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Isolation and Sequence Analysis of *dacB*, Which Encodes a Sporulation-Specific Penicillin-Binding Protein in *Bacillus subtilis*

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A novel penicillin-binding protein (PBP 5*) with D,D-carboxypeptidase activity is synthesized by *Bacillus subtilis*, beginning at about stage III of sporulation. The complete gene (*dacB*) for this protein was cloned by immunoscreening of an expression vector library and then sequenced. The identity of *dacB* was verified not only by the size and cross-reactivity of its product but also by the presence of the nucleotide sequence that coded for the independently determined NH_2 terminus of PBP 5*. Analysis of its complete amino acid sequence confirmed the hypothesis that this PBP is related to other active-site serine D,D-peptidases involved in bacterial cell wall metabolism. PBP 5* had the active-site domains common to all PBPs, as well as a cleavable amino-terminal signal peptide and a carboxy-terminal membrane anchor that are typical features of low-molecular-weight PBPs. Mature PBP 5* was 355 amino acids long, and its mass was calculated to be 40,057 daltons. What is unique about this PBP is that it is developmentally regulated. Analysis of the sequence provided support for the hypothesis that the sporulation specificity and mother cell-specific expression of *dacB* can be attributed to recognition of the gene by a sporulation-specific sigma factor. There was a good match of the putative promoter of *dacB* with the sequence recognized by sigma factor E (σ^E), the subunit of RNA polymerase that is responsible for early mother cell-specific gene expression during sporulation. Analysis of PBP 5* production by various *spo* mutants also suggested that *dacB* expression is on a σ^E-dependent pathway.

The penicillin-binding proteins (PBPs) are a family of membrane-bound enzymes that are active in the metabolism of prokaryotic cell walls (16). They are evolutionarily related to some of the soluble β-lactamases and D,D-peptidases secreted by bacteria and also have some structural features in common with a penicillin-binding transmembrane protein involved in signal transduction (24, 46, 62). The active-site serine of all of these penicillin-interactive proteins is part of the conserved domain S-X-X-K, which is located near the amino terminus of the β-lactamases and the mature form of those PBPs with a molecular mass of less than 50 kDa but more towards the middle of the sequence in the larger PBPs (24). Closer to the carboxy terminus, all of these proteins also have the sequence K-T-G, H-T-G, or K-S-G, which is an essential part of the tertiary structure of the active site (24). The class A β-lactamases and the PBPs also have a third domain (S-X-N) in common, which is located between the other two (46).

Our studies have focused on PBP 5*, which is different from all of the other PBPs described so far because it is developmentally regulated. This protein, which has D,D-carboxypeptidase activity in vitro, is not synthesized by *Bacillus subtilis* until about stage III of sporulation (43, 49, 50). Although it has a lower molecular weight than any of the six vegetative PBPs in this species, it is not a derivative of one of them (10, 50). Its location in the outer forespore membrane and the timing of its synthesis together suggest that PBP 5* is required specifically for synthesis of the spore form of peptidoglycan known as cortex (9, 43).

Although PBP 5* is produced only by sporulating cells and probably is active on a unique substrate or under unique conditions, it has been our working hypothesis that this PBP is related to other active-site serine D,D-peptidases involved in bacterial cell wall metabolism. The results of the present study, in which we cloned and sequenced the gene for PBP 5*, confirm this hypothesis. The amino acid sequence of PBP 5*, derived from the nucleotide sequence of its structural gene, *dacB*, had all of the active-site motifs common to other PBPs. We also learned that PBP 5* has a cleavable N-terminal signal sequence and a carboxy-terminal membrane anchor, which are typical features of low-molecular-weight PBPs (17).

A second hypothesis that we addressed with this study was that the sporulation specificity of *dacB* gene expression is due to the presence of a promoter sequence that can be recognized only by a sporulation-specific form of RNA polymerase. We found that this was indeed likely to be the case, because the only good match for the sequence upstream of *dacB* with the known consensus sequences for *B. subtilis* promoters corresponded to the sequence recognized by the sigma factor E-associated holoenzyme, which first appears at stage II during sporulation (29). The results of our studies on PBP 5* production by various sporulation mutants also fully support this hypothesis.

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids. *Escherichia coli* Y1089(r^-) and Y1090(r^-) were obtained from Promega Corp., Madison, Wis., to serve as bacterial hosts for λgt11 and recombinant phage derivatives (20, 60). The λgt11 library of *B. subtilis* DNA was a gift from Chet Price (47). *E. coli* JM101 (28) and vector pTZ19R (27) were acquired from U.S. Biochemical Corp., Cleveland, Ohio, for use in sequencing of cloned DNA fragments. Plasmid pCP115 (35) was acquired from the *Bacillus* Genetic Stock Center, Columbus, Ohio. *E. coli* HB101 (4) was the host strain used to propagate pCP115 and recombinant derivatives of this plasmid. The *spoIIE* mutants were obtained from P. Piggot (33);

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the *spoIIIG*, *spoIVB*, and *spoIVCB* mutants were obtained from R. Losick (25); and all of the others listed (see Table 2) were obtained from the *Bacillus* Genetic Stock Center. *B. subtilis* 168 *trpC2* (18, 44) is our standard laboratory strain, which was used as the source of membranes and PBPs for this study.

