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Nucleotide Sequence and Expression of the β-Lactamase Gene from *Staphylococcus aureus* Plasmid pI258 in *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*

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The structural gene for β-lactamase in the *Staphylococcus aureus* plasmid pI258 was cloned into a *Staphylococcus aureus-Bacillus subtilis-Escherichia coli* shuttle vector, pWN101, and the nucleotide sequence of the gene was determined. pWN101 was structurally stable and the β-lactamase gene was expressed efficiently from its native promoter and ribosome-binding site in all three hosts.

Plasmid-encoded β-lactamases (EC 3.5.2.6.) from *Staphylococcus aureus* are relatively small, single-chain exoproteins whose synthesis is inducible by a variety of β-lactam compounds. The enzymes are well characterized both enzymologically and structurally (1, 14, 22). They are extremely active, having both high substrate affinity and high turnover numbers (in the range of 10^4/min). Moreover, they can be accurately assayed by a variety of very sensitive techniques (13, 18, 24). The promoter and the region encoding the signal peptide for the pI258 β-lactamase (16) gene have been mapped and sequenced previously (9, 10). Here, we report completion of the nucleotide sequence of the structural gene, as well as the construction of a *Staphylococcus aureus-Bacillus subtilis-Escherichia coli* shuttle vector containing the gene. We found that the vector was structurally stable in all these hosts and that the gene was expressed efficiently using its native promoter and ribosome-binding site.

To establish the pI258 β-lactamase gene (*blaZ*) in *S. aureus* and *B. subtilis*, as well as in *E. coli*, we ligated *Hin*dIII digests of the *blaZ*-containing *E. coli* plasmid pAO7 (19) and the *S. aureus* and *B. subtilis* plasmid pC194 (4, 5) (Fig. 1). All seven *Hin*dIII clones obtained by transformation of *E. coli* AB259 (HfrH *thi-1 rel-1* λ⁻) (8) had the two plasmids joined in the same orientation. The resulting cointegrate plasmid, pWN101, was used to transform *B. subtilis* BD224 (*trpC2 recE4 thr-5*) (BGSC 1A46) frozen competent cells (3) and *S. aureus* RN4220 (7) (a mutant that is an efficient acceptor of *E. coli* DNA) protoplasts (11), with selection for chloramphenicol resistance (5 μg/ml). For each host, plasmid DNA from several transformants was characterized by restriction nuclease analysis and was found to be intact. However, the plasmid showed moderate hereditary instability in all three host strains, since colonies grown on agar plates in the absence of the selective antibiotic exhibited sectoring in the *N*-phenyl-1-naphthylamine-azo-*O*-carboxybenzene test (15) (data not shown).

The DNA sequence of the *blaZ* gene and the flanking regions is shown in Fig. 2. The deduced amino acid sequence of the exoenzyme is identical to that determined previously from analysis of tryptic, chymotryptic, peptic, and CNBr peptides of β-lactamase (1). The putative promoter region, ribosome-binding site, repressor-binding site, and deduced amino acid sequence of the signal peptide are identical to those described previously (9). No sequence characteristic of typical Rho-independent transcription terminators (25) was found in the 170-base-pair sequence following the translation termination codon of *blaZ*. Although the β-lactamase of *S. aureus* shows 40% homology with that of *Bacillus licheniformis* (2), its nucleotide sequence shows very low homology with that of *B. licheniformis*. This results from the fact that codon usage in the two organisms is quite different (data not shown).

The β-lactamase activities in both whole cultures and supernatants of pWN101-harboring *E. coli*, *B. subtilis*, and *S. aureus* and pI258-harboring *S. aureus* (17) were determined as described by O'Callaghan et al. (18). As shown in Fig. 3, β-lactamase specific activity peaked in the mid-exponential phase for *S. aureus* and *B. subtilis* and in the early exponential phase for *E. coli*. Production in *E. coli* seemed anomalous in that specific enzyme activity declined throughout exponential-phase growth. It is known that class A β-lactamase in gram-positive bacteria forms a mature membrane-bound hydrophobic form as well as a mature soluble exoenzyme (12). Under the conditions of these

