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# Sec- and Tat-Dependent Translocation of β-Lactamases across the *Escherichia coli* Inner Membrane▽†

N. Pradel,[1]* J. Delmas,[1] L. F. Wu,[2] C. L. Santini,[2] and R. Bonnet[1]

*Laboratoire de Bactériologie, JE2526, UFR Médecine, Université d'Auvergne, Clermont-Ferrand,[1] and Laboratoire de Chimie Bactérienne, UPR9043, CNRS, Marseille,[2] France*

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**β-Lactamases represent the major resistance mechanism of gram-negative bacteria against β-lactam antibiotics. The amino acid sequences of these proteins vary widely, but all are located in the periplasm of bacteria. In this study, we investigated the translocation mechanism of representative β-lactamases in an *Escherichia coli* model. N-terminal signal sequence analyses, antibiotic activity assay, and direct measurement of translocation of a green fluorescent protein (GFP) reporter fused to β-lactamases revealed that most were exported via the Sec pathway. However, the *Stenotrophomonas maltophilia* L2 β-lactamase was exported via the *E. coli* Tat translocase, while the *S. maltophilia* L1 β-lactamase was Sec dependent. These results show the possible Tat-dependent translocation of β-lactamases in the *E. coli* model system. In addition, the mutation of the cytoskeleton-encoding gene *mreB*, which may be involved in the spatial organization of penicillin-binding proteins, decreased the MIC of β-lactams for β-lactamase-producing *E. coli*. These findings provide new knowledge about β-lactamase translocation, a putative new target for addressing β-lactamase-mediated resistance.**

β-Lactams are one of the most frequently prescribed antibiotic families, especially against gram-negative bacteria such as *Escherichia coli*. The production of β-lactamases is the predominant cause of resistance to β-lactam antibiotics in all gram-negative bacteria. These enzymes are encoded either (i) by chromosomes and are specific to a bacterial species or (ii) by plasmids, transposons, and/or integrons, which facilitate their spread in the bacterial world. The β-lactamases encoded by genes on these mobile elements are involved in antibiotic multiresistance, which is an emerging and major concern in *E. coli* (26).

Mature β-lactamases are located in the periplasmic space, where they cleave the amide bond in the β-lactam ring, rendering β-lactam antibiotics harmless to bacteria. β-Lactamases are classified into four classes, designated A, B, C, and D, with classes A and C being the most frequently occurring among bacteria (1, 6). Class A, C, and D β-lactamases are serine-utilizing hydrolases, while class B enzymes use a catalytic zinc center. The three classes of serine β-lactamases are evolutionarily related and belong to a superfamily that also includes DD-peptidases and other penicillin-binding proteins. All these proteins contain an S-x-x-K motif, with serine being the active site residue (23). Although clearly related, the sequences of the three classes of serine β-lactamases vary considerably outside the active site, even in the N-terminal part of the precursor form, which ensures the efficient translocation of the enzyme across the inner membrane.

Two major export systems, Sec and Tat, are involved in the translocation of proteins into the periplasm of gram-negative bacteria. The Sec apparatus threads exported proteins in a locally unfolded state across the membrane. In contrast, the hallmark of the Tat system is its ability to translocate folded proteins across the plasma membrane (25). The preproteins contain a characteristic signal sequence at the N terminal that is required for secretion. The signal sequence, on average, contains 24 amino acids, which form three characteristic regions, including (i) a positively charged amino terminus, (ii) a hydrophobic core region, and (iii) a carboxy-terminal domain that contains the cleavage site for signal peptidase. Upon export into the periplasm, the signal sequence is cleaved, yielding the mature protein, which folds into the correct conformation (30). The signal peptides of the proteins exported by the Tat pathway share similar overall structures with the Sec-dependent signal peptides but generally possess a twin-arginine (RR) motif in the n-region, a weakly hydrophobic h-region, and a positively charged Sec avoidance signal just before the cleavage site. Recent studies show that a naturally occurring Lys-Arg (KR) motif, RNR motif, or engineered KR, RK, and KK motifs in the n-region preserve the ability to mediate Tat pathway transport (19, 20). In addition, an increase in the hydrophobicity of the h-region, or loss of the Sec avoidance signal by removal of the charged residue, converts a Tat signal into a Sec-targeting signal peptide. Software has been developed on the basis of these characteristics to determine the probability that a protein uses the Sec or the Tat pathway (4, 34). However, the folding of the mature part of the protein seems also to be important for targeting to the translocation system (8, 31, 35). Green fluorescent protein (GFP) fluorescence is a direct and sensitive indicator of protein folding and localization (12, 45). The fusion of proteins with GFP has been successfully used to study the role of signal sequences and of

* Corresponding author. Mailing address: Laboratoire de Bactériologie, Faculté de Médecine, Université d'Auvergne, 28 Place Henri-Dunant, 63 001 Clermont-Ferrand, France. Phone: (33) 473 754 920. Fax: (33) 473 754 922. E-mail: nathalie.pradel@u-clermont1.fr.