Membrane preparation and PBP assay. Membranes of *B. subtilis* were prepared by sonic disruption of the cells and differential centrifugation as described previously (43). Labeling of the membranes with [^{3}H]benzylpenicillin was performed in accordance with the optimum binding conditions established for *B. subtilis* (11, 43). After labeling, the membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis and the radioactive PBPs were detected by fluorography (3).

E. coli membranes were also prepared by sonication and differential centrifugation, but the assay for their PBPs was performed by a different protocol (45). Proteins from the inner membrane were selectively dissolved with the detergent Sarkosyl (ICN Pharmaceuticals, Inc., Plainview, N.Y.) and separated from the insoluble material by centrifugation prior to gel electrophoresis (45).

Quantitation of the PBPs on fluorographs was done as described previously (43), with a scanning densitometer (Biomed Instruments, Inc., Fullerton, Calif.).

Purification of PBP 5* and production of antibodies. Sporulation of *B. subtilis* was induced by the nutrient exhaustion technique (43). The best recovery of PBP 5* was achieved by harvesting the culture after 4 h of sporulation (t_4). The PBPs were copurified by penicillin affinity chromatography (2) of the Triton X-100-solubilized membranes from t_4 cells. It was possible to enrich greatly for PBP 5* by two modifications of the original technique. The solubilized membranes were preincubated with 10 μg of cephalothin per ml to block the binding of all of the cephalothin-sensitive PBPs to the affinity column and partially block the binding of PBP 5, which is slightly less resistant than PBP 5* to cephalothin (43). After recovery from the affinity column, the dilute cephalothin-resistant PBPs were concentrated on a DE52 column. Further purification of PBP 5* was achieved by eluting most of the contaminating PBP 5 from the DE52 column with a buffer containing only 0.05 M NaCl prior to elution of PBP 5* with 0.5 M NaCl.

Antiserum was produced by Research Biogenics, Inc., Bastrop, Tex., by subcutaneous injection of a single rabbit with a PBP preparation that was estimated to be at least 91% PBP 5*. The major contaminant was PBP 5. The initial injection of 100 μg was given with Freund's complete adjuvant; subsequent boosters of 100 μg each were given with incomplete adjuvant at 2 weeks, and 1, 2, and 3 months. Antibody production was monitored by enzyme-linked immunoassays of Western blots (immunoblots; see below) of *B. subtilis* membranes containing PBP 5*. A primary response was observed after 1 month. The antibody titer appeared to reach a plateau after the 3-month injection and remained at a high level for several months thereafter. The preimmune serum did not react with PBP 5*.

Enrichment for monospecific antibodies. The antiserum cross-reacted with a number of membrane proteins from both *E. coli* and *B. subtilis*. Most of these reactions could be eliminated by preabsorption of the serum overnight in the cold with an extract of *E. coli* or vegetative *B. subtilis* cells without diminishing the reaction of the serum with PBP 5*. However, the most effective enrichment for anti-PBP 5* was blot affinity purification as described by Olmsted (31). A single strip of a nitrocellulose blot containing pure PBP 5* could be used for up to 15 rounds of antibody binding and elution before it physically deteriorated. The eluates were pooled and stored in the refrigerator after addition of 0.1% gelatin and 0.02% sodium azide. The anti-PBP 5* appeared to be completely specific for PBP 5*; it did not cross-react on a Western blot with other PBPs or any other membrane proteins from *B. subtilis* or *E. coli*.

Immunoassays. Enzyme-linked immunoassays of membrane proteins or purified PBPs were done on nitrocellulose blots to which the samples had been electrophoretically transferred (52) from polyacrylamide gels. After a blot had been blocked with 2% nonfat dry milk (23), it was incubated for 2 to 5 h at room temperature with the primary antibody. Several different secondary-antibody preparations were used during the course of this study. Horseradish peroxidase-linked goat anti-rabbit antibody (Bio-Rad Laboratories, Richmond, Calif.) was used for evaluation of samples of the crude antiserum and during affinity purification of anti-PBP 5*. Alkaline phosphatase-linked goat anti-rabbit antibody and the more sensitive anti-rabbit immunoglobulin G (Fc); Promega Corp., Madison, Wis.) were used for subsequent assays. The ProtoBlot Immunoscreening System (Promega Corp.) was used to screen the λgt11 library with anti-PBP 5*.

DNA methods. Except where noted otherwise, methods involving phage, plasmid, or chromosomal DNA were done as described by Sambrook et al. (37). When kits were used, the manufacturer's directions were followed.

Construction of recombinant plasmid pCB1. The entire *Eco*RI fragment of *B. subtilis* DNA from one of the positive λgt11 recombinant clones was recovered and cloned into the *Eco*RI site of pCP115, a 4.4-kb modified derivative of pBR322 (35, 42). The recombinant plasmid was named pCB1. It was used to transform competent cells of *E. coli* HB101 with selection for the *amp* marker on the plasmid. Plasmid minipreps (61) were done to identify a transformant with the recombinant plasmid, and the presence of the 2.3-kb insert was verified by restriction analysis of the purified plasmid DNA.

DNA sequencing. Restriction fragments of the original 2.3-kb *Eco*RI clone from *B. subtilis* were subcloned into vector pTZ19R and then sequenced by the dideoxynucleotide chain-termination method (38) by using a Sequenase kit and M13 universal and reverse primers purchased from U.S. Biochemical Corp. Six custom-synthesized oligonucleotides were also used as primers: five of them were made on the premises by using a Beckman System 1 Plus DNA Synthesizer, and the sixth primer was synthesized by Operon Technologies, Inc., Alameda, Calif.

