Cytoplasmic-Membrane Anchoring of a Class A β-Lactamase and Its Capacity in Manifesting Antibiotic Resistance

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Bacterial β-lactamases are the major causes of resistance to β-lactam antibiotics. Three classes of these enzymes are believed to have evolved from ancestral penicillin-binding proteins (PBPs), enzymes responsible for bacterial cell wall biosynthesis. Both β-lactamases and PBPs are able to efficiently form acyl-enzyme species with β-lactam antibiotics. In contrast to β-lactamases, PBPs are unable to efficiently turn over antibiotics and therefore are susceptible to inhibition by β-lactam compounds. Although both PBPs and gram-negative β-lactamases operate in the periplasm, PBPs are anchored to the cytoplasmic membrane, but β-lactamases are not. It is believed that β-lactamases shed the membrane anchor in the course of evolution. The significance of this event remains unclear. In an attempt to demonstrate any potential influence of the membrane anchor on the overall biological consequences of β-lactamases, we fused the TEM-1 β-lactamase to the C-terminal membrane-anchor of penicillin-binding protein 5 (PBP5) of Escherichia coli. The enzyme was shown to express well in E. coli and was anchored to the cytoplasmic membrane. Expression of the anchored enzyme did not result in any changes in antibiotic resistance pattern of bacteria or growth rates. However, in the process of longer coinoculation, the organism that harbored the plasmid for the anchored TEM-1 β-lactamase lost out to the organism transformed by the plasmid for the nonanchored enzyme over a period of 8 days of continuous growth. The effect would appear to be selection of a variant that eliminates the problematic protein through elimination of the plasmid that encodes it and not structural or catalytic effects at the protein level. It is conceivable that an evolutionary outcome could be the shedding of the sequence for the membrane anchor or alternatively evolution of these enzymes from nonanchored progenitors.

Introduction of β-lactam antibiotics to the clinic rapidly gave rise to emergence of resistant bacteria (10, 30). Over the past 60 years, resistance to this class of antibiotics has been disseminated widely, the vast majority of which is due to production of β-lactamases (10). There are approximately 600 known β-lactamases (3), which fall into four functional classes (1). Three out of four (classes A, C and D) are serine dependent (4, 10). These enzymes undergo acylation by the antibiotic, followed by a hydrolytic deacylation event that completes the catalytic cycle. Class B β-lactamases are zinc ion dependent and do not pursue covalent chemistry in their catalysis. The three classes of serine-dependent enzymes are believed to have originated from penicillin-binding proteins (PBPs) (10, 17, 21, 29). PBPs are biosynthetic enzymes involved in the last steps of cell wall assembly (11, 32). They are believed to be more ancient than β-lactamases in an evolutionary sense (24). Whereas the catalytic domains have similar folds in each case (18, 22), a major difference is that PBPs are anchored on the cytoplasmic membrane, while β-lactamases are not (the few potential exceptions are discussed below).

It is significant that both PBPs and serine-dependent β-lactamases share an active site acylation step in their catalytic cycles (29, 36). Actually, the active site acylation is believed to have been handed down from the ancestral PBP to the nascent β-lactamase (17, 22). It has been presumed that in the evolution of PBP from their biosynthetic functions to β-lactamases as antibiotic resistance enzymes two key events took place. One was the advent of a hydrolytic deacylation catalytic machinery (22, 29). The other was the shedding of the membrane anchor. The first endowed the nascent resistant enzyme with the ability to turn over the antibiotic by the two-step hydrolytic activity (14). The second is believed to allow the resistance enzyme to serve as a vanguard in countering the ingress of the antibiotic from the milieu.

The evolution of the hydrolytic deacylation mechanism for the advent of antibiotic resistance is intuitively logical, for which detailed catalytic insight for class A β-lactamases is accumulating (15, 23, 25). In contrast, the loss of a membrane anchor is less obvious. It might appear reasonable to assume that if this structural element were to be eliminated from the PBP for the enzyme to become a β-lactamase, it would have given the anchorless enzyme an advantage over its membrane-bound progenitor. Perhaps the loss of the membrane anchor allowed the β-lactamases to intercept and hydrolyze the incoming molecules of β-lactam before those could reach PBPs on the outer surface of the plasma membrane.

