JOURNAL OF BACTERIOLOGY, Apr. 1995, p. 2074–2079 Vol. 177, No. 8
0021-9193/95/$04.00+0

Identification and Cloning of the Gene Encoding Penicillin-Binding Protein 7 of *Escherichia coli*

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Received 19 September 1994/Accepted 10 February 1995

Penicillin-binding protein (PBP) 7 of *Escherichia coli* is a poorly characterized member of the family of enzymes that synthesize and modify the bacterial cell wall. The approximate chromosomal position of the gene encoding this protein was determined by measuring the expression of PBPs during lytic infection of *E. coli* by each of the 476 miniset members of the Kohara λ phage genomic library. Phages λ363 and λ364, encompassing the region from 47.7 to 48 min of the chromosome, overproduced PBP 7. One open reading frame, *yohB*, was present on both these phages and directed the expression of PBPs 7 and 8. The predicted amino acid sequence of PBP 7 contains the consensus motifs associated with other PBPs and has a potential site near the carboxyl terminus where proteolysis by the OmpT protein could occur, creating an appropriately sized PBP 8. The PBP 7 gene (renamed *pbpG*) was interrupted by insertion of a kanamycin resistance gene cassette and was moved to the chromosome of *E. coli*. No obvious growth defects were observed, suggesting that PBP 7 is not essential for growth under normal laboratory conditions.

Escherichia coli contains seven well-characterized penicillin-binding proteins (PBPs) designated PBPs 1a, 1b, 2, 3, 4, 5, and 6, in order of their decreasing molecular weights as determined with sodium dodecyl sulfate (SDS)-polyacrylamide gels. However, beginning with the earliest observations, other PBP bands have been observed. Two of these, PBPs 7 and 8, are smaller than the rest (molecular masses, approximately 31 and 29 kDa, respectively) and have a confusing history. One problem has been the lack of reproducibility with which they are observed in bacterial preparations (24, 25). Another source of confusion has been their nomenclature: PBP 7 was originally the name of the smaller of the two (25); they have been called PBPs 7a and 7b (5); and, in cases in which only one of the two was visible, their designation as PBP 7 or 8 has been arbitrary. While the seven larger PBPs are well characterized, PBPs 7 and 8 have been generally ignored.

However, interest has been rekindled in these small PBPs. Tuomanen and Schwartz (27) found that PBP 7 was consistently bound by antibiotics that are able to lyse nongrowing *E. coli* cells, a property not normally associated with β-lactams. The nature of this sensitivity remains unclear. Also, an increase in the expression of PBP 8 has been correlated with increased resistance of *E. coli* to the antibiotics cephaloridine and ceftazidime (15). Recently, we clarified the relationship of the two proteins by showing that PBP 8 is a proteolytic product of PBP 7, an artifact of sample preparation due to the action of the OmpT protease (12). Also, Romeis and Höltje have purified PBP 8 and shown that it is a DD-endopeptidase with a strict requirement for intact sacculi as a substrate (22). In addition, these authors showed that PBP 8 is not an integral membrane protein because it can be dissociated from membrane preparations by treatment with 1 M NaCl (22).

The known PBPs play roles in cell wall synthesis and modification, and some have specific roles in cell division (10, 24, 25). We would like to know whether PBP 7 contributes to these functions in *E. coli*, and we report here the identification and cloning of the gene encoding PBP 7 and the construction of viable mutants unable to produce this protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, media, and enzymes. Bacterial strains used as cloning hosts were as follows: *E. coli* CSQ (strain W1485, *supE lacI*^{q}), *E. coli* SP1070 (*his supF dacA*::Km *dacC*; originally named JBS1002) (6) from J. Broome-Smith, *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.), *E. coli* KS300 (F$^-$ *araD139 galE galK* Δ*lacX74 rpsL thi recA1* Δ*phoA*[*PvuII*]) (26) from J. Beckwith, and *E. coli* SF100 (KS300 *recA*$^+$ Δ*ompT*) (3) from G. Georgiou. Plasmid pHP45Ω-Km was received from J. Frey (9), and the pBCSK vectors were from Stratagene. The Kohara λ phage miniset (13) was provided to us by Y. Kohara. Of the original 476 λ clones in this set, 28 members did not correspond to the *E. coli* genomic map (7); the corrected versions of these clones were obtained from F. Blattner. Bacterial cells were grown in one of the following media (16): Luria-Bertani (LB) medium; LB-maltose medium (LB plus 0.2% maltose, 10 mM $MgSO_4$, and 10 mM $CaCl_2$); or minimal M9 medium plus 0.2% glucose and 40 μg of histidine per ml. Antibiotics were added as required at the following concentrations: tetracycline, 12.5 μg/ml; chloramphenicol, 50 μg/ml; and kanamycin, 50 μg/ml. Restriction enzymes and T4 DNA ligase were from New England Biolabs, Inc., Beverly, Mass. Protein assays were performed with the enhanced microBCA assay (Pierce Chemical Co., Rockford, Ill.).

