Reduced Heat Resistance of Mutant Spores after Cloning and Mutagenesis of the Bacillus subtilis Gene Encoding Penicillin-Binding Protein 5

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Part of the gene encoding penicillin-binding protein 5 from Bacillus subtilis 168 was cloned in Escherichia coli with a synthetic oligonucleotide as a hybridization probe. The gene was designated dacA by analogy with E. coli. The nucleotide sequence was determined, and the predicted molecular mass was 45,594 daltons (412 amino acids). A comparison of the predicted amino acid sequence with that of the E. coli penicillin-binding protein 5 indicated that these enzymes showed about 25% identity. The B. subtilis dacA gene was mutated by integration of a plasmid into the structural gene by homologous recombination. A comparison of the mutant and control strains revealed that (i) the mutant lacked detectable penicillin-binding protein 5, (ii) the d-alanine carboxypeptidase activity of membranes isolated from the mutant was only 5% of that measured in membranes from the control strain, (iii) the mutant cells showed apparently normal morphology only during exponential growth, and after the end of exponential phase the cells became progressively shorter, (iv) the mutant sporulated normally except that the forespore occupied about two-thirds of the mother cell cytoplasm and, during its development, migrated towards the center of the mother cell, and (v) purified mutant spores were 10-fold less heat resistant but possessed normal refractivity and morphology. Preliminary chemical analysis indicated that the structure of the cortex of the mutant was different.

The peptidoglycan layer (cortex) surrounding the bacterial endospore is indispensable for its remarkable heat resistance and dormancy (12). The chemical structure of this unique polymer, in particular its relatively low degree of peptide cross-linkage, is radically different from that of exponentially growing cells (44, 50). The contributions of these special features of cortex structure to the creation or maintenance of the dehydrated, heat-resistant state of the spore cytoplasm are unknown but may be investigated by selectively inactivating the enzymes involved in cortex formation.

Synthesis of the cortex during sporulation of Bacillus megaterium and B. subtilis (morphological stages III to V) is accompanied by dramatic changes in the level and number of penicillin-binding proteins (PBPs) (35, 45, 46), the enzymes that catalyze the final polymerization and cross-linkage of peptidoglycan (37, 53). PBPs can be divided into two groups, with relative molecular masses (Mr) above and below 50 kilodaltons. The higher-Mr PBPs of Escherichia coli (37) and probably of Bacillus spp. (5, 17, 32, 53) are essential, and binding of β-lactam antibodies results in cell death. The lower-Mr PBPs, however, are apparently nonessential for the maintenance of normal growth and morphology in exponentially growing cells of E. coli (27, 37) and B. subtilis (3).

The lower-Mr PBPs of E. coli (37) and B. subtilis (22) possess d-alanine carboxypeptidase activity in vitro. Exponentially growing and stationary-phase cells of B. subtilis contain a single membrane-bound d-alanine carboxypeptidase, PBP 5 (3, 17, 22, 35, 45, 48), whereas E. coli has three PBPs, 4, 5, and 6 (37). We have recently shown that B. subtilis contains a second d-alanine carboxypeptidase (44a), designated PBP 5a, which is unique to sporulating cells (35, 45). These enzymes probably function in vivo as d-alanine carboxypeptidases (16, 26, 29, 37). It is possible that their activities regulate the degree of peptide cross-linkage by altering the levels of tetrapeptide-pentapeptide substrates available for transpeptidation or by cleaving existing peptide cross-links. There are no clear experimental data about the precise roles of the lower-Mr PBPs in vivo.

We describe here the molecular cloning and insertion mutagenesis of the structural gene of B. subtilis PBP 5. The results indicate that this enzyme is required for normal morphology during stationary phase and sporulation but not apparently in exponentially growing cells. Most interesting, PBP 5 is also essential for full development of the heat resistance of B. subtilis endospores.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. B. subtilis 168 Sueoka (trpC2) (23), grown and sporulated in modified Schaeffer medium (23), was used throughout this study. E. coli/TG1[K-12, Δ(lac-pro) supE thi hsdS D5E traD36 proA1 B° lacF° lacZAM15], T. J. Gibson, Ph.D. thesis, University of Cambridge, Cambridge, U.K., 1984) was the host for all M13 phage and plasmid transformations. The plasmid vectors pUC12 (49) and pJH101 (10) and bacteriophage M13mp8 (28) have been described. The gene bank from partially Sau3A-digested B. subtilis chromosomal DNA was constructed by using the bacteriophage vector λ2001 grown in E. coli Q358 and Q359 (19).
Manipulation of DNA. General methods used in the preparation and manipulation of M13 phage DNA and plasmid DNA have been described previously (2, 25). Restriction fragments were purified from agarose gels by elution into dialysis bags (25) or onto Whatman DE81 paper (9). Total chromosomal DNA was isolated from B. subtilis by the method described by Chater et al. (7), except that the polyethylene glycol precipitation step was omitted and, after one extraction with phenol-chloroform-isoamyl alcohol (25:24:1), the DNA was further purified on a cesium chloride gradient (25).