This model would be more efficient in gram-negative bacteria, where the diffusion of the β-lactamases is limited to the periplasmic space because of the existence of the outer membrane. It also explains why gram-positive bacteria developed other mechanisms of resistance to β-lactam antibiotics in addition to the production of β-lactamases. If the soluble β-lactamases are more efficient in their function as resistant enzymes, then their anchored counterparts might be handicapped, presenting a selective opportunity for the evolutionary loss of the anchor. The validity of this thesis was put to test in...
the present study by generating a membrane-anchored TEM-1 β-lactamase (from Escherichia coli) and comparing it to the unanchored native version.

**MATERIALS AND METHODS**

**Construction of plasmids.** Two separate constructs were created by fusing the TEM-1 β-lactamase gene to the fragment encoding the anchor region of PBP5 from *E. coli*, either directly to the 20-amino-acid anchor (28) (GNNFGKIIDYIMKFHHWFG) or to the anchor plus the five subsequent amino acids (QEIPTEM-1). The β-lactamase gene to the fragment encoding the anchor region of PBP5 (the spacer sequence is underlined). The five-amino-acid spacer is part of the PBP5 sequence adjacent to the membrane

**TABLE 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA sequence</th>
<th>PCR fragment length (bp)</th>
</tr>
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<tr>
<td>BamSH1</td>
<td>GTGCCCGGATCCCTTCAATTGGC</td>
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</tr>
<tr>
<td>TEMnStop</td>
<td>CCAAGCTTATACCATGAGGCG</td>
<td>70</td>
</tr>
<tr>
<td>PBP5A-D1</td>
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<tr>
<td>PBP5A-D2</td>
<td>CAAGAATTCGCCGAAGTAACTTC</td>
<td>85</td>
</tr>
<tr>
<td>PBP5A-R</td>
<td>TATAAGCTTGGTTTACCGTGCGG</td>
<td>70 or 85</td>
</tr>
<tr>
<td>E16Nd</td>
<td>CTAGCAACCTGTCGGCTCGTATAC</td>
<td>5,103</td>
</tr>
<tr>
<td>E16Nr</td>
<td>GTAAAGTCGGCAACCAGGTTCGAG</td>
<td></td>
</tr>
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</table>

**TABLE 2. Plasmids used in this study**

<table>
<thead>
<tr>
<th>No.</th>
<th>Plasmid</th>
<th>Copy no. (copies/cell)</th>
<th>Selection markera</th>
<th>β-Lactamase expressedb</th>
<th>Expt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pTZ-ATEM-NS</td>
<td>75</td>
<td>Km, Amp</td>
<td>TEM-1 with PBP5 anchor</td>
<td>Initial MIC testing</td>
</tr>
<tr>
<td>2</td>
<td>pTZ-ATEM</td>
<td>75</td>
<td>Km, Amp</td>
<td>TEM-1 with PBP5 membrane anchor and 5-AA spacer (A-TEM-1)</td>
<td>Initial MIC testing; plasmid elimination</td>
</tr>
<tr>
<td>3</td>
<td>pTZ19-3</td>
<td>75</td>
<td>Km, Amp</td>
<td>Wild-type TEM-1</td>
<td>Initial MIC testing; plasmid elimination</td>
</tr>
<tr>
<td>4</td>
<td>pTZ-TEM Mut</td>
<td>75</td>
<td>Km</td>
<td>Decacylation-deficient TEM-1</td>
<td>Quantification of TEM-1 in the plasma membrane and periplasm</td>
</tr>
<tr>
<td>5</td>
<td>pTZ-ATEM Mut</td>
<td>75</td>
<td>Km</td>
<td>Decacylation-deficient TEM-1</td>
<td>Quantification of TEM-1 in the plasma membrane and periplasm</td>
</tr>
<tr>
<td>6</td>
<td>pACYC184-TEM</td>
<td>15</td>
<td>Amp, Cm, Tc</td>
<td>Wild-type TEM-1</td>
<td>MIC testing with β-lactams</td>
</tr>
<tr>
<td>7</td>
<td>pACYC184-ATEM</td>
<td>15</td>
<td>Amp, Cm, Tc</td>
<td>Wild-type TEM-1 with PBP5 membrane anchor and 5-AA spacer (A-TEM-1)</td>
<td>MIC testing with β-lactams</td>
</tr>
</tbody>
</table>