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TABLE 1. Target genes and primers used in PCR in this study

| Target Gene | Primer | Oligonucleotide sequence (5′ to 3′) |
|---|---|---|
| Kanamycin resistance | Kn8760-F | TTT AGT ACT AAA GCC ACG TTG TGT CTC AA |
| | Kn8760-R | TAA GCC CGA ACG GCT TAG AAA AAC TCA TCG AGC A |
| *L1* | L1EH-F | AAC GAA TTC GTT AAC ATG CGT TCT ACC CTG CTC GCC TTC GCC |
| | L1BN-R | AGC GGA TCC GCT AGC GCG GGC CCC GGC CGT TTC CTT GGC |
| | L1ss-R | AGC GGA TCC GCT AGC CCG CAG CTG CGG CAG TGG TGT CTC GGC |
| *L2* | L2EH-F | AAC GAA TTC GTT AAC ATG CTC GCC CGT CGC CGA TTC CTG C |
| | L2BN-R | AGC GGA TCC GCT AGC TCC GAT CAA CCG GTC GGC AAT CCG |
| | L2ss-R | AGC GGA TCC GCT AGC GGC GGT CTT GCC CGC CGC ACG GGC |
| *blaC* | BlaCEH-F | AAC GAA TTC GTT AAC ATG CGC AAC AGA GGA TTC GGT CG |
| | BlaCBN-R | AGC GGA TCC GCT AGC TGC AAG CAC ACC GGC AAC GCA CG |
| | BlaCss-R | AGC GGA TCC GCT AGC CGG ACG GGC CCC GCT CGC ATG CCG |
| *ampC* | AmpCEH-F | AAC GAA TTC GTT AAC ATG CGC GAT ACC AGA TTC CCC TGC |
| | AmpCBN-R | AGC GGA TCC GCT AGC GCG CTT CAG CGG CAC CTT GCC C |
| | AmpCss-R | AGC GGA TCC GCT AGC CTT CAG GCG ATC CGC CGG GGC C |
| *tem-1* | TemEH-F | AAC GAA TTC GTT AAC ATG AGT ATT CAA CAT TTT CGT GTC GCC |
| | TemBN-R | AGC GGA TCC GCT AGC CCA ATG CTT AAT CAG TGA GGC ACC |
| | Temss-R | AGC GGA TCC GCT AGC ATC TTT TAC TTT CAC CAG CGT TTC TGG G |
| *CTX-M-14* | C14EH-F | AAC GAA TTC GTT AAC ATG GTG ACA AAG AGA GTG CAA CGG ATG ATG TTC GC |
| | C14BN-R | AGC GGA TCC GCT AGC CAG CCC TTC GGC GAT GAT TCT CGC CGC |
| | C14ss-R | AGC GGA TCC GCT AGC CGC CAG CTT TTG CTG CAC CGC ACT CGT CTG CGC |

the mature part of proteins in exportation via the Sec or the Tat pathway (12, 37, 44).

Little is known about the translocation process of β-lactamases. It is accepted that they have evolved from membrane associated penicillin-binding proteins (PBPs) (23, 24). PBPs are Sec dependent, and MreB and FtsZ cytoskeleton helical structures are thought to be involved in their spatial organization (10, 38, 48). They locate at different sites in the membrane, according to PBP type and to bacterial species (38, 39). By extension, it was inferred that β-lactamases are translocated by the Sec system. However, BlaC and BlaS β-lactamases were recently shown to be translocated by the Tat system in *Mycobacterium* spp. (28). Moreover, the mature part of TEM-1 β-lactamases is compatible with Sec and Tat processing, and TEM-1 is currently used as a reporter protein for the two pathways (27, 42, 44).

In this work, we used in silico N-terminal signal sequence analyses, antibiotic activity assays, and a GFP reporter to investigate the periplasmic translocation of various β-lactamases.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** *E. coli* strains MC4100A [F′ *lac*Δ(*lacZYA-argF*)*U169 araD139 rpsL150 thi flbB5301 deoC7 ptsF25 relA1 rbsR ara*$^+$], B1LK0A (like MC4100A, Δ*tatC*), and CU164A (*secY39-cs zhd-33*::Tn*10 ara*$^+$) were described by Santini et al. (36) and Ize et al. (22). The other strains used in this study were *E. coli* MC1000 [*araD139* (*ara leu*)*7697 lacX74 galU galK atrA*] and its derivative YLS-3 (Δ*mreB*) (40). *E. coli* TG1 strain [Δ(*lac-pro*) *supE thi hsdD5*/F′] *traD36 proA*$^+$*B*$^+$ *lacI*$^q$ *lacZ*ΔM15] was used in cloning experiments. Strains used as templates to obtain the β-lactamase genes were *Escherichia coli* CF001, producing the class A penicillinase TEM-1 (18), *Pseudomonas aeruginosa* ATCC 27853 reference strain, producing the class C β-lactamase AmpC (43), and an *Escherichia coli* strain producing the extended-spectrum β-lactamase CTX-M-14 (11). The genes encoding the class B β-lactamase L1 and the class A β-lactamase L2 (2, 47) were obtained from a clinical strain of the *Stenotrophomonas maltophilia* species which was isolated at the teaching hospital of Clermont-Ferrand, France.