Oligonucleotide synthesis. The 17-mer oligonucleotide probe was synthesized and purified as described previously (6). The sequence, 5'-GTCATCTTTTTTGTCAT-3', complementary to the mRNA was predicted from the peptide Met-Thr-Lys-Met-Thr (amino acid positions 37 through 42) adjacent to the active-site serine residue of B. subtilis PBP 5 (51). The degenerate positions at the threonine and lysine residues were chosen as the most likely on the basis of the codon usage data from a published nucleotide sequence (34). Restriction fragment and colony blot screening was carried out at 30°C in 6x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.2) (see below). These conditions allowed stable oligonucleotide hybridization to occur with up to two mismatches between the probe and the DNA (43).

Blotting and hybridization. Restriction fragments from agarose gels (25), M13 phage colonies or plaques, or pUC colonies (6) were transferred to nitrocellulose, prehybridized with 6x SSC containing 5x Denhardt solution, 0.5% sodium dodecyl sulfate, and 250 μg of tRNA per ml for 30 to 60 min at 30°C and hybridized for 30°C with labeled (6) oligonucleotide probe (10^6 cpm/ml) for the appropriate time (16 h for agarose gels and 2 h for colony and plaque hybridizations) in a sealed plastic bag (50 μl of prehybridization solution per cm² of nitrocellulose and 25 μl of hybridization solution per cm²). The filters were then washed with three changes of 6x SSC containing 0.5% sodium dodecyl sulfate at 30°C for 10 min and autoradiographed for 10 to 16 h.

Nucleotide sequencing and computer analysis. The 2.0-kilobase (kb) HindIII fragment of pJT3 was sequenced by the M13 dideoxynucleotide method (2). The isolated fragments were circularized by ligation, sheared by sonication, and used in the construction of a library of M13mp8 clones containing inserts at the SmaI site, as described previously (2). Eighty clones were randomly picked and sequenced to cover both strands at an average of five readings per base pair. Entry, assembly, and editing of the data was done with the GELIN, DBAUTO, and DBUTIL programs (38, 40) on a Digital Equipment VAX computer. Analysis of the sequence for open reading frames, promoter sequences, and hairpin loop structures was performed with the program ANALYSEQ (41, 42). The program DIAGON (38) was used for nucleotide and amino acid sequence comparisons. Further amino acid alignments were made with the program of Wilbur and Lipman (54). The method of Kyte and Doolittle (20) was used to analyze hydrophathy along the amino acid sequence of proteins.

Insertional mutagenesis. The mutagenesis strategy of plasmid integration by a Campbell-type insertion at the region of homology (13) has been used previously to construct mutant strains and can also be used to (i) map genes on the chromosome, (ii) clone chromosomal DNA adjacent to the integrated plasmid ("chromosome walking"), and (iii) define the limits of a transcriptional unit (1, 10, 13, 21, 30, 31, 56). Integrative disruption of the PBP 5 structural gene was achieved by transformation (31) of B. subtilis with a derivative of plasmid pJH101 containing an internal part of the PBP 5 gene (the 175-base-pair [bp] AlaI fragment shown in Fig. 1) and selection of chloramphenicol-resistant (Cm') recombinants on nutrient agar containing 2 μg of chloramphenicol per ml. Similarly, Cm' recombinants that were not defective in PBP 5 were obtained by using pJH101 containing the entire 2.0-kb HindIII fragment, which extends beyond the 3' boundary of the PBP 5 gene (see Fig. 3). For chromosome walking experiments, the chromosomal DNA purified from the Cm' recombinants was digested with SphI and ligated in dilute solution, and the recircularized plasmids were recovered in E. coli by transformation and selection for ampicillin-resistant (Ap') recombinants essentially as described (56).

Purification of spores and determination of heat resistance. Dormant spores of B. subtilis cultured for about 24 h at 37°C in modified Schaeffer medium (23) were purified by washing the spore pellet three times with deionized water at 4°C, suspending it in 0.5 times the original culture volume of 50 mM Tris (pH 7.5) containing 50 μg of lysozyme per ml, and incubation for 60 min at 37°C followed by one wash with 0.1 M NaCl and three washes with deionized water. The final spore pellets were suspended in 0.5 times the original culture volume of deionized water. Purified spore preparations contained at least 98% phase-bright spores. Spore heat resistance was determined by incubating 25 μl of the purified spore preparation in 2.5 ml of deionized water at 80°C for 15 min and calculating the percent survivors from viable-cell counts. Assays were performed in triplicate, and two independently purified spore preparations were tested for each strain.

