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dacD, an *Escherichia coli* Gene Encoding a Novel Penicillin-Binding Protein (PBP6b) with DD-Carboxypeptidase Activity

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In the course of a study of genes located at min 44 of the *Escherichia coli* genome, we identified an open reading frame with the capacity to encode a 43-kDa polypeptide whose predicted amino acid sequence is strikingly similar to those of the well-known DD-carboxipeptidases penicillin-binding proteins PBP5 and PBP6. The gene product was shown to bind [^{3}H]benzylpenicillin and to have DD-carboxypeptidase activity on pentapeptide muropeptides in vivo. Therefore, we called the protein PBP6b and the gene *dacD*. As with other *E. coli* DD-carboxypeptidases, PBP6b is not essential for cell growth. A quadruple *dacA dacB dacC dacD* mutant was constructed and shown to grow as well as its isogenic wild-type strain, indicating that the loss of any known PBP-associated DD-carboxypeptidase activity is not deleterious for *E. coli*. We also identified the homologous gene of *dacD* in *Salmonella typhimurium* as one of the components of the previously described *phsBCDEF* gene cluster.

Penicillin-binding proteins (PBPs) are cytoplasmic membrane enzymes involved in the last steps of peptidoglycan biosynthesis (29). They are able to bind β-lactams covalently at a conserved active serine residue because of their structural homology with the natural substrate (D-Ala-D-Ala) for transpeptidation (23, 42). Up to 10 PBPs have been identified in *Escherichia coli* by using different labelled β-lactam antibiotics and different electrophoretic conditions (PBP1a, -1b, -1c, -2, -3, -4, -5, -6, -7, and -8) (38, 42). PBP1c was seen only by using a ^{125}I-Bolton-Hunter derivative of ampicillin (38), and PBP8 has been shown to be an OmpT-mediated proteolytic degradation product of PBP7 (22). The genes coding for the eight classical PBPs (*ponA*, *ponB*, *pbpA*, *pbpB*, *dacB*, *dacA*, *dacC*, and *pbpG*, respectively) have previously been cloned and sequenced.

The high-molecular-mass PBPs (PBP1a, -1b, -2, and -3) are believed to be dual transpeptidase-transglycosylases. They catalyze glycan chain elongation and peptidoglycan cross-linkings (17). These enzymes are essential for cell growth and survival, since they are the primary targets of β-lactam antibiotic action (44, 46). Their inactivation by antibiotics or by mutation results in cell death. PBP2 is essential for the maintenance of the rod shape of cells during elongation (44), whereas PBP3 is essential for the formation of the septum during cell division (41).

The low-molecular-mass PBPs (PBP4, -5, -6, and -7) are dispensable, as their inactivation by mutation does not affect bacterial growth and division. PBP7 is a DD-endopeptidase (39); PBP4 is essentially a DD-endopeptidase, but it has also a weak DD-carboxypeptidase activity (26). PBP5 and PBP6, which account for 80% of the penicillin-binding capacity of cells, are the major DD-carboxypeptidases (42). The physiological role of the low-molecular-mass PBPs remains to be elucidated.

Here we present *dacD*, an *E. coli* gene which encodes a novel PBP with a molecular mass equivalent to those of PBP5 and PBP6. Like those proteins, the novel PBP, PBP6b, has DD-carboxypeptidase activity. We have also identified the gene homologous to *dacD* in *Salmonella typhimurium*.

MATERIALS AND METHODS

Strains, plasmids, media, and antibiotics. The *E. coli* K-12 strains used in this study are described in Table 1. Plasmid pMR100 (4) is a pUC19 derivative carrying the *Eco*RI fragment shown in Fig. 1. Plasmid pPR328 is a pUC9 derivative obtained by substituting a chloramphenicol resistance cassette for the ampicillin resistance determinant (36). Plasmids pAB5 and pAB10 were constructed by cloning the 4.3-kb *Eco*RI fragment into the polylinker of pPR328 in opposite orientations; in pAB5, *dacD* can be transcribed from its own promoter as well as from *lacZp*. λNK1324 is a λ vector carrying a Cm Tn*10* minitransposon (24).

Liquid and solid Luria-Bertani (LB) and M63 minimal media were prepared as previously described (31). Antibiotics were used at the following final concentrations: ampicillin, 40 μg ml^{-1}; tetracycline, 20 μg ml^{-1}; kanamycin, 30 μg ml^{-1}; spectinomycin, 25 μg ml^{-1}; and chloramphenicol, 30 μg ml^{-1}. Isopropyl-β-D-galactopyranoside (IPTG) was added at a concentration of 1 mM to induce the *lac* promoter.