Expression of proteins encoded by the Kohara *E. coli* genomic library. Fresh, high-titer lysates of each phage in the Kohara miniset λ library were prepared in the following manner. A 10-μl sample of stock lysate of each member of the λ library was added to 75 μl of *E. coli* SP1070 (Δ*dacA* Δ*dacC*) that had been grown to late log phase in LB-maltose medium. This mixture was incubated without shaking for 20 min at 37°C. Portions (3 ml) of LB-maltose were added to each tube, and the tubes were incubated overnight at 37°C in a roller drum. The following morning, 30 μl of chloroform ($CHCl_3$) was added, the tubes were incubated with rolling for 15 min at 37°C, and whole cells and debris were removed from each culture by centrifugation at 4,000 × *g* for 6 to 10 min. Samples (3 ml) of supernatant were moved to two 1.7-ml microcentrifuge tubes, each containing 8 μl of $CHCl_3$, for storage at 4°C.

To express PBPs from genes cloned on the Kohara phages, *E. coli* SP1070 was grown overnight in LB-maltose and was inoculated into LB medium plus 10 mM $MgSO_4$ to give an initial A_{600} of 0.02. When the A_{600} reached 0.200 to 0.250, 3 ml of the SP1070 culture was added to 500 μl of freshly prepared phage lysate (10^{10} to 10^{11} PFU/ml) and incubated with rotation for 45 min at 37°C. Whole cells were collected from these samples by centrifugation at 16,000 × *g* for 10 min. The supernatant was discarded, and the cell pellet was resuspended in 10 μl of 100 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$–1% Triton X-100 prior to storage at −20°C. Each 10-μl sample was thawed and labeled with ^{125}I-penicillin X as described by Henderson et al. (12). For rapid screening of PBP expression in

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cells infected by the Kohara phages, ^{125}I-labeled penicillin X samples were separated on precast SDS–12% polyacrylamide gel electrophoresis (PAGE) minigels (Bio-Rad, Hercules, Calif.) at 150 V for 70 min. For greater resolution, selected samples were separated on large-format SDS–12% PAGE gels (16.5 by 20 cm) (8) which were run at a 25-mA constant current per gel. Gels were dried and exposed to X-Omat AR film (Eastman Kodak, Rochester, N.Y.) for 1 to 10 days.

PCR amplification and cloning of the PBP 7 gene. The gene for PBP 7 was amplified by the PCR procedure. Chromosomal DNA was prepared from *E. coli* MC1061 by the method of Wilson (29). To 50 ng of chromosomal DNA were added the primers 5′-GCCCTCGAGGCCGCCAGCGCCACA-3′ and 5′-AGT GGATCCAAAATTACGGATGGCAGAGT-3′, which were designed to introduce an *Xho*I and a *Bam*HI site at the 5′ and 3′ ends of the amplified fragment, respectively. One unit of *Taq* polymerase (Boehringer Mannheim, Indianapolis, Ind.) was added to 50 ng of chromosomal DNA in 50 μl of buffer (10 mM Tris-HCl [pH 8.8], 50 mM KCl, 1.5 mM Mg_2Cl, 100 μg of bovine serum albumin, 250 μM [each of the four] deoxynucleoside triphosphates, 600 ng of each primer). Amplification was performed by incubation of the reaction mixture for 5 min at 94°C and cycling of the temperature 33 times (1 min at 94°C, 1 min at 61°C, and 2 min at 72°C), followed by a final 3-min incubation at 72°C. The resulting 2.1-kb fragment was purified, digested with *Xho*I and *Bam*HI, and cloned into plasmid pBCSK$^+$, yielding plasmid pMT34.

Insertional mutagenesis of *pbpG*. A *Bam*HI site was introduced by linker ligation into either the *Sma*I or the *Eco*RV site of the cloned *pbpG* gene, and a kanamycin resistance gene from pHP45Ω-Km (9) was inserted into the new *Bam*HI site. These plasmid-borne *pbpG* mutants were transferred to the chromosome of *E. coli* CSQ by λ-mediated transduction (14) using as an intermediate Kohara phage λ363, which contains the wild-type *pbpG* gene.

Sequence analysis. For the initial similarity search, DNA sequences were compared with other sequences in the GenBank and EMBL libraries, with the BLAST program (1), available as an electronic mail server at the National Center for Biotechnology Information (electronic mail address, blast@ncbi.nlm.nih.gov). Additional analysis of sequence data was performed with the PC/Gene suite of programs (IntelliGenetics, Inc., Mountain View, Calif.) and with DNA Inspector IIe (Textco, West Lebanon, N.H.).

