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Characterization of *dacC*, Which Encodes a New Low-Molecular-Weight Penicillin-Binding Protein in *Bacillus subtilis*

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The *pbp* gene (renamed *dacC*), identified by the *Bacillus subtilis* genome sequencing project, encodes a putative 491-residue protein with sequence homology to low-molecular-weight penicillin-binding proteins. Use of a transcriptional *dacC-lacZ* fusion revealed that *dacC* expression (i) is initiated at the end of stationary phase; (ii) depends strongly on transcription factor σ^H; and (iii) appears to be initiated from a promoter located immediately upstream of *yoxA*, a gene of unknown function located upstream of *dacC* on the *B. subtilis* chromosome. A *B. subtilis dacC* insertional mutant grew and sporulated identically to wild-type cells, and *dacC* and wild-type spores had the same heat resistance, cortex structure, and germination and outgrowth kinetics. Expression of *dacC* in *Escherichia coli* showed that this gene encodes an ~59-kDa membrane-associated penicillin-binding protein which is highly toxic when overexpressed.

The polymerization and cross-linking of peptidoglycan in bacteria is catalyzed by a group of enzymes known as penicillin-binding proteins (PBPs). In *Bacillus subtilis*, a gram-positive bacterium that forms heat-resistant endospores upon nutrient deprivation, PBPs are required not only for synthesis of peptidoglycan in vegetative cells but also for synthesis of the sporulation septum and the spore's primordial germ cell wall and cortex (7).

Low-molecular-weight PBPs are usually monofunctional DD-peptidases, which regulate the number of peptide cross-links formed in the peptidoglycan (11, 12). To date, three genes from *B. subtilis* (*dacA*, *dacB*, and *dacF*) encoding polypeptides with high sequence homology to low-molecular-weight DD-peptidases have been cloned and characterized (8, 45, 49). The most well characterized of these is the PBP5*-encoding gene, *dacB*, which is transcribed around stage III of sporulation from a σ^E-dependent promoter (8, 37), suggesting a role for PBP5* in spore cortex synthesis. Indeed, spores of *dacB* null mutants are heat sensitive (6, 30), and their cortex has more peptide side chains, a higher degree of cross-linking, and less muramic acid lactam residues than that of wild-type spores (4, 29, 30). The gene encoding PBP5, *dacA* (45), accounts for most if not all DD-carboxypeptidase activity in exponentially growing *B. subtilis* (22, 45, 47) and is present in lower amounts in stationary-phase and sporulating cells (39). Inactivation of PBP5 is not lethal for the cell (5, 45) and also has no effect on spore heat resistance (6, 30). However, overexpression of *Bacillus stearothermophilus dacA* in *Escherichia coli* results in cell lysis (10), and attempts to transform *E. coli* with a plasmid containing *B. subtilis dacA* were unsuccessful (45). The *dacF* gene product has not yet been identified biochemically, but studies using *dacF-lacZ* transcriptional fusions showed that *dacF* is transcribed in the forespore compartment of the sporulating cell (49) and that this transcription is σ^F dependent (36). Disruption of *dacF* has no obvious effect on spore formation, spore cortex structure, or spore properties (4, 29, 49), and thus the function of this gene is unclear.

Recently, the *B. subtilis* genome sequencing project (20, 46) identified the *pbp* gene (here renamed *dacC*) encoding a putative 491-residue low-molecular-weight PBP with highest sequence homology to *E. coli* PBP4 (19) and PBP4 from *Actinomadura* strain R39 (14). In this work we show that *dacC* expression is dependent on transcription factor σ^H and that *dacC* does indeed encode a new membrane-bound PBP, which migrates at the position of *B. subtilis* PBP4*, between PBP4 and PBP5, on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Therefore, we have named this protein PBP4a. While a *dacC* mutation had no phenotypic effect in *B. subtilis*, overexpression of *dacC* was toxic to *E. coli*.

Transcriptional regulation of *dacC*. To study the transcriptional regulation of *dacC*, strain PS2323 carrying a transcriptional *dacC-lacZ* fusion at the *dacC* locus was constructed (all *B. subtilis* strains used in this study are listed in Table 1). PCR was used to generate a 654-bp fragment containing a part of the *B. subtilis* genome starting 162 nucleotides (nt) upstream and ending 480 nt downstream of the putative *dacC* translational initiation codon. The primers used for PCR were Y1 and Y2 (Table 2), and the template was chromosomal DNA from strain PS832. Digestion of the 654-bp PCR product with *Bam*HI and *Eco*RI yielded a 649-bp fragment which was ligated into *Bam*HI/*Eco*RI-digested plasmid pUC19 to generate plasmid pTMY1 (Fig. 1A). DNA sequencing confirmed that the DNA sequence of the insert was correct. The 649-bp *Bam*HI/*Eco*RI fragment from pTMY1 was ligated into *Bam*HI/*Eco*RI-digested plasmid pJF751a (43) to generate plasmid pTMY2 (Fig. 1A), which was used to transform PS832 to generate strain PS2323, which contains a transcriptional *dacC-lacZ* fusion at the *dacC* locus. After Southern blot analysis was used to verify that the chromosome structure of PS2323 was as expected (data not shown), cells were sporulated at 37°C in 2× SG medium (24), 1-ml samples were withdrawn at various times, and the β-galactosidase activities of the samples were measured using the substrate 4-methylumbelliferyl-β-D-galactoside (27). As shown in Fig. 2A, *dacC-lacZ* expression began shortly after the end of exponential growth and peaked about 2 h into sporulation. However, no β-galactosidase activity was detected