Sequence analysis. The nucleotide sequence of *dacD* was determined as previously indicated (4). Sequences were compiled, arranged, and analyzed with the PC-Gene software package. Comparisons of nucleotide sequences were performed by using the GAP program of the University of Wisconsin package, version 8, and polypeptide sequences were compared by using the BLAST program.

Construction of a truncated *dacD* allele (*dacD1*). RYC1000 cells harboring pMR100 were mutagenized with λNK1324 by the method of Berg (7). Clones grown on selective plates containing chloramphenicol were pelleted and used to prepare plasmid DNA. This DNA was used to transform RYC1000 cells, and Cm^r Ap^r clones were selected and examined for Microcin B17 (MccB17) resistance to discard those harboring plasmids with inserts within *sbmC* (wild-type *sbmC* in high copy number confers resistance to MccB17 [4]). Inserts outside *sbmC* were mapped by restriction analysis. One Cm insert mapping to *dacD* (plasmid pMR115) was sequenced by using the internal *dacD* primer 5′-TCAC CCAGCAAAACCGTA-3′. The insertion was shown to be at nucleotide position 789 of the *dacD* gene.

The *dacD*::Cm allele (*dacD1*) was substituted for the wild-type allele on the chromosome of strain JC7623, as previously described (50). *Hin*dIII-linearized pMR115 was transformed in JC7623, and Cm^r Ap^r clones were selected. The allelic replacement was verified by P1 transduction experiments using the close *his* and *zee-152*::Tn*10* (4) markers as references. Then, the *dacD*::Cm allele was moved to strains MC4100, UGM599, and JBS1002 by P1 transduction, resulting in strains UGM600, UGM601, and UGM602, respectively. By using P1, the

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TABLE 1. *E. coli* K-12 strains used in this work

Strain	Genotype and/or phenotype	Source or reference
JC7623	F$^-$ *thr leu proA2 lacY1 galR argE rpsL supE mtl xyl his recB21 recC22 sbcB15*	50
JBS1002[a]	*his supF srl*::Tn*10 recA56* Δ*dacC1* Δ*dacA*::Km	8
D456	*his supF* Δ*dacA*::Km *dacB*::Spc Δ*dacC1*	15
MC4100	F$^-$ Δ(*argF-lac*)*U169 araD139 deoC1 flbB5301 ptsF25 relA1 thiA rpsL150*	12
RYC1000	MC4100 Δ*rbs-7 recA56 gyrA*	16
UGM599	MC4100 Δ*dacA*::Km	This work
UGM600	MC4100 *dacD1*	This work
UGM601	UGM599 *dacD1*	This work
UGM602	JBS1002 *dacD1*	This work
UGM603	UGM602 *dacB*::Spc	This work

[a] In our hands, JBS1002 is RecA$^+$ (UV^r).

dacB::Spc allele from strain D456 was also introduced into UGM602 to prepare UGM603.

Maxicell experiments. To identify the *dacD* polypeptide, RYC1000 maxicells harboring different plasmids (pPR328, pAB5, pAB10, pMR100, and pMR115) were prepared as previously described (40). Then they were labelled with 40 μCi of [^{35}S]methionine ml^{-1} for 8 min at 37°C. Labelled polypeptides were separated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) (27) and visualized by autoradiography.

Membrane preparation and PBP assay. JBS1002 cells carrying different plasmids (pPR328, pAB5, and pAB10) were grown in LB medium supplemented with chloramphenicol (25 μg ml^{-1}) at 37°C. When A_{600} was 0.7, IPTG was added and cultures were incubated for 1 h more. Cells were harvested, chilled in an ice-water bath, and collected by centrifugation at 7,000 × *g* for 10 min at 4°C. The cell pellet was washed once with ice-cold 50 mM sodium phosphate buffer (pH 7.0; Pi buffer), pelleted as described above, and resuspended in the same buffer. Membranes were obtained by the disruption of cells in the French press and differential centrifugation as previously described (34). Membranes were suspended in Pi buffer at a protein concentration of 20 μg ml^{-1} and frozen at −20°C until used in the PBP assay.

The PBP assay was carried out essentially as described previously (38). A 100-μg sample of membranes was labelled with [^{3}H]benzylpenicillin (20 Ci/mmol; Amersham) at a final concentration of 25 μM for 10 min at 37°C. The reaction was stopped by adding sample buffer and boiling for 3 min. Samples were directly subjected to SDS–10% PAGE. The gel was fixed, stained with Coomassie blue R, embedded in sodium salicylate, dried, and exposed for fluorography for 16 days.