Membrane association of PBPs 7 and 8. The membrane association of PBP 7 was determined by a modification of the method of Romeis and Höltje (22). Alterations of the method included the substitution in buffer I of 10 mM Tris-HCl (pH 8.0) for 10 mM Tris-maleate (pH 6.8), the use of DNase I of type II (Sigma, St. Louis, Mo.), and the processing of a sample of 2 ml. The locations of the PBPs were tested in two strains: *E. coli* SF100 (which produces only PBP 7) and *E. coli* KS300 (which produces PBPs 7 and 8).

Purification of PBP 8. PBP 7 is cleaved by the OmpT protease to yield the proteolytic fragment PBP 8 (12), which was purified for peptide sequencing. *E. coli* SP1070 (Δ*dacA* Δ*dacC*) was grown in a 500-liter fermentor to mid-log phase in minimal M9 medium supplemented with 0.2% glucose and 40 μg of histidine per ml. Cells were grown, harvested, and frozen in liquid nitrogen by the staff of the University of Wisconsin Biochemistry Fermentation Facility (Madison, Wis.). A portion of the frozen cell paste (150 g) was thawed and suspended in 300 ml of 100 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$ containing 40 μg of each of the following per ml: RNase A, DNase I (type I), and phenylmethylsulfonyl fluoride. The mixture was broken by two passes through a French pressure cell (American Instruments Co., Urbana, Ill.) at 16,000 lb/in^2, and the lysate was incubated at 37°C for 30 min. Cell debris was removed by centrifugation at 3,000 × *g* for 10 min at 4°C. Between 20 and 30% of the cellular PBP 8 was pelleted by this procedure, and this material was processed further. The resulting membrane pellet was suspended to a final volume of 100 ml in 100 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$–100 mM NaCl–2% Triton X-100 (solubilization buffer) and was solubilized by being stirred overnight at 4°C. Unsolubilized material was removed by centrifugation at 16,000 × *g* for 20 min.

Procion Blue H-EGN 125 (Pro Chemical and Dye, Somerset, Mass.) was coupled to Toyopearl HW65F resin (TosoHaas, Montgomeryville, Pa.) by the procedure of Mottl and Keck (17, 18). The solubilized 16,000 × *g* supernatant (93 ml) was combined with an equal volume of dye-coupled resin that had been equilibrated with solubilization buffer. The sample and dye resin were mixed in a rotating water bath for 30 min at room temperature and poured into a sintered-glass (coarse grade) Büchner funnel. Proteins that remained unbound were removed from the resin by being washed with 1 liter of 100 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$–100 mM NaCl–0.1% Triton X-100 (rinse buffer). Proteins bound to the dye resin were eluted with 300 ml of 1.0 M NaCl in rinse buffer. The eluted protein was concentrated at room temperature to 32 ml in an Amicon Model 202 stir cell (Amicon, Beverly, Mass.) that was fitted with a YM-10 membrane having a molecular mass cutoff of 10 kDa.

Ampicillin affinity chromatography was performed by a modification of the procedure of Hara et al. (11). Ampicillin was bound to Affi-15 resin (Bio-Rad) and unreacted sites were blocked and equilibrated in 100 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$–500 mM NaCl–0.1 mM dithiothreitol–0.1% Triton X-100 (affinity buffer). Dithiothreitol (final concentration, 0.1 mM) was added to the 32 ml of concentrated protein from the dye chromatography step. This solution was added to 6.2 ml of equilibrated Affi-15–ampicillin resin and was mixed gently on a rocking platform for 2 h at 37°C. The mixture was poured into a 15-ml sintered-glass (coarse grade) Büchner funnel, and proteins that remained unbound were removed by being washed under a vacuum with 500 ml of affinity buffer at room temperature. The affinity resin was packed into a column (1-cm diameter), and bound proteins were eluted with hydroxylamine. Affinity buffer containing 1 M hydroxylamine was added to the resin and allowed to equilibrate for 30 min, and then a volume of 10 ml was eluted. This step was repeated twice more, when the resin had been in contact with the hydroxylamine for 60 and 120 min. Each 10-ml elution sample was dialyzed against six 1-liter changes of 100 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$–500 mM NaCl. Each dialyzed sample was concentrated to 0.5 ml by centrifugation at 5,000 × *g* through a Centricon-30 concentrator (Amicon) at 4°C.

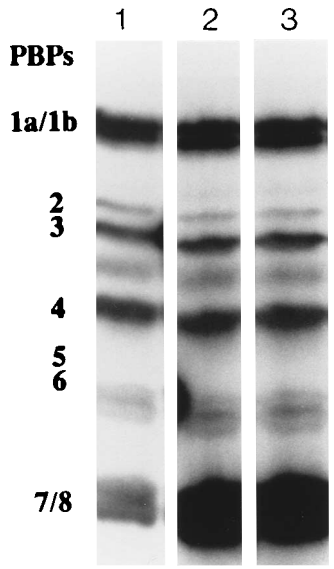

FIG. 1. Overexpression of *E. coli* PBP 7 from Kohara λ phage. *E. coli* SP1070 (Δ*dacA* Δ*dacC*) (6) was infected with λ Kohara phage. Infected cells were labeled with ^{125}I-penicillin X, and the proteins were separated by SDS-PAGE. The positions of the PBPs are indicated on the left. Lane 1, uninfected *E. coli* SP1070; lane 2, *E. coli* infected with λ363; lane 3, *E. coli* infected with λ364.