Plasmid pMP159 used to amplify the *Mycobacterium tuberculosis blaC* gene was provided by Martin S. Pavelka, Jr. (14). Other plasmids used were pBK-CMV (Stratagene, Amsterdam, The Netherlands) and p8761 (this study). This last plasmid encodes GFP-mut2 fused to a twin-arginine signal peptide under the control of the arabinose promoter of pBAD24 (16). Strains were routinely grown at 37°C in Luria-Bertani (LB) medium or on LB plates. Glucose (0.2%), arabinose (0.2%), and kanamycin (50 μg/ml) were added as required to the medium. Precultures were grown from single colonies and used at 100-fold dilutions for inoculation of the experimental cultures.

**Cell induction assays.** Only conditional *sec* mutants are available. Hence, in this study we used the cold-sensitive *secY-cs* mutant CU164A, in which protein export is partially slowed down at 37°C and is severely affected upon exposure to 23°C. To study the impact of these mutations on the process of β-lactamase–GFP fusion, bacteria were incubated in LB-kanamycin-glucose (0.2%) medium at permissive temperatures (37°C for cold-sensitive strains) with shaking, until early exponential phase. The cultures were put on ice for 3 min and then kept at 23°C (nonpermissive temperature) for 1 h. The cells were harvested by centrifugation, washed once with LB, and suspended in LB-kanamycin-arabinose (0.2%) medium and incubated a further 3 h at 23°C with shaking.

**Construction of the plasmids expressing β-lactamases.** GFP fusions were constructed using plasmid p8761. Introduction of the *kanR* gene from *E. coli* GGB112 strain (provided by J.-M. Ghigo, Institut Pasteur, Genetics Biofilms Laboratory, France) at the ScaI-BglI sites (in the *ampR* gene) of the p8760 plasmid (37), yielded plasmid p8761. The entire β-lactamase-encoding genes or the first 120 to 135 bp encoding the signal peptides were amplified from the corresponding strains with the primers listed in Table 1. Amplification was performed with the *Taq* DNA polymerase according to the manufacturer's instructions (MP Biomedicals, Illkirch, France). The amplified fragments were purified using Wizard SV gel and PCR clean-up system (Promega, Lyon, France), double-digested by EcoRI and NheI, and cloned into the corresponding sites of the plasmid p8761. The resulting p61TEM-1, p61CTX-M-14, p61BlaC, and p61AmpC plasmids, respectively, encoded the entire TEM-1 (286 amino acids [aa]), CTX-M-14 (291 aa), BlaC (307 aa), or AmpC (397 aa), followed by two residues (AS), and then the GFP-mut2 (238 aa) reporter. The resulting p61ssTEM-1, p61ssCTX-M-14, p61ssBlaC, p61ssL1, p61ssL2, and p61ssAmpC plasmids encoded the corresponding predicted TEM-1, CTX-M-14, BlaC, L1, L2, and AmpC N-terminal signal sequences, followed by the first 7 to 10 residues of the mature moiety of β-lactamases, and then the GFP-mut2. The expression was verified using medium containing 0.2% arabinose and subsequent GFP-specific immunoblotting. In addition, the fluorescence capacity of the GFP-fusion proteins was visualized under a confocal LSM510 Meta fluorescent microscope. For cloning and constitutive expression of β-lactamases in pBK-CMV, PCRs were performed as described above, using the Platinum *Taq* DNA polymerase high-fidelity system (Invitrogen, Cergy Pontoise, France). Plasmid pBK-CMV was digested by SmaI and ligated with each β-lactamase-specific PCR product. All constructions were checked by double DNA sequencing (Genome Express, Grenoble, France).

**Cellular fractionation, electrophoresis, and immunological procedures.** Crude extracts were prepared by centrifugation of 1 ml of culture at an optical density at 600 nm ($OD_{600}$) of 0.5. The pellets were suspended in 0.1 ml Tris-HCl buffer (40 mM, pH 8) and 0.02 ml of 1% protamine sulfate. Samples were boiled for 15

min, and cellular debris was removed by centrifugation. Periplasm and spheroplasts were prepared by lysozyme/EDTA/cold osmoshock and ultracentrifugation as described previously (33, 36). The Complete protease inhibitor (Roche Diagnostics, Mannheim, Germany) was added at the maximum recommended concentration in all buffers used.

Protein samples were separated by polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% SDS (sodium dodecyl sulfate) on 10% or 12.5% acrylamide gels and immobilized onto polyvinylidene difluoride (PVDF) membranes. The immunoblot assay was performed using an enhanced chemiluminescence blotting system according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, United Kingdom). The antiserum used was polyclonal rabbit anti-GFP at a dilution of 1:20,000.

**MIC assays.** MICs were determined by a liquid microdilution method in Müller-Hinton broth (Sanofi Diagnostics Pasteur, Marnes la Coquette, France) with an inoculum of $10^4$ CFU and were interpreted as recommended by the CA-SFM (7). The antibiotics were provided as powders by Glaxo-SmithKline (amoxicillin and ceftazidime), Roussel-Uclaf (cefotaxime), Wyeth Laboratories (piperacillin), and Ely Lilly (cephalothin). A two-dilution difference between two strains expressing the same β-lactamase was considered significant.