Sequence analysis was performed with the aid of the Genetics Computer Group software package from the University of Wisconsin (12). The strength of stem-loop structures and interactions at potential ribosome-binding sites was calculated as described by Tinoco et al. (48).

NH_2-terminal sequence determination of PBP 5*. Partially purified PBP 5* was separated from all apparent contaminating proteins by electrophoresis on a preparative sodium dodecyl sulfate-polyacrylamide gel. The protein was then electrophoretically transferred from the gel onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, Mass.; 26). The protein band corresponding to PBP 5* was detected by light staining of the membrane with Coomassie blue (59) and excised from the blot. The amino terminus of the protein was sequenced directly from the membrane strip with an Applied Biosystems gas phase sequenator at the Protein and Nucleic Acid Chemistry Facility at the Yale University School of Medicine.

Reagents. All restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs, Inc., Beverly, Mass. Recovery and purification of DNA fragments from agarose were done with the aid of a Gene Clean II kit purchased from Bio101, Inc., La Jolla, Calif. All ingredients for acrylamide and agarose gel electrophoresis were purchased from Bio-Rad Laboratories. Reagents for color development after immunoscreening with alkaline phosphatase-linked antibody were purchased from Promega Corp. [^{3}H]benzylpenicillin was a gift from P. J. Cassidy of Merck Sharp & Dohme Laboratories, West Point, Pa. [α-^{35}S]dATP was from Amersham Corp., Arlington Heights, Ill. Triton X-100 and the reagents for penicillin affinity chromatography were purchased from Sigma Chemical Co., St. Louis, Mo.

Nucleotide sequence accession number. The 2,377-bp sequence reported here is available in the GenBank data base under accession no. M84227.

RESULTS

Cloning of *dacB*, the gene for PBP 5*. Construction of a library of *Eco*RI-digested genomic DNA of *B. subtilis* in the λgt11 expression vector has been described previously (47). At the time this project was initiated, the library had a titer of 2.4×10^8 PFU/ml and 75% of the phage had inserts. When the library was plated on *E. coli* Y1090 and screened with monospecific polyclonal anti-PBP 5* (20), positive plaques were readily obtained. Their phage were purified (55), and four of them were examined in further detail. To verify that plaques of these λgt11 clones reacted specifically with the antibody to PBP 5*, the immunoscreening procedure was repeated on each of the purified phage with the crude antiserum (41). The antibody that was recovered from plaque lifts reacted only with PBP 5* on Western blots of *B. subtilis* membranes and not at all with samples of *E. coli* membranes.

A preliminary characterization of the antigen was possible after preparation of *E. coli* Y1089 lysogens of the positive clones. Small-scale crude lysates of these lysogens were analyzed on Western blots for the presence of the *B. subtilis* PBP (20, 41). A single protein that cross-reacted with anti-PBP 5* was detected in each lysate, and it had roughly the same electrophoretic mobility as authentic PBP 5* (Fig. 1, lane b). This protein did not react with mouse anti-β-galactosidase (data not shown), nor was it produced by *E. coli* lysogenized with the original vector (Fig. 1, lane c). We concluded from these results that not only was it likely that the entire gene for PBP 5* had been cloned, but also at least some of the upstream sequence must have been present too, since the PBP was not produced as a β-galactosidase fusion protein.

DNA was prepared from each of four λgt11 clones. Digestion with *Eco*RI released a single cloned fragment of approximately 2.3 kb. The fragments from the four clones had the same restriction pattern. The tentative conclusion from all of the observations made so far is that they are identical. Only one clone was used for subsequent studies.

Localization of *B. subtilis* PBP 5* in *E. coli*. Although the PBP 5* that was produced by the *E. coli* lysogens was most readily detected by immunoscreening, it was also faintly detectable by the [^{3}H]penicillin-binding assay. Thus, at least some of the cloned PBP was enzymatically active (i.e., it could bind penicillin). Was it also properly localized in the foreign host? To answer this question, it was necessary to have the cloned gene on a high-copy-number plasmid rather than a bacteriophage vector. We constructed recombinant plasmid pCB1 as described in Materials and Methods and maintained it in *E. coli* HB101. Penicillin-binding assays were done on cell membranes from the recombinant strain and a control strain bearing the original nonrecombinant plasmid pCP115 after they were grown in LB medium to the late-exponential phase. At least some of the PBP 5* produced in *E. coli* was located in the same inner membrane fraction as the *E. coli* PBPs (Fig. 2, lanes b and e). *E. coli* PBPs 5 and 6 both migrated to almost the same location as *B. subtilis* PBP 5* on a gel, which probably accounts for the presence of a detectable PBP(s) even in the control strain (Fig. 2, lane a). However, the facts that (i) there was much more PBP in the recombinant sample at this position on the gel than in the control and (ii) the identity of PBP 5* was confirmed by immunoassay (Fig. 2, lane e) support the conclusion that this foreign protein was not only active but also associated with the correct membrane of its *E. coli* host.