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TABLE 1. *B. subtilis* strains used in this study

Strain	Relevant genotype[a]	Source, reference, or construction[b]
PS832	Wild type, trp$^+$ revertant of 168	Laboratory stock
AG518	*pheA1 trpC2 abrB*::Tn*917* Erm^r	A. D. Grossman
AG558[d]	*pheA1 trpC2 spo0H*::pJ0H7d Cm^r	A. D. Grossman
DZR168	*pheA1 trpC2 spo0A*::Erm^r	A. D. Grossman
MO1615	*spo0H*::Kan^r	P. Stragier
PS258	*trpC2 codY*::Erm^r	A. L. Sonenshein
RL1061	PY79 Δ*spoIIGB*::Erm^r	R. Losick
SM69-1	PY79 Δ*spoIIA*::Sp^r	S. Meyer
PS1805	Δ*pbpE*::Erm^r	31
PS1840	*spo0A*::Erm^r	DZR168→PS832
PS2322	*amyE*::*dacC-lacZ* Cm^r	pTMY3→PS832
PS2323	*dacC-lacZ* Cm^r	pTMY2→PS832
PS2324	*dacC*::pTMY4 Sp^r	pTMY4→PS832
PS2459	*dacC-lacZ spo0H*::Kan^r Cm^r	MO1615→PS2323
PS2521	*dacC-lacZ* Δ*spoIIGB*::Erm^r Cm^r	RL1061→PS2323
PS2522	*dacC-lacZ* Δ*spoIIA*::Sp^r Cm^r	SM69-1→PS2323
PS2627	*dacC-lacZ* Erm^r	pCm::Erm[c]→PS2323
PS2628	*spo0H*::pJ0H7d Cm^r	AG558→PS832
PS2629	*dacC-lacZ spo0H*::pJ0H7d Erm^r Cm^r	AG558→PS2627
PS2630	*dacC-lacZ abrB*::Tn*917* Erm^r Cm^r	AG518→PS2323
PS2631	*abrB*::Tn*917* Erm^r	AG518→PS832
PS2632	*dacC-lacZ spo0A*::Erm^r Cm^r	PS1840→PS2323
PS2760	*amyE*::*yoxA-lacZ* Cm^r	pLP2→PS832
PS2795	*dacC-lacZ spo0H*::Kan^r *spo0A*::Erm^r Cm^r	PS1840→PS2459
LP41	*dacC-lacZ codY*::Erm^r Cm^r	PS258→PS2323

[a] Abbreviations: Cm^r, resistance to 5 μg of chloramphenicol per ml; Erm^r, resistance to 0.5 μg of erythromycin and 12.5 μg of lincomycin per ml; Sp^r, resistance to 100 μg of spectinomycin per ml; Kan^r, resistance to 10 μg of kanamycin per ml.

[b] *B. subtilis* was transformed as described previously (2), and transformants were selected on 2× SG agar plates containing appropriate antibiotics.

[c] Described in reference 40.

[d] This strain contains a truncated copy of *spo0H* under the control of its normal promoter and an intact copy of *spo0H* under the control of P_{spac} and is therefore *spo0H* in the absence of IPTG (18).

in purified spores of PS2323 (results not shown). The level of *dacC-lacZ* expression detected was also much lower than for most sporulation genes; when *o*-nitrophenyl-β-D-galactopyranoside was used as a substrate, the maximum β-galactosidase activity measured with strain PS2323 (*dacC-lacZ*) was only 7 to 8 Miller units compared with 2 to 3 Miller units for cells of the wild-type strain, PS832 (data not shown).

The timing of its expression suggests that *dacC* may be a stationary-phase- or early-sporulation-specific gene. To test this hypothesis, mutations disrupting the genes encoding σ^H (*spo0H*::Kan^r), σ^E (Δ*spoIIGB*::Erm^r), or the σ^F operon (Δ*spoIIA*::Sp^r) were introduced into strain PS2323 and *dacC-lacZ* expression was monitored as described above. The *spo0H*::Kan^r mutation (PS2459) completely abolished *dacC-lacZ* expression, while the effects of the *spoIIGB* (PS2521) and *spoIIA* (PS2522) mutations were minimal (Fig. 2A). To further analyze the dependence of *dacC* expression on σ^H, a *dacC-lacZ* fusion strain (PS2629) which contains a truncated copy of the *spo0H* gene under control of its normal promoter and an intact copy of *spo0H* under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter P_{spac} (18, 50) was generated and analyzed. Consistent with the observed lack of *dacC-lacZ* expression in the *spo0H*::Kan^r mutant (PS2459) no significant β-galactosidase activity was detected when strain PS2629 was grown in 2× SG medium without an inducer (Fig. 2B). In contrast, when 1 mM IPTG was added to an exponentially growing culture of strain PS2629 at an optical density at 600 nm (OD_{600}) of 0.5, the β-galactosidase activity measured increased significantly, but only after more than 2 h of induction, when the cells were well into stationary phase (Fig. 2B). A likely explanation for this delay is that *spo0H* expression is regulated posttranscriptionally so that induction of *spo0H* expression does not lead to an immediate rise in functional σ^H levels (16, 48).