**Enzyme activity tests.** For enzyme activity tests, cells were first grown to exponential phase in LB medium supplemented with kanamycin at 37°C with shaking and then kept at 23°C with shaking for 3 h. Specific activity was determined on sonicated 25-μg protein crude extract fraction with a computerized microacidimetric method using the 702 SM Titrino apparatus (Metrohm, Switzerland) in 100 mM NaCl, with 200 μg/ml benzylpenicillin (penicillin G) as the reporter substrate.

## RESULTS

**In silico analysis of β-lactamase signal sequences.** Signal sequences of β-lactamases were analyzed with the TATFIND 1.2 and TatP software (4, 34). Eighty-nine different signal sequences were identified among 400 β-lactamases (see Data Set S1 in the supplemental material). The predicted sizes of the signal sequences varied from 14 to 34 amino acids. Most of them were preferentially compatible with a Sec translocation. Of these 89 signal sequences, 14 (16%) gave a positive result for a possible Tat signal sequence with at least one of the two types of software (see Data Set S1 in the supplemental material). These putative Tat-dependent β-lactamases belonged to class A (11 of 46), B (1 of 10), or C (2 of 23) and, except for CMY-1, were chromosome encoded. The species producing these β-lactamases were not directly related phylogenetically. They belonged to the families *Enterobacteriaceae*, *Xanthomonadaceae*, *Mycobacteriaceae*, *Streptomycetaceae*, *Phyllobacteriaceae*, *Pseudomonadaceae*, *Sphingomonadaceae*, *Rhizobiaceae*, and *Comamonadaceae*. The Tat-dependent translocation of β-lactamases, however, seemed more likely to occur in plant pathogens such as *Xanthomonas*, *Stenotrophomonas*, or *Mesorhizobium* than in clinical bacteria.

Only seven signal sequences (8%) were identified as Tat dependent with both types of software (see Data Set S1 in the supplemental material). The corresponding β-lactamases belonged to class A and were produced by *Stenotrophomonas maltophilia* (L2 β-lactamase), *Mycobacterium tuberculosis*, *Streptomyces albus* (BlaC β-lactamases), *Xanthomonas* (accession numbers NP_643470, YP_365024, YP_450290, and AAC43036), and *Mesorhizobium* (YP_674772). The hydrophobicity of the h-region of these putative Tat-dependent β-lactamases was between 0.43 and 6.31 (15 to 22 aa). These values were highly variable compared to those of typical Tat-specific targeting signal (21). The BlaS β-lactamase of *Mycobacterium smegmatis*, reported as a Tat substrate in this species (28), gave a negative prediction with one of the two programs.

TABLE 2. MIC assay results

| β-Lactamase and strain tested | MIC (μg/ml) of: | | | | |
|---|---|---|---|---|---|
| | Amoxicillin | Piperacillin | Cephalothin | Ceftazidime | Cefotaxime |
| TEM-1 | | | | | |
| WT | 1,024 | 512 | 32 | ≤0.06 | ≤0.06 |
| *secY-cs* | 1,024 | 256 | 32 | ≤0.06 | ≤0.06 |
| Δ*tatC* | 512 | 128 | 16 | ≤0.06 | ≤0.06 |
| Δ*mreB* | 256 | 32 | 8 | ≤0.06 | ≤0.06 |
| CTX-M-14 | | | | | |
| WT | 2,048 | 512 | 1,024 | 1 | 16 |
| *secY-cs* | 2,048 | 512 | 1,024 | 1 | 16 |
| Δ*tatC* | 2,048 | 512 | 512 | 1 | 16 |
| Δ*mreB* | 1,024 | 128 | 128 | 0.5 | 4 |
| L1 | | | | | |
| WT | 1,024 | 64 | 128 | 8 | 1 |
| *secY-cs* | 512 | 32 | 128 | 8 | 1 |
| Δ*tatC* | 512 | 32 | 128 | 8 | 1 |
| Δ*mreB* | 64 | 32 | 32 | 0.06 | 0.06 |
| L2 | | | | | |
| WT | 512 | 128 | 256 | 4 | 2 |
| *secY-cs* | 512 | 128 | 256 | 4 | 2 |
| Δ*tatC* | 4 | ≤2 | ≤2 | ≤0.06 | ≤0.06 |
| Δ*mreB* | 256 | 32 | 64 | 0.06 | 0.06 |
| AmpC | | | | | |
| WT | 512 | 32 | 1,024 | 0.25 | 0.5 |
| *secY-cs* | 512 | 32 | 512 | 0.25 | 0.5 |
| Δ*tatC* | 512 | 32 | 256 | 0.25 | 0.5 |
| Δ*mreB* | 128 | 16 | 256 | 0.06 | 0.12 |
| Empty plasmid | | | | | |
| WT | 4 | 2 | 4 | ≤0.06 | ≤0.06 |
| *secY-cs* | 4 | 2 | 4 | ≤0.06 | ≤0.06 |
| Δ*tatC* | 4 | ≤2 | ≤2 | ≤0.06 | ≤0.06 |
| Δ*mreB* | 4 | ≤2 | ≤2 | ≤0.06 | ≤0.06 |

Altogether, these observations suggest that few β-lactamases are compatible with the Tat translocation pathway. They also suggest that different β-lactamases could use different secretion systems in the same bacterium; for example, L1 (predicted as Sec dependent) and L2 (predicted as Tat dependent) in *S. maltophilia*. Four putative Sec substrates (the widespread β-lactamases TEM-1 and CTX-M-14 of *E. coli*, AmpC of *P. aeruginosa*, and L1 of *S. maltophilia*) and two putative Tat substrates (BlaC of *M. tuberculosis* and L2 of *S. maltophilia*) were selected to analyze their translocation in an *E. coli* model system.