Nucleic acid sequence of the *dacB* region. The DNA sequence of the *Eco*RI fragment from one λgt11 clone was determined (Fig. 3). The largest open reading frame (ORF) extended from nucleotide (nt) 584 to nt 1735. Evaluation of its amino acid sequence (see below) confirmed that this ORF corresponded to *dacB*. There was an AUG start codon at nt

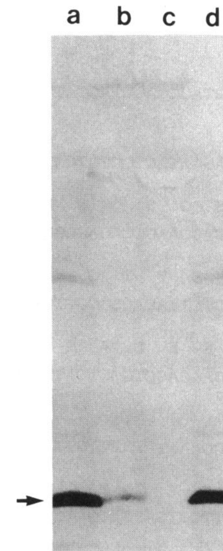

FIG. 1. Production of full-length PBP 5* after induction of *E. coli* (λgt11 *dacB*). The PBP was detected by enzyme-linked immunoassay of a Western blot with purified anti-PBP 5*. Lanes: a and d, *B. subtilis* membranes prepared from cells at t_4 of sporulation; b, lysate of *E. coli* Y1089 (λgt11 *dacB*); c, lysate of *E. coli* Y1089 (λgt11). Arrow, location of authentic PBP 5*.

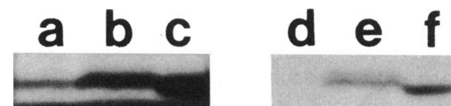

FIG. 2. Location of *B. subtilis* PBP 5* in the inner membrane of *E. coli*. Lanes: a, b, and c, fluorograph of a gel loaded with [^{3}H]penicillin-labelled membranes; d, e, and f, Western blot of the same membrane samples assayed with anti-PBP 5*; a and d, inner membrane samples of *E. coli*(pCP115); b and e, inner membrane samples of *E. coli*(pCB1); c and f, *B. subtilis* membranes with authentic PBP 5*.