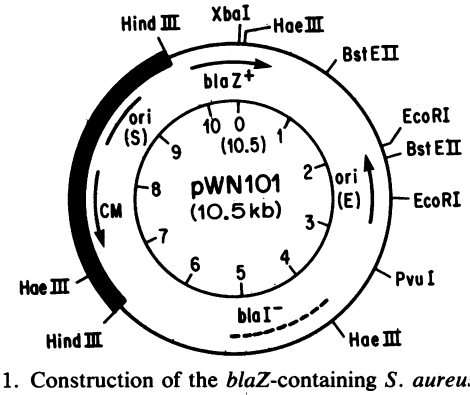

FIG. 1. Construction of the *blaZ*-containing *S. aureus-B. subtilis-E. coli* shuttle vector pWN101. pWN101 was constructed by joining the *E. coli* plasmid pAO7 (——) and the *S. aureus* and *B. subtilis* plasmid pC194 (▬) at the unique *Hin*dIII site of each plasmid. *ori*(S) indicates the replication origin region of pWN101 in *S. aureus* and *B. subtilis*, and *ori*(E) indicates the region in *E. coli*. *blaI*⁻, the mutant repressor gene for *blaZ*, is indicated by a dotted line since it has not been mapped accurately. CM, Gene for chloramphenicol resistance; kb, Kilobases.

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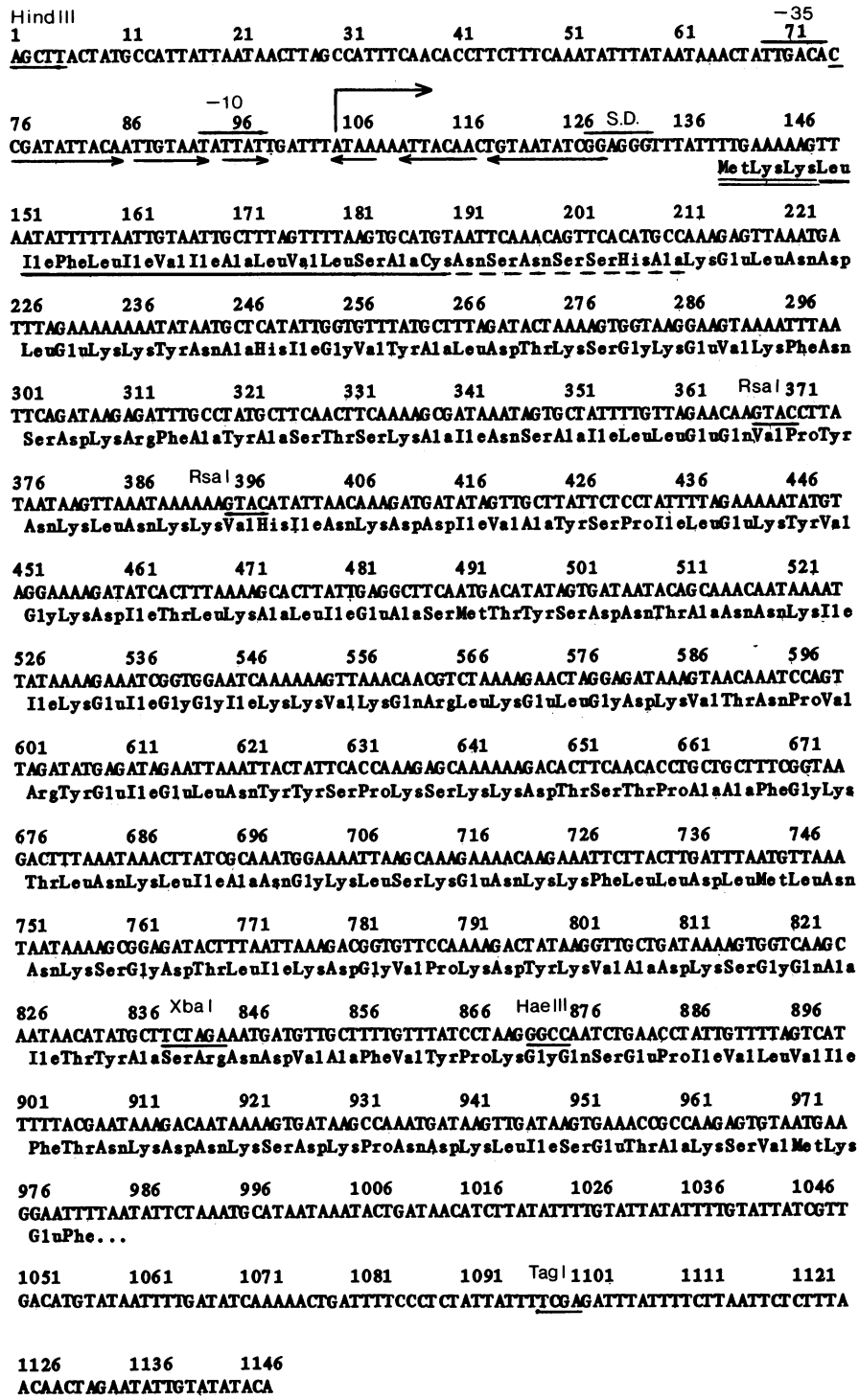

