomial pathogen that mostly affects patients with serious underlying diseases.1 This bacterium has a remarkable capability to acquire and develop multiple cellular mechanisms that confer resistance to virtually all antimicrobial agents applicable to Acinetobacter infections. Until recently, carbapenems were considered to be the gold standard in the treatment of infections caused by multidrug-resistant A. baumannii (MDRAB) strains. However, the rate of carbapenem resistance among MDRAB strains has dramatically increased worldwide since the early 2000s, resulting in a need to search for other therapeutic strategies.2 Sulbactam is one of the few antimicrobial agents that are currently considered a plausible option to combat MDRAB infections.1,3 Although this β-lactam has been primarily known as an inhibitor of Ambler class A penicillinases, it also has intrinsic and clinically relevant antimicrobial activity against Acinetobacter spp.4,5 Previous studies have shown that sulbactam (given alone or in a commercially available combination with either ampicillin or cefoperazone) retains in vitro activity against a substantial number of MDRAB strains including carbapenem-resistant and colistin-resistant isolates.6–8

In contrast to the other antimicrobial agents relevant for the therapy of A. baumannii infections, virtually nothing is known about the molecular basis of resistance to sulbactam. This is rather surprising considering the ongoing discussion on the potential of sulbactam to combat MDRAB3 and the fact that the resistance of acinetobacters to this drug has been known for more
than 30 years. To our knowledge, no published studies have addressed the issue of the mechanism of sulbactam resistance, although Joly-Guillou et al. reported as long ago as 1995 that TEM-1-producing Acinetobacter strains showed increased MICs of sulbactam compared with penicillinase-negative strains. Notably, several recent studies have found high proportions of blaTEM-1-carrying isolates among MDRAB strains. The inspection of in vitro antibiotic susceptibilities and genotypic data in our collection of A. baumannii strains collected over the last two decades revealed a strong association between the presence of the gene encoding TEM-1 and sulbactam resistance. Based on these findings, we designed the present study to test the hypothesis that TEM-1 is responsible for resistance to sulbactam in A. baumannii.

Methods

Strains and their genotypic characteristics

The 17 clinical A. baumannii strains investigated in the present study are listed in Table 1. These were selected to represent different combinations of quantitative susceptibilities to sulbactam and molecular typing characteristics as revealed by multilocus sequence typing (MLST) and by ApaI macrorestriction profiling of genomic DNA. Based on MLST, the strains were classified into the following sequence types (STs): ST1, ST7 (both of these belonging to clonal complex CC1/EU clone I), ST2 (EU clone II), ST3 (EU clone III), ST39, ST46 and ST52 (Table 1). The strains belonging to one of two main lineages (EU clone I and II) differed from each other in their ApaI patterns except for two pairs (NIPH 2862/NIPH 2884 and NIPH 2873/ANC 3943), each pair including EU clone II strains with identical ApaI patterns. Strains NIPH 2862 and NIPH 2884 were collected in one hospital over the same period of time but clearly differed in their sulbactam MICs (Table 1). Strain ANC 3943 (representative of a carbapenem-resistant A. baumannii population currently prevailing in the Czech Republic) differed from NIPH 2873 only in carbapenem MIC (Table 1) and the presence of ISAba1 upstream of the blaOXA-51 gene. All 17 strains were PCR-negative for the presence of the genes encoding the following β-lactamases known to occur in A. baumannii: NDM, VIM, IMP, VEB-1, PER-1, OXA-10, OXA-23-like, OXA-40-like, OXA-58-like, OXA-235-like and OXA-143-like (for primers and references, see Table S1, available as Supplementary data at JAC Online). Only strains NIPH 2862, ANC 3943 and ANC 4030 harboured ISAba1 upstream of the blaOXA-51-like gene, which is in accordance with their increased carbapenem MICs (Table 1).

Susceptibility testing

Susceptibilities to nine β-lactam agents or their combinations (Table 1), which are primarily effective against A. baumannii, were assayed by Etest (bioMérieux, Marcy l’Etoile, France) using Mueller–Hinton II agar (BBL, BD, Belgium) and following the manufacturer’s guidelines and the CLSI criteria for interpretation (Table 1). As clinical breakpoints for sulbactam are not available, we used a provisional susceptibility breakpoint of ≤4 mg/L derived from the CLSI breakpoint for ampicillin/sulbactam (≤8/4 mg/L).