Peptidoglycan purification and HPLC analysis. Cultures (150 ml) of strains JBS1002(pPR328), JBS1002(pAB5), and JBS1002(pAB10) were grown in LB medium supplemented with 40 μg of chloramphenicol ml^{-1} at 37°C. When A_{600} was 0.3, the expression of *lac* promoter was induced by the addition of IPTG for 1 h (final A_{600}, 1.3). Cultures were rapidly cooled down to 4°C, and cells were harvested by centrifugation (10,000 × *g*, 15 min, 4°C). Peptidoglycan was purified and analyzed by high-performance liquid chromatography (HPLC) as previously described (20), using a Hypersil RP18 column (3-mm particle size; 250 by 4 mm) (Teknochroma, Barcelona, Spain). Muropeptides were identified by their retention times after periodic calibration with a mixture of known, purified muropeptides. Quantification was performed by automatic integration of the absorption peak areas as previously described (33, 37).

Nucleotide sequence accession number. The nucleotide sequence of *dacD* has been deposited in the EMBL database under accession number X90412. The same sequence with the name phsE-Ecoli has been deposited by others under accession number P33013.

RESULTS

Nucleotide sequence of *dacD* and its polypeptide product. In the course of recent work aimed at the characterization of *sbmC*, a stationary-phase-induced *E. coli* SOS gene involved in MccB17 susceptibility (4), we cloned a large chromosomal fragment from min 44 of the *E. coli* genetic map (3) which includes the 4.3-kb *Eco*RI DNA fragment shown in Fig 1. The right half of this fragment, from the *Hpa*I site to the *Eco*RI site, was completely sequenced on both strands (4). This DNA sequence contains the 5′ end of the *sbcB* gene (32) and two open reading frames (ORF) of 1,170 and 471 bp which are transcribed in counterclockwise direction on the *E. coli* genetic map. The short ORF is the *sbmC* gene (4).

The large ORF is located between *sbcB* and *sbmC*, and the nucleotide sequence of its 5′ end is shown in Fig. 2. Translation of this ORF could start at either of the TTG codons at positions 71 and 77 (Fig. 2). These codons are preceded by the

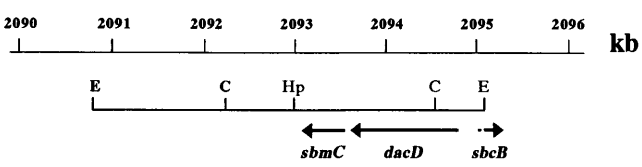

FIG. 1. Physical map of the 4.3-kb *Eco*RI fragment, including *dacD*, and its location on the map of Kohara et al. (25). The positions and directions of transcription of genes are indicated. Restriction sites are as follows: C, *Cla*I; E, *Eco*RI; and Hp, *Hpa*I.

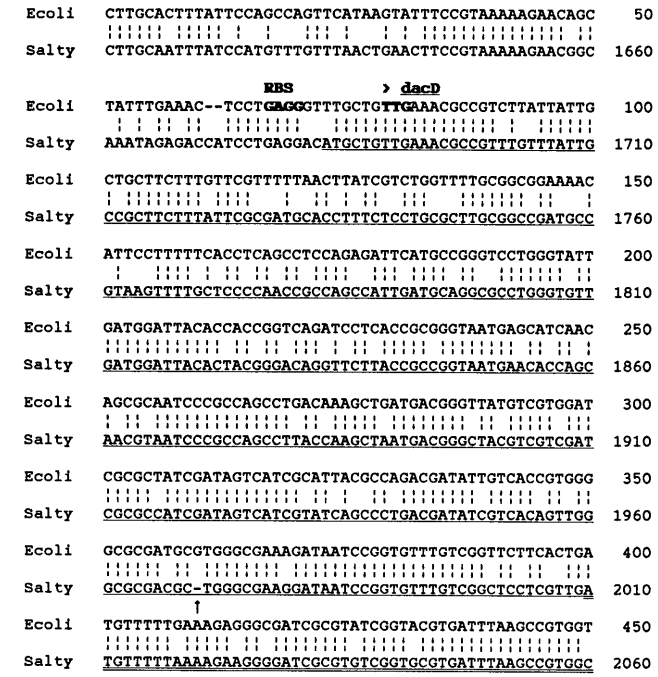

FIG. 2. Comparison of nucleotide sequences of the 5′ regions of *dacD* from *E. coli* and *S. typhimurium*. The 1,456-base-long nucleotide sequence of the *sbcB-sbmC* region of *E. coli* (Ecoli) was compared with the 2,940 nucleotides of the *phsBCDEF* cluster of *S. typhimurium* (Salty), as determined by Alami and Hallenbeck (1), by using the GAP program of the University of Wisconsin package. The putative ribosome binding site (RBS) and start codon of *dacD* are shown in bold. A vertical arrow indicates the gap (-) found in the *S. typhimurium* nucleotide sequence. Previously proposed ORF in *S. typhimurium* are singly (*pshE*) and doubly (*pshF*) underlined. Dotted lines indicate identity.