N-terminal amino acid sequencing. PBP 8 (1.5 to 2 nmol) was separated by SDS–12% PAGE, the gel was stained with Coomassie blue G-250, and the proteins were electroblotted onto a ProBlott membrane (Applied Biosystems, Foster City, Calif.) by means of a Trans-Blot Cell (Bio-Rad). The N-terminal amino acid sequence was determined by Macromolecular Resources (Department of Biochemistry, Colorado State University, Fort Collins, Colo.) with an Applied Biosystems 473A protein sequencer with standard Edman chemistry.

RESULTS AND DISCUSSION

Overexpression of PBPs by a λ library of the *E. coli* genome. The Kohara miniset library is composed of 476 individual λ phages that contain an ordered array of cloned DNA fragments representing approximately 99% of the chromosome of *E. coli* (13). Because genes cloned into λ are often overexpressed during the lytic cycle of the phage (20), we infected *E. coli* with each λ phage of the Kohara miniset and assayed for the overproduction of PBPs. The well-characterized *E. coli* PBPs were overexpressed from phage clones known to carry the appropriate genes (data not shown). The only exception was PBP 4, which was consistently expressed to a much lower degree. Infection with two Kohara phages, λ363 and λ364, directed overexpression of PBPs 7 and 8 (Fig. 1, lanes 2 and 3). Between PBPs 3 and 4 there is an unidentified protein band that is labeled with ^{125}I-penicillin X (Fig. 1). This protein appears when cells are lysed by any of several means (data not shown), and we are attempting to determine whether it is a proteolytic product of a known PBP or whether it represents an additional, novel PBP.

Identification and subcloning of the gene encoding PBPs 7 and 8. The chromosomal map position of the overlap between

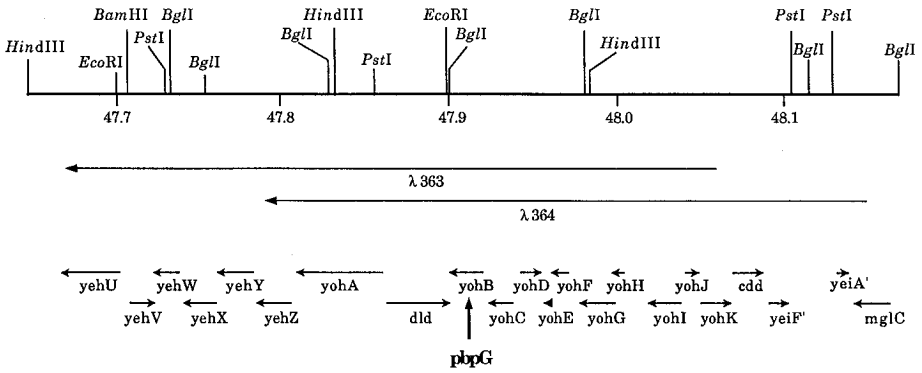

FIG. 2. Map of the region from 47.6 to 48.2 min of the *E. coli* chromosome. The chromosomal area cloned in the λ Kohara phages λ363 and λ364 is shown. The unidentified open reading frame *yohB* has been redesignated *pbpG*. Arrows indicate directions of transcription.

λ363 and λ364 corresponds to the region from 47.8 to 48 min on the *E. coli* chromosome (Fig. 2) (23). During the course of this work, the DNA sequence for the region from 47 to 48 min of the *E. coli* chromosome was reported (21). Richterich et al. (21) identified potential open reading frames in this area and one, *yohB* at 47.92 min (Fig. 2), yielded a predicted protein sequence similar to known carboxypeptidases and PBPs. Because *yohB* was contained on DNA cloned in λ363 and λ364, we used the sequence information to subclone this gene to determine whether it encoded PBPs 7 and 8.

An 11.5-kb *Eco*RI DNA fragment was isolated from λ364 and was cloned into the *Eco*RI site of pBCSK$^+$. *E. coli* containing the resulting plasmid, pTAH100, overexpressed PBPs 7 and 8 (Fig. 3, lane 3). *yohB* was further subcloned by excising a 1.1-kb *Xba*I fragment from pTAH100 and ligating it into the *Xba*I site of pBCSK$^+$, creating pTAH101, in which *yohB* was the only open reading frame present. *E. coli* containing pTAH101 overexpressed PBPs 7 and 8 (Fig. 3, lane 4), indicating that PBPs 7 and 8 were encoded by *yohB*. The *yohB* gene was also subcloned by PCR-mediated amplification directly from the chromosome. The resulting clone, pMT34, carried *yohB* and *yohC* on the amplified DNA fragment, and cells containing pMT34 also overexpressed PBPs 7 and 8 (data not shown). Therefore, *yohB* was redesignated *pbpG* (for penicillin-binding protein 7, with the number 7 represented by G, the seventh letter of the alphabet). Overexpression of PBP 7 did not affect bacterial growth (data not shown).