a. Amp, ampicillin; Km, kanamycin; Cm, chloramphenicol; Tc, tetracycline.

b. 5-AA, five-amino-acid.

The deacylation-deficient TEM-1 mutant (Glu166Asn) (7) was previously created in our lab by mutating the blaTEM-1 gene in pTZ19-3 by using a QuikChange site-directed mutagenesis from Stratagene with the primers E16Nd and E16Nr (Table 1). The resulting plasmid containing mutant β-lactamase was named pTZ-TEMmut (Table 2). Plasmid pTZ-TEMmut was digested with AciI endonuclease. The two sites for this endonuclease in this given construct flank the Glu166Asn encoding region. The resulting fragment was used to substitute the AciI-AciI fragment in the plasmid pTZ-TEM. Orientation of the insert in the resulting construct was verified by restriction endonuclease analysis. The resulting construct named pTZ-TEMmut (Table 2) expressed deacylation-deficient TEM-1 with the membrane anchor on its C terminus.

**MIC determination.** All MICs were determined by using the serial dilution method in LB media using 96-well microtiter plates. The initial bacterial load was 5 × 10⁶ CFU per ml. Bacteria were incubated with antibiotics overnight at 37°C.

**Determination of the A-TEM copy-number in the plasma membrane.** The deacylation-deficient A-TEM-1 described above was used for detection and quantification of the A-TEM-1 in the membrane fraction. Strains expressing the deacylation-deficient A-TEM-1 (pTZ-TEMmut) and its anchorless version were grown overnight in 50 ml of LB medium, harvested by centrifugation, and resuspended in 50 mM phosphate buffer (pH 7.0). Bacterial cell were disrupted by sonication for 30 min on ice using Branson Sonifier 450 cell disruptor (VWR Scientific). Cell debris were separated from the supernatant containing membranes by centrifugation at 21,000 × g for 30 min. The pelleted membranes were washed once in the same buffer and resuspended in 3 ml of 50 mM phosphate buffer (pH 7.0). Aliquots of the membrane fractions were taken and incubated with 10 μM Bocillin FL (39) (Invitrogen) at 37°C for 20 min. To detect the fluorescent acyl-enzyme species, the gel was scanned by using Storm 840 imaging system (GE), and the intensities of the bands corresponding to the acyl-enzyme species were measured by using ImageQuant software (GE). The fluorescence intensities of the bands corresponding to the A-TEM-1 and combined PBPs 2 and 3 were measured and compared.

**Determination of the relative amount of A-TEM-1 in the periplasm.** To evaluate the efficiency of the membrane anchoring of A-TEM-1, periplasmic extraction of this enzyme was compared to the periplasmic concentration of the wild-type TEM-1 in the control strain. *E. coli* IM83 cells harboring plasmids pTZ-ATEMmut and pTZ-TEMmut were grown in LB medium overnight. Bacteria were separated from the supernatant by centrifugation and resuspended at 3,000 × g for 15 min. The cell pellets were weighed. The periplasmic fraction was isolated as described previously (27). The solutions containing the liberated periplasmic contents of the strains expressing deacylation-deficient A-TEM-1 and TEM-1 were concentrated. Aliquots containing these enzymes were incubated with 10 μM Bocillin FL (Invitrogen) at 37°C for 20 min and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were scanned by using the Storm 840 imaging system (GE), and the intensities of the bands corresponding to the acyl-enzyme species were measured by using ImageQuant software (GE) and compared.