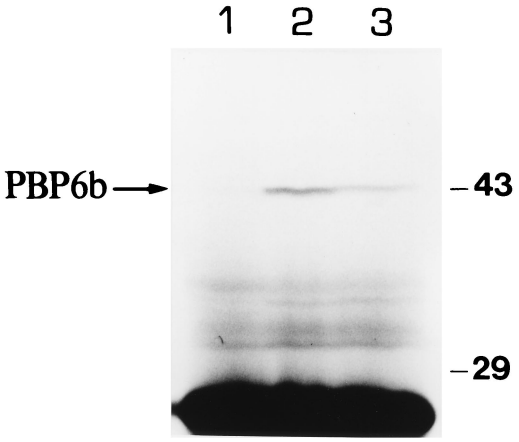

FIG. 3. Identification of the *dacD* gene product. Maxicells were prepared, labelled, and run on an SDS–10% polyacrylamide gel as described in Materials and Methods. Polypeptides synthesized from plasmids pPR328 (lane 1), pAB5 (lane 2), and pAB10 (lane 3) are shown. The position of PBP6b migration is shown by an arrow. The positions of molecular mass markers (in kilodaltons) are on the right.

same typical ribosome binding site (GAGG). Because the first TTG is separated by only 2 nucleotides from the ribosome binding site, most probably it is the second one which is used in vivo for starting translation. Assuming this, the ORF would be 1,164 nucleotides long and it would encode a polypeptide of 388 amino acids with a predicted molecular mass of 43,346 Da and an isoelectric point of 6.25. A putative rho-dependent transcription terminator consisting in a 12-bp inverted repeat is found just downstream of this ORF.

When the deduced amino acid sequence of the ORF was compared with those included in the nonredundant database at the Spanish National Center for Biotechnology Information by using the BLAST network service, high-level similarities to PBP5 and PBP6 of *E. coli* were found. Specifically, the following three well-defined amino acid motifs in the β-lactam-binding domain of PBPs, which are also found in β-lactamases and DD-endopeptidases, are present in the predicted polypeptide: the tetrad SXXK, the triad (S/Y)XN, and the triad (K/H)(T/S)G (17). These boxes were identified as SLTK, SGN, and KTG in the predicted protein, and they were conserved and located in equivalent positions in PBP5 and PBP6. The amino-terminal end of the polypeptide has the typical features of a cleavable hydrophobic sequence, including a signal peptidase cleavage site (18). It suggests that the protein is exported outside the cytoplasm, with the signal sequence removed during or after export. This is the case for PBP5 and PBP6, both of which are synthesized as preproteins (35). Therefore, we propose to name the ORF *dacD* and its polypeptide product PBP6b.

To demonstrate that *dacD* is translated into protein, the polypeptides synthesized from high-copy-number plasmids bearing *dacD* were analyzed. As shown in Fig. 3, both plasmids, pAB5 and pAB10, directed the synthesis of a polypeptide with an apparent molecular mass of 41 to 43 kDa which was not detected in cells harboring the vector pPR328. This polypeptide was confirmed to be PBP6b by showing that pMR115, a plasmid carrying the truncated *dacD1* allele, did not synthesize a protein of this size (data not shown). Notice that the amount of the 41- to 43-kDa polypeptide was slightly larger in cells with pAB5 than in cells with pAB10. This finding is in accordance with the orientation of *dacD*. In pAB10, *dacD* is transcribed

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PBP5    MNTIFSARIMKRLALTTALCTAFISAAHADDLNIKTMIPGVPQIDAESYI    50
PBP6    M-TQYSS-LLRGLAAGSAFLFLFAPTAFAAEQTVEA-----PSVDARAWI    43
PhsE    MK--------RRLFIAASLFAMHLSPALAADAV--SFAPQPPAIDAGAWV    40
PBP6b   MK--------RRLIIAASLFVFNLSSGFAAENI--PFSPQPPEIHAGSWV    40
        *       . . *  ....     ... *..    .     * ..* ...

PBP5    LIDYNSGKVLAEQNADVRRDPASLTKMMTSYVIGQAMKAGKFKETDLVTI   100
PBP6    LMDYASGKVLAEGNADEKLDPASLTKIMTSYVVGQALKADKIKLTDMVTV    93
PhsE    LMDYTTGQVLTAGNEHQQRNPASLTKLMTGYVVDRAIDSHRISPDDIVTV    90
PBP6b   LMDYTTGQILTAGNEHQQRNPASLTKLMTGYVVDRAIDSHRITPDDIVTV    90
        *.**..*..*.. *.. . .******.**.**...*... ... .*.**.