Inactivation of *pbpG*. A kanamycin resistance gene cassette was inserted into the coding sequence of the cloned *pbpG* gene, and *E. coli* containing this plasmid no longer overproduced PBP 7 or 8 (data not shown). The insertion mutation was moved into the chromosome of *E. coli* by the λ transduction method of Kulakauskas et al. (14). Kanamycin resistant transductants did not express PBP 7 or 8 (Fig. 4). Although the chromosomal *pbpG* gene had been disrupted, the bacteria grew normally, suggesting that PBP 7 is a nonessential protein when *E. coli* is grown in rich or minimal media under normal laboratory conditions.

Characteristics of the *pbpG* gene product. The *pbpG* gene

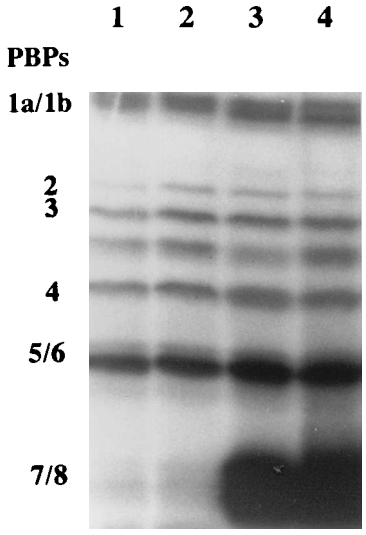

FIG. 3. Overexpression of PBPs 7 and 8 from the cloned *pbpG* gene. *E. coli* XL1-Blue was transformed with various plasmids, whole cells were labeled with ^{125}I-penicillin X, and the proteins were separated by SDS-PAGE. Each lane shows the PBPs expressed by *E. coli* containing the following plasmids: lane 1, pBCSK$^+$; lane 2, pBCSK$^-$; lane 3, pTAH100; lane 4, pTAH101.

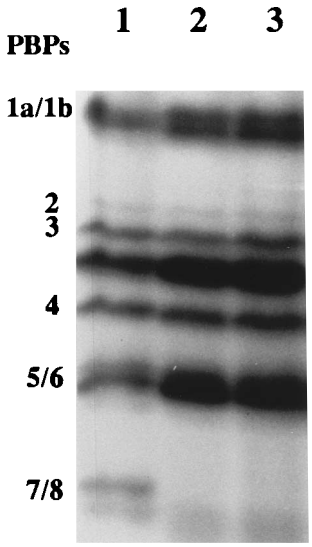

FIG. 4. Deletion of PBPs 7 and 8 by interposon insertion mutagenesis of *pbpG*. A mutated *pbpG* gene was interrupted and transduced into the chromosome of *E. coli* CSQ. Whole cells of *E. coli* transductants were labeled with ^{125}I-penicillin X, and the proteins were separated by SDS-PAGE. The following *E. coli* strains were labeled (the restriction site into which the kanamycin resistance cassette was inserted is indicated in brackets): lane 1, CSQ; lane 2, TH105K (CSQ *pbpG*::ΩKm [*Eco*RV]); lane 3, TH103K (CSQ *pbpG*::ΩKm [*Sma*I]).

