A Gene Encoding a Holin-Like Protein Involved in Spore Morphogenesis and Spore Germination in *Bacillus subtilis*

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We report here studies of expression and functional analysis of a *Bacillus subtilis* gene, *ywcE*, which codes for a product with features of a holin. Primer extension analysis of *ywcE* transcription revealed that a single transcript accumulated from the onset of sporulation onwards, produced from a σ^A -type promoter bearing the TG dinucleotide motif of “extended” –10 promoters. No primer extension product was detected in vivo during growth. However, specific runoff products were produced in vitro from the *ywcE* promoter by purified σ^A -containing RNA polymerase ($E\sigma^A$), and the in vivo and in vitro transcription start sites were identical. These results suggested that utilization of the *ywcE* promoter by $E\sigma^A$ during growth was subjected to repression. Studies with a *lacZ* fusion revealed that the transition-state regulator AbrB repressed the transcription of *ywcE* during growth. This repression was reversed at the onset of sporulation in a Spo0A-dependent manner, but Spo0A did not appear to contribute otherwise to *ywcE* transcription. We found *ywcE* to be required for proper spore morphogenesis. Spores of the *ywcE* mutant showed a reduced outer coat which lacked the characteristic striated pattern, and the outer coat failed to attach to the underlying inner coat. The mutant spores also accumulated reduced levels of dipicolinic acid. *ywcE* was also found to be important for spore germination.

During the initial stages of sporulation in *Bacillus subtilis*, the rod-shaped cell is asymmetrically divided into a smaller prespore and a larger mother cell. Migration of the septal membranes towards the proximal cell pole, a process called engulfment, and their eventual fusion at the cell pole release the prespore as a free protoplast within the mother cell cytoplasm (15). The engulfed prespore (or forespore) is separated from the mother cell cytoplasm by two membranes of opposing polarities, which derive from the invagination of the cell envelope earlier during polar division (15). The arrangement of the spore membranes dictates the pattern of synthesis of the various spore structures, whose assembly may start soon after activation of the mother cell line of gene expression but becomes more evident following forespore engulfment (13, 15, 16, 28). Synthesis of an inner layer of peptidoglycan, known as the primordial cell wall, occurs across the prespore inner membrane and requires prespore-specific gene expression (16). In contrast, synthesis of the spore cortex peptidoglycan occurs across the prespore outer membrane and requires the expression of several mother cell-specific genes (16). Another protective layer that forms around the developing spore is a protein coat that confers protection against peptidoglycan-breaking enzymes and noxious chemicals and is also important for spore germination (13, 28). Formation of both the cortex and coat layers requires the synthesis of SpoIVA (49, 55), which localizes at or near the forespore outer membrane (14).

It is not known how SpoIVA influences cortex synthesis. However, SpoIVA is required for proper localization and maintenance around the developing spore of several morphogenetic proteins that act to guide assembly of the spore coat (14, 41).

SpoVID localizes to the surface of the developing spore in a SpoIVA-dependent manner and is required for attachment of the nascent coat to the spore surface (41). In *spoVID* mutants, the coat forms swirls throughout the mother cell cytoplasm, and the resulting spores, which have an exposed cortex layer, are highly susceptible to lysozyme (5). SpoVID acts in part through another coat morphogenetic protein called SafA (40, 56). SafA has a cell wall-binding LysM domain (4) encompassing its first 50 amino-terminal residues and has been shown by immunogold labeling to localize at the interface between the cortex and coat regions (40). The C-terminal half of SafA shares similarity to known inner coat proteins, and it has been proposed that SafA helps to link the cortex, to which it would bind via its LysM domain, to the nascent coat via interactions with other coat proteins involving its C-terminal portion (41). SpoVID directly interacts with SafA and is required for the targeting of SafA to the surface of the developing spore (40). One intriguing aspect of the function of SafA is how its LysM domain is put in contact with the spore cortex peptidoglycan. SafA is made in the mother cell, whereas the cortex accumulates in the compartment defined by the inner and outer spore membranes (15, 16). Several proteins with secretory signal sequences are made in either the prespore or the mother cell and are presumably translocated across the prespore membranes into the cortical region. The cortex-lytic enzyme SleB, which also has a potential peptidoglycan-binding domain, for example, is synthesized in the forespore with a signal peptide that is cleaved, and mature SleB is found associated with spore

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TABLE 1. Bacterial strains used for this study