PBP5    GNDAWATGNPVFKGSSLMFLKPGMQVPVSQLIRGINLQSGNDACVAMADF   150
PBP6    GKDAWATGNPALRGSSVMFLKPGDQVSVADLNKGVIIQSGNDACIALADY   143
PhsE    GRDAWAKDNPVFVGSSLMFLKEGDRVSVRDLSRGLIVDSGNDACVALADY   140
PBP6b   GRDAWAKDNPVFVGSSLMFLKEGDRVSVRDLSRGLIVDSGNDACVALADY   140
        *.****..**.. ***.**** * .*.* .* .*. ..******.*.**.

PBP5    AAGSQDAFVGLMNSYVNALGLKNTHFQTVHGLDADGQYSSARDMALIGQA   200
PBP6    VAGSQESFIGLMNGYAKKLGLTNTTFQTVHGLDAPGQFSTARDMALLGKA   193
PhsE    IAGGQPQFVAMMNSYVKKLNLQDTHFETVHGLDAPGQHSSAYDLAVLSRA   190
PBP6b   IAGGQRQFVEMMNNYAEKLHLKDTHFETVHGLDAPGQHSSAYDLAVLSRA   190
         **.*  *...**.*.. * * .* *.******* ** *.* *.*....*

PBP5    LIRDVPNEYSIYKEKEFTFNGIRQLNRNGLLWDNSLNVDGIKTGHTDKAG   250
PBP6    LIHDVPEEYAIHKEKEFTFNKIRQPNRNRLLWSSNLNEDGMKTGTTAGAG   243
PhsE    IIHGEPEFYHMYSEKSLTWNGITQQNRNGLLWDKTMHIDGLKTGHTSGAG   240
PBP6b   IIHGEPEFYHMYSEKSLTWNGITQQNRNGLLWDKTMNVDGLKTGHTSGAG   240
        .*.. *. * ...**..*.* * * *** ***..... **.*** *. **

PBP5    YNLVASATEGQMRLISAVMGGRTFKGREAESKKLLTWGFRFFETVNPLKV   300
PBP6    YNLVASATQGDMRLISVVLGAKTDRIRFNESEKLLTWGFRFFETVTPIKP   293
PhsE    FNLIASAVDGQRRLIAVVMGAKSSKGREEQARKLLQWGQQNFATVQILHS   290
PBP6b   FNLIASAVDGQRRLIAVVMGADSAKGREEEARKLLRWGQQNFTTVQILHR   290
        .**.***..*..***..*.*. . . * ... *** ** . *.**  ..

PBP5    GKEFASEPVWFGDSDRASLGVDKDVYLTIPRGRMKDLKASYVLNSSELHA   350
PBP6    DATFVTQRVWFGDKSEVNLGAGEAGSVTIPRGQLKNLKASYTLTEPQLTA   343
PhsE    GKKVGSERIWYGDKEKIALGTEQDFWMALPKAEIPHIKAKYVLDRKELEA   340
PBP6b   GKKVGTERIWYGDKENIDLGTEQEFWMVLPKAEIPHIKAKYTLDGKELTA   340
        . .  ....*.**..  .**....  ...*.. . ..**.*.*.  .* *

PBP5    PLQKNQVVGTINFQLDGKTIEQRPLVVLQEIPEGNFFGKIIDYIKLMFHH   400
PBP6    PLKKGQVVGTIDFQLNGKSIEQRPLIVMENVEEGGFFGRVWDFVMMKFHQ   393
PhsE    PIAAHQQVGEIELYDRDKLIAQWPLVTLESVGKGGMFSRLSDY----FQH   386
PBP6b   PISAHQRVGEIELYDRDKQVAHWPLVTLESVGEGSMFSRLSDY----FHH   386
        *.   * **.*..   .* ....**..... .*..*... *.    *..

PBP5    WFG----   403
PBP6    WFGSWFS   400
PhsE    K-----A   388
PBP6b   K-----A   388
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FIG. 4. Comparison of the sequences of *E. coli* PBP5, PBP6, and PBP6b and *S. typhimurium* PhsE. An asterisk indicates a residue that is perfectly conserved in the alignment, and a dot indicates a residue that is well conserved. The predicted cleavage site of precursors for the new proteins is shown by an arrow. The three well-defined motifs in the β-lactam-binding domain of PBPs are shaded.

from its own promoter, whereas in pAB5 it may be transcribed from its promoter as well as from *lacZp*.