FIG. 2. Nucleotide sequence of *blaZ* gene and deduced amino acid sequence of *S. aureus* β-lactamase precursor. The −35 and −10 transcriptional initiation signals and the Shine-Dalgarno (S.D.) sequence are indicated. A prominent inverted repeat, which may be involved in regulation, is indicated by the horizontal arrows below the sequence. The transcription initiation site is marked with an arrow above the sequence (9, 10). The regions of the signal peptide (12) are underlined as follows: ═══, positively charged residues adjacent to the initiator methionine; ——, hydrophobic stretches; -----, hydrophilic region between the N terminus of the membrane-bound enzyme and the stable secreted form.

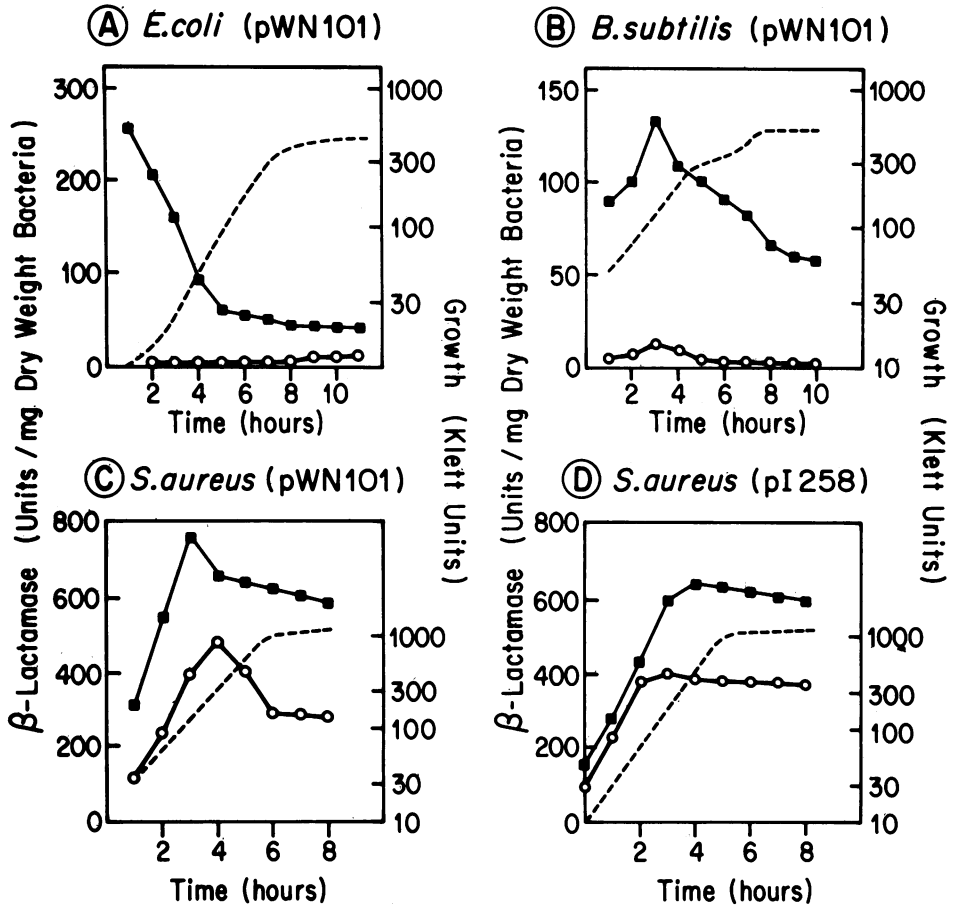

FIG. 3. Expression of β-lactamase from pWN101 and pI258 (17). (A) *E. coli*(pWN101); (B) *B. subtilis*(pWN101); (C) *S. aureus*(pWN101); (D) *S. aureus*(pI258). Cultures were sampled at various stages of growth (-----), and both the whole culture (■) and supernatant (○) were assayed for β-lactamase activity at pH 5.8 spectrophotometrically (18). One unit is 1 μmol of penicillin hydrolyzed per ml per h at 30°C (20). One Klett unit corresponded to about 4.5 μg (dry weight) of bacteria per ml. This bacterial concentration corresponded to about 2×10^7 cells per ml for *S. aureus* and 10^7 cells per ml for *E. coli* and *B. subtilis*.

experiments, in *S. aureus* about 50 to 60% of the β-lactamase remained bound to the cells and about 40 to 50% was secreted into the medium, as described previously (12, 21). However, in *B. subtilis* more than 90% of the β-lactamase activity was bound to the cells, and only very small amounts of the enzyme were detected in the supernatant fluid. Although this difference was probably due to proteolytic degradation of the secreted enzyme, as previously observed by Saunders et al. (23), it was unaffected by the use of a *B. subtilis* mutant (kindly provided by E. Chang) lacking the two major exoprotease activities (data not shown). Therefore, this enzyme could be degraded by a minor protease in *B. subtilis*.

pWN101 was structurally stable, and the *blaZ* gene was expressed efficiently in all three host species. These findings suggest that the *S. aureus* β-lactamase may be useful as an indicator enzyme. Recently, we have made deletions in the control region of *blaZ* and inserted a multiple cloning site in front of the Shine-Dalgarno sequence and the structural gene of *blaZ*, respectively, to create a series of fusion vectors, including a novel type of translational fusion vector. Preliminary results show that these derivatives of pWN101 are suitable for study of gene regulation in *S. aureus*.