-35 -10

GAATTCGAGCTCGGTACCGAGCTAGCATTCTTATCAATGGTTGTCAATACCGTGAACGATTACCTAAATCAAACGACGCTTGAGTCCTTACAGGCTGAAT 100

M P S S P D

TGCCTATTGAAAAAGGATACTGCTGTGATGTGCTTAGCACTCTGAGAAGAATGGCAGTATTTTGTGAAGGAGGCGCAGAAGCGTGCCGTCGTCTCCTGAT 200

A G A F S G S Q S E K T L Y N V Y H Q C I E E F F M P K K A Q W C

GCAGGAGCCTTTTCAGGAAGCCAGAGCGAAAAAACACTTTATAATGTGTATCACCAATGCATTGAAGAGTTCTTTATGCCGAAAAAAGCACAGTGGTGTG 300

E N S R A S Y T G V S A I E F Y H A V P A S L E Q L L L P L S A A F

AAAATAGCCGGGCGTCTTATACCGGGGTCAGCGCAATTGAGTTTTATCATGCTGTTCCTGCGTCGCTTGAGCAATTATTATTGCCGTTGAGCGCGGCCTT 400

L K M R E E L A H Y E A S G S S M A P I R *

TCTGAAGATGAGGGAAGAGCTGGCTCATTACGAAGCGTCCGGATCAAGCATGGCGCCAATCAGATAACGTTTACTCTCCCTTTTTCAGGGAGAGTTTTTT 500

-35 -10 M R I F

TATGTTTGCTTCCAGAAATATAATTGTTATTCATAACTGATGGACATGCGCATAAACTTGTACAAACCACCACAAGGACGTGAGCAAACATGCGCATTTT 600

K K A V F V I M I S F L I A T V N V N T A H A A I D V S A K S A I

CAAAAAAGCAGTATTCGTGATCATGATTTCTTTTCTTATTGCAACCGTAAATGTGAATACAGCACATGCTGCTATAGATGTCAGTGCAAAAAGCGCGATC 700

I I D G A S G R V L Y A K D E H Q K R R I A **S** I **T** **K** I M T A V L A

ATTATTGACGGTGCGTCAGGCAGAGTGTTATATGCCAAGGATGAGCATCAAAAAAGGCGGATCGCGAGCATCACAAAAATTATGACCGCCGTCCTCGCCA 800

I E S G K M D Q T V T V S A N A V R T E G S A I Y L T E G Q K V K L

TCGAATCCGGCAAAATGGATCAAACAGTGACGGTTTCGGCAAATGCTGTCAGAACAGAAGGCTCCGCCATTTATTTAACAGAAGGTCAAAAAGTGAAGCT 900

K D L V Y G L M L R **S** **G** **N** D A A V A I A E H V G G S L D G F V Y M

GAAAGATCTTGTGTATGGCCTGATGCTAAGATCAGGAAACGATGCCGCTGTCGCGATTGCTGAGCATGTCGGTGGGAGCCTCGATGGGTTTGTTTATATG 1000

M N Q K A E Q L G M K N T R F Q N P H G L D D H E N H Y S T A Y D

ATGAATCAAAAAGCCGAGCAGCTTGGTATGAAAAACACCCGTTTTCAAAACCCGCACGGATTGGACGACCATGAAAATCATTATTCAACAGCCTATGACA 1100

M A I L T K Y A I E A E R L S K D F R H K N I Q A E T M E S V W K N

TGGCCATTTTAACTAAGTATGCTATCGAAGCTGAAAGACTATCAAAAGATTTCAGGCACAAAAATATACAAGCGGAAACGATGGAAAGCGTATGGAAAAA 1200

K N K L L T M L Y P Y S T G G **K** **T** **G** Y T K L A K R T L V S T A S K

CAAAAACAAACTGCTGACCATGCTTTATCCGTACAGCACAGGAGGAAAAACAGGCTATACAAAACTTGCAAAACGGACACTGGTCTCTACGGCTTCAAAG 1300

D G I D L I A V T I N D P N D W D D H M K M F N Y V F E H Y Q T Y

GACGGCATTGACCTGATAGCCGTCACCATTAATGACCCTAATGATTGGGATGACCATATGAAGATGTTTAACTATGTGTTTGAGCATTATCAAACCTATT 1400

L I A K K G D I P K L K G T F Y E S K A F I K R D I T Y L L T E E E

TAATTGCAAAAAAAGGGGATATCCCAAAGCTGAAAGGCACATTTTATGAATCGAAGGCTTTTATCAAACGTGATATCACCTATTTGCTGACCGAAGAGGA 1500

K E N V K I N T T L L K P K K A W E K D A S K I P D I V G H M E I

AAAAGAAAATGTAAAAATTAATACCACACTGCTCAAGCCGAAAAAAGCATGGGAAAAAGATGCCTCGAAAATCCCTGACATCGTAGGGCACATGGAGATC 1600

M F N D A T I A K V P I Y Y E N E R H Q K P K K Q F F E T F K S I

ATGTTCAACGATGCAACCATCGCAAAAGTGCCGATCTATTATGAAAATGAGCGGCATCAAAAACCGAAGAAACAATTTTTTGAAACGTTTAAATCAATCT 1700

F L N A A G G A K W S I *

TTCTGAATGCGGCAGGCGGAGCAAAATGGTCAATATAATTTGGGTCAGTTTAACCGTGATCGGACTCGTTTTTGCGATGTGCAACGGAACTCTGCAAGAT 1800

GTAAATGAAGCCGTGTTTAAGGGTGCGAAAGAAGCGATTACGATCTCATTTGGATTGATGAGCGTGCTTGTTTTCTGGCTTGGCTTAATGAAAATCGCCG 1900

AGCAGTCAGGGCTTTTGGATATTTTCAGCCGGATGTGCAGACCTTTTATATCTAAACTGTTCCCTGATATACCGCGGATCACCCGGCGATGGGCTATATT 2000

TTATCTAATCTTATGGCGAATTTCTTCGGCCTCGGAAATGCAGCAACTCCGCTTGGCATTAAAGCAATGGAACAAATGAAAAAGCTGAACGGAAACCGAT 2100

CGGAAGCAAGCCGGTCGATGATTACTTTTTTAGCTGTTAATACGTCCTGCATCACACTCATCCCGACAACGGTGATCGCTGTCCGAATGGCTTATTCCTC 2200

CAAAACGCCGACAGATATCGTCGGGCCAAGCATTTTGGCAACGCTTATTTCCGGAATCGGTGCCATTATCATTGATCGGTATTTTTATTACCGCCGCAAA 2300

AAGAAGGGAAGGTGAGCGGATGGAAATCATCAACTGGCTGTCTTTAGCCATGATTCCAATCATTATTGCCGGAATTC 2377

FIG. 3. Nucleotide sequence of the *dacB* region from *B. subtilis* and deduced amino acid sequences of ORF-X (nts 169 to 464) and *dacB* (nts 584 to 1735). The 17 amino acids that were identified by sequencing the NH_2 terminus of the mature form of PBP 5* are underlined (amino acid residues 28 through 44 of the preprotein). The three active-site motifs are in boldface. The stop codon for each gene is indicated with an asterisk. The most likely ribosome-binding site for each gene is underlined, and the inverted repeats that may serve as termination sequences are underlined with arrows. The sequences upstream of each gene that most closely match the promoter consensus sequence recognized by sporulation-specific σ^E are labelled −10 and −35.

590, which was preceded by a potential ribosome-binding site ($\Delta G = -15.2$ kcal [1 cal = 4.184 J]/mol), beginning at nt 574. There were 12 nts from the center of this sequence to the first nucleotide of the putative start codon, which is within the normal range for spacing of these two features (19). There was a typical UAA stop codon at nt 1736, followed by a sequence at nts 1878 to 1903 that could form a stem-loop structure ($\Delta G = -12.1$ kcal/mol), which may serve as a rho-independent transcription termination site for the *dacB* gene. This was the strongest potential termination site in the last 700 nt of the sequence. In addition, it had the typical characteristics of being GC rich and having a string of uracils within 24 nts of its center (36).

We evaluated other ORFs on both strands in all reading frames that did not overlap significantly with *dacB*. Only one of them had a start codon that was preceded by a potential ribosome-binding site. ORF-X extended from nt 168 to nt 464. There was a GUG start codon at nt 183, which was 12 nts downstream from the center of a likely ribosome-binding site ($\Delta G = -17.8$ kcal/mol) and not in the same reading frame as *dacB*. The UAA stop codon was followed by a potential transcription termination site ($\Delta G = -17.2$ kcal/mol) at nt 473 to 495. ORF-X had no homology of apparent significance with any sequence in the protein or nucleic acid data bases. It is not known whether ORF-X is actually expressed.

The sequences preceding ORF-X and *dacB* were examined for possible promoters. In each case, the best match among the published consensus sequences for *B. subtilis* promoters (14, 15, 29) was with the −10 (ATACAAT) and −35 (GTCATA) sequences recognized by sigma factor E (σ^E).