**Determination of the amount of the deacylation-deficient TEM-1 in the periplasm of the strain with high-copy-number plasmid.** The amount of deacylation-deficient TEM-1 in the periplasm of the cells harboring pTZ-TEMmut was...
determined by using the Bocillin FL-based technique described above. Several different concentrations of the homogenous deacylation-deficient TEM-1 were loaded on the same gel to be used as calibration standards. The deacylation-deficient TEM-1 was purified according to a protocol used for other β-lactamases (12).

**Determination of the relative amount of TEM-1 in the periplasm of the strains with high- and low-copy-number number plasmids.** The concentrations of periplasmic TEM-1 in E. coli JM83, harboring either pTZ19-3 or pACYC184 plasmids, were compared to determine how vector copy number affects the expression level of TEM-1. The periplasmic contents from both strains were isolated as described above. Both samples were subjected to SDS-PAGE, the gel was stained with Coomassie brilliant blue, and the intensity of the band corresponding to TEM-1 was measured and quantified by using ImageQuant software (GE).

The levels of expression of the TEM-1 β-lactamase in the strains harboring plasmids pTZ19-3 or pACYC184 were also compared by using a spectrophotometric assay. In this case the enzyme produced by pTZ19-3 plasmid was diluted to the extent that its rate of nitrocefin hydrolysis matched that of TEM-1 produced by pACYC184 plasmid. Depletion of nitrocefin was monitored spectrophotometrically at 510 nm.

**Plasmid competition and growth rates comparison.** We determined the growth rates of E. coli strains producing anchored and nonanchored β-lactamase and evaluated the effect of anchoring on plasmid stability. Aliquots of 100 µl each of E. coli JM83 cell cultures containing plasmids pTZ19-3 and pTZ-ATEM (ca. 5.4 × 10⁶ CFU/ml for each) were grown in 5 ml of the LB medium. Every 24 h the bacterial culture was diluted 1,000-fold, and 100 µl of this mixture was reincubated into 5 ml of the fresh LB medium. Every 12 h the bacterial culture was diluted and plated on petri dishes containing LB agar supplemented with 30 µg of kanamycin/ml. Plasmid DNA was isolated from 33 separate colonies on days 2, 4, 6, and 8 of the experiment and analyzed by restriction endonuclease digestion and gel electrophoresis in order to identify the plasmids. This experiment was repeated three times for a total of 99 distinct colonies. Doubling times during log phase were determined for both strains by a method reported in the literature (16) using equations 1 and 2:

\[
\log N_t = \log N_0 + n \log 2
\]

\[
g = \left(\frac{t_2 - t_1}{n}\right) \log 2
\]

where \(N_t\) is the number of bacteria at time \(t\), \(N_0\) is the number of bacteria at time \(t_0\), \(n\) is the number of doublings, and \(g\) is the doubling time.

We also determined the plasmid elimination rates of E. coli JM83 strains producing the anchored and unanchored wild-type TEM-1 β-lactamase. Cells containing plasmids pTZ19-3 and pTZ-ATEM were grown overnight in LB media, the densities of the bacterial cultures were adjusted to 5.4 × 10⁶ CFU/ml, and 100-µl portions of these cultures were used to inoculate 5 ml of LB medium. Both of these strains were grown separately, and the bacterial cultures were diluted the same way as described for the plasmid competition experiment. Every 2 days the cultures were diluted 50,000-fold and plated on petri dishes containing LB agar without kanamycin. The colonies from these plates were transferred onto petri dishes containing kanamycin supplemented (30 µg/ml) LB agar using a replica-plating tool. The difference between the number of colonies on the plates without kanamycin and with kanamycin corresponds to the number of colonies loosing the plasmids.