Identifying the homologous gene of *dacD* in *S. typhimurium*. The search for homologies also revealed that the 281-residue carboxyl stretch of PBP6b is 89% identical to the predicted polypeptide PhsF. PhsF has been described as a component of the *phsBCDEF* cluster from *S. typhimurium* (1). We then compared the nucleotide sequence of *dacD* with that of the *S. typhimurium phsBCDEF* cluster by using the GAP program of the University of Wisconsin package. This comparison revealed that a single-nucleotide gap at position 1970 of the *S. typhimurium* sequence produced a frameshift in the *phsE* ORF which resulted in premature termination of the ORF (Fig. 2). After correction of the gap, *phsE* was enlarged, covering the proposed *phsF*. The corrected *phsE* gene of *S. typhimurium* and *dacD* are 87% identical at the nucleotide level, with most of the nucleotide changes found at the third position of codons (Fig. 2). This indicates that the corrected *phsE* gene of *S. typhimurium* is the homolog of the *E. coli dacD* gene.

The predicted amino acid sequences of PBP5, PBP6, PBP6b, and *S. typhimurium* PhsE precursors are aligned in Fig. 4.

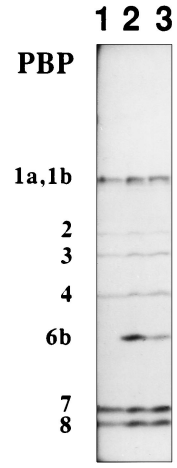

FIG. 5. Identification of the *dacD* gene product as a PBP. A 100-μg sample of cell envelopes from an exponentially growing culture of strain JBS1002 containing plasmid pPR328 (lane 1), pAB5 (lane 2), or (pAB10) (lane 3) was labelled with 25 mM [^{3}H]benzylpenicillin for 10 min at 37°C. The reaction was stopped by the addition of SDS-PAGE sample buffer, and the proteins were fractionated in an SDS–10% PAGE gel and detected by fluorography after 16 days of exposure. The positions of detected PBPs are marked on the left.

Comparisons of these sequences show that PBP5 and PBP6 are 62% identical (9) and that PBP6b is 47.4% identical to PBP5, 47.7% identical to PBP6, and 87.6% identical to PhsE. These high-level homologies among the four polypeptides allow us to predict the position of the cleavage site of precursors for the novel PBPs (Fig. 4).

PBP6b binds penicillin. Since PBP6b has nearly the same molecular mass as PBP5 and PBP6, we used a strain, JBS1002, that bears null *dacA* and *dacC* alleles to evaluate the penicillin-binding ability of PBP6b. As shown in Fig. 5, all known *E. coli* PBPs, except PBP5 and PBP6, are present in cell membrane extracts from JBS1002 (lane 1). The same protein pattern (the same bands of equal intensity) was observed when cells harbored pAB5 or pAB10, but a new band of 41 kDa appeared (Fig. 5, lanes 2 and 3). This result demonstrated that PBP6b is indeed a PBP. Note that as in the maxicell experiment, the amount of protein detected with benzylpenicillin in membrane extracts from cells carrying pAB5 was larger. The fact that PBP6b was not detected in extracts from JBS1002(pPR328) (*dacD*$^+$) (Fig. 5, lane 1) suggests that *dacD* is expressed at a very low level.

PBP6b is a DD-carboxypeptidase. Peptidoglycans from strains JBS1002(pPR328), JBS1002(pAB5), and JBS1002 (pAB10) were analyzed by HPLC as described in Materials and Methods. Comparisons of muropeptide profiles (Fig. 6) indicate that specific muropeptides are present in the same amounts in these three strains. Only the proportion of pentapeptide muropeptides (peaks 1, 2, and 3) changed, dropping drastically when expression of the *dacD* gene was induced by IPTG. The molar percentage of pentapeptide muropeptides in strain JBS1002(pPR328) was 8.5 ± 0.4 mol%. It decreased to 3.3 ± 0.3 mol% when the copy number of *dacD* was increased (pAB10) and to 1.9 ± 0.3 mol% when the gene in high copy number was transcribed from its own promoter as well as from *lacZp* (pAB5). These results indicate that the product of the *dacD* gene, PBP6b, has DD-carboxypeptidase activity.

PBP6b is not essential for *E. coli* growth. Previous work has shown that known low-molecular-mass PBPs with DD-carboxypeptidase activity are not required for growth of otherwise wild-type cells. Single deletion *dacA* (43), *dacB* (15), and *dacC* (10) mutants, as well as double *dacA dacC* deletion mutants (8) and triple *dacA dacB dacC* deletion mutants (15), grow as well as wild-type strains do under usual laboratory conditions. These findings may be interpreted as follows: PBP6b or other unknown PBPs with DD-carboxypeptidase activity compensate for the loss of major DD-carboxypeptidase PBPs, or alternatively, the DD-carboxypeptidase activity associated with these PBPs is dispensable for cells.