**Analysis of ampicillin resistance phenotypes in wild-type *E. coli* and in the corresponding translocation mutants.** In *E. coli*, the Tat translocation apparatus is composed of the main TatA, TatB, and TatC proteins, but the structure and composition of the Tat complex can vary according to the species. However, a recent study showed that Tat translocases from diverse gram-negative bacteria are functionally capable of replacing *E. coli* Tat translocase (51*). E. coli* strains can therefore be used as a model to assess the translocation of β-lactamases of diverse origins. *E. coli* MC4100A (wild type [WT]) and its derivatives B1LK0A (Δ*tatC*) and CU164A (*secY-cs*) were therefore transformed with pBK-CMV derivative plasmids, which constitutively expressed TEM-1, CTX-M-14, AmpC, L1, and L2 β-lactamases.

The expression of these β-lactamases was assessed by the determination of the MICs (Table 2) and by enzyme assay (Table 3). Wild-type and Δ*tatC* strains presented high and similar levels of resistance to amoxicillin (512 to 2,048 μg/ml), except with L2. The L2-producing WT strain presented a

TABLE 3. Enzyme assay results

| β-Lactamase | Strain | Activity (mmol/s/mg) |
|---|---|---|
| TEM-1 | WT | 1.456 |
| | *secY-cs* | 0.350 |
| | Δ*tatC* | 0.900 |
| CTX-M-14 | WT | 0.051 |
| | *secY-cs* | 0.014 |
| | Δ*tatC* | 0.055 |
| L1 | WT | 5.000 |
| | *secY-cs* | 1.100 |
| | Δ*tatC* | 4.000 |
| L2 | WT | 0.514 |
| | *secY-cs* | 0.385 |
| | Δ*tatC* | 0.164 |
| AmpC | WT | 0.026 |
| | *secY-cs* | 0.005 |
| | Δ*tatC* | 0.016 |
| Empty plasmid | WT | <0.001 |
| | *secY-cs* | <0.001 |
| | Δ*tatC* | <0.001 |

higher level of resistance to amoxicillin than the corresponding Δ*tatC* strain (512 versus 4 μg/ml) (Table 2). The same difference was obtained with other β-lactams (piperacillin, cephalothin, ceftazidime, and cefotaxime). These results suggest a Tat-dependent translocation of L2 in *E. coli*, in accordance with its predicted twin-arginine signal sequence. For the expected Sec-dependent β-lactamases, the MICs were similar for the *secY-cs* mutants and the WT strains. This result can be explained by the partially slowed Sec-dependent export in the *secY-cs* mutant at the permissive temperature used for the experiments (37°C). Hence, to reliably assess the effect of the *secY-cs* mutation we used an enzyme assay (Table 3). TEM-1, CTX-M-14, L1, and AmpC had lower specific activity against penicillins in the *secY-cs* mutant than in the WT and Δ*tatC* strains (for example, 1.1 μmol/s/mg versus 5 and 4 μmol/s/mg for L1). Altogether, the results suggest a Sec-dependent translocation of TEM-1, CTX-M-14, L1, and AmpC and a Tat-dependent translocation mechanism for L2.

**Translocation study of full-length β-lactamases fused with GFP.** The translocation of full-length β-lactamases fused at their C terminus to GFP was analyzed with the plasmids p61TEM-1, p61CTX-M-14, p61AmpC, and p61BlaC. Expression of the fusions was tightly controlled by an arabinose-inducible p*ara* promoter. The stability of the constructs was checked by immunoblotting. Little or no GFP moiety was found in the protein extracts, providing evidence of the high stability of the fusions (data not shown).

Crude extracts of the wild-type MC4100A and the corresponding *secY-cs* and Δ*tatC* mutant strains harboring each plasmid were prepared after aerobic growth. Translocation of the full-length β-lactamase fusions was analyzed by immunoblotting, using an anti-GFP antibody. As shown in Fig. 1, the anti-GFP polyclonal antibody recognized the precursor (PreBL-GFP) and the mature form (BL-GFP) of TEM-1–, CTX-M-14–, AmpC-, and BlaC-GFP fusions. The translocation was accompanied by signal sequence cleavage, as indicated by the band of lower molecular mass, corresponding to the size of the mature moiety. TEM-1–GFP, CTX-M-14–GFP, and AmpC-GFP were mainly found in the mature form for the WT and the Δ*tatC* mutant and in the precursor form for the *secY-cs* mutant (Fig. 1). These results suggest a Sec-dependent translocation of TEM-1, AmpC, and CTX-M-14 β-lactamases. Unexpectedly, processing of BlaC-GFP was observed for the Δ*tatC* strain, and only the precursor form was observed for the *secY-cs* strain, suggesting a Sec-dependent translocation of BlaC-GFP in the *E. coli* model. Immunoblot assays were performed on cell fractions of the WT, Δ*tatC*, and *secY-cs* strains to track subcellular localization of the precursor and mature forms (Fig. 2). We confirmed the periplasmic localization of the mature form of TEM-1–GFP in the WT and Δ*tatC* strains. GFP fusion was absent, or present in a significantly reduced amount, in the periplasm of the *secY-cs* mutant. To rule out the possibility of the effect being due to the entire β-lactamase–GFP construction, we deciphered the contribution of the β-lactamase signal sequences in determining the translocation pathway.

