Secretion cloning vectors in *Escherichia coli*

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The DNA fragment coding for the signal peptide of the OmpA protein, a major outer membrane protein of *Escherichia coli*, has been inserted into the high-level expression vectors, pIN-III. A foreign DNA fragment can be cloned in any one of the three reading frames at the unique *EcoRI*, *HindIII* or *BamHI* sites immediately after the *ompA* signal peptide coding sequence. The cloned foreign gene is under the control of both the *lpp* promoter and the *lac* promoter-operator. The expression of the gene is regulated by the *lac* repressor produced by the same vectors. Using the pIN-III-*ompA* vector, the DNA fragment coding for only the mature portion of β -lactamase was inserted into the *EcoRI* site. Upon induction of gene expression, β -lactamase was secreted into the periplasmic space. The *ompA* signal peptide was correctly removed resulting in the production of β -lactamase with four extra amino acid residues (Gly-Ile-Pro-Gly) at its amino terminus due to the linker sequence in the vector. After a 3-h induction, β -lactamase was accumulated to 20% of total cellular protein without any detectable accumulation of pro- β -lactamase. Using oligonucleotide-directed site-specific mutagenesis, we have also removed the linker sequence and upon induction of gene expression, β -lactamase with the authentic NH₂-terminal sequence was produced, in even larger amounts than the β -lactamase with the linker sequence.
Key words: β -lactamase/*ompA*/pIN-III/signal peptide

Introduction

In the *Escherichia coli* system it is possible to guide a cloned gene product to three different locations in the cell; the cytoplasm, outside the cytoplasmic membrane, or into the outer membrane (Masui *et al.*, 1983). The secretion of a cloned gene product across the cytoplasmic membrane can be achieved by fusing an appropriate signal peptide to the amino-terminal end of the gene product. Such a secretion cloning system has significant advantages over the other cloning systems. Firstly, the amino-terminal amino acid residue of the secreted product can be identical to the natural gene product as a result of the cleavage of the signal peptide by a specific signal peptidase. By contrast, the gene product has in general a methionine residue at its amino terminus when it is produced in the cytoplasm. Secondly, protease activity is considered to be much less in the periplasmic space than in the

cytoplasm. Therefore, the gene product is more stable in the former than the latter. Thirdly, many toxic enzymes or proteins such as nucleases and proteases cannot be produced in the cytoplasm. They have to be secreted to the outside of the cytoplasmic membrane while being synthesized.

We have constructed secretion vectors with which a cloned gene product is produced in large amounts and very efficiently secreted across the cytoplasmic membrane. This was achieved by inserting the DNA fragment coding for the signal peptide of the OmpA protein, a major outer membrane protein, into the pIN-III high-level expression cloning vectors developed in our laboratory (Masui *et al.*, 1984). The primary cloned gene product thus contains the *ompA* signal peptide at its amino terminus, which should allow the product to be secreted across the cytoplasmic membrane. Upon translocation of the gene product across the cytoplasmic membrane, the signal peptide is cleaved to release the processed product in the space between the cytoplasmic membrane and the outer membrane (periplasmic space). When the β -lactamase gene was cloned into the new secretion vectors, pIN-III-*ompA*, β -lactamase was produced at the level of 20% of total cellular protein, and was accumulated in the periplasmic space. It was found that the *ompA* signal peptide was very efficiently removed at its normal cleavage site.

Results

Construction of the secretion vectors

We have previously described the construction of the high level expression vector pIN-III (Masui *et al.*, 1984). We have used this vector as the basis for the construction of secretion vectors. As shown in Figure 1, the 27-bp DNA fragment between the unique *XbaI* and the unique *EcoRI* sites of the pIN-III-A3 vector (Masui *et al.*, 1984) was replaced with the DNA fragment carrying the coding region for the entire *ompA* signal peptide. The DNA sequence of the *ompA* gene has been determined (Movva *et al.*, 1980a; Beck and Bremmer, 1980); the OmpA protein is primarily synthesized as a secretory precursor, pro-OmpA protein, which has the signal peptide of 21 amino acid residues at the amino-terminal end (Movva *et al.*, 1980b). We have chosen the *ompA* signal peptide for the secretion vectors (i) because it functions efficiently to secrete a large amount of the OmpA protein and (ii) because it does not require any modification of the cleavage site during the secretion process, in contrast to the prolipoprotein. In the case of the prolipoprotein, modification of the cysteine residue at the cleavage site with a glyceride group is essential for the cleavage of the signal peptide (Inouye *et al.*, 1983; Tokunaga *et al.*, 1982).