Transcription of *spo0H* occurs at a very low level during vegetative growth due to repression of *spo0H* transcription by the AbrB repressor (9, 48). This repression is relieved by the key regulator of sporulation initiation, Spo0A (9, 41), which becomes activated by phosphorylation via a complex signal transduction pathway when cells are deprived of nutrients (17). In addition to affecting *spo0H* transcription, AbrB also represses transcription of some other sporulation genes by binding directly to their promoter regions (34). To analyze the influence of Spo0A and AbrB on *dacC* transcription, *spo0A*::Erm^r and *abrB*::Tn*917* mutations were introduced into PS2323 to generate strains PS2632 and PS2630, respectively, and the *dacC-lacZ* expression of these strains was measured. Neither the *spo0A*::Erm^r nor the *abrB*::Tn*917* mutation significantly altered the level of *dacC-lacZ* expression, although the onset of expression was slightly earlier in the strain with the *abrB* mutation (Fig. 2C), perhaps due to a change in the timing of *spo0H* transcription in this strain. The lack of effect of a *spo0A* mutation on *dacC-lacZ* expression is in contrast to what has been reported for most σ^H-dependent genes expressed around the onset of sporulation (15). The lack of *dacC-lacZ* expression in a *spo0A spo0H* double mutant (data not shown) excluded the possibility that the *dacC-lacZ* expression observed in the *spo0A*::Erm^r mutant resulted from the activation of another promoter in the *dacC* region. However, when cells were grown in nutrient sporulation broth (DSM [35]) instead of 2× SG, both the *spo0A*::Erm^r and the *abrB*::Tn*917* mutations abolished *dacC-lacZ* expression while in a wild-type background, and *dacC-lacZ* expression occurred with a similar timing as in 2× SG but the level of expression was about twofold lower (data not shown). We have no simple explanation for these findings, but we have found that omitting glucose from the 2× SG medium had no effect on *dacC-lacZ* expression (results not shown). A mutation in *codY*, a gene encoding a repressor of other stationary phase-induced genes (38), also had no obvious effect on *dacC-lacZ* transcription when cells were grown in 2× SG medium (data not shown).

While σ^H may affect transcription of some genes indirectly by stimulating transcription of *spo0A* (33), the strong dependence of *dacC* expression on *spo0H* transcription suggests that the *dacC* promoter may be directly recognized by $E\sigma^H$ (18). To identify potential regulatory sequences upstream of *dacC*, plasmid pTMY3 was introduced into PS832 to generate strain PS2322, which contains a *dacC-lacZ* fusion at the *amyE* locus. Plasmid pTMY3 is a derivative of pDG268 (3) that contains the 649-bp *Bam*HI/*Eco*RI fragment from pTMY1 (Fig. 1A). No significant β-galactosidase activity was detected in this strain (results not shown), suggesting that the *dacC* promoter

TABLE 2. PCR primers used in this study

Primer	Nucleotide sequence[a]
Y1	5′-CGGAATTCCTTACACATGGGTGACAAACGCAGTG-3′
Y2	5′-CGGATCCACGTATCATCACCGATCAGATTGCC-3′
pbpy-5′	5′-CATATGAAAAAAAGCATAAAGCTTTATG-3′
pbpy-3′	5′-GGATCCCTATTATTGATTTGCCAAAATG-3′
pbpy-P5	5′-CATATGGCTGAAAAACAAGATGCACTTTC-3′
pbpy-P6	5′-GGATCCCTAATTTAGAAGAATAGAGAAAACAAG-3′
yoxa-P2a	5′-CGCGGATCCTTGATTCATTGCTAG-3′
yoea-3′	5′-TCCCCCGGGTGCCTGTCGCTTTC-3′

[a] Restriction endonuclease sites are underlined.