Features of the amino acid sequence of PBP 5*. The NH_2-terminal sequence of the purified protein extracted from the cell membrane of *B. subtilis* was A-I-D-V-S-A-K-X-A-I-I-I-D-G-G-A-S-G. This sequence was located 27 amino acid residues downstream from the putative translational start site for *dacB* (Fig. 3). The NH_2-terminal peptide that was not present in the mature protein had all of the features of a cleavable NH_2-terminal signal sequence; i.e., the amino terminus of the sequence had a net positive charge, the middle part of the sequence was composed primarily of hydrophobic residues, the +1 position was alanine, there was an acidic residue in the immediate NH_2-terminal region of the mature protein, and the −3, −1 rule was satisfied (53, 54).

Mature PBP 5* was 355 amino acids long and its mass was calculated as 40,057 daltons, which is consistent with earlier determinations of its apparent size (6, 43). Analysis of codon usage within the *dacB* gene revealed no bias that would suggest that this protein is expressed at an unusually high level (40). However, a comparison of codon usage in *dacB* with usage in the vegetatively expressed *dacA* gene, which codes for PBP 5, did identify at least 10 codons for which the bias in *dacB* is typical of genes involved in sporulation (40) and the bias in *dacA* is not. There were no examples of codon usage in which the reverse was true. The amino acid composition of both the *dacA* and *dacB* genes was similar to that of the average protein from *B. subtilis* (56).

PBP 5* is normally bound to the mother cell (sporangial) and outer forespore membranes (9). By analogy with other low-molecular-weight PBPs, it is likely that PBP 5* is anchored in these membranes at its carboxy end and is exposed very little, if at all, to the cytoplasm. However, examination of its primary structure revealed no stretch of uncharged amino acids long enough to span the membrane

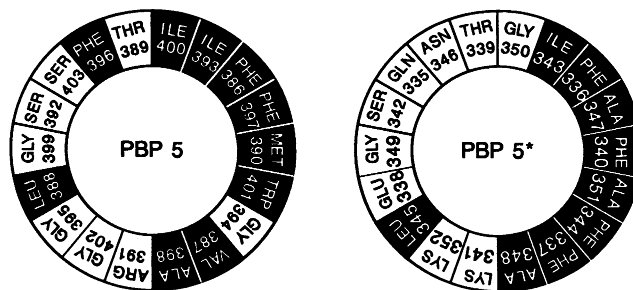

FIG. 4. Carboxy-terminal amino acid sequences of *B. subtilis* PBP 5 (residues 386 through 403) and PBP 5* (residues 335 through 352) depicted on helical wheels (39). The hydrophobic amino acids are shaded.

bilayer as an α-helix (21 residues are typically required; 13). Thus, the mechanism of its attachment is probably the same as that of two carboxypeptidase-PBPs from *E. coli* and two vegetative carboxypeptidase-PBPs from different *Bacillus* species, all of which possess a carboxy-terminal amphiphilic α-helix that associates with the membrane (22, 57). The likelihood of this possibility was explored by drawing a portion of the amino acid sequence as a helical wheel (39). It was observed that the carboxy terminus of PBP 5* (from Gln-335 through Lys-352) could form an 18-amino-acid amphiphilic helix (Fig. 4) which was 33% identical to the sequence that corresponds to the membrane anchor of *B. subtilis* PBP 5 (Phe-386 through Ser-403).

Comparison of PBP 5* with other PBPs. Comparison of the amino acid sequence of PBP 5* (mature form) with the full-length sequences of other low-molecular-weight PBPs revealed at least 30% identity with each one, except for PBP 4 from *E. coli* (Table 1). The low homology (17%) with PBP 4 was expected, since this PBP shows poor homology with all other PBPs (30). However, even 30% is a relatively low level of overall identity when one considers that PBPs 5 and 6 from *E. coli*, both of which are low-molecular-weight PBPs with D,D-carboxypeptidase activity, are 63% identical to one another (5). Nevertheless, these results are consistent with the fact that polyclonal antibodies raised against PBP 5* did not detectably cross-react with any other PBP. A somewhat greater degree of identity was observed when specific sequences within the active site were compared (Fig. 5).

All PBPs and class A β-lactamases that have been sequenced have three conserved domains that are believed to

TABLE 1. Comparison of the 355-amino-acid PBP 5* sequence with those of other low-molecular-weight PBPs[a]

Protein	No. of amino acids excluding leader sequence	% Identity to PBP 5*
E. coli PBP 4	457 (269)[b]	17 (22)[b]
B. subtilis PBP 5	399	31
E. coli PBP 5	374	30
E. coli PBP 6	373	31
B. subtilis spoIIA PBP	366	34
Streptomyces sp. strain K15 PBP	262	35

[a] Full-length comparisons were done by the Bestfit program (gap weight = 3) with the Genetics Computer Group sequence analysis software package (12).

[b] The values in parentheses were obtained by removal of an internal domain of 188 amino acids that appears to be unique to PBP 4 (30).