The major portion of the DNA fragment to be inserted into the pIN-III-A3 vector was derived from the *ompA* gene as the 65-bp *HhaI* fragment. This fragment was isolated from a clone of the *ompA* gene, pAM003 (P.Green and M.Inouye, unpublished) as follows: the pAM003 DNA was digested with *EcoRI* and the resulting 871-bp fragment encompassing the entire *ompA* gene was purified. The 65-bp *HhaI* fragment

Fig. 1. Schematic representation of the construction of the secretion vectors pIN-III-ompA-1 (*ompA-2* and *ompA-3*). Details of the procedures and conditions are found in Results and Materials and methods. The small arrow indicates the position of the single base deletion described in the text. Dots indicate the Shine-Dalgarno sequence. Abbreviations are: Ba, *Bam*HI; Ec, *Eco*RI; Hc, *Hinc*II; Hh, *Hha*I; Hi, *Hind*III; Hp, *Hpa*I; Ps, *Pst*I; Sa, *Sac*I; and Xb, *Xba*I; *lpp*^p, *lpp* promoter, and *lac*^{po}, *lac* promoter-operator.

was then isolated from the *Eco*RI fragment as shown in Figure 1. This *Hha*I fragment encompasses the region from nucleotide -8 to +57 of the *ompA* gene (+1 represents the A residue of the initiation codon, ATG, of the *ompA* gene) (Movva *et al.*, 1980a; Beck and Bremmer, 1980). To reconstruct the Shine-Dalgarno sequence for ribosome-binding, two oligonucleotides were synthesized; oligonucleotide *a* consists of 15 nucleotides and contains the Shine-Dalgarno sequence, GAGG (dotted sequence in Figure 1). The 6-nucleotide sequence, GATAAC, upstream of the Shine-Dalgarno sequence is identical to that in the *ompA* gene (Movva *et al.*, 1980a; Beck and Bremmer, 1980). Therefore, this oligonucleotide reproduces the identical sequence to the *ompA* gene up to nucleotide -18. Oligonucleotide *b* is complementary to *a*, and the hybrid between oligonucleotides *a* and *b* creates an *Xba*I sticky end at the 5' end and a *Hha*I sticky end at the 3' end (Figure 1). In a similar fashion, the cleavage site region for the *ompA* signal peptide was constructed. Oligonucleotide *d* consists of 14 nucleotides and encompasses the *ompA* sequences from nucleotide +56 to +63, which codes for -Ala¹⁹-Gln-Ala²¹. The Ala residue at position 21 is the carboxy-terminal residue of the *ompA* signal peptide. To construct an *Eco*RI site immediately after

the cleavage site as reading frame 3 [*Eco*RI cleaves off the third base of a codon in phase as defined previously (Nakamura and Inouye, 1982; see Figure 2A)], the nucleotide sequence, 3'CCTTAA5' was added after nucleotide +63. This sequence corresponds to 5'GGAATT3' in the sense strand, which codes for Gly-Ile. Oligonucleotide *c* consists of eight bases and when the complementary hybrid is formed with oligonucleotide *d*, a *Hha*I site and an *Eco*RI site are created at the 5' end and the 3' end, respectively (Figure 1).

The pIN-III-ompA-3 vector was then constructed by ligating all the fragments described above to the large linear fragment of pIN-III-A3 resulting from the digestion of the pIN-III-A3 DNA with *Xba*I and *Eco*RI (see Figure 1). Since the *Hha*I fragment from the *ompA* gene can be inserted in two different orientations, the transformant with the *Hha*I fragment of the right orientation was selected by examining the position of the *Taq*I site within the *Xba*I-*Bam*HI fragment. If the *Hha*I fragment is inserted in the right orientation, two fragments of different sizes are generated by *Taq*I digestion of the *Xba*I-*Bam*HI fragment. On the other hand, if the *Hha*I fragment is inserted in the wrong orientation, the *Taq*I digestion gives rise to two DNA fragments of almost the same size. One such transformant carrying the *Hha*I frag-

Fig. 2. Partial DNA sequences, and the derived protein sequences of the pertinent region of the various plasmids described in Figures 1 and 3. (A) pIN-III-*ompA*-1, pIN-III-*ompA*-2 and pIN-III-*ompA*-3. (B) pJG105 and pJG108. Abbreviations are the same as in Figures 1 and 3. Arrows indicate the cleavage site of the OmpA signal peptide. The linker sequence in pJG105 is underlined.