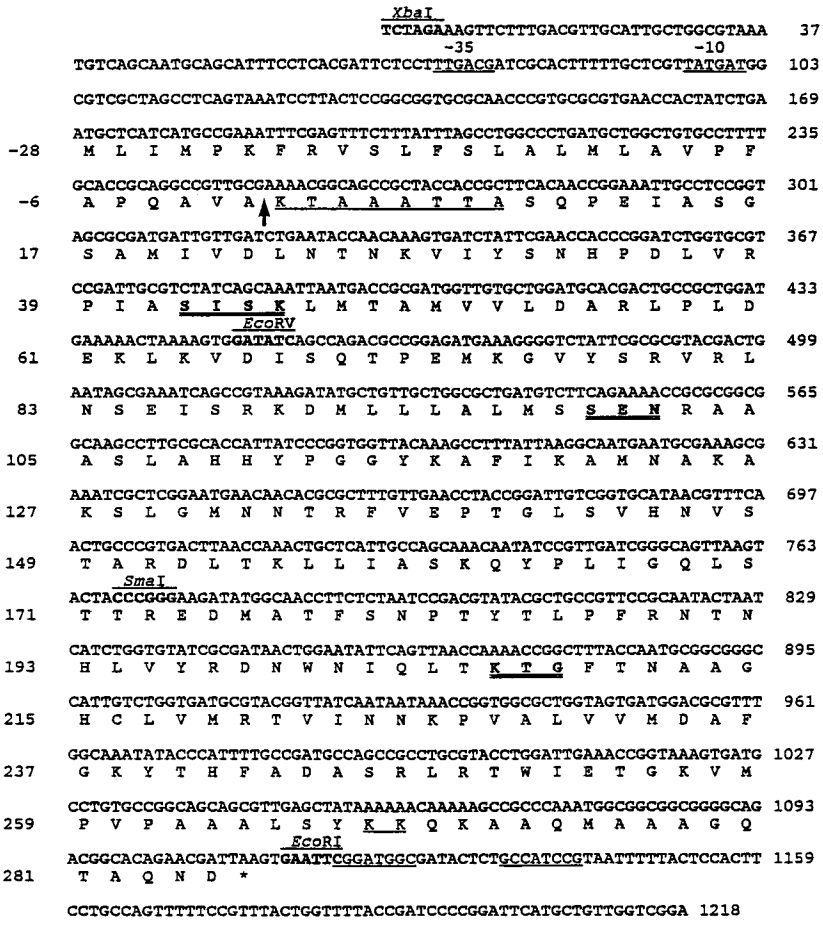

FIG. 5. Nucleotide sequence and deduced amino acid sequence of the portion of the *E. coli* genome containing the *pbpG* gene. Nucleotide numbering is presented on the right, and amino acid numbering is presented at the left. The open reading frame (nt 170 to 1108) can potentially encode a polypeptide of 313 amino acids. The arrow between amino acids −1 and +1 indicates the predicted position at which signal peptidase can cleave the protein. Peptide sequencing confirmed that the underlined residues (amino acids 1 to 8) form the amino terminus of PBP 8. The probable site at which OmpT protease cleaves PBP 7 to form PBP 8 is also underlined (the two lysine residues at positions 268 and 269). The −35 and −10 regions of a potential promoter sequence are underlined (nt 72 to 77 and nt 96 to 101, respectively), as are sequences that can form the stem (nt 1119 to 1142) of a potential ρ-independent terminator, which includes a run of T residues just downstream of the stem (nt 1146 to 1150). Interposon antibiotic resistance cassettes were inserted into the *Eco*RV (nt 449) and *Sma*I (nt 768) restriction sites. These sites are shown in boldface type and overlined. The motifs SXXK, (S/Y)XN, and (K/H)(T/S)G, at amino acids 42 to 45, 99 to 101, and 206 to 208, respectively, are shown in boldface type and double underlined. The sequence is derived from that reported by Richterich et al. (21) and includes only the region surrounding the *yohB* open reading frame (here renamed *pbpG*).

begins at nucleotide (nt) 37649 and ends at nt 36708 on the complementary strand of the sequence reported by Richterich et al. (21). The sequence of the cloned *Xba*I-*Eco*RI DNA fragment is displayed in Fig. 5, with some downstream sequence appended. The first AUG codon of *pbpG* is located 170 nt downstream of the *Xba*I site (Fig. 5). If this is the actual start site for translation, then *pbpG* encodes a 313-amino acid, 34.2-kDa protein. This is larger than the 31,000-molecular-weight PBP 7 observed by SDS-PAGE (12, 25). However, PBPs 7 and 8 are periplasmic enzymes (22), suggesting that they may possess a cleavable signal sequence. This would be consistent with the structure of other well-characterized low-molecular-weight PBPs, which are cleaved at their amino termini as the proteins mature, although these PBPs remain membrane associated (10). According to the algorithm of von Heijne (28), the sequence of the amino terminus of the predicted PbpG protein satisfies the criteria, including the −3 and −1 alanine rule, for the existence of a single peptidase cleavage site after the 28th amino acid. This predicts that PbpG should be cleaved between the alanine and lysine residues (Fig. 5).

To determine whether this cleavage occurs, PBP 8 was purified by dye affinity chromatography (17, 18) and ampicillin affinity chromatography. The amount of PBP 8 relative to that of total protein was determined at each step by separation of labeled proteins by SDS-PAGE and measurement of the intensity of the PBP 8 band on the autoradiogram. PBP 8 was enriched 81-fold after the dye affinity step and over 4,000-fold after the ampicillin affinity step (Table 1), giving a final yield that was ~2.5 times greater than that of a previous purification scheme (22). Approximately 1.5 to 2 nmol of PBP 8 was isolated by electroblotting the protein from a polyacrylamide gel onto a nylon membrane, and the N-terminal sequence of PBP 8 was determined. The sequence KTAAATTA (Fig. 5, underlined amino acids 1 to 8) confirmed that the lysine at the predicted site of cleavage was the true amino terminus of the PBP 8 protein. Thus, if PBPs 7 and 8 have a common amino terminus (see below), then the mature PBP 7 protein is predicted to contain 285 amino acids with a calculated molecular mass of 31,164 Da, consistent with the observed molecular weight for PBP 7. This result suggests that PBPs 7 and 8 are