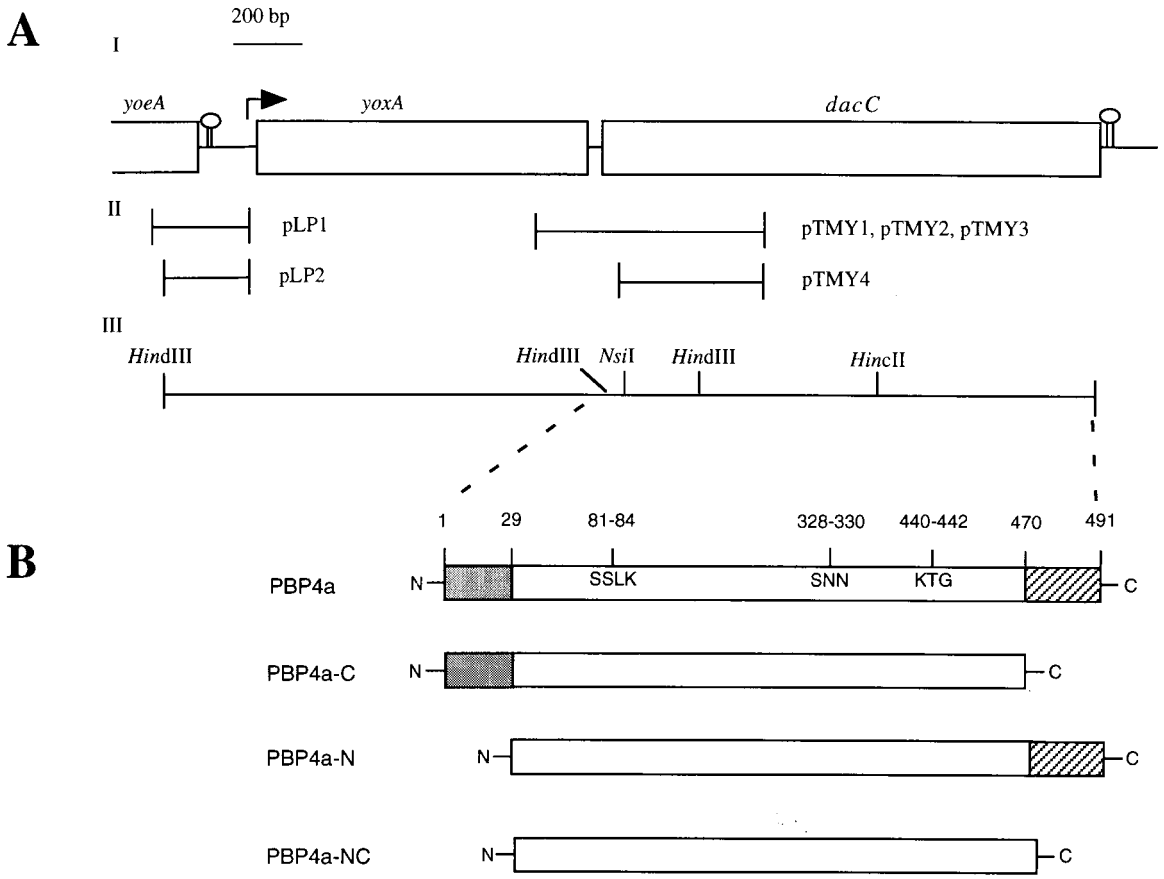

FIG. 1. Diagram of the *dacC* locus, and constructs and protein variants generated. (A) Map of the *dacC* locus. (I) Putative ORFs are indicated by open boxes, potential transcription terminators are shown as stem-loop structures, and the arrow depicts the predicted transcription initiation site and direction of transcription. (II) Fragments used in plasmid constructs for insertional mutagenesis and for generation of transcriptional *dacC-lacZ* fusions. (III) Map of selected restriction endonuclease cleavage sites. (B) Schematic depiction of PBP4a variants generated in this work. Amino acids 1 to 29 (gray) constitute a cleavable signal peptide as described in the text. The three regions that constitute the penicillin-binding site (^{81}SSLK^{84}, ^{328}SNN^{330}, and ^{440}KTG^{442}) were inferred by sequence alignment of PBP4a with PBP4 from *Actinomadura* strain R39 (14) and *E. coli* PBP4 (19) using GCG software (Wisconsin Package Version 9.1; Genetics Computer Group, Madison, Wis.). Amino acids 470 to 491 (hatched) were predicted by a computer analysis (DNA Strider 1.2) to form an amphipathic α-helix, potentially serving as a membrane anchor. Numbers refer to amino acids of the PBP4a primary sequence. The figure is not drawn to scale.