Other methods. The PBPs present in isolated membranes were detected with [3H]benzylpenicillin (10 μg/ml; 31 Ci/mmole (46). The D-alanine carboxypeptidase activity present in 40 μg of membrane protein (24, 46) was measured by the release of D-[14C]alanine from UDP-N-acetyl-muramyl-L-alanyl-d-glutamyl-meso-2,6-diaminopimelyl-d-alanyl-d-[14C]alanine ([14C]-labeled UDP-MurNAc-pentapeptide) in 200 μl of 50 mM potassium phosphate buffer (pH 5.5) containing 10 mM MgCl₂ and 5 μl of 0.3 mM UDP-MurNAc-pentapeptide at 40°C/min. The reaction was carried out at 37°C for 2 h, and the percentage of D-[14C]alanine released was determined by paper chromatography as described previously (47). Spore cortex was purified from a single spore crop as described previously (18), and the amount of total hexosamine and free amino groups was determined by the method of Ghuyse et al. (11).

Chemicals. Restriction endonucleases were from Bethesda Research Laboratories and New England Biolabs. T4 DNA polymerase was obtained from PL Biochemicals. The Klenow fragment of DNA polymerase I and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim. T4 DNA ligase was a generous gift of Tim Hunt. Deoxyadenosine 5'-[α-32P]-thio)triphosphate (410 Ci/mmole) was from Amersham International and radioactive UDP-MurNAc-pentapeptide and [phenyl-4-3H]benzylpenicillin (31 Ci/mmole) were generous gifts of Peter Reynolds and Merck Sharp and Dohme, respectively.

RESULTS and DISCUSSION

Cloning and partial sequencing of the PBP 5 gene. A 17-mer oligonucleotide probe (5'-GTCATCTTTTTTGTCAT-3'), predicted from the peptide Met-Thr-Lys-Met-Thr (amino acid positions 37 to 42) adjacent to the active-site serine residue (serine-36) of B. subtilis PBP 5 (51), was used
to probe restriction enzyme digests of \textit{B. subtilis} 168 Sueoka chromosomal DNA. The labeled probe hybridized to single, predominant 1.0-, 2.0-, 2.9-, 3.8-, and 7.6-kb bands after digestion with \textit{BclI}, HindIII, \textit{SphI}, \textit{ClaI}, and EcoRI, respectively (not shown). Chromosomal 2.0-kb HindIII fragments were gel purified and cloned into the HindIII site of pUC12 in \textit{E. coli}. Two positively hybridizing clones were obtained by screening about 600 recombinant \textit{E. coli} colonies with labeled probe. Restriction analysis of these recombinant plasmids revealed that one of them, called pJT33, contained a single 2.0-kb HindIII fragment. To confirm that this fragment contained part of the PBP 5 gene, \textit{Alul}-digested pJT33 DNA was subcloned into the replicative form of plasmid M13mp8 DNA (digested with \textit{SmaI} and dephosphorylated). Four clones from about 300 recombinants hybridized with labeled probe. These were sequenced with universal primer, and one, called M13/8175, contained a single 175-bp \textit{Alul} fragment with the predicted amino acid sequence (Fig. 1, nucleotide positions 1 to 178) that corresponds exactly with the published PBP 5 amino-terminal sequence (51). The PBP 5 gene was designated \textit{dacA} by analogy with the corresponding \textit{E. coli} gene.

The 2.0-kb HindIII fragment was sequenced. Figure 1 shows part (1.2 kb) of the nucleotide sequence of the HindIII fragment and the predicted amino acid sequence of \textit{B. subtilis} PBP 5. The HindIII fragment lacked the 5' portion of the \textit{dacA} gene encoding the N-terminal 16 amino acids (51). The carboxy-terminal sequence agreed with that published previously (52). A possible transcriptional terminator, centered at position 1262, was noted (data not shown).

Attempts to clone the rest of the \textit{B. subtilis} \textit{dacA} gene into pUC12 and M13mp8 from \textit{BclI}-digested chromosomal DNA with the above strategy and a gene bank in the bacteriophage vector \textit{X}001 screened with the 32P-labeled 2.0-kb HindIII fragment failed.