Strain	Genotype/phenotype	Origin or reference
MB24	<i>trpC2 metC3</i> ; wild type	Laboratory stock
AH2357	<i>trpC2 metC3 ΔspoIIAC::em</i> ; Em ^r	Laboratory stock
AH38	<i>trpC2 metC3 spoIIIGB55</i>	Laboratory stock
AH77	<i>trpC2 metC3 ΔsigK::erm</i> ; Em ^r	Laboratory stock
AH2544	<i>trpC2 metC3 ΔabrB::neo</i> ; Neo ^r	Laboratory stock
AH3795	<i>trpC2 metC3 ΔspoIIIG</i>	Laboratory stock
FB111	<i>ΔcwlJ::tet</i> ; Tet ^r	51
FB112	<i>ΔsleB::sp</i> ; Sp ^r	42
FB113	<i>ΔcwlJ::tet ΔsleB::sp</i> ; Tet ^r Sp ^r	42
AH630	<i>trpC2 metC3 ΔywcE::neo</i> ; Neo ^r	This work
AH3113	<i>trpC2 metC3 Δspo0A::Pspac-spo0A</i> ; Cm ^r	This work
AH3362	<i>trpC2 metC3 Δspo0H::Pspac-spo0H</i> ; Cm ^r	This work
AH3279	<i>trpC2 metC3 ΔamyE::ywcE-gfp_{mut2}</i> ; Neo ^r	This work
AH3338	<i>trpC2 metC3 ΔamyE::PywE-lacZ</i> ; Cm ^r	This work
AH3339	<i>trpC2 metC3 ΔspoIIAB::em ΔamyE::PywE-lacZ</i> ; Cm ^r Em ^r	This work
AH3340	<i>trpC2 metC3 spoIIIGB55 ΔamyE::PywE-lacZ</i> ; Cm ^r	This work
AH3341	<i>trpC2 metC3 ΔspoIIIG ΔamyE::PywE-lacZ</i> ; Cm ^r	This work
AH3342	<i>trpC2 metC3 ΔsigK::erm ΔamyE::PywE-lacZ</i> ; Cm ^r Em ^r	This work
AH3344	<i>trpC2 metC3 ΔthrC::PywE-gfp_{mut2}</i> ; Em ^r	This work
AH3382	<i>trpC2 metC3 ΔamyE::PywE-lacZ</i> ; Sp ^r	This work
AH3384	<i>trpC2 metC3 Δspo0A::Pspac-spo0A ΔamyE::PywE-lacZ</i> ; Cm ^r Sp ^r	This work
AH3385	<i>trpC2 metC3 Δspo0H::Pspac-spo0H ΔamyE::PywE-lacZ</i> ; Cm ^r Sp ^r	This work
AH3386	<i>trpC2 metC3 ΔabrB::neo ΔamyE::PywE-lacZ</i> ; Neo ^r Sp ^r	This work
AH3387	<i>trpC2 metC3 Δspo0A ΔabrB::neo ΔamyE::PywE-lacZ</i> ; Cm ^r Sp ^r Neo ^r	This work
AH3410 ^a	<i>trpC2 metC3 ywcE::neoΩpGR182</i> ; Cm ^r	This work
AH3414	<i>trpC2 metC3 ywcE::neoΩpGR182 amyE::PywE-gfp</i> ; Neo ^r Cm ^r	This work
AH3443	<i>trpC2 metC3 ΔcwlJ::tet</i> ; Tet ^r	This work
AH3444	<i>trpC2 metC3 ΔsleB::sp ΔywcE::neo</i> ; Sp ^r Neo ^r	This work
AH3445	<i>trpC2 metC3 ΔcwlJ::tet ΔywcE::neo</i> ; Tet ^r Neo ^r	This work

^a The Ω symbol denotes the single reciprocal integration of the indicated plasmid at the *ywcE* locus.

membranes, cortexes, and coats (38). SpoIVH, which is required for cortex synthesis, is produced in both the prespore and the mother cell with a signal peptide which can be replaced by that of SleB (31). SafA does not have a signal sequence, and it has been suggested that other mechanisms could facilitate its transfer from the mother cell cytoplasm into the cortex region of the developing spore, including the involvement of holins (40). Holins are involved in the precise temporal control of cell lysis upon bacteriophage infection by forming a pore that permits access of phage-encoded lysins to the cell wall compartment (59). There are two main classes of holins. Class I includes proteins with three membrane-spanning domains, a dual start motif, and a highly charged C-terminal region. The prototype member of this class is the λ S protein (18, 59) (see Fig. 1C). Class II holins, such as the S protein from the lambdaoid bacteriophage 21, are smaller (60 to 85 residues), with only two predicted transmembrane domains (3, 59).