is upstream of the 649-bp *Bam*HI/*Eco*RI fragment in PS2322. The DNA sequence of the region upstream of *dacC* suggests that *dacC* may be in a two-gene operon with a gene of unknown function termed *yoxA* (20). Strikingly, the region immediately upstream of the putative *yoxA* translational initiation codon contains sequences (5′-TGAAT-3′ and 5′-GGAGGAA AT-3′) separated by 14 bp that match perfectly the −10 and −35 consensus sequences of σ^H-dependent promoters (15, 33). To investigate whether this putative σ^H promoter is functional, a 290-bp fragment containing the region from 13 nt upstream of the putative *yoxA* initiation codon to 153 nt upstream of the putative *yoeA* stop codon (*yoeA* is gene of unknown function located upstream of *yoxA* [20]) was PCR amplified from chromosomal DNA of strain PS832 using primers yoxa-P2a and yoea-3′ (Table 2). The PCR product was ligated into pCR2.1 (Invitrogen), generating plasmid pLP1, and the DNA sequence of the insert was confirmed. Digestion of plasmid pLP1 with *Bam*HI and *Hin*dIII yielded a 236-bp fragment, which was cloned into *Bam*HI/*Hin*dIII-digested pDG268 (3), generating plasmid pLP2 (Fig. 1A), which was then introduced into strain PS832, generating strain PS2760, which contains the *yoxA-lacZ* fusion at the *amyE* locus. Southern blot analysis confirmed that the chromosome structure of strain PS2760 was as expected (results not shown). Measurement of the β-galactosidase activity in a sporulating culture of PS2760 showed that the timing and level of expression of the *yoxA-lacZ* fusion in this strain were identical to those of strain PS2323, which contains a *dacC-lacZ* fusion at the *dacC* locus (data not shown). This strongly suggests that the promoter controlling *dacC* is located within the 236-bp *Bam*HI/*Hin*dIII fragment in plasmid pLP2 and further supports the idea that *dacC* transcription depends directly on σ^H. However, due to the low level of *dacC* transcription, we did not attempt to further localize the *dacC* transcription start site.

Generation and analysis of an insertional *dacC* mutant. To begin to study the function of *dacC*, we constructed strain PS2324 containing a disrupted *dacC* gene by transformation of strain PS832 with plasmid pTMY4 (Fig. 1A). Plasmid pTMY4 was constructed by digesting plasmid pTMY1 with *Nsi*I and *Sal*I, which released an ~400-bp fragment from within the coding region of *dacC*, and ligating this ~400-bp fragment into *Pst*I/*Sal*I-digested plasmid pJL73 (23). Transformation of strain PS832 with plasmid pTMY4 yielded strain PS2324, in which *dacC* has been disrupted; Southern blot analysis verified that the genomic structure of PS2324 was as expected (data not shown).

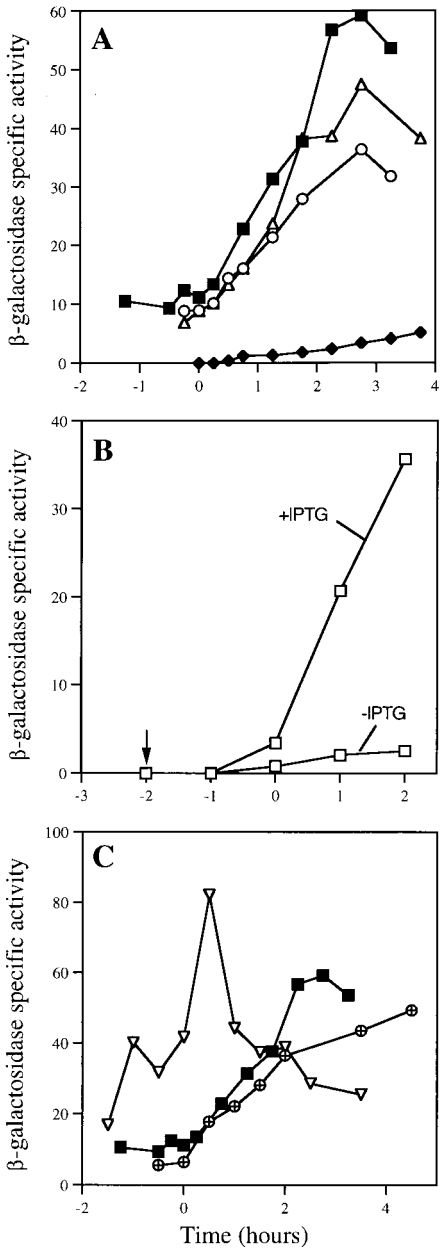

FIG. 2. Transcriptional regulation of *dacC*. *B. subtilis* strains containing transcriptional *dacC-lacZ* fusions at the *dacC* locus were grown and sporulated at 37°C in 100 ml of 2× SG medium with no antibiotics (24), the OD_{600} values of the cultures were measured, and 1-ml samples were withdrawn for measurement of β-galactosidase activity as described in the text at the times indicated (t_0 is defined as the end of exponential phase). Values on the *y* axis are fluorescence units per OD_{600} unit of the cultures. (A) β-Galactosidase activities in strains lacking sigma factors. Symbols and strains (relevant genotypes) are as follows: ■, PS2323 (wild type); ◆, PS2459 (*spo0H*::Kan^r); ○, PS2522 (Δ*spoIIA*::Sp^r); and △, PS2521 (Δ*spoIIGB*::Erm^r). (B) β-Galactosidase activity in strain PS2629 (P_{spac}-*spo0H*) with or without IPTG (arrow indicates time of IPTG addition). (C) β-Galactosidase activities in transcription factor mutant strains. Symbols and strains (relevant genotypes) are as follows: ▽, PS2632 (*spo0A*::Erm^r); ⊕, PS2630 (*abrB*::Tn*917*); and ■, PS2323 (wild type).