TABLE 1. Purification of PBP 8

Sample	Vol (ml)	Concn (mg/ml)	Total protein	Activity (10^7 U)[a]	Recovery (%)	Sp act (10^4 U)[a]	Purification (fold)
Whole cells	460	35.0	16.1 g	20	100	1.2	0
3,000 × *g* pellet	100	6.6	0.66 g	5.4	20.7	8.1	5.1
16,000 × *g* supernatant	93	3.6	0.34 g	2.5	9.7	7.5	4.7
Procion Blue H-EGN	32	0.39	12.5 mg	1.6	6.2	138	81.2
Affi-15–ampicillin	1.5	0.04	0.06 mg	0.39	1.5	6,500	4,060

[a] Activity was measured by optical scanning of the radioactive protein bands separated by SDS-PAGE; the area under each protein peak reflects the amount of ^{125}I-penicillin X bound. Activity is expressed in arbitrary units of area and equals the specific activity [area/(micrograms of protein per lane)] multiplied by the total amount (in micrograms) of protein in each sample.

probably processed in a manner similar to that of the classical low-molecular-weight PBPs.

PBP 8 is an artifact derived from PBP 7 after cleavage by the OmpT protease (12). OmpT has been reported to cleave proteins between pairs of dibasic amino acids (Arg-Arg, Lys-Lys, Arg-Lys, and Lys-Arg) (4). There are two such sites in the 285-amino-acid sequence of PbpG: Arg-88–Lys-89 and Lys-268–Lys-269. The latter site is located 17 amino acids from the carboxyl terminus of the protein. If the OmpT protease cleaves PBP 7 between these two lysines, then the resulting 268-amino-acid protein has a molecular mass of 29,452 Da, consistent with the 29-kDa size of PBP 8 as measured by SDS-PAGE (12, 25). Combined with the preceding results, this information suggests that PBPs 7 and 8 have a common amino terminus and that PBP 8 differs from PBP 7 by an OmpT-mediated truncation of the carboxyl end of PBP 7.

Calculations for PBPs 7 and 8 gave a pI value of 10.4 for both proteins. This is significantly greater than 8.3, the value for PBP 8 as reported by Ayala et al. (2). We separated purified PBP 8 by two-dimensional nonequilibrium pH gel electrophoresis and compared the pI of PBP 8 with those of a set of standard proteins (Bio-Rad). PBP 8 had a pI greater than 8.6, the pI of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, by a factor that suggested a final pI value of approximately 10 (data not shown). Thus, the measured pI is consistent with the calculations from the predicted protein sequence. The difference between these values and those of Ayala et al. (2) may simply reflect the availability of accurate protein standards.

PBP 7 is not an integral membrane protein. With the exception of PBP 4 (10), the PBPs of *E. coli* are membrane bound. Analysis of the amino acid sequence of PBP 7 revealed no defined transmembrane helix, amphiphilic helix, or lipid anchor. Romeis and Höltje (22) found that PBP 8 could be removed from bacterial membranes by 1 M NaCl and that PBPs 7 and 8 could be released from cells by osmotic shock. They thus concluded that the proteins were located in the periplasm and were not integral membrane proteins. To confirm this possibility, membrane fractions were isolated from *E. coli* SF100 (*ompT*) and incubated in 1 M NaCl. PBP 7 was solubilized from membranes by exposure to 1 M NaCl (data not shown). Therefore, PBPs 7 and 8 appear to be soluble proteins that associate with the bacterial membrane in a salt-dependent manner.

PBP 7 contains the active-site motifs that characterize other PBPs. Four peptide motifs are shared among the PBPs and β-lactamases, which together make up a family of serine active-site penicillin-interacting proteins (10). Three-dimensional crystallographic analyses place the consensus-like sequences at the active sites of these enzymes, and the distance between the four motifs is somewhat conserved in the primary structure of the proteins (10). The active-site serine occurs in the first motif, SXXK (where X is any amino acid), which is located 33 to 60 amino acids from the amino terminus of the smaller penicillin-interacting proteins. PBP 7 contains a potential active-site motif, SISK, within this range (Fig. 5, amino acids 42 to 45). The second motif, (S/Y)XN, is located approximately 48 to 93 amino acids from the active-site serine (10), except in the case of PBP 4, in which the two motifs are separated by 243 amino acids (19). The predicted amino acid sequence of PBP 7 also includes such a sequence, SEN (Fig. 5, amino acids 99 to 101), 57 amino acids from the active-site serine at position 42. The third motif is simply a peptide segment with one or two dicarboxylic acids (D and/or E), located approximately 30 to 60 amino acids after the (S/Y)XN sequence. In PBP 7, there are four potential dicarboxylic acid candidates between the SEN and KTG sequences: E-138, D-152, E-174, and D-175. However, the aspartic acid at position 152 is located in a run of 5 amino acids, S(T/S)ARD, that are completely conserved between PBPs 5, 6, and 7 (Fig. 6). The distance of this residue from the previous motif is 41 amino acids, consistent with the distance for other D/E motifs in penicillin-interacting proteins (10). Finally, the fourth PBP motif, (K/H)(T/S)G, occurs 40 to 90 amino acids from the D/E motif. In PBP 7, the KTG sequence is located 55 amino acids from D-152 (Fig. 5, amino acids 206 to 208). Thus, the predicted protein sequence of PBP 7 conforms in every respect to the consensus motifs and their distributions in previously characterized PBPs.