The *B. subtilis* genome appears to code for several holin-like proteins (35). For this study, we analyzed the expression and function of the *ywcE* gene of *B. subtilis* (35), because its predicted product is structurally related to class I holins (see Fig. 1C) and because *ywcE* is expressed during sporulation. We found that transcription of *ywcE* takes place from a σ^A -type promoter that is repressed during growth and induced at the onset of sporulation in a Spo0A-dependent manner. A functional YwcE-green fluorescent protein (GFP) fusion protein localizes to both the cell and spore membranes. *ywcE* mutants form spores with reduced levels of pyridine-2,6-dicarboxylic acid (DPA) that have an outer coat layer that lacks the normal

striated pattern. Our results also indicate that *ywcE* expression is important for spore germination.

MATERIALS AND METHODS

Bacterial strains, media, and general techniques. The bacterial strains used for this study are congenic derivatives of the wild-type strain MB24 and are listed in Table 1. The *Escherichia coli* strain DH5 α (Gibco BRL) was used as the host for the construction and propagation of all plasmids described herein. Luria-Bertani (LB) medium was routinely used for the growth of both *B. subtilis* and *E. coli* strains at 37°C. Difco sporulation medium (DSM) was used to induce sporulation by nutrient exhaustion (10). Genetic manipulations of *E. coli* and *B. subtilis* and spore resistance or germination properties were assessed as previously described (10, 24). Other general methods were performed as previously described (10, 24, 25, 27). The levels of DPA were measured as previously described (10).

Construction of a *ywcE* insertional mutant. Plasmid pAH103 has been described before (26). It was created by inserting a 1,755-bp EcoRI-EcoRV DNA fragment isolated from pLactT2 between the EcoRI and HincII sites of plasmid pUS19 (26). The insert in pAH103 encompasses the 3' end of the *qaxD* gene, the complete *ywcE* gene, and the 5' end of the *rodA* gene (not represented in Fig. 1). After plasmid pAH103 was digested with HindII, a neomycin resistance (Nm^r) determinant released from pBEST501 (32) with SmaI was inserted in the same orientation as the *ywcE* gene, thus producing pAH105. To transfer the Nm^r determinant to the *B. subtilis* chromosome, pAH105 was linearized with ScaI and used to transform competent MB24 cells. A transformant, verified by PCR to result from a double-cross-over event at the *ywcE* region, was named AH630 (Table 1).

Construction of *ywcE-lacZ* and *ywcE-gfp_{mut2}* transcriptional fusions. A 386-bp fragment carrying DNA upstream from the *ywcE* start codon was PCR amplified with primers *ywcE*-392D(EcoRI) and *ywcE*-6R(BglII) (Table 2), simultaneously digested with EcoRI and BglII, and cloned between the EcoRI and BamHI sites of pSN32 (39), yielding pGR172 (Fig. 1). PstI-linearized pGR172 was used to transfer the *ywcE-lacZ* fusion to the *amyE* locus of wild-type strain MB24 to produce the Cm^r Amy⁻ strain AH3338 (Fig. 1; Table 1). The chloramphenicol (Cm) resistance determinant was subsequently changed to a spectinomycin

TABLE 2. Oligonucleotides used for this study

Primer	Sequence (5' to 3') ^a
<i>ywcE</i> -461R(<i>gfp_{mut2}</i>)	GTGAAAAGTTCTTCTCCTTTACTGTTGTTGTTGCTGCTTCGCTTTCTGCGAAGATACGGAAGGCCG
<i>ywcE</i> -356R	CAAATAAAGAGAGGCGTTGC
<i>ywcE</i> -392D(HindIII)	GCGGCGCTTTTAAAGCTTCACGAAATATGACG
<i>ywcE</i> -392D(EcoRI)	GCGGCGCTTTTGAATTCCACGAAATATGACG
<i>ywcE</i> -6R(BglIII)	CCCAGATCTCCTTTCATTTTGTGATAACG
<i>gfpR</i>	GGCGAATTCCTATTGTATAGTTCATCCATGC
<i>spoIIG</i> -131D(EcoRI)	GGAATTCATCGTCGAGATGATTATGG
<i>gfpR</i> (BglIII)	GAAGATCTTCTTATTGTATAGTTCATCCATGCG
<i>gfp</i> -30D	AGTAAAGGAGAAGAAGCTTTTCACTGGAG
<i>spo0A</i> -563D(HindIII)	GGTGAAGCTTGTTAACTACATTTGGGGAGG
<i>spo0A</i> -1064R(SpeI)	CCGATTTTCATGGATAATACTAGTGATGCTC

^a Native or engineered restriction sites are underlined.