**Processing of β-lactamase signal sequence-GFP fusions.** The ability of signal sequences to mediate GFP export was investigated with fusions of β-lactamase signal sequences with GFP. DNA fragments encoding TEM-1 (first 34 residues), CTX-M-14 (first 38 residues), AmpC (first 35 residues), BlaC (first 35 residues), L1 (first 31 residues), and L2 (first 34 residues) signal sequences were cloned into p8761 to obtain the corresponding plasmids p61ssTEM-1, p61ssCTX-M-14, p61ssAmpC, p61ssBlaC, p61ssL1, and p61ssL2. Crude extracts were prepared after aerobic growth of the WT and the Δ*tatC* and *secY-cs* mutants. Figure 3 shows the corresponding immunoblotting results using antisera against GFP. Two recognized polypeptides were observed for all the constructs: the faster-migrating mature form (GFP; 33 kDa) and the slower-migrating precursor form of the signal sequence-GFP fusions (ssGFP; 36 to 37 kDa). Only the precursors were detected in the

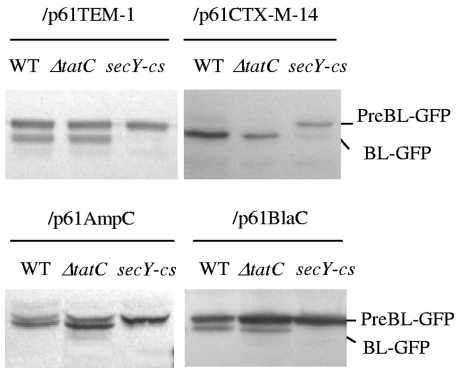


FIG. 1. Processing of β-lactamase–GFP fusions. Crude extracts were prepared from strains MC4100A (WT), B1LK0A (Δ*tatC*), and CU164A (*secY-cs*) grown aerobically at 37°C in LB medium supplemented with glucose and then induced for 3 h, at 23°C with shaking, in LB medium supplemented with arabinose. Proteins (20 μg each) were resolved on polyacrylamide (12.5%)–SDS denaturing gels, immobilized on PVDF membranes, and analyzed by immunoblotting using anti-GFP antiserum. Precursor (PreBL-GFP) and mature (BL-GFP) forms of the fusion proteins are indicated to the right of the panels.

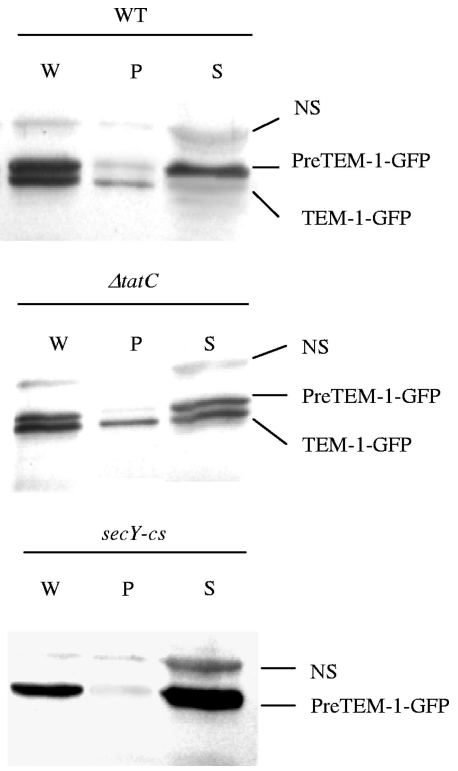


FIG. 2. Cellular distribution of the TEM-1–GFP fusion protein expressed from the p61T plasmid in MC4100A (WT), B1LK0A (Δ*tatC*), and CU164A (*secY-cs*) strains. Periplasm (10 μg of protein each) and spheroplasts (20 μg of protein each) samples were prepared from cells grown in the presence of arabinose. W, crude extracts; P, periplasmic fraction; S, spheroplasts. Nonspecific bands (NS) and precursor (PreTEM-1-GFP) and mature (TEM-1-GFP) forms of the fusion proteins are indicated to the right of the panels.