**RESULTS AND DISCUSSION**

**Anchoring of TEM-1 β-lactamase to the plasma membrane.** In the present study, we chose the anchor of PBPS of E. coli, an extensively studied PBPS, for incorporation into the TEM-1 β-lactamase structure. This low-molecular-mass PBPS has a C-terminal membrane anchor. Due to the fact that the TEM-1 β-lactamase has the N-terminal leader sequence experiencing cleavage in the process of translocation across the plasma membrane, we incorporated the anchor domain at the C terminus of our protein. Although the structure of the membrane-spanning domain of PBPS is unknown (presumed helical), the crystal structure for the rest of the protein has been determined, and the position of the membrane anchor has been reported (6).

We created two forms of the gene for anchored TEM-1 (A-TEM-1): one giving rise to a protein with the 20-amino-acid C-terminal anchor of PBPS (plasmid pTZ-ATEM-NS) and the other with the C-terminal anchor with an extra 5-amino-acid spacer sequence (the 25 C-terminal amino acids of PBPS) between the β-lactamase and the anchor (plasmid pTZ-ATEM). We expected that both of these forms of A-TEM-1 would be anchored on the outer surface of the plasma membrane of the host cells. Anticipated to act like a hinge, the five-amino-acid spacer between the TEM-1 and the membrane anchor was meant to give more flexibility in the positioning of the β-lactamase on the surface of the membrane. We were concerned that without the spacer the β-lactamase might not be fully functional, when held flush against the surface of the membrane. Our concerns were confirmed when E. coli JM83 expressing the TEM-1 β-lactamase without the spacer (construct pTZ-ATEM-NS) demonstrated a significantly smaller MIC for ampicillin relative to the version with the spacer (construct pTZ-ATEM) (500 and 16,000 µg/ml, respectively). Although the β-lactamase activity generated from the construct pTZ-ATEM-NS has been relatively compromised, at 500 µg/ml, the MIC is still substantially higher than 4 µg/ml for background strain E. coli JM83, which is devoid of β-lactama. In another control, E. coli JM83 harboring the plasmid pTZ19-3, expressing the wild-type nonanchored TEM-1 β-lactamase, had the same level of resistance to ampicillin as the strain harboring pTZ-ATEM (16,000 µg/ml). The high β-lactamase activity of A-TEM-1 with the spacer (plasmid pTZ-ATEM) was the reason for using this A-TEM-1 gene in all our subsequent experiments. Several additional constructs were created using this gene for different experiments (Table 2).

**Quantification of A-TEM-1.** The high ampicillin MIC observed for the construct with the spacer (pTZ-ATEM), indistinguishable from that for the strain harboring the nonanchored version, was unexpected. We wondered whether the high MIC for the strain with A-TEM-1 could be explained by poor anchoring of the enzyme to the plasma membrane. If the membrane anchor were to be ineffective, most of the A-TEM-1 could be transported to the periplasm the same way as the wild-type nonanchored TEM-1. We set out to investigate the extent of anchoring by determining the concentrations of the A-TEM-1 and wild-type nonanchored TEM-1 β-lactamase in both the periplasm and the cytoplasmic membrane. For these quantitative determinations, we were in need of a suitable mutant version of the TEM-1 β-lactamase that would experience acylation by the antibiotic but would not undergo deacylation.