To gain more insight on this issue, we constructed a truncated *dacD1* allele which contains a Cm Tn*10* minitransposon inserted at nucleotide position 789 of the gene. This allele was used to construct *dacD*, *dacA dacD*, *dacA dacC dacD*, and *dacA dacB dacC dacD* strains (UGM600, UGM601, UGM602, and UGM603, respectively). All these *dacD*::Cm *E. coli* derivatives produced colonies of normal size and morphology in LB and M63 media, and in LB liquid medium, they had the same growth rate. Differences in cell morphology between strains were not observed when cells were examined with an optical microscope. These results show that like other DD-carboxypeptidases, PBP6b is dispensable. More important, they suggest that the loss of the DD-carboxypeptidase activity associated with these four PBPs is not detrimental for *E. coli* cells.

DISCUSSION

Three PBPs with DD-alanine carboxypeptidase activity were isolated from *E. coli* (47). These enzymes, PBP4, PBP5, and PBP6, are the major penicillin-binding components in cells, accounting for 80 to 85% of the penicillin-binding capacity (42). PBP4 also has a DD-endopeptidase activity and is thought to be involved in the turnover of peptide cross-bridges in murein (21, 26). PBP5 is the most studied of these carboxypeptidases and has been shown to catalyze hydrolysis of the D-alanine-D-alanyl peptide bond of the pentapeptide side chain in the murein sacculus, resulting in a tetrapeptide (30, 45). Like PBP5, PBP6 was found to have DD-carboxypeptidase activity by using various natural and synthetic substrates, and its specific activity was estimated to be three times lower than that of PBP5 (2). The identification of PBP6b in this work enlarges the list of *E. coli* PBPs with DD-carboxypeptidase activity.

PBP6b has been shown to be encoded by *dacD*, a gene located at min 44 of the *E. coli* map between *sbcB* and *sbmC* (4). PBP6b appears to be a D-alanine carboxypeptidase, acting on pentapeptide muropeptides in vivo. This was indicated by comparisons of the muropeptide profiles from JBS1002 (Δ*dacA* Δ*dacC*) and isogenic derivative strains bearing *dacD* in high copy number. The molar proportion of pentapeptide muropeptides was unusually high (8.5 mol%) in the walls of JBS1002 cells, as would be expected in the absence of PBP5, the major DD-carboxypeptidase of *E. coli*. In JBS1002 derivative strains overproducing PBP6b, the proportion of pentapeptide decreased, with the extent of the decrease being dependent on the increase of PBP6b in cells (Fig. 6). In fact, the pentapeptide level in JBS1002(pAB5) cells was typical of that found in wild-type cells.

PBP6b shares several molecular features with PBP5 and PBP6. These three enzymes have nearly the same molecular weight and composition, and their predicted amino acid sequences are highly similar. These three PBPs appear to be associated with the inner membrane, anchored by their respective carboxyl ends, which have the capacity to form the amphiphilic alpha-helical secondary structure required for anchoring (19).

The existence of such highly similar proteins at the sequence level, with the same apparent enzymatic activity on the same substrates, raises the issue of their role(s) in murein biosyn-