(Spec) resistance determinant by the transformation of AH3338 with plasmid pJL62 (36), producing the Cm^s Spec^r AmyE[−] strain AH3382 (Table 1). To construct a *ywcE*-*gfp_{mut2}* transcriptional fusion, we first constructed a vector for transcriptional fusions to *gfp* to be integrated at the *thrC* locus of the chromosome. Briefly, a 973-bp fragment carrying the *spoIIGA* promoter region, the *spoVG* Shine-Dalgarno sequence, and the *gfp* coding region was amplified from pMS201 (a gift from Monica Serrano) with primers *spoIIG*-131D(EcoRI) and *gfpR*(BglIII) (Table 2) and introduced into EcoRI-BamHI-digested pDG1664 (20), yielding pGR168. The 398-bp PCR product amplified with primers *ywcE*-392D(EcoRI) and *ywcE*-6R(BglIII) (see above) was digested with EcoRI and BglIII and introduced between the EcoRI and BamHI sites of pLITMUS29 (New

England Biolabs), yielding pGR174. A 412-bp EcoRI-HindIII fragment obtained from pGR174 was then introduced into EcoRI-HindIII-digested pGR168, yielding pGR176. This plasmid was used to transfer the *ywcE*-*gfp* transcriptional fusion to the *thrC* locus of wild-type strain MB24, creating the Erm^r Spec^s strain AH3344 (Fig. 1; Table 1).
Construction of a *ywcE*-*gfp* translational fusion. A C-terminal fusion between *ywcE* and *gfp_{mut2}* was obtained using the splicing-by-overlap-extension technique (30). Briefly, a 653-bp PCR fragment including the *ywcE* gene and its promoter region was amplified from the chromosome of *B. subtilis* strain MB24 using primers *ywcE*-392D(HindIII) and *ywcE*-461R(*gfp_{mut2}*) (Table 2). The *gfp_{mut2}* gene (710 bp) was PCR amplified using pEIA18 (46) as a template and primers

FIG. 1. *ywcE* locus and YwcE protein. Panel A shows the *ywcE* region of the *B. subtilis* chromosome. The *ywcE* gene is divergently oriented relative to *rodA* (only the 5' end of this gene is represented) and convergent with *qoxD* (only its 3' end is shown). A stem-loop structure between *ywcE* and *qoxD* represents a transcriptional terminator. Restriction enzyme sites used to insert a neomycin resistance cassette within the *ywcE* gene or used for in vitro transcription experiments are represented. The relative positions of the *ywcE* -10 and -35 promoter elements as well as that of the transcriptional start site are shown. The lines below the partial physical and genetic map represent inserts in the indicated plasmids. Panel B shows the sequence of the *ywcE* promoter. The -35 and -10 elements, the transcriptional start site (+1), the ribosome binding site, and the initiation codon are shown in bold. The arrows represent two regions of dyad symmetry located just upstream of the translation initiation signals. The sequence is numbered relative to the first nucleotide in the initiation codon. Panel C represents the primary structures of YwcE and of the S protein of phage λ. The lines below the amino acid sequence (in single-letter code) represent the locations of predicted transmembrane segments. Basic (+) and acidic (-) amino acids are indicated. Asterisks indicate the highly charged C-terminal regions of both proteins.

gfp-30D and *gfpR* (Table 2). A linker encoding four asparagines was engineered in the *ywcE*-461R(*gfp_{mut2}*) primer (17). The *ywcE*-*gfp_{mut2}* fusion was amplified with primers *ywcE*-392D(HindIII) and *gfpR* using an equimolar mix of both the *ywcE* and *gfp_{mut2}* PCR products as the template. The resulting fragment of 1,363 bp was digested with EcoRI and HindIII and inserted between the same sites in pMLK83 (33), generating pGR155. Strain AH3279 (Neo^r) resulted from the integration of pGR155 into the *amyE* locus of wild-type MB24 (Table 1). pGR182 was constructed by amplifying an internal fragment of a neomycin gene from pBEST501 (32) using the primers NeoD (NotI) (5'-GTCGTCAGACTGATGCGGCCGCTATTTCGG-3') and NeoR (XbaI) (5'-CTGCCATTGCTACCTCTAGAGTCAAGGATG-3'). The amplified product was digested with NotI and EcoRI and inserted between the same sites of pMS38 (60). The resulting plasmid, pGR182, was used to transform AH3319 to Cm^r. A Neo^r transformant, AH3410, was then transformed with pGR155 to yield the AmyE⁻ strain AH3414.

Conditional allele of *spo0A*. A 502-bp DNA fragment corresponding to 470 bp of the 5' coding sequence of *spo0A* was amplified using primers *spo0A*-563D and *spo0A*-1064R (Table 2), digested with HindIII and SpeI, and inserted between the HindIII and XbaI sites of pDH88 (23), yielding pGR96. Plasmid pGR96 was integrated into the host chromosome by means of a single-crossover (Campbell-type) recombinational event that occurred in the region of homology, giving strain AH3113 (Table 1).

RNA purification and primer extension analysis. Total *B. subtilis* RNA