Membranes from vegetative cells of strains PS2324 (*dacC*) and PS832 (wild type) and cells of the same strains harvested 2 h into sporulation (t_2 of sporulation) were purified and incubated with fluorescein-hexanoic-6-aminopenicillanic acid (FLU-C_6-APA), proteins were separated by SDS–10% PAGE, and PBPs were visualized with a fluorimager (FluorimagerSI; Vistra) as described previously (32). Identical PBP profiles were obtained for the two strains (results not shown), suggesting that PBP4a is present at levels too low to be detected in *B. subtilis* and/or has a low affinity for penicillin. Strain PS2324 grew and sporulated, its spores germinated at rates comparable to those of the wild-type strain, and *dacC* spores were as heat resistant as wild-type spores (results not shown). In addition, analysis of spore cortex structure by reversed-phase high-pressure liquid chromatography (4, 29) showed no significant structural differences between the cortices from *dacC* and wild-type spores (results not shown). Thus, *dacC* appears to be dispensable for *B. subtilis* under normal growth conditions.

Expression of *dacC* variants in *E. coli*. Given the lack of detection of the *dacC* gene product in *B. subtilis*, we overexpressed *dacC* in *E. coli* in order to determine if it indeed encodes a PBP. We also decided to overexpress several truncated forms of *dacC*-encoded protein to study their function and localization in *E. coli*. The four *dacC*-encoded variants overexpressed in *E. coli* are depicted in Fig. 1B. PBP4a corresponds to full-length *dacC*-encoded protein (491 residues), PBP4a-C is PBP4a lacking residues 470 to 491, PBP4a-N is PBP4a lacking residues 2 to 29, and PBP4a-NC is PBP4a lacking both residues 2 to 29 and 470 to 491. Residues 1 to 29 are found to constitute a cleavable signal peptide (see below), while residues 470 to 491 are predicted to form an amphipathic α-helix (data not shown) that may constitute a C-terminal membrane anchor commonly found in low-molecular-weight PBPs (12). For PCR amplification of the regions encoding the PBP4a variants, primers (Table 2) were as follows: PBP4a, pbpy-5′ and pbpy-3′; PBP4a-C, pbpy-5′ and pbpy-P6; PBP4a-N, pbpy-P5 and pbpy-3′; and PBP4a-NC, pbpy-P5 and pbpy-P6. PCR products were ligated into pCR 2.1 (Invitrogen), and the inserts were sequenced to confirm their identity, removed by digestion with *Bam*HI and *Nde*I, ligated into *Bam*HI/*Nde*I-digested pET11a (42), and used to transform *E. coli* BL21 (DE3)/pLysS (42). The resulting *E. coli* strains were termed PS2599 (PBP4a), PS2690 (PBP4a-C), 2691 (PBP4a-N), and PS2692 (PBP4a-NC).

Recombinant *E. coli* strains were grown at 37°C to an OD_{600} of ~0.5 in 50 ml of 2× YT medium (per liter: 16 g of tryptone, 10 g of yeast extract, 5 g of NaCl) with chloramphenicol (20 μg/ml) and ampicillin (50 μg/ml), and IPTG was added to 0.5 mM. After 2 h of further incubation, samples (1 ml) from induced cultures were pelleted by centrifugation, and proteins were solubilized in 100 μl of SDS-sample buffer (21) and analyzed by SDS–10% PAGE. A strong 59-kDa band was present in the lanes containing proteins from induced PS2599 and PS2691, while lysates of induced PS2690 and PS2692 gave a doublet of 57 and 58 kDa (57–58-kDa doublet) and a strong 57-kDa band, respectively; these bands were not present in lysates of induced cells which carried only the vector (Fig. 3A). In some gels, the protein whose synthesis was induced in strain PS2599 also migrated as a 59–60-kDa doublet (Fig. 3B, lane 2), suggesting that PBP4a undergoes posttranslational processing, presumably removal of the signal sequence, and that the efficiency of processing varies from experiment to experiment. Although 57 to 59 kDa is larger than the theoretical molecular mass of PBP4a (52.9 kDa including the N-terminal signal peptide), the absence of any strong 57- to 59-kDa band in the extract from strain PS2602, which harbors only the vector (Fig. 3A, lane 1), strongly suggests that the 57- to 59-kDa bands are the PBP4a variants. To confirm this, the proteins on a gel run parallel to the one shown in Fig. 3A were transferred to a polyvinylidene difluoride membrane and the amino-terminal sequences of the 57- to 59-kDa bands were determined (28)