ment in the right orientation was selected. DNA sequencing from the *Xba*I site to the *Bam*HI site of this plasmid DNA revealed that the pIN-III-*ompA*-3 DNA had exactly the same sequence as predicted from the construction except for the loss of one G:C pair in the upstream *Hha*I site as indicated by arrows in Figure 1 (data not shown). The reason for this mutation is not known at present. This mutation resulted in the loss of the upstream *Hha*I site and the space between the Shine-Dalgarno sequence and the initiation codon became seven bases, one base shorter than that of the *ompA* gene.

To construct two other reading frames, A1 and A2, the 77-bp *Xba*I-*Hha*I fragment was first purified from the pIN-III-*ompA*-3 as illustrated in Figure 1. This fragment was then religated with two synthetic oligonucleotides to the large *Xba*I-*Eco*RI fragment derived from the pIN-III-A3 vector (Figure 1). For the construction of the pIN-III-*ompA*-1 vector, oligonucleotides *e* (9-mer) and *f* (15-mer) were used, which reconstructed the *Hha*I site and the *Eco*RI site. The DNA sequence of the pIN-III-*ompA*-1 DNA at the cleavage region was confirmed as shown in Figure 2A (data not shown). The pIN-III-*ompA*-2 was also constructed in a similar fashion with use of synthetic oligonucleotides *g* (10-mer) and *h* (16-mer). Its DNA sequence was also confirmed as shown in Figure 2A. The amino acid sequences at the cleavage region for all three vectors are also shown in Figure 2A.

Construction of pIN-III-*ompA*-3- β -lactamase

To test the vectors constructed above, we attempted to clone the gene for β -lactamase (*bla*) into pIN-III-*ompA*-3. For this purpose the *bla* gene in the vector itself was first inactivated by inserting the gene conferring chloramphenicol resistance into the unique *Pst*I site in the *bla* gene as shown in Figure 3. The gene was obtained from pIN-II-B3-Cm^r (J.Coleman and

Fig. 3. Schematic representation of the construction of plasmid pJG105 carrying the DNA coding for β -lactamase without the β -lactamase signal peptide. Abbreviations are: Bs, *Bst*EII; Ha, *Hae*II. Other abbreviations are the same as in Figure 1.

M.Inouye, unpublished). The *Pst*I fragment carrying the Cm^r gene was inserted into the *Pst*I site of pIN-III-*ompA*-3 (Figure 3). Into the *Eco*RI site of the resulting pIN-III-*ompA*-3-Cm^r was inserted the *bla* gene, which was obtained from pTG2. pTG2 was derived by changing 5'GCTCAC3' to GGTCAC at the signal peptide cleavage region of the *bla* gene of pBR322 (Kadonaga *et al.*, 1984). This single base change created a *Bst*EII site (see Figure 2B). Thus digestion of pTG2 with *Bst*EII and *Hae*II generated a DNA fragment coding for β -lactamase without the signal peptide. Treatment of this fragment with the Klenow fragment of DNA polymerase in the presence of 4 dNTP filled in the 5' end hang-over sequence at the *Bst*EII site and removed the 3' end hang-over sequence at the *Hae*II site (Figure 3). As a result both ends became blunt-ended, to which 12-mer *Eco*RI lingers (dCCGGAATTCGG) were ligated. The fragment was then digested with *Eco*RI and inserted into the *Eco*RI site of pIN-III-*ompA*-3. The transformants were initially selected by chloramphenicol resistance. Those transformants which had the *bla* gene fragment in the right orientation were then selected by their ampicillin resistance. It was found that they were ampicillin-resistant even in the absence of isopropyl- β -

Fig. 4. SDS polyacrylamide gel electrophoresis of total cell fractions of *E. coli* JA221 *lpp*⁻/F' *lacI*^q/pJG105 and pJG108. Cells were grown at 37°C in M9 media to a Klett reading of 30 at which time IPTG was added to a final concentration of 2 mM to half the culture. After 3 h, cells were harvested, washed twice with 10 mM Tris-HCl, pH 7.0 and submitted to SDS-PAGE as described under Materials and methods. Lanes 1 and 3, 0.1 ml of pJG105 harboring cells grown with (lane 3) and without (lane 1) IPTG, lanes 2 and 4, 0.1 ml of pJG108-harboring cells grown with (lane 4) or without (lane 2) IPTG, lane 5, pure β -lactamase.