PCR and sequencing

Genes encoding TEM-1 or its variants (hereafter termed blaTEM) were amplified by PCR using primers C1 and C2 as previously described. For the blaTEM-positive strains, a second PCR to amplify the entire gene including the promoter region was carried out using primers TEM prom F and R. Amplicons were sequenced using primers TEM prom F and R and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). DNA sequences were obtained on an ABI 3130xl genetic analyser (Applied Biosystems) and analysed using KODON software (Applied Maths, St-Martens-Latem, Belgium).

Gene copy number assay

To assess differences between the number of the blaTEM genes in blaTEM-positive strains, a copy number assay based on quantitative PCR (qPCR) was performed using primer pairs targeting the blaTEM gene (RT TEM-F, 5′-TTTTCGCCATTCTGTCCT-3′; RT TEM-R, 5′-GGGGCAGAAGCTCTCAAGG ATC-3′) and the rpoB (housekeeping) gene as previously described. To prepare a DNA standard for qPCR, the rpoB amplicon was cloned into the pCR plasmid using a TOPO TA Cloning Kit (Invitrogen). The resulting recombinant plasmid pCR (pCR8) harboured a single copy of blaTEM-1 and a 902 bp fragment of rpoB. Dilutions of pCR (pCR8) over the range 8·6 × 10^−1 to 2·6 × 10^−10 copies/μL were used as DNA templates to generate standard curves. qPCR was performed using a C1000 Real-Time system (Bio-Rad) and Quantitect SYBR Green (Qiagen, Hilden, Germany) according to the manufacturer’s guidelines. Real-time cycling parameters for both genes were: an initial hot start of 95°C for 5 min and then 40 cycles of 95°C for 15 s, followed by both annealing and extension at 55°C for 1 min. The relative copy number difference was determined using the 2^CT calculation as previously described.

Semi-quantitative reverse transcription PCR (qRT–PCR)

For qRT–PCR, cells of the blaTEM-positive strains were cultured in Luria–Bertani (LB) broth until mid-log phase (optical density measured at 600 nm (OD_{600}) = 0.5), harvested by brief centrifugation and resuspended in the RNASKET buffer medium (Qiagen). Extraction of total RNA and reverse transcription were performed as previously described. To detect transcripts of the blaTEM and rpoB genes, the same primers as those in the gene copy number assay were used. Expression was monitored using real-time PCR as described above. Triplicate samples and non-template and non-reverse transcriptase controls were included in each run. All qRT–PCR assays were performed three times. Dissociation curves were obtained to identify the PCR products. Expression of the blaTEM gene was quantified relative to that of the rpoB gene, and the relative expression was calculated as previously described.

Transformation with the blaTEM-1 gene

The recombinant plasmid pAT801 harbouring the entire blaTEM-1 gene with the P3 type promoter was extracted from a donor Escherichia coli strain using a Plasmid Mini Kit (Qiagen) and transformed into both E. coli Top10 and the sulbactam-susceptible, blaTEM-1-negative A. baumannii strain NIPH 56 (Table 1 and Table S2, available as Supplementary data at JAC Online) by electroporation. Transformants were selected on LB agar plates containing 100 mg/L ticarcillin after overnight incubation at 37°C and tested for the blaTEM-1 gene by PCR and for susceptibilities to β-lactams. The presence of pAT801 in the transformants was assayed by plasmid analysis as previously described.

GenBank accession number

The sequence of the blaTEM-19 gene was deposited in the GenBank database under accession number JX042489.