Comparison of PBP 7 with other PBPs. Alignment of PBP 7 with known PBPs indicated that it was most similar to PBPs 5 and 6 of *E. coli* (Fig. 6 and data not shown). PBP 7 shares 67 identical amino acids with PBP 5 and 77 identical amino acids with PBP 6. Thus, over its 285-amino-acid length, PBP 7 is 24 and 27% identical to PBPs 5 and 6, respectively. Clusters of extended identity or similarity appear in the vicinity of the three major active-site motifs (Fig. 6, amino acids shown in boldface type at positions 42 to 45, 99 to 101, and 206 to 208). In addition, the three PBPs share 5 highly conserved amino acids (STARD) that appear in PBP 7 at positions 148 to 152, which may correspond to the fourth, dicarboxylic acid motif.

If the sequence of PBP 4 is altered to conform to that of the class A β-lactamases, as in the analysis by Mottl et al. (19), the modified PBP 4 shares 43 (15%) identical amino acids with PBP 7. Two of the four motifs, SXXK and SXN, are perfectly aligned, and the KTG sequence is misaligned by only 1 amino acid (data not shown). Little or no similarity was observed when PBP 7 was compared to the *E. coli* MepA protein, another bacterial endopeptidase.

Summary. We have identified and cloned the gene that encodes PBP 7, a poorly characterized protein in *E. coli*. PBP 7, though soluble, is at least partially associated with the bacterial membrane, and *E. coli* grows normally when the protein is overexpressed or when the *pbpG* gene is inactivated. Although the protein is not essential under usual laboratory con-

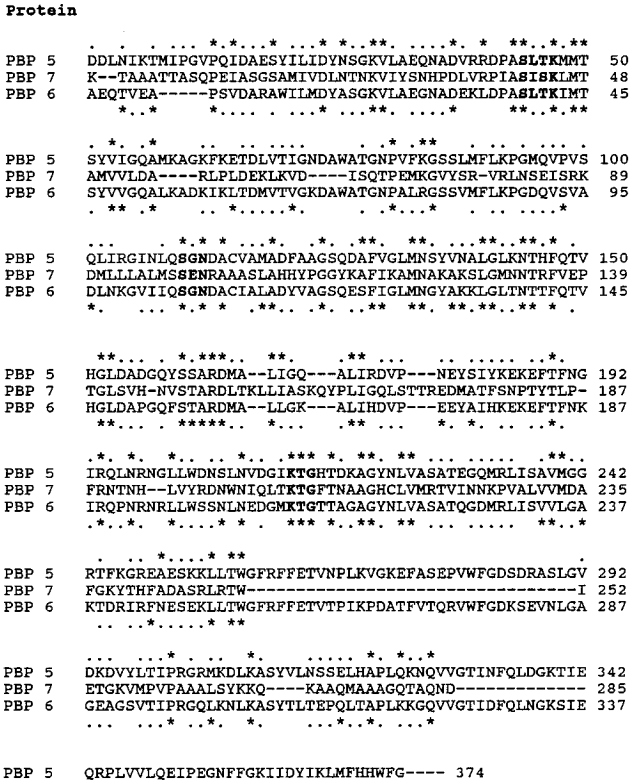

FIG. 6. Alignment of amino acid sequences of PBP 7 and *E. coli* PBPs 5 and 6. Asterisks indicate the positions of amino acids that are identical between PBP 7 and PBPs 5 or 6. Dots represent conservative amino acid replacements. Dashes are introduced to maximize alignment. Each PBP is numbered beginning from the first residue following the signal peptidase cleavage site.

ditions, further investigation of PBP 7 should be valuable because of its correlation with the lytic action of the penem class of β-lactams (27). In addition, PBP 7 may play a specialized role in remodeling the cell wall since the enzyme exhibits unique substrate requirements (22).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM40947 from the National Institutes of Health and by SmithKline Beecham Pharmaceuticals.

M.T. especially thanks J.-V. Höltje, U. Schwarz, and T. Romeis for scientific direction and help.

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