D-thiogalactoside (IPTG), a *lac* inducer, and that they were IPTG-sensitive. This indicates that even in the absence of IPTG, a small amount of β -lactamase was produced, which was enough to make cells ampicillin-resistant, while in the presence of IPTG, over-production of β -lactamase was lethal to the cells. One of the transformants was designated as pJG105, and its DNA sequence was determined to be the same as predicted from the construction procedure as shown in Figure 2B (data not shown).

Expression of the β -lactamase gene

When cells carrying pJG105 were grown in the presence of IPTG, a protein similar in apparent mol. wt. to β -lactamase was produced and became the major product (~20% of total cellular protein) after 3 h of induction (Figure 4, lane 3). On the other hand, very little product was observed in the absence of inducer (Figure 4, lane 1). The efficient control of gene expression was essential since the overproduction of the protein eventually became lethal to the cells which appeared to be swollen and no longer rod-shaped when viewed under a microscope. The new product exhibited β -lactamase activity and could be immunoprecipitated with anti- β -lactamase serum (data not shown).

To characterize further the product, cells carrying pJG105 were induced with IPTG for 20 min and labeled with [³H]proline for 5 min. The cells were then washed, lysed and immunoprecipitated with anti- β -lactamase serum. The immunoprecipitates were subjected to sodium dodecyl sulfate

Fig. 5. Sequential Edman degradation of the β -lactamase product of pJG105 and pJG108. A 20 ml culture of *E. coli* JA221 *lpp*⁻/F' *lacI*^q/pJG105 or pJG108 was labeled at 37°C for 5 min in the presence of 2 mM IPTG and [³H]proline or [³H]glycine (pJG105) or [³H]-lysine (pJG108). The total cell fractions were then prepared, solubilized and treated with anti- β -lactamase serum. The immunoprecipitates were submitted to SDS-PAGE. The band corresponding to β -lactamase was excised from each gel and the protein was eluted and subjected to sequential Edman degradation. (A) pJG105 labeled with [³H]proline (panel 1) or [³H]glycine (panel 2); (B) pJG108 labeled with [³H]lysine.

polyacrylamide gel electrophoresis (SDS-PAGE), the band corresponding to the β -lactamase product was excised from the gel and the protein was eluted and subjected to sequential Edman degradation. As shown in Figure 5A, panel 1, the only significant radioactivity appeared at positions 3 and 6 indicating that the *ompA* signal peptide was cleaved at its normal processing site to give β -lactamase with an extra four amino acid extension (Gly-Ile-Pro-Gly) at its amino terminus (see Figure 2B). This was further confirmed by performing the Edman degradation on samples labeled with [³H]glycine, and finding that the radioactivity appeared at positions 1 and 4 as expected (Figure 5A, panel 2). It should be emphasized that the fact that no proline radioactivity appeared at the second position (Figure 5A), indicated that there was practically no cleavage at the amino-terminal end of the histidine residue, which is the normal amino terminus of β -lactamase. Furthermore, the result in Figure 4 illustrated that almost all the product was processed without any detectable accumulation of its secretory precursor.

Localization of the β -lactamase product

In *E. coli*, the usual cellular locale of pBR322-encoded β -lactamase is known to be the periplasmic space (Koshland and Botstein, 1980). However, treatment of cells carrying pJG105, after induction with IPTG for 3 h, with osmotic

shock or lysozyme-EDTA failed to release the product into the medium. On the other hand, brief sonication followed by low speed centrifugation lead to the recovery of the majority of the product which appeared to be contaminated mainly with outer membrane proteins. Treatment of the aggregate with 0.3% sodium lauryl sarkosinate followed by high speed centrifugation lead to the solubilization of β -lactamase which was greater than 96% pure as judged by SDS-PAGE (data not shown). The results indicate that overproduction of the protein leads to its aggregation in the periplasmic space. This aggregation was not due to crosslinking via the cysteine residues in β -lactamase since migration of the product on SDS-PAGE in the absence of reducing agent was actually faster than in the presence of reducing agent. This indicated that only the normal disulfide bond of β -lactamase is formed and this provides further evidence that the β -lactamase has been translocated to the periplasmic space since it has been shown that the disulfide bond of β -lactamase is formed during or after its translocation into the periplasmic space (Pollitt and Zalkin, 1983).