As mentioned earlier, class A β-lactamases acquired Glu-166 as the basic residue that activates a water molecule to give deacylation of the acyl-enzyme species (15). Mutation of this residue to any amino acids that cannot activate the hydrolytic water molecule generates a protein that still would experience acylation by β-lactam antibiotics but is unable to undergo deacylation rapidly (9, 34). We replaced Glu-166 with Asn in both the wild-type (construct pTZ-TEM Mut) and anchored TEM β-lactamase (construct pTZ-ATEM Mut). When E. coli JM83 cells were transformed with these plasmids, the cells produced the deacylation-deficient forms of TEM-1 and A-TEM-1 β-lactamases, respectively. Membrane and periplasmic fraction were prepared from these strains. Aliquots from these
samples were incubated with Bocillin FL, a fluorescent penicillin, and samples were subjected to SDS-PAGE and quantification. Our data unequivocally demonstrated the presence of the anchored enzyme A-TEM-1 in the membrane fraction (Fig. 1A and B), where the nonanchored TEM-1 was not detectable (Fig. 1B). Comparison of the intensities of the bands corresponding to the anchored TEM-1 and the host cell PBPs 2 and 3 in the membrane fractions (Fig. 1A) allowed us to quantify the anchored β-lactamase. The copy numbers for *E. coli* PBPs 2 and 3 are known from the literature (8) to be 120 ± 14 and 132 ± 17 molecules per cell, respectively (8). Using the PBPs as reference points, based on the level of fluorescence we determined the A-TEM-1 copy number in the membranes to be 12,600 ± 430 molecules per cell. We hasten to add that more than half of the 4,285 distinct proteins of *E. coli* are believed to be membrane bound (38).

We also determined concentrations of the A-TEM-1 and TEM-1 β-lactamases in the periplasmic fraction isolated from the strains harboring plasmids pTZ-TEM*mut* and pTZ-ATEM*mut* (Fig. 1C). Our data revealed the concentration of nonanchored native TEM-1 β-lactamase in the periplasm of *E. coli* JM83 (pTZ-TEM*mut*) at 185 ± 3.5 μM, corresponding to 7,300 ± 135 molecules per cell. This concentration is not atypical of those of other critical proteins in bacteria (20), (33). The concentration of the A-TEM-1 β-lactamase in the periplasm of *E. coli* JM83(pTZ-ATEM*mut*) was only 0.32 ± 0.06 μM (or 13 ± 2 molecules per cell). This analysis shows that merely ~0.1% of the A-TEM-1 β-lactamase exists in the soluble periplasmic fraction. The presence of this minute amount of the A-TEM-1 β-lactamase in the soluble periplasmic fraction can be attributed to release of some of the protein from the membrane during isolation of the periplasmic content. This experiment has demonstrated that high MIC for ampicillin for *E. coli* JM83 (pTZ-ATEM) cannot be attributed to the lack of anchoring of the β-lactamase on the surface of the plasma membrane.

All of the experiments described above were performed using high-copy-number plasmids (pTZ19-3, pUC19, and their derivatives containing A-TEM-1). The copy number for these plasmids is about 75 per cell (19, 26). Hence, high copy number translates into high expression of the β-lactamases. It is likely that the predecessors of the modern β-lactamases might not have been expressed at high levels and that the difference in the biological activities between the anchored and unanchored β-lactamases could conceivably have been more pronounced. To reduce the amounts of TEM-1 and A-TEM-1 in cells, we recloned the genes encoding these two proteins into the low-copy-number vector pACYC184. The resulting constructs were named pACYC184-TEM and pACYC184-ATEM, respectively. It is reported that pACYC184 produces just 15 replicates of itself in the cell (31). As expected, *E. coli* JM83(pACYC184-TEM) produced five times less of the TEM-1 β-lactamase than *E. coli* JM83(pTZ19-3). Both *E. coli* JM83(pACYC184-TEM) and *E. coli* JM83(pACYC184-ATEM) had MICs for ampicillin of 4,000 μg/ml. This is a fourfold decrease from 16,000 μg/ml documented for the same *E. coli* harboring the high-copy-number constructs (pTZ19-3 and pTZ-ATEM). Thus, the fivefold difference in the concentration of the β-lactamase manifested itself in a 4-fold difference in MICs. To evaluate the β-lactamase activity of the organisms with the low-copy-number constructs *E. coli* JM83(pACYC184-TEM) and *E. coli* JM83(pACYC184-ATEM), the MICs were determined for several β-lactam antibiotics (Table 3). Still, similar to the case of the high-copy-number constructs, the A-TEM-1 and TEM-1 β-lactamases expressed in lower quantities manifested the same level of resistance against all tested antibiotics (Table 3).