Results and discussion

To test the hypothesis that the TEM-1 β-lactamase is responsible for sulbactam resistance in A. baumannii, we first explored the relationship between sulbactam susceptibility and the presence of the blaTEM-1 gene in the 17 clinical strains. The MICs of sulbactam...
<table>
<thead>
<tr>
<th>Strain</th>
<th>City, country, year of isolation</th>
<th>Specimen</th>
<th>STa (EU clone)</th>
<th>Apal profile</th>
<th>blaTEM</th>
<th>blaTEM promoter</th>
<th>Relative expression of blaTEM</th>
<th>Copy number of blaTEM</th>
<th>MICs (mg/L)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANC 4030</td>
<td>Prague, Czech Republic, 2011</td>
<td>urine</td>
<td>2 (EU II)</td>
<td>1</td>
<td>blaTEM</td>
<td>P4</td>
<td>110.83 ± 0.17</td>
<td>6.02 ± 0.23</td>
<td>&gt;128</td>
<td>&gt;128</td>
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<tr>
<td>NIPH 1605</td>
<td>Sedlčany, Czech Republic, 2001</td>
<td>urine</td>
<td>1 (EU I)</td>
<td>2</td>
<td>blaTEM</td>
<td>P4</td>
<td>16.52 ± 0.32</td>
<td>0.88 ± 0.01</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>HK302</td>
<td>Zurich, Switzerland, 1977</td>
<td>urine</td>
<td>1 (EU I)</td>
<td>3</td>
<td>blaTEM</td>
<td>P4</td>
<td>18.8 ± 0.38</td>
<td>1.07 ± 0.07</td>
<td>16</td>
<td>16</td>
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<tr>
<td>NIPH 7</td>
<td>Prague, Czech Republic, 1991</td>
<td>respiratory tract burn</td>
<td>7 (EU I)</td>
<td>4</td>
<td>blaTEM</td>
<td>P4</td>
<td>16.5 ± 0.24</td>
<td>0.96 ± 0.05</td>
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<td>16</td>
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<td>sputum</td>
<td>2 (EU II)</td>
<td>5</td>
<td>blaTEM</td>
<td>P4</td>
<td>12.2 ± 0.15</td>
<td>0.64 ± 0.02</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>NIPH 2873</td>
<td>Prague, Czech Republic, 2005</td>
<td>wound</td>
<td>2 (EU II)</td>
<td>5</td>
<td>blaTEM</td>
<td>P4</td>
<td>13.94 ± 0.13</td>
<td>0.5 ± 0.08</td>
<td>8</td>
<td>16</td>
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<td>sputum</td>
<td>2 (EU II)</td>
<td>6</td>
<td>blaTEM</td>
<td>P4</td>
<td>21.73 ± 0.20</td>
<td>0.5 ± 0.11</td>
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<td>16</td>
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<td>blood</td>
<td>1 (EU I)</td>
<td>7</td>
<td>blaTEM</td>
<td>P4</td>
<td>10.77 ± 0.57</td>
<td>1.19 ± 0.12</td>
<td>8</td>
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<td>urinary catheter</td>
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<td>blaTEM</td>
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<td>14.77 ± 0.25</td>
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<td>9</td>
<td>blaTEM</td>
<td>P3</td>
<td>8.27 ± 0.21</td>
<td>1.5 ± 0.01</td>
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<td>4</td>
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<td>P4</td>
<td>6.09 ± 0.17</td>
<td>0.76 ± 0.04</td>
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<td>Genoa, Italy, 1998</td>
<td>tracheostomy</td>
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<td>tracheostomy</td>
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<td>11</td>
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<td>NT</td>
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<td>2</td>
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<td>NIPH 2554</td>
<td>Prague, Czech Republic, 2005</td>
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<td>1 (EU I)</td>
<td>12</td>
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<td>none</td>
<td>NT</td>
<td>NT</td>
<td>0.5</td>
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<td>cannula</td>
<td>39</td>
<td>13</td>
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<td>none</td>
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<td>NT</td>
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<td>ATCC 19606T</td>
<td>Not known, before 1949</td>
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<td>52</td>
<td>14</td>
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<td>none</td>
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<td>NT</td>
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<td>NIPH 56</td>
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<td>burn</td>
<td>1 (EU I)</td>
<td>15</td>
<td>none</td>
<td>none</td>
<td>NT</td>
<td>NT</td>
<td>0.5</td>
<td>1</td>
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<td>NIPH 56 (pAT801)</td>
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<td></td>
<td>blaTEM-1</td>
<td>73.17 ± 0.22</td>
<td>99 ± 0.18</td>
<td>32</td>
<td>64</td>
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NT, not tested; NP, not published.

aSTs based on the Institute Pasteur MLST scheme (www.pasteur.fr/mlst).
bApaI macrorestriction profiles of genomic DNA.
cExpression of blaTEM genes normalized to rpoB expression.
dCopy number of blaTEM transcripts normalized to rpoB transcripts.