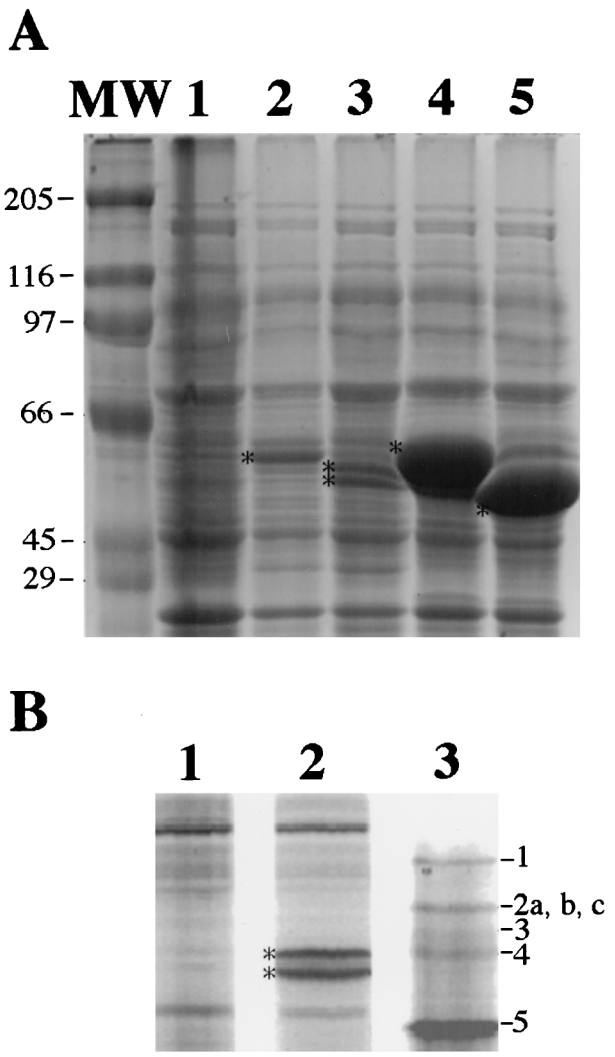

FIG. 3. Expression of *dacC* variants and penicillin-binding activity of PBP4a. (A) Expression of *dacC* variants in *E. coli*. Recombinant *E. coli* strains were grown and induced, protein was solubilized as described in the text, and 7 μl of each sample was analyzed by SDS–10% PAGE and staining with Coomassie blue. Lanes, corresponding strains, and proteins they express (parentheses) are as follows: 1, PS2602 (vector alone); 2, PS2599 (PBP4a); 3, PS2690 (PBP4a-C); 4, PS2691 (PBP4a-N); and 5, PS2692 (PBP4a-NC). Lane MW contains molecular weight markers (molecular masses are in kilodaltons). Asterisks denote migration positions of the PBP4a variants. (B) Analysis of PBPs in membranes from induced *E. coli* strains PS2602 (lane 1; vector alone) and PS2599 (lane 2; PBP4a). Cells were grown and induced as for panel A for 90 min, 25 ml of culture was harvested by centrifugation, membranes were isolated from sonicated cells by centrifugation (100,000 × *g*, 1 h) and incubated for 30 min at 30°C with 100 μM FLU-C_6-APA, proteins (~10 μg) were analyzed by SDS–10% PAGE, and PBPs were visualized with a FluorimagerSI (Vistra). A lane containing labeled PBPs from vegetative *B. subtilis* cells of strain PS832 (32) is shown for comparison (lane 3; the PBPs corresponding to each band are indicated on the right). Asterisks denote position of the 59–60-kDa PBP4a doublet.

(for proteins migrating as a doublet, the lower band was sequenced). The sequence obtained for the major induced bands for all PBP4a variants was AEKQD, corresponding to residues 30 to 34 of PBP4a, indicating that residues 1 to 29 constitute a signal peptide. Fractionation of sonicated cells by a high-speed centrifugation method (44) showed that most (>90%) of PBP4a, PBP4a-N, and PBP4a-C were membrane associated (presumably in the inner membrane) while PBP4a-NC was present as inclusion bodies in *E. coli* (data not shown). Thus, removal of either the N-terminal signal peptide or the C-terminal putative membrane anchor did not prevent PBP4a from being membrane associated; the membrane association of PBP4a-C despite the lack of the putative membrane anchor could be due to the hydrophobic character of the protein or to expression of the protein in a heterologous system. However, removal of both of these regions resulted in loss of solubility and membrane association. In addition, removal of residues 1 to 29 at the N terminus dramatically increased the amount of PBP4a protein produced in *E. coli* (Fig. 3A, compare lanes 4 and 5 with lanes 2 and 3), while removal of residues 470 to 491 at the C terminus of PBP4a had essentially no effect on expression levels (Fig. 3A, compare lanes 2 and 3).

The PBP4a signal peptide contains three lysines within the amino-terminal six residues, a hydrophobic core region of 15 residues that terminates with a proline, and alanine residues at positions −3, −1, and +1 relative to the cleavage site. These features are similar to those of other *B. subtilis* signal peptides (26), suggesting that the PBP4a variants were processed in *E. coli* as one might expect them to be processed in *B. subtilis*.