**Comparison of the anchored TEM-1 with the wild-type TEM-1.** As stated earlier, the set of mutations that would lead to the emergence of β-lactamases from the parental PBPs would have been an incremental process. In light of the fact that modern class A β-lactamases are diffusion limited in their catalytic abilities (5, 13), we know that these enzymes have reached high catalytic competence. This is the zenith of the evolutionary selection events, where diffusion of the substrate into the active site and that of the product away from it actually limit catalysis and not the bond-making and bond-breaking processes, which are typically the more challenging steps. Hence, many mutations over the evolutionary time scale have occurred to arrive at the modern extant β-lactamases.

We realize that evolution of β-lactamase from the ancestral PBP would have generated a nascent β-lactamase activity, which by the necessity of the incompleteness of the process at the outset would have resulted in a poor antibiotic resistance enzyme. We decided to test whether such poor catalytic competence could have been the impetus for shedding of the membrane anchor and sending of the less-competent catalyst into the plasma membrane. We decided to test whether such poor catalytic competence could have served as a vanguard for the protection of the critically important membrane-anchored PBPs. The less-competent β-lactamases could conceivably have been more pronounced. To reduce the amounts of TEM-1 and A-TEM-1 in cells, we recloned the genes encoding these two proteins into the low-copy-number vector pACYC184. The resulting constructs were named pACYC184-TEM and pACYC184-ATEM, respectively. It is reported that pACYC184 produces just 15 replicates of itself in the cell (31). As expected, *E. coli* JM83(pACYC184-TEM) produced five times less of the TEM-1 β-lactamase than *E. coli* JM83(pTZ19-3). Both *E. coli* JM83(pACYC184-TEM) and *E. coli* JM83(pACYC184-ATEM) had MICs for ampicillin of 4,000 μg/ml. This is a fourfold decrease from 16,000 μg/ml documented for the same *E. coli* harboring the high-copy-number constructs (pTZ19-3 and pTZ-ATEM). Thus, the fivefold difference in the concentration of the β-lactamase manifested itself in a 4-fold difference in MICs. To evaluate the β-lactamase activity of the organisms with the low-copy-number constructs *E. coli* JM83(pACYC184-TEM) and *E. coli* JM83(pACYC184-ATEM), the MICs were determined for several β-lactam antibiotics (Table 3). Still, similar to the case of the high-copy-number constructs, the A-TEM-1 and TEM-1 β-lactamases expressed in lower quantities manifested the same level of resistance against all tested antibiotics (Table 3).

**TABLE 3. MICs in *E. coli* JM83 for β-lactam antibiotics**

<table>
<thead>
<tr>
<th>Construct</th>
<th>MIC (μg/ml) in <em>E. coli</em> JM83*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>LOT</td>
</tr>
<tr>
<td><em>E. coli</em> JM83(pACYC184-ATEM)</td>
<td>64</td>
</tr>
<tr>
<td><em>E. coli</em> JM83(pACYC184-TEMM)</td>
<td>64</td>
</tr>
<tr>
<td><em>E. coli</em> JM83 alone (no plasmid)</td>
<td>4</td>
</tr>
</tbody>
</table>

*Abbreviations: LOT, cephalothin; LOR, cefaloridine; CAZ, ceftiazidine; OXA, oxacillin; AMP, ampicillin.*

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<sup>A</sup> Antimicrob. Agents Chemother. Downloaded from http://aac.asm.org/ on September 7, 2017 by guest
la tamase would still destroy the incoming antibiotic, before it has the opportunity to approach its target PBPs for inhibition.