Antibiotic abbreviations and breakpoints (mg/L) according to the CLSI: SUL, sulbactam; SAM, ampicillin/sulbactam (2:1), S (≤8/4), R (≥32/16); CAZ, ceftazidime, S (≤8), R (≥32); CTX, cefotaxime, S (≤8), R (≥32); FEP, cefepime, S (≤8), R (≥32); IPM, imipenem, S (≤4), R (≥16); MEM, meropenem, S (≤4), R (≥16); PIP, piperacillin, S (≤16), R (≥128); TIM, ticarcillin/clavulanic acid, S (≤16/2), R (≥128/2).

fNIPH 56 transformed by plasmid pAT801 harbouring the blaTEM-1 gene.
and other β-lactams for these strains are shown in Table 1. Eight strains were susceptible (<4 mg/L) to sulbactam, while five and four strains showed sulbactam MICs of 8 mg/L and ≥16 mg/L, respectively. The susceptibility value to ampicillin/sulbactam (2:1) corresponded to that observed for sulbactam alone in each strain (Table 1), which confirms that sulbactam is responsible for the effect of this drug combination against A. baumannii. PCR detection of the genes encoding TEM-1 or its variants revealed an amplicon in all strains with a sulbactam MIC ≥8 mg/L. In addition, amplicons were produced by NIPH 1717 and NIPH 2884, with a sulbactam MIC of 2 mg/L and 1 mg/L, respectively. Sequencing of the amplicons showed the presence of the ßltem-1 gene in all strains, except NIPH 2884, in which the ßltem-19 gene was identified (GenBank accession no. JX042489). Thus, with the exception of strain NIPH 1717, the presence of the ßltem-1 gene was associated with either resistance or intermediate susceptibility to sulbactam, while the absence of this gene correlated with sulbactam susceptibility.

To assess the relationship between the level of sulbactam resistance and ßltem-1 expression at the transcriptional level, qRT–PCR was carried out for the 11 ßltem-1/TEM-19-positive strains. The results shown in Table 1 indicate that the ßltem-1 genes were expressed in all strains. Moreover, there was a positive correlation between ßltem-1 gene expression and MICs of sulbactam, as indicated by a Pearson’s correlation coefficient value of 0.92 between the values (log2) of the relative expression of ßltem-1 and the corresponding sulbactam MICs (log2) calculated for the ßltem-1-positive strains. The copy number of the ßltem-1 genes was further assessed as a parameter potentially influencing the expression of the genes (Table 1). In all but one strain, the copy number of the ßltem-1/TEM-19 genes relative to the rpoB housekeeping gene ranged from 0.5 to 1.5, which estimates that one copy of ßltem-1 was present in the genome of each of these strains. This finding is in accordance with recent data indicating that a single chromosomal copy of the ßltem-1 gene is commonly present in the A. baumannii genome. For example, EU clone 1 strains were shown to carry typically one copy of this gene located in the AbarR3 resistance island.15,16,25 In line with this, all TEM-1-positive EU clone I strains included in the present study (NIPH 7, NIPH 10, NIPH 1605 and HK302) were calculated to have ßltem-1 copy numbers ranging from 0.88 to 1.19 (Table 1). Strain ANC 4030 (EU clone II) was the only one with a significantly higher ßltem-1 copy number (6.02). Compared with the other strains, ANC 4030 also showed a markedly higher relative expression level of the ßltem-1 gene (110.83) and a high sulbactam MIC (>128 mg/L). Moreover, the normalized relative expression (per copy of the ßltem-1 gene) was 18.4 for ANC 4030, which falls into the range of values found for the other ßltem-1-positive strains (8.27–21.73). Thus, multiple copies of ßltem-1 are likely to contribute to the high level of sulbactam resistance in ANC 4030.

Different levels of TEM-1 activity have been associated with distinct promoter sequences in E. coli.26 We therefore examined the possible influence of promoter type on the level of sulbactam resistance and on the expression of the ßltem-1 genes in the ßltem-1/TEM-19-positive strains. Two promoter types were found to be linked to the ßltem-1/TEM-19 genes: promoter P3 in one strain (NIPH 1717) and promoter P4 in the other 10 strains (Table 1). Compared with P3, P4 has been previously reported to be associated with up to 32-fold increases in MICs of β-lactams for E. coli.26 Notably, our P3-carrying strain had the lowest sulbactam MIC (2 mg/L) of the ßltem-1-positive strains, although we did not find significant differences in the relative expression of ßltem-1 between NIPH 1717 and the other ßltem-1-positive strains.