PBP4a binds penicillin. To analyze whether recombinant PBP4a binds penicillin, membranes from cells of induced

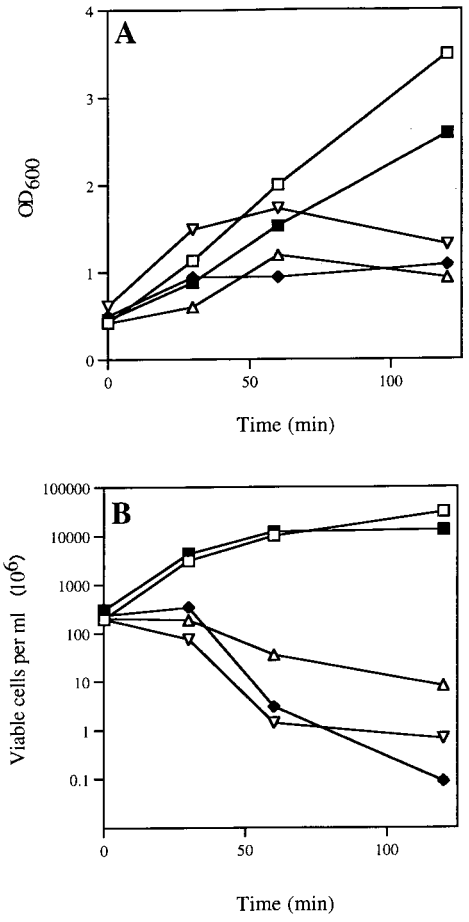

FIG. 4. Effects of PBP4a variants on growth and viability of *E. coli*. Recombinant *E. coli* strains were grown and induced with IPTG as described in the text, and the OD_{600} (A) and viability (B) were measured after induction. The viability was measured by plating dilutions on 2× YT agar plates containing chloramphenicol (20 μg ml^{-1}) and ampicillin (50 μg ml^{-1}). Symbols, strains, and proteins they express (parentheses) are as follows: □, PS2602 (vector); ▽, PS2599 (PBP4a); △, PS2690 (PBP4a-C); ◆, PS2691 (PBP4a-N); and ■, PS2692 (PBP4a-NC).

strain PS2599 or PS2602 were incubated with FLU-C_6-APA, proteins were separated by SDS–10% PAGE, and bands were visualized by fluorimaging (Fig. 3B). A labeled 59–60-kDa doublet was present in membranes from strain PS2599 (Fig. 3B, lane 2) but not in labeled membranes from strain PS2602 harboring only the vector (Fig. 3B, lane 1), suggesting that the 59–60-kDa doublet is PBP4a. Comparison with FLU-C_6-APA-labeled membranes from vegetative cells of strain PS832 or from cells of the same strain harvested at t_2 of sporulation showed that recombinant PBP4a from *E. coli* membranes migrated at the same position as *B. subtilis* PBP4*, between PBP4 and PBP5 (Fig. 3B, lane 3, and data not shown). However, we could not detect PBP4a in membranes isolated from sporulating cells of strain PS1805, which lacks PBP4* (data not shown).

PBP4a variants affect growth and viability of *E. coli*. Overexpression of *dacA* from *B. subtilis* or *B. stearothermophilus* appears to be toxic for *E. coli* (10, 45). To investigate whether this was also the case for *dacC*, cultures of strains PS2599, PS2690, PS2691, PS2692, and the control strain PS2602 were grown to an OD_{600} of ~0.5 and induced with 0.5 mM IPTG and the OD_{600} was monitored. All cultures continued to grow for 30 min after induction, but after 60 min, the OD_{600} of cells expressing PBP4a, PBP4a-C, and PBP4a-N stopped increasing, while the culture expressing PBP4a-NC continued to grow at the same rate as the strain carrying the vector alone (Fig. 4A). Consistent with these observations, determination of the number of viable cells in the induced cultures showed decreased viability of strains PS2599, PS2690, and PS2691 30 min after induction while the number of viable cells in induced cultures of strains PS2602 and PS2692 increased throughout the 120-min induction period (Fig. 4B). Thus, expression of PBP4a, PBP4a-C, and PBP4a-N is toxic for *E. coli*, while expression of PBP4a-NC is not. The lack of toxicity of PBP4a-NC may be related to its presence as inclusion bodies in *E. coli*.

Enzymatic activity of PBP4a? During the course of growth and induction of recombinant *E. coli*, microscopic examination of cultures expressing PBP4a, PBP4a-C, or PBP4a-N revealed the presence of lysed cells, and in cultures expressing PBP4a or PBP4a-C, spherical cells were occasionally observed (results not shown). Lysed and/or spherical cells have previously been observed in recombinant *E. coli* cells that overexpress proteins with DD-carboxypeptidase activity (10, 25), suggesting that PBP4a may have a similar enzymatic activity. Preliminary efforts to determine the enzymatic activity of PBP4a by reversed-phase high-pressure liquid chromatography analysis of purified cell walls from induced recombinant *E. coli* (13) or by using PBP4a-NC purified from inclusion bodies from strain PS2692 in in vitro assays (1) were unsuccessful. Thus, the enzymatic activity of PBP4a, if any, remains to be determined.

In summary, we have shown that (i) *dacC* transcription depends strongly on transcription factor σ^H and appears to be initiated from a promoter immediately upstream of the *yoxA* gene; (ii) disruption of *dacC* has no dramatic effects on *B. subtilis* growth, sporulation, and spore properties; and (iii) *dacC* encodes a membrane-bound PBP which is toxic when overexpressed in *E. coli*.

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