Removal of the linker sequence

In the actual application of the pIN-III-*ompA* vectors, it is desirable to remove the extra sequence due to the linker after construction of a primary clone of a given gene. This can be achieved easily by oligonucleotide-directed site-specific mutagenesis. For this purpose, a 24-mer oligonucleotide, (GTAGCGCAGGCCACCCAGAAACG), was synthesized and used to remove the 12-base linker sequence according to the method described by Moringa *et al.* (1984) (see Figure 2B). One transformant which showed a strong hybridization signal with the ^{32}P -labeled oligonucleotide was designated pJG108, and its DNA sequence was confirmed as shown in Figure 2B (data not shown). The cells carrying pJG108 were more sensitive to IPTG and produced more β -lactamase than the cells carrying pJG105 (Figure 4, lane 2). This provides another indication that it is the overproduction of β -lactamase which is lethal to the cell rather than slow processing of the pro- β -lactamase. In addition, the product from pJG108 migrated slightly faster in SDS-PAGE than the product from pJG105 (Figure 4, lane 1). From the sequential Edman degradation of the product labeled with ^3H lysine, the radioactivity appeared at the 7th and 9th steps (Figure 5B) demonstrating that the *ompA* signal was again cleaved correctly (Figure 2B). The product from pJG108 which constituted >20% of total cellular protein after 3 h of induction could not be released by osmotic shock or lysozyme-EDTA treatments, and was found to be aggregated in a similar fashion to the product from pJG105.

Discussion

We have constructed very efficient secretion vectors (pIN-III-*ompA*) and have tested them by cloning the gene coding for β -lactamase. The main features of the secretion vectors are (i) the use of the strong *lpp* promoter, (ii) the addition of a *lac* promoter operator fragment to ensure that expression is dependent on the addition of a *lac* inducer, (iii) the use of the DNA fragment coding for the OmpA signal peptide to direct the secretion of the product of a cloned gene and (iv) the construction of three unique cloning sites in three different reading frames. We envisage the use of these vectors in a two step process. In step 1 a gene is cloned into one of the three unique cloning sites and in step 2 the linker-derived sequences can be removed if necessary to give a product identical to the

natural gene product. We have cloned the gene coding for β -lactamase and examined its expression. The inserted gene lacking the coding sequence for the β -lactamase signal peptide, was inserted into the *EcoRI* site of pINIII-*ompA*-3 using synthetic *EcoRI* linkers. Our results indicate that the cloned gene is expressed at a very high rate and that the product is β -lactamase with a four amino acid extension (Gly-Ile-Pro-Gly) at its amino terminus introduced by the use of *EcoRI* linkers. Furthermore, the *ompA* signal peptide was cleaved at its normal processing site and the cleaved product was translocated into the periplasmic space where it was found to form insoluble aggregates most likely due to its overproduction (~20% of total cellular protein after 3 h of induction). We have also deleted the linker-derived sequence using oligonucleotide-directed site-specific mutagenesis and found that the *ompA* signal peptide was again cleaved correctly and that mature product was found to have an NH_2 -terminal sequence identical to that of β -lactamase of pBR322, and was produced in larger amounts than the β -lactamase containing the extra linker sequence. After 3 h of induction, β -lactamase constituted >20% of total cellular protein. It is clear from our results that this vector system can be useful for high level production of secreted proteins and that the *ompA* signal peptide can be functional when attached to sequences unrelated to OmpA protein. In addition in the case of β -lactamase, the efficiency of expression is at least 100-fold more than the same construction in the same vector system using the β -lactamase signal sequence instead of the *ompA* signal sequence (data not shown). We have also cloned the genes for human α -interferon and staphylococcal nuclease A and our preliminary data indicate that both genes are expressed and that the products do not contain the OmpA signal peptide.

We believe that we have constructed a very efficient secretion vector which is ideally suited for high level expression of foreign genes coding for normally secreted proteins although not all proteins may be secreted in this system. We are planning to test this system with a variety of foreign genes, including genes coding for peptide hormones, in order to establish its universality.