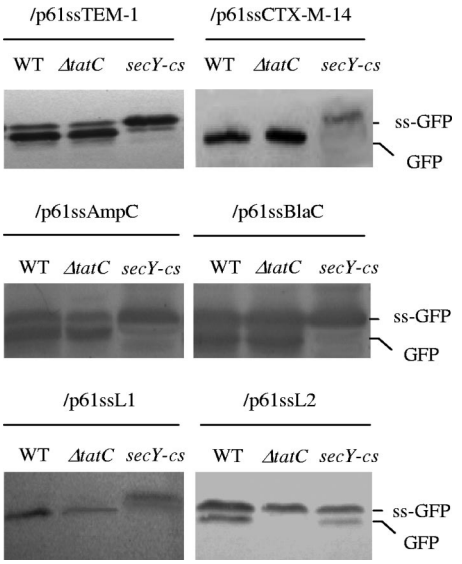


FIG. 3. Influence of the β-lactamase signal sequences on GFP translocation in the WT MC4100A, and mutant B1LK0A (Δ*tatC*), and CU164A (*secY-cs*) strains. Crude extracts (25 μg of protein each) were separated onto 12.5% acrylamide-bisacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and analyzed by immunoblotting using anti-GFP antiserum. Precursors (ss-GFP) and processed mature GFP are indicated on the right.

crude extracts of the *secY-cs* strain expressing ssTEM-1-GFP, ssCTX-M-14-GFP, ssAmpC-GFP, and ssL1-GFP and in the predicted Tat-dependent construction ssBlaC-GFP, which is consistent with the results obtained with the entire BlaC sequence. The mature form of these fusions was observed for the WT and the Δ*tatC* strains. In contrast, the ssL2-GFP precursor was only detected in the crude extracts of the Δ*tatC* strain, whereas the mature form was observed in the WT and the *secY-cs* strains. The β-lactamase signal sequences contained therefore enough information to address GFP to the *E. coli* Sec or Tat system. All signal sequences except L2 were addressed to the Sec translocation system.

**Folding and localization of β-lactamase–GFP fusions.** GFP fluorescence of the fusions was used in the wild-type, Δ*tatC*, and *secY-cs* strains as an indicator of localization and folding state. Chromophore formation was monitored by fluorescence microscopy. Fused to Sec-translocated proteins, the GFP moiety is exported into the periplasm without formation of the fluorochrome. In contrast, GFP fused to a Tat-translocated protein folds in the cytoplasm and appears as a peripheral halo after its export into the periplasm (37). As expected for Tat-dependent GFP fusions, peripheral fluorescence was observed in the wild-type and the *secY-cs* mutant cells expressing ssL2-GFP (Fig. 4A and C). Little cytoplasmic fluorescence was observed for the wild-type and Δ*tatC* cells expressing TEM-1–GFP, and a higher cytoplasmic fluorescence was observed in the *secY-cs* mutant cells (Fig. 4D, E, and F). Similar results were obtained with the β-lactamases AmpC, CTX-M-14, BlaC, and the corresponding signal sequences, as expected for Sec-

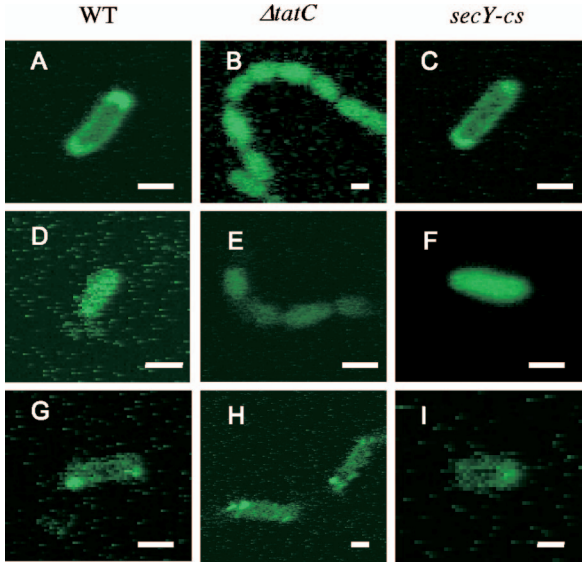


FIG. 4. Localization of GFP fusions in wild-type, Δ*tatC*, and *secY-cs* cells. (A to C) Fluorescence images of WT/p61ssL2 (ssL2-GFP), Δ*tatC*/p61ssL2 (ssL2-GFP), and *secY-cs*/p61ssL2 (ssL2-GFP) when cells were grown aerobically at 37°C until exponential growth phase and then induced for 3 h with arabinose at 23°C with shaking. (D to I) Fluorescence images of WT/p61TEM-1 (TEM-1–GFP), Δ*tatC*/p61TEM-1 (TEM-1–GFP), and *secY-cs*/p61TEM-1 (TEM-1–GFP) when cells were grown aerobically at 37°C until exponential growth phase and then induced for 3 h with arabinose at 23°C with shaking. In panels G, H, and I, variations were observed from the analysis of TEM-1–GFP expression. Bar, 1 μm.