It has been reported (23) that it is difficult to establish the pI258 *blaZ* gene in *B. subtilis* on multicopy plasmid vectors by using either pUB110- or pC194-based vectors or pC194-based shuttle vectors. Instead, chromosomal integration has been used for establishment and expression of the *S. aureus blaZ* gene in *B. subtilis*. It has been suggested (23) that the instability of *blaZ* in *B. subtilis* on multicopy vectors is due to either the *blaZ* strong promoter or the existence of an approximately 10-base-pair direct repeat in the *blaZ* promoter region. In this work, the *blaZ*-containing restriction fragment was established in the pC194-based vector pWN101, and no structural instability was observed. Therefore, it seems that the 10-base-pair direct repeat is not directly responsible for the reported instability of *bla*-containing plasmids. The difference between the results reported here and those reported previously may be attributed to the use of different *Bacillus* strains and different shuttle vectors. It should be noted that the copy number of pWN101 became much lower (about 5 copies per cell) than that of pC194 (about 30 copies per cell) in *B. subtilis*, even when the *blaZ* promoter was deleted, but it maintained the same copy number as that of pC194 (about 30 copies per cell) in *S. aureus* (data not shown). It has been reported that it is not possible to clone an *Eco*RI fragment containing a highly expressed allele of a *B. licheniformis* β-lactamase gene (*penP*) into the *Eco*RI site of pUB110, but it is possible to clone the same fragment into a low-copy-number plasmid

vector (6). In this study, the ability to establish *blaZ* in *B. subtilis* on a derivative of the high-copy-number plasmid pC194 may have been due to the spontaneous decrease in copy number of this plasmid in *B. subtilis*.

The β-lactamase gene was expressed by pI258 at about twice the rate of that by pWN101 in *S. aureus*. This could have been due to a difference in regulation, perhaps related to interruption of the *Hin*dIII site 5′ to the gene in pWN101 (15).

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