Comparison of the complete amino acid sequences of PBP 5 of \textit{B. subtilis} and \textit{E. coli}. There are several regions of similarity between the amino acid sequences of \textit{B. subtilis} PBP 5 and \textit{E. coli} PBP 5 (4) (Fig. 2), accounting for an overall 25% identity. This suggests (8) that these proteins are homologous. The similar regions (Fig. 2) include one around the active-site serine residues (positions 36 and 44 for \textit{B. subtilis} and \textit{E. coli}, respectively), extending from positions 7 to 56 in \textit{B. subtilis} PBP 5 (47% identical amino acids). This alignment is the same as described previously (37). The average hydropathy (20) along the sequences of the two proteins is very similar (data not shown).

Insertional mutagenesis. The 175-bp \textit{Alul} and 2.0-kb HindIII fragments were each subcloned into the integrational plasmid pTH101 (10) to produce plasmids pJT15 and pJT2000, respectively. These plasmids cannot replicate in \textit{B. subtilis}, so Cm' transormants of the recombinant-proficient strain \textit{B. subtilis} 168 Sueoka can only arise by homologous recombination by a Campbell-type mechanism between the cloned \textit{B. subtilis} DNA and the chromosome. The likely products from the recombination of plasmids
pJT175 and pJT2000 with the chromosome are shown in Fig. 3. Recombination of pJT175 results in insertion of the plasmid into the dacA gene and yields no intact gene (Fig. 3b), whereas recombination of pJT2000 maintains an intact copy of dacA (Fig. 3c). These Cm\(^r\) strains, designated *B. subtilis* JT175 (trpC2 cam-I) and *B. subtilis* JT2000 (trpC2 cam-2) were grown in the presence of a low chloramphenicol concentration (2 to 5 \(\mu\)g/ml) to reduce plasmid amplification.

![Diagram](http://jb.asm.org/)

**FIG. 2.** Comparison of the complete amino acid sequence of *B. subtilis* PBP 5 (amino-terminal 16 amino acid residues [51]) with the sequence of *E. coli* PBP 5 (including the signal peptide, amino acids -29 through -1) [4]. Identical amino acids are boxed, and gaps introduced into the sequences are shown as dashes.

![Diagram](http://jb.asm.org/)

**FIG. 3.** Schematic representations of the *B. subtilis* 168 chromosome region around the 2.0-kb HindIII fragment that contains part of the dacA gene (a), the chromosome region of the Cm\(^r\) strain JT175, showing the integration of pJT175 into the 2.0-kb HindIII fragment (b), and the chromosome region of the Cm\(^r\) strain JT2000, showing the integration of pJT2000 (c). (See Fig. 1 and text for details). The open boxes represent the 2.0-kb HindIII fragment. The filled boxes represent the 175-bp *Ala* and 2.0-kb HindIII fragments, each subcloned into the integrational plasmid pJH101 to give plasmids pJT175 and pJT2000, respectively. The dashed and solid lines represent the regions of the chromosome around the 2.0-kb HindIII fragments and the plasmid pJH101, respectively. The hatched boxes represent the cat (chloramphenicol acetyltransferase) gene that confers Cm\(^r\) in *B. subtilis* and the amp (\(\beta\)-lactamase) gene of pBR322. ori (origin of replication) is from pBR322 [10]. Abbreviations for the restriction enzymes: *A*, *Ala*; *B*, *Bcl*; *H*, HindIII; *E*, EcoRI; *Sa*, *SacI*; *Sp*, *SphI*.
complete B. subtilis dacA gene cannot be stably cloned with M13 phage or pBR-derived vectors because it is toxic to E. coli.

Analysis of the mutant phenotype. Membranes isolated from exponentially growing cells of mutant B. subtilis JT175, which lacked detectable PBP 5 (Fig. 4, track b), contained only about 5% of the D-alanine carboxypeptidase activity present in membranes from control strain JT2000 (not shown). The growth and sporulation of strains JT175 and JT2000 in modified Schaeffer medium (23) supplemented with 5 μg of chloramphenicol per ml were monitored by phase-contrast microscopy and by measuring the optical density at 600 nm. The generation time of both strains growing exponentially was identical (30 min at 37°C), and there were no apparent differences in morphology (not shown). It is evident that during exponential growth, either the loss of 95% of the membrane-associated D-alanine carboxypeptidase activity can be compensated for or the remaining activity is sufficient to maintain normal growth and morphology. It has been shown previously that 95% inhibition of D-alanine carboxypeptidase activity with 6-amino penicillanic acid in exponentially growing B. subtilis did not affect cell growth or the extent of peptide cross-linkage in vivo (3, 33). Similarly, an E. coli mutant lacking PBP 5, which is the major D-alanine carboxypeptidase, appeared to grow normally during exponential phase (36). The source of the residual D-alanine carboxypeptidase activity detected in B. subtilis JT175 membranes is unknown. None of the higher-Mr PBPs apparently catalyze D-\[^{14}C\]alanine release from UDP-MurNAc-pentapeptide substrate (17). It seems unlikely that the residual 5% D-alanine carboxypeptidase activity was due to a truncated form of PBP 5, because plasmid integration was at the region of the sequence encoding the active site (Fig. 3b). Also, PBP 5a, which is a D-alanine carboxypeptidase in vitro (44a) is not detectable in exponentially growing cells (45, 45, 46).