To evaluate experimentally the involvement of TEM-1 production in sulbactam resistance, the sulbactam-susceptible strains A. baumannii NIPH 56 and E. coli Top10 were transformed by electroporation with the pAT801 plasmid harbouring the ßltem-1 gene. Transformants were successfully obtained, as evidenced by the detection of the ßltem-1 gene and plasmid pAT801 by PCR and plasmid analysis, respectively, in the ticarcillin-resistant derivatives of the parental strains. In the transformed strains A. baumannii NIPH 56 (pAT801) and E. coli Top10 (pAT801), sulbactam MICs increased from 0.5 mg/L to 32 mg/L and from 16 mg/L to ≥256 mg/L, respectively. A. baumannii NIPH 56 (pAT801) also acquired resistance to ticarcillin, ticarcillin/clavulanate and piperacillin, but no change in susceptibility values was recorded for piperacillin/tazobactam, ceftazidime, cefotaxime, cefepime, imipenem, meropenem and aztreonam as evidenced by Etest and/or disc diffusion (Tables 1 and Table S2, available as Supplementary data at JAC Online). Notably, a more than 32-fold increase in the MIC of ticarcillin/clavulanate was observed for A. baumannii NIPH 56 (pAT801) compared with NIPH 56, indicating that clavulanate did not significantly inhibit the activity of TEM-1. Previous studies have shown that a high-level expression of the ßltem-1 gene at the transcriptional level resulted in the overproduction of TEM-1 and consequently in clavulanate resistance in E. coli.27,28 In line with this, the overexpression of the ßltem-1 gene in A. baumannii NIPH 56 (pAT801) was supported by the high copy number and relative expression values of the gene (Table 1).

The finding of the ßltem-19 gene in one of the strains studied deserves comment. To our knowledge, this gene has not yet been reported to occur in A. baumannii. The ßltem-19 gene differs from ßltem-1 by a single nucleotide (706G→A), leading to an amino acid change.29 This change takes place in the enzyme catalytic domain30 and may lead to a more than 64-fold decrease in hydrolytic activity against sulbactam in E. coli.31 As we found the ßltem-19 gene in A. baumannii NIPH 2884, which was genotypically closely related and epidemiologically linked to A. baumannii NIPH 2882 harbouring the ßltem-1 gene, the conversion of ßltem-1 into the ßltem-19 gene may have occurred during the in-hospital spread of the bacterium. The sulbactam MIC for NIPH 2884 (1 mg/L) was 8-fold lower than that for NIPH 2882, suggesting that the shift from ßltem-1 to ßltem-19 may have restored clinical susceptibility to this antibiotic.

Besides its intrinsic antibiotic activity against acinetobacters, sulbactam acts as a time-dependent irreversible inhibitor protecting β-lactams from hydrolysis by various serine β-lactamases, including TEM-1. It has been suggested that TEM-1 first hydrolyses ~7000 sulbactam molecules and then sulbactam functions as an irreversible inhibitor of the enzyme.32,33 Furthermore, the concentration inhibiting 50% of the TEM-1 activity for sulbactam was shown to be much higher (900 nM) than those for clavulanate and tazobactam (60 nM and 97 nM, respectively). More specifically, ~10000 molecules of sulbactam are needed to inactivate a single TEM-1 molecule.33 Therefore, it is likely that TEM-1 effectively protects Acinetobacter spp. cells against sulbactam unless the drug reaches concentrations that inhibit the enzyme activity.

In conclusion, our findings are congruent with the assumption that the production of TEM-1 results in sulbactam resistance or non-susceptibility in A. baumannii. This is especially supported by
the correlation between the level of sulbactam resistance and the expression of the \( b\)lo\( \text{TEM-1} \) gene, by the transferability of sulbactam resistance via a \( \text{b}\)lo\( \text{TEM-1} \)-carrying plasmid, and by the susceptibility of a clinical strain expressing TEM-19, a low-activity variant of TEM-1. Although other mechanisms of resistance to sulbactam are expected to occur in acinetobacters, the high prevalence of TEM-1 producers among MDRAB strains makes this particular mechanism of major clinical importance.

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**Supplementary data**

Tables S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**