Materials and methods

Bacterial strains and plasmids

E. coli strain JA221 (*lpp*⁻ *hdsM*⁺ *trpE5* *leuB6* *lacY* *recA1*/F' *lacI*^q *lac*⁺ *pro*⁺) (Nakamura and Inouye, 1979) was used in all experiments as the host cell. Cells were grown in the following media depending on the experiment: M9 media supplemented with glucose (4 mg/ml), tryptophan (20 $\mu\text{g}/\text{ml}$), leucine (20 $\mu\text{g}/\text{ml}$), thiamine (2 $\mu\text{g}/\text{ml}$), chloramphenicol (10 $\mu\text{g}/\text{ml}$) and/or ampicillin (50 $\mu\text{g}/\text{ml}$); and L broth supplemented with chloramphenicol (10 $\mu\text{g}/\text{ml}$) and/or ampicillin (50 $\mu\text{g}/\text{ml}$).

Plasmids pIN-III-A3 (Masui *et al.*, 1984), pIN-II-B3-Cm^r (J.Coleman and M.Inouye, unpublished) pTG2 (a generous gift from J.Knowles, Harvard University) were used in the plasmid constructions.

DNA manipulations

Isolation of plasmid DNA and routine manipulations were carried out as previously described (Nakamura and Inouye, 1979). Restriction enzymes and T4 DNA ligase were obtained from Bethesda Research Laboratories. DNA polymerase, Klenow fragment, was from New England Nuclear. An *EcoRI* 12-mer linker oligonucleotide (dCCGGAATTCCGG) was purchased from New England Biolabs. DNA sequencing was carried out by the method of Maxam and Gilbert (1977).

Detailed procedures for plasmid constructions

Oligonucleotides were synthesized as described previously (Hsiung *et al.*, 1983). Annealing of complementary oligonucleotides was performed by mixing 0.4 μmol of each oligonucleotide in 100 μl of a solution containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 and incubating at 100°C for 2 min followed by cooling at 4°C for 2 h. For construction of pIN-III-*ompA*-3 (Figure 1), the

ligation mixture contained 0.008 pmol pIN-III-*ompA*-3 (Figure 1), the ligation mixture contained 0.008 pmol pIN-III-A3 DNA previously digested with *Xba*I-*Eco*RI, 1 pmol each of the annealed synthetic nucleotides (*a* + *b* and *c* + *d*) and 0.07 pmol of the 65-bp *Hha*I fragment from pAM003, in a 24 μ l reaction mixture containing 42 mM Tris-HCl, pH 7.5, 8.5 mM MgCl₂, 0.5 mM ATP, 10.4 mM dithiothreitol, and 4 units of T4 DNA ligase. The mixture was incubated at 12.5°C for 16 h followed by transformation into JA221 *lpp*⁻/*F'* *lac*I^q. The desired transformants were identified using colony hybridization (Vlasuk et al., 1983) with oligonucleotide *a* and *c* as probes, and the correct orientation of the fragments was determined as described in the Results section. For the construction of pIN-III-*ompA*-1 and *ompA*-2, the ligation solution contained 0.6 pmol of the large linear DNA fragment after digestion of pIN-III-*ompA*-3 with *Xba*I-*Eco*RI, 27 pmol of the *Xba*I-*Hha*I fragment from the same vector (see Figure 1) and 20 pmol of the annealed synthetic oligonucleotides (*e* + *f* for A-1 and *g* + *h* for A-2) in 15 μ l of 6.6 mM Tris-HCl, pH 7.5, containing 6.6 mM MgCl₂, 0.6 mM ATP, 10 mM dithiothreitol, and 3 units of T4 DNA ligase. The mixture was incubated at 12.5°C for 16 h, and transformed into JA221 *lpp*⁻/*F'* *lac*I^q. One candidate each for the A-1 and A-2 reading frames were selected and found to contain three of the *Hha*I-*Eco*RI synthetic linker oligonucleotides. Plasmid DNA was then digested with *Eco*RI and the large linear fragments were treated with T4 DNA ligase and transformed into JA221 *lpp*⁻/*F'* *lac*I^q. Three transformants were picked for pIN-III-*ompA*-1 and pIN-III-*ompA*-2 and DNA sequencing of the *Xba*I-*Bam*HI fragments was performed.