In contrast, from the end of exponential growth (defined as the time at which the last vegetative, symmetrical cell division occurs) the mutant cells appeared to be significantly shorter than the control cells. This morphological effect was observed in at least five separate cultures grown at 30°C (Fig. 5). The forespore of the mutant, formed as a result of asymmetric cell division at stage II, migrated towards the center of the mother cell during stages IV to VI (Fig. 5B and BIV to BVI) to occupy about two-thirds of the parent cell cytoplasm. During the normal course of B. subtilis sporulation (Fig. 5A and AIV to AVI), the forespore occupied about one-third of the sporangium and was usually situated at one end. Apart from the position of the forespore in the mutant, its morphology was similar to that of the control strain.

Inactivation of PBP 5 therefore appears to affect the normal formation of the mother cell wall after the end of exponential growth. This might be due to inhibition of cell wall synthesis, or normal cell division may continue longer in the mutant, although there was no significant difference in the number of viable cells between the mutant and control strains at stage IV of sporulation (data not shown). Since the levels of the other PBPs, in particular PBP 2b, change during this time (35, 45, 46) and the sporulation-specific cell division is unique in that it is asymmetric and the septum contains little if any peptidoglycan (14), the balance of cell wall enzyme activities probably differs from that during exponential growth. It is possible that the PBPs that are catalyzing transpeptidation and peptidoglycan synthesis after the end of exponential growth require tetrapeptide substrate, which would normally be produced by the action of PBP 5.
Alternatively, higher levels of pentapeptide might allow cell division to continue for a longer period.

The levels of sporulation-specific PBP 5a (Fig. 4), the proportion of cells that contained forespores (70 to 80%), and the release of dormant spores indicated that the extent and synchrony of sporulation were the same in both strains.

**Spore heat resistance.** Although there were no significant morphological differences as viewed by phase contrast microscopy (compare AVII and BVII in Fig. 5), purified spores of the mutant strain JT175 were approximately 10-fold less heat resistant than spores of the control strain JT2000: only 8% of the mutant spores survived incubation at 80°C for 15 min, compared to 80% for the control strain. This could be due to a reduced amount of spore cortex in the mutant spores. Imae et al. (15) have shown that the amount of spore cortex is closely related to the heat resistance of *B. sphaericus* spores. Mutant spores that were similar in refractivity and shape to wild-type spores and yet contained only 50% of the wild-type amount of cortex were approximately 10-fold less heat resistant (15). As discussed above, the absence of PBP 5 could reduce the amount of tetrapeptide available to the cortex transpeptidase, which may be PBP 3 (35, 45, 46), and therefore less peptidoglycan would be synthesized. Another explanation for the reduction in spore heat resistance is that the structure of the cortex has been altered. Determination of the ratio of free amino groups (i.e., meso-diaminopimelic acid residues not cross-linked to D-alanine residues) to total hexosamine in pure cortex from mutant spores revealed that it contained approximately 10 ± 2% (standard error) more free amino groups than the cortex from strain JT2000 spores (data not shown). Since in the wild-type spore cortex only 35% of the muramic acid residues are substituted with tetrapeptide (50), this increase in the level of free amino groups represents a significant reduction in the degree of peptide cross-linkage (about 28%) of the mutant cortex. Alternatively, the increased number of free amino groups may indicate an increase in the number of muramic acid residues that are substituted with peptide. It is likely, however, that inactivation of PBP 5 resulted in a perturbation of cortex structure, substantially reducing its capacity to create or maintain the heat-resistant state of the spore cytoplasm.

These data suggest that the vegetative enzyme PBP 5 does have a role in cortex synthesis. The fact that the defect in cortex structure and spore heat resistance occurs despite the presence of a functional sporulation-specific PBP 5a (45), which is a D-alanine carboxypeptidase *in vitro* (44a), implies that these two enzymes have different roles in cortex formation.

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**LITERATURE CITED**