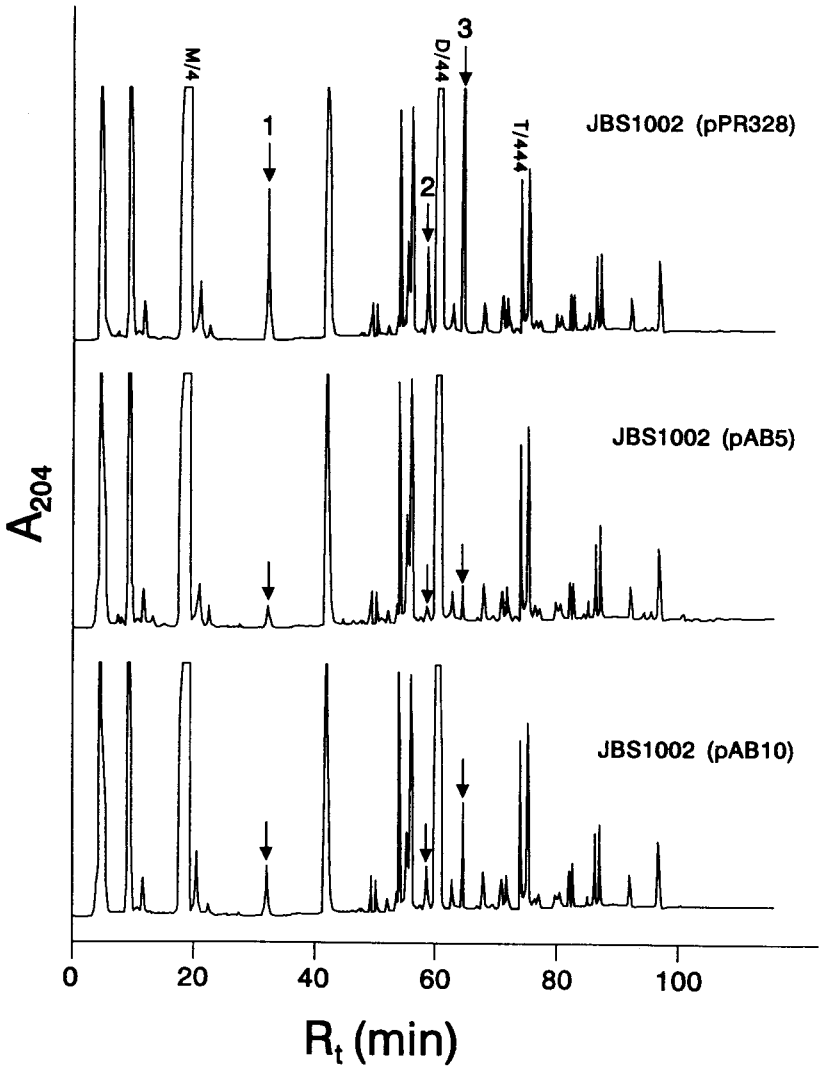

FIG. 6. Analysis of peptidoglycan composition by HPLC. Peptidoglycans from *E. coli* JBS1002(pPR328), JBS1002(pAB5), and JBS1002(pAB10) were purified and subjected to HPLC analysis as described in Materials and Methods. M/4, disaccharide tetrapeptide; D/44, bis-disaccharide tetra-tetrapeptide; and T/444, tris-disaccharide tetra-tetra-tetrapeptide (20, 37). Numbered peaks identify the following muropeptides: 1, disaccharide pentapeptide; 2, bis-disaccharide tetra-pentapeptide with Gly as the C-terminal amino acid; and 3, bis-disaccharide tetra-pentapeptide. R_t, retention time.

thesis. Does each have a specific role in peptidoglycan biosynthesis? Might they fulfill the same physiological function(s)? These questions have been and remain a matter of controversy. The finding that two of them, PBP5 and PBP6, when produced at high levels, enable cells producing a thermolabile PBP3 (mutant *ftsI23*) to grow at 42°C (6) is in accordance with the view that those PBPs are interchangeable. However, evidence that PBP5 and PBP6 play different physiological roles is growing. For example, overproduction of PBP5 caused cells to become spherical or to lyse (28), whereas overproduction of PBP6 and PBP6b did not result in these deleterious effects (48) (this work). The cell division thermosensitive mutant *ftsK44* did recover the ability to grow at 42°C by inactivation of *dacA* but not by inactivation of *dacC* or *dacB* (5). In contrast with the earlier claim of Amanuma and Strominger (2), DD-carboxypeptidase activity could not be associated with PBP6 by using various natural and synthetic substrates (48). Finally, the synthesis of PBP6, but apparently not that of PBP5, is regulated by the growth phase. The levels of PBP6 increase at the onset of stationary phase and are maintained throughout this phase (11, 13). Together, these data support the view that each DD-carboxypeptidase PBP plays a specific function in the cell.

Whatever these functions are, we know that they are not essential for *E. coli* growth in the laboratory, since inactivation of the genes encoding them by deletion or insertion does not result in major deleterious effects for cells. In fact, a strain, UGM602, with *dacA*, *dacC*, and *dacD* inactivated grows as well as its isogenic wild-type strain does.

dacD appears to be subject to tight regulation since a protein of 41 kDa could not be detected from JBS1002 (Δ*dacA* Δ*dacC*) cells by the penicillin-binding assay. Indeed, *dacD* may be responsible for the 5% residual DD-carboxypeptidase activity retained in a triple *dacA dacB dacC* mutant (14). Waxman and Strominger suggested that as little as 5% of residual carboxypeptidase activity may be sufficient to impart a normal degree of peptide cross-linking (49). The fact that the quadruple *dacA dacB dacC dacD* mutant grows as well as its isogenic double and triple mutants do may be interpreted as follows. (i)

E. coli has an additional PBP-associated DD-carboxypeptidase that remains to be discovered. Normally cryptic, this PBP would be expressed in the absence of all others. (ii) The PBP-associated DD-carboxypeptidase activity is dispensable for cell growth and division under standard laboratory conditions. (iii) If required for cell viability, the DD-carboxypeptidase activity might be performed by a periplasmic enzyme that lacks the ability to bind β-lactams.

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