Whereas we are unable to set up for testing a system that would mimic the incremental evolutionary steps, we can nonetheless test this concept by the use of a poor substrate for the modern enzyme. That is to say, the modern enzyme would not be competent at hydrolytic turnover of the poorer substrate; hence, the situation would parallel that for the nascent primordial β-lactamase. For this purpose we selected cephalothin. This β-lactam antibiotic is turned over by the TEM-1 β-lactamase with a $k_{cat}/K_{m}$ of $7.7 \times 10^7$ M$^{-1}$ s$^{-1}$, a level of attenuated catalytic competence for the enzyme of two orders of magnitude over the corresponding value for a preferred penicillin substrates (2, 35). There was no difference between the MICs for the E. coli JM83 harboring either pACYC184-ATEM or pACYC184-TEM in both cases. Both the wild-type and the anchored TEM-1 β-lactamase expressed in the cells demonstrated equally poor resistance against cephalothin, as manifested by MICs of 64 μg/ml (Table 3); the control E. coli JM83 strain expressing no β-lactamases had an MIC of 4.0 μg/ml.

To determine whether the expression of the anchored TEM-1 β-lactamase affects the growth rate of the organism or the ability of the organism to maintain the expression vector, we performed additional experiments. The doubling times for both E. coli JM83(pTZ-ATEM) and E. coli JM83(pTZ19-3) during the exponential phase of the growth were determined to be 40 ± 7 and 40 ± 5 min, respectively. Therefore, there is no perceptible difference in the doubling times.

To address whether the presence of the anchor presented any disadvantage to the organism over longer growth time, we performed an additional experiment. We mixed equal amounts of E. coli JM83(pTZ-ATEM) and E. coli JM83(pTZ19-3) and grew them in LB broth with daily reinoculation of the mixture into fresh medium (50,000-fold dilution each time). Every 2 days aliquots of the culture were diluted and plated on petri dishes containing LB agar supplemented with 30 μg of kanamycin/ml. Plasmid DNA was isolated from the individual colonies and analyzed using restriction endonucleases. The results of this analysis (Fig. 2A) show that cells expressing the A-TEM-1 β-lactamase were completely eliminated in competition with the cells expressing the wild-type TEM-1 β-lactamase after 8 days of incubation.

Whereas no perceptible difference in doubling times between the two strains was noted, this experiment revealed that strain E. coli JM83(pTZ-ATEM) lost out to E. coli JM83 (pTZ19-3), regardless. These results indicate that the difference could be due to a lower stability of the pTZ-ATEM plasmid relative to pTZ19-3. To compare stability of the plasmids, E. coli JM83(pTZ-ATEM) and E. coli JM83 (pTZ19-3) strains were grown separately without the addition of any antibiotic for 8 days with daily reinoculation. Both strains were diluted and plated on petri dishes with LB agar every 2 days. The colonies from these plates were transferred onto petri dishes containing kanamycin-supplemented (30 μg/ml) LB agar by using a replica-plating tool. The difference between the number of colonies growing on regular agar and on kanamycin-supplemented agar corresponds to the number of the cells losing the plasmid (pTZ19-3 or pTZ-ATEM). By the eighth day of the experiment, pTZ-ATEM was completely eliminated by the host cells, whereas 9% of the cells still contained pTZ19-3 (Fig. 2B). Although the reason for the relatively lower stability of the plasmids expressing anchored TEM-1 remains unknown, we speculate that the insertion of A-TEM-1 into the cytoplasmic membrane could potentially create a disadvantage to bacteria harboring the anchored enzyme by disturbing the delicate homeostatic balance. The response of the host cell in this case would be to select for a variant that eliminates the problematic protein through elimination of the plasmid that encodes it. The case of a chromosomally encoded β-lactamase would obviously be different.

We have shown herein that anchoring of the resistance enzyme on the plasma membrane confers effective resistance to β-lactam antibiotics. The effects of this membrane sequestration were not measurable in the short term. When longer-term effects—days not hours—were explored, there was a selection force for the loss of the plasmid that harbored the gene for the anchored enzyme. We acknowledge that the outcome for a chromosomally encoded gene might be different. The nascent β-lactamase could obviously perform its role as an antibiotic resistance enzyme in the absence of its membrane anchor; hence,
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