Protein		Active-site motif		% Identity to PBP 5*
		SXXK Motif		
B. subtilis PBP 5*	23	KDEHQKRRIASITKIMTAVLA	43	100
E. coli PBP 4	32	YHSQQMALPASTQKVITALAA	52	33
B. subtilis PBP 5	26	KNADKRLPIASMTKMMTEYLL	46	43
E. coli PBP 5	34	QNADVRRDPASLTKMMTSYVI	54	33
E. coli PBP 6	29	GNADEKLDPASLTKIMTSYVV	49	38
B. subtilis spoIIA-PBP	31	KNSNERLAPASMTKIMTMLLI	51	43
Streptomyces K15 PBP	25	KAADTRRSTGSTTKIMTAKVV	45	43
		SXN Motif		
B. subtilis PBP 5*	79	DLVYGLMLRSGNDAAVAIAEH	99	100
E. coli PBP 4	277	DLLKIMLKKSDNMIADTVFRM	297	24
B. subtilis PBP 5	91	ELYQATAIYSANAAAIAIAEI	111	43
E. coli PBP 5	101	QLIRGINLQSGNDACVAMADF	121	52
E. coli PBP 6	96	DLNKGVIIQSGNDACIALADY	116	48
B. subtilis spoIIA-PBP	92	EMLKGIAIASGNDASVAMAEF	112	48
Streptomyces K15 PBP	87	QLLYGLMLPSGCDAAYALADK	107	62
		KTG Motif		
B. subtilis PBP 5*	184	MLYPYSTGGKTGYTKLAKRTL	204	100
E. coli PBP 4	388	AGVDGKVSAKTGSLQGVYNLA	408	14
B. subtilis PBP 5	218	YKKATVDGLKTGSTDSAGSCF	238	28
E. coli PBP 5	204	DNSLNVDGIKTGHTDKAGYNL	224	33
E. coli PBP 6	199	SSNLNEDGMKTGTTAGAGYNL	219	33
B. subtilis spoIIA-PBP	198	KFYPGVDGVKTGYTGEAKYCL	218	52
Streptomyces K15 PBP	204	SSYSGAIGVKTGSGPEAKYCL	224	38

FIG. 5. Alignment of the active-site motifs in low-molecular-weight PBPs. The numbers associated with each sequence correspond to its position within the mature PBP. The proteins, except for PBP 5*, are listed in descending order based on the number of residues in the mature protein.

be essential for either the three-dimensional structure or the catalytic activity of the penicillin-interactive site on the protein (17, 24, 46). PBP 5* appeared to be typical in this regard. The highly conserved active-site motif SXXK was found near the amino terminus at residues 33 to 36. The SXN motif was located at residues 88 to 90, and the KTG motif was at residues 193 to 195. The positions of these conserved sequences relative to each other and to the two ends of the protein were similar to the arrangements found in other membrane-bound low-molecular-weight PBPs (24, 30).

For comparison with PBP 5*, we included among the low-molecular-weight PBPs one whose existence has not been confirmed. The *spoIIA*-PBP transcript is synthesized as part of the *spoIIA* operon relatively late in sporulation (58). Its derived amino acid sequence bears significant homology to the low-molecular-weight PBPs, and it has the three conserved active-site motifs (Fig. 5). The sequence used in our comparison with PBP 5* did not include the 23 NH_2-terminal residues of the *spoIIA* PBP, as this peptide had all of the characteristics of a cleavable signal sequence, and it was assumed to be one. It should also be noted that our list of low-molecular-weight PBPs (Table 1 and Fig. 5) includes PBP 4 of *E. coli* and the *Streptomyces* sp. strain K15 PBP, which are both essentially soluble proteins with no apparent membrane anchor, although they do interact with the cell membrane (30, 32). The *Streptomyces* sp. strain K15 PBP is also exceptional in that it functions as a D,D-transpeptidase rather than as a D,D-carboxypeptidase.

Dependence of PBP 5* production on *spo* genes. The presence of a promoter recognition sequence for sporulation-specific σ^E prompted us to examine more thoroughly the production of PBP 5* in various sporulation mutants. Results of previous such studies were consistent with the notion that *dacB* was on a σ^E-dependent pathway, but they

TABLE 2. Dependence of PBP 5* production on *spo* genes

spo mutation	Production of PBP 5*
spo0A12	−
*spo0A*Δ*677*	−
spo0H4	−
spoIIB131	+
spoIID66	+
spoIIE20	−
spoIIE21	−
spoIIGB41	−
spoIIGB55	−
Δ*spoIIGB*::*erm*	−
spoIIIA35	+
spoIIIC94	+
spoIIID83	+
spoIIIE36	+
*spoIIIG*Δ*1*	+
spoIVA178	+
spoIVB165	+
spoIVCB23	+

were incomplete and, in some cases, inconclusive (43, 50). For example, the absence of PBP 5* was reported for a *spoIIE* mutant that was deficient in processing of the precursor of σ^E, but there was no examination of mutants altered in *spoIIGB*, the actual structural gene for this sigma factor (50). We examined three different *spoIIGB* mutants, including one with a deletion, and in each case there was no trace of PBP 5* by t_6 of sporulation (Table 2). Our interpretation of the results with these mutants and others was verified by immunoassay using purified anti-PBP 5*. This is an important modification of previous studies in which the presence or absence of PBP 5* was documented only by the penicillin-binding assay. In some cases, the penicillin-binding assay can be misleading because a protein with roughly the same mobility as PBP 5*, but probably derived from one of the higher-molecular-weight PBPs, is present in the sample (for example, see Fig. 3g in reference 50). On several occasions, we observed the appearance during sporulation of a polypeptide that cross-reacted with anti-PBP 5, but not anti-PBP 5*, which was easy to confuse with PBP 5* on a fluorograph of radiolabelled PBPs (data not shown).