Construction of pJG105

pIN-III-*ompA*-3 (0.1 μ g) and pIN-II-B3-Cm^r (0.005 μ g), both previously digested with *Pst*I, were ligated in 14 μ l of 6.6 mM Tris-HCl, pH 7.5, containing 6.6 mM MgCl₂, 0.4 mM ATP and 0.5 units of T4 DNA ligase. The mixture was incubated at 12.5°C for 16 h and transformed into JA221 *lpp*⁻/*F'* *lac*I^q. One of the chloramphenicol-resistant transformants was chosen and termed pIN-III-*ompA*-3-Cm^r. The source of the β -lactamase gene was plasmid pTG2. 20 μ g of pTG2 were digested with 20 units of *Hae*II in 200 μ l of 50 mM Tris-HCl, pH 8.0, containing 10 mM MgCl₂ and 50 mM NaCl. After 90 min at 37°C, 20 units of *Bst*EII were added and the incubation continued at 60°C for 90 min. The mixture was then frozen at -70°C and then brought to room temperature at which time 1 μ l of 1 mM dNTP's and 2 units of DNA polymerase, Klenow fragment, were added. Following a 30-min incubation at room temperature, the mixture was applied to a preparative polyacrylamide gel and the band of lowest mobility was excised from the gel and the DNA was recovered. The purified *Bst*EII-*Hae*II fragment (0.5 μ g) was then mixed with 50 pmol of the *Eco*RI linker oligonucleotide (dCCGGAATTCCGG) in 10 μ l of 6.6 mM Tris-HCl, pH 7.5, containing 6.6 mM MgCl₂, 1 mM ATP, and 2 units of T4 DNA ligase. After 16 h at 12.5°C, the solution was made 50 mM in Tris-HCl, pH 7.5, 10 mM in MgCl₂, 1 mM in β -mercaptoethanol and 150 mM in NaCl, in a final volume of 50 μ l. Ten units of *Eco*RI were then added and the mixture was incubated at 37°C for 2 h, followed by purification of the DNA by polyacrylamide gel electrophoresis. To the purified DNA (0.1 μ g) was added 0.05 μ g of pIN-III-*ompA*-3-Cm^r, previously digested with *Eco*RI, in 10 μ l of 6.6 mM Tris-HCl, pH 7.5, containing 6.6 mM MgCl₂, 0.4 mM ATP and 0.5 units of T4 DNA ligase. The mixture was incubated for 16 h at 12.5°C and then transformed into JA221 *lpp*⁻/*F'* *lac*I^q. One of the ampicillin and chloramphenicol-resistant transformants was designated pJG105 and used for further characterization.

Oligonucleotide-directed site-specific mutagenesis

The procedure used was essentially that of Moringa et al. (1984). The plasmid used for the mutagenesis was pJG106 which is identical to pJG105 (Figure 3) except that the *Pst*I sites flanking the Cm^r gene were removed (data not shown). The pJG106 plasmid DNA was digested with *Nde*I or with *Xba*I and *Pst*I and added to the synthetic 24-mer oligonucleotide (GTAGCGCAGGCCACCCAGAAACG) and mutagenesis was carried out as previously described (Moringa et al., 1984). For screening of transformants, the ³²P-labeled oligonucleotide was used, as previously described (Moringa et al., 1984) except that hybridization was carried out at 50°C. One positive transformant was chosen and designated pJG107. The pJG107 plasmid DNA was then digested with *Xba*I and *Bam*HI and the small fragment was ligated with the large fragment resulting from the digestion of pJG105 with the same enzymes. After transformation into JA221 *lpp*⁻/*F'* *lac*I^q one transformant was picked and designated pJG108 and used for subsequent characterization.

Expression of the β -lactamase gene

For labeling experiments, 20 ml cultures were grown in M9 media to a Klett reading of 50 (blue filter) at which time IPTG (Sigma) was added to 2 mM. After 20 min, 200 μ Ci [³H]proline, [³H]glycine, or [³H]lysine (Amersham Corp.) were added for a period of 5 min and labeling was stopped by quick chilling to 0°C.

Cell disruption was accomplished using a Heat-Systems-Ultrasonics Cup Horn Sonicator. Preparation of cell fractions and SDS-PAGE were as previously described (Inouye et al., 1982). Preparation of periplasmic fractions was according to published procedures (Neu and Heppel, 1965). Purification of β -lactamase and preparation of anti- β -lactamase serum and its use for immunoprecipitation were exactly as described previously (Ghrayeb and Inouye, 1984). Sequential Edman degradations were performed using a JEOL Sequence Analyzer JAS-47K as previously described (Inouye et al., 1977). The oligonucleotide (GTAGCGCAGGCCACCCAGAAACG) used for site-specific mutagenesis was synthesised using a Systec DNA synthesizer.

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