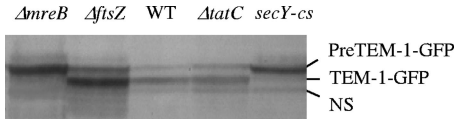


FIG. 5. Processing of the TEM-1–GFP fusion protein expressed from the p61TEM-1 plasmid in MC4100A (WT), B1LK0A (Δ*tatC*), CU164A (*secY-cs*), YLS3 (Δ*mreB*), and YLS24 (Δ*ftsZ*) cells. Cells were first grown aerobically (with shaking) at 37°C until exponential growth phase and then induced for 3 h with arabinose at 23°C with shaking. Crude extracts were separated onto 12.5% acrylamide-bisacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Nonspecific bands (NS) and precursor (PreTEM-1-GFP) and mature (TEM-1-GFP) forms of the fusion proteins are indicated to the right of the panels.

dependent GFP fusions (data not shown). Peripheral spots of fluorescence were also observed for 20% of cells expressing TEM-1–GFP in WT, Δ*tatC*, and *secY-cs* cells (Fig. 4G, H, and I). Such localizations have been previously reported for MreB-dependent proteins (3, 5, 13, 15, 39).

**Analysis of the roles of MreB and FtsZ cytoskeletons in β-lactamase translocation.** The roles of the FtsZ and MreB cytoskeletons in β-lactamase processing were investigated by using the YLS3 (Δ*mreB*) and YLS24 (Δ*ftsZ*) *E. coli* mutants. A single polypeptide corresponding to the TEM-1–GFP precursor (63 kDa) was detected for the *mreB* mutant expressing TEM-1–GFP (Fig. 5). In agreement with this result, MIC assays showed that each Δ*mreB* strain was more susceptible to the antibiotics tested than was the wild-type counterpart (Table 2). The results suggest that the *mreB* mutation directly or indirectly has an effect on β-lactamase processing and activity. In contrast, the mature form of TEM-1–GFP was detected in the *ftsZ* mutant, as in the wild-type strain, evidence that the *ftsZ* mutation did not affect TEM-1–GFP processing.

## DISCUSSION

The mechanisms of the translocation of β-lactamases to their active site have been very infrequently investigated. Eighty-nine N-terminal signal sequences representative of 400 β-lactamases were analyzed in this study. The majority of these signal sequence were plausible Sec-targeting signal peptides. The 14 expected Tat-dependent sequences originated preferentially from phytopathogenic bacteria and were more commonly located on the chromosome than on a transmissible plasmid. The Tat system is required as a general translocation system in certain bacterial species, such as phytopathogenic bacteria (9, 17, 49, 50). Hence, in such bacteria, β-lactamases may use the efficiency of the Tat system to their advantage.

The export of six representative β-lactamases was investigated in the *E. coli* model system. As expected by in silico analysis, β-lactamase specific activity and GFP reporter fusions showed TEM-1, CTX-M-14, AmpC, and L1 β-lactamases as preferentially Sec dependent. Their signal sequences were able to direct GFP by the Sec pathway. In contrast, the L2 β-lactamase of *Stenotrophomonas maltophilia* was not exported in an *E. coli* Δ*tatC* mutant. This is the first report of a Tat-dependent β-lactamase translocation process in a bacterium other than *Mycobacterium*. In addition, the BlaC β-lactamase, reported as Tat dependent in *Mycobacterium tuberculosis* by Flores et al. (14) and by our in silico analysis, was Sec dependent in *E. coli*. These results suggest that the β-lactamase translocation pathway was dependent not only on the signal sequence but also on the bacterial species. The differences in the BlaC translocation process could be explained by the differences between the Tat proteins of *M. tuberculosis* and *E. coli*. TatCs were 32% identical, whereas no similarity between the two species was found for TatA and TatB. Another explanation is that β-lactamase folding kinetics occurring in *M. tuberculosis* could be distinct from those occurring in *E. coli*, depending on the physiological environment. Environmental conditions (for example, pH and temperature) encountered by the bacteria, reducing and oxidizing conditions of the cytoplasm, and diverse chaperones could affect the folding kinetics of the proteins (29, 46).

TEM-1–GFP formed punctuated fluorescence patterns in the lateral wall and at the division site, as reported for the MreB cytoskeleton and for the Sec and Tat machineries (15, 32, 40, 41). Moreover, failure in β-lactamase processing was revealed by immunoblotting and MIC analysis in the Δ*mreB* strain. Bacterial cytoskeleton proteins form helical filaments along the long axis of the cell just beneath the cytoplasmic membrane. A similar helical localization pattern has been observed for the MreB-dependent PBP2 protein in *Caulobacter crescentus* (13). Increasing evidence tends to support the idea that cytoskeletons can determine the spatial protein transport path from the cytoplasm to the cell wall (3, 5, 15, 39, 40). Because PBPs have different localizations and action sites in the peptidoglycan layer, there may be similar specific localizations for the derived β-lactamases (38). Thus, MreB filaments might serve as an organizer or tracking device to direct β-lactamases to the Sec and/or to the Tat system.

In conclusion, this work provides evidence that β-lactamase Tat targeting is rare, except in species using the Tat system as a major general export pathway, probably to enhance translocation efficiency. In addition, although β-lactamase signal sequences are sufficient to mediate α Sec- or a Tat-dependent export in *E. coli*, the β-lactamase translocation pathway could also depend on the characteristics of bacterial species. Finally, we have made the first investigations into the putative role of the MreB cytoskeleton in β-lactamase processing.

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