The results presented here (Table 2) verify earlier reports on *spo0A*, *spo0H*, *spoIIE20*, *spoIIIA*, *spoIVA*, and *spoIVB* (43, 50) and extend the analysis to include mutants in the structural genes for not only σ^E (*spoIIGB*) but also forespore-specific σ^G (*spoIIIG*) and late mother cell-specific σ^K (*spoIIIC* and *spoIVCB*). We conclude that *dacB* is indeed on a σ^E-dependent pathway.

DISCUSSION

The gene for sporulation-specific PBP 5* from *B. subtilis* was cloned and sequenced. We named it *dacB*, which reflects the fact that it is the second D-alanyl–D-alanine carboxypeptidase–PBP gene to be identified in this species. Its sequence is the first complete one to be obtained for any PBP gene from *Bacillus* spp. What we learned from an analysis of *dacB* confirms our hypothesis that PBP 5* is related to other D,D-peptidases–PBPs, although so far it is the only PBP that is synthesized exclusively during sporulation. Among the structural features that PBP 5* has in common with most other low-molecular-weight PBPs are a cleavable NH_2-terminal signal sequence, a carboxy-

terminal sequence that can form a potential membrane anchor as an amphilphilic α-helix, three conserved active-site domains, and overall sequence identity of at least 30%. These new data are consistent with other features that were already known, namely, that PBP 5* is membrane bound in *B. subtilis*, reacts enzymatically with β-lactam antibiotics, and has D,D-carboxypeptidase activity in vitro (10, 43, 49, 50).

There are no features of the amino acid sequence of PBP 5* that suggest that there are any major structural differences between this sporulation-specific PBP and vegetative PBPs. In addition, a comparison of PBP 5* with a second potentially sporulation-specific PBP (the PBP-like protein encoded within the *spoIIA* operon; 58) did not reveal significantly greater homology between them than between either one of them and vegetative PBP 5 from the same species. These results suggest that if there are any differences between these proteins that relate to their sporulation or vegetative specificity, they are more likely to be found at the functional rather than the structural level. It seems reasonable to expect that the intracellular conditions during sporulation or the novel structure of the cortical peptidoglycan would dictate the need for one or more sporulation-specific PBPs with some properties different from the vegetative ones. However, these differences may become evident only when more detailed comparative enzymologic investigation is done on the purified PBPs.

Sequence analysis has also provided support for the hypothesis that *dacB* expression is dependent on recognition of the gene by one of the sporulation-specific sigma factors. Both the temporal regulation of expression and the compartmental location of synthesis of a number of proteins essential to sporulation are determined by alternative forms of sigma factors that sequentially modify the specificity of RNA polymerase as sporulation progresses. As expected, there was no complete recognition sequence for the vegetative RNA polymerase in the 570-nucleotide sequence preceding *dacB*. On the other hand, not far upstream from the ribosome-binding site of *dacB* there was a good match with the −10 and −35 consensus promoter regions recognized by stage II sporulation sigma factor σ^E. The same −10 region also matched the consensus sequence for σ^K, but PBP 5* is synthesized by sporulating cells before the stage when this sigma factor is formed (25, 43). Thus, we can tentatively conclude that expression of *dacB* is on a σ^E-dependent pathway.

There is strong support for the above conclusion in the results of studies of PBP 5* production by various *spo* mutants (Table 2; 50). PBP 5* is not synthesized when σ^E is absent (*spoIIGB* mutants) or unprocessed (*spoIIE* mutants). In addition, localization of PBP 5* in the outer forespore membrane rather than the inner forespore membrane led us to suggest earlier that PBP 5* was probably synthesized exclusively in the mother cell compartment (9). Now, we can point to the likely mechanism for this compartmentalization, since it is σ^E-containing RNA polymerase that is responsible for early mother cell-specific gene expression during sporulation (21).

It was somewhat unexpected that a full-length, enzymatically active PBP 5* could be synthesized from the cloned gene in *E. coli* with no apparent effect on host viability. This result was in sharp contrast to the problems encountered when attempts were made to clone *dacA*, the *B. subtilis* gene for vegetative PBP 5 (51). In fact, the 5′ end of the *dacA* gene has not been cloned. One possible explanation for the difference is that expression from the vegetative promoter of *dacA* may have been so efficient in *E. coli* that it either was lethal to the cell or favored genetic rearrangements that deleted the promoter, whereas expression from the minor sporulation promoter of *dacB* did not lead to excessive or toxic levels of PBP 5*.

Although the PBPs of *B. subtilis* were first described 2 decades ago (1), not much is known about their specific roles in vivo. Probably the single greatest limitation in these studies has been the absence of a selection for PBP mutants (6). This is just the opposite of the situation with *E. coli*, where the ease of obtaining mutants paved the way very early for significant progress towards an understanding of its PBPs (45). With the cloned *dacB* gene in hand and the ability to manipulate it, we are beginning to overcome this handicap. For example, we have recently succeeded in inactivating the *dacB* gene by insertion of a plasmid bearing an internal fragment of the gene. The null mutant produced heat-sensitive spores (8), which is consistent with our hypothesis (43) that PBP 5* is required for cortex synthesis. Preliminary mapping of the inactivated gene placed *dacB* between *lys* (210°) and *aroC* (206°) on the *B. subtilis* genetic map, which is a region that contains no reported *spo* loci (8, 34). The location of *dacB* is also quite distant from that of *dacA*, the only other PBP gene of *B. subtilis* that has been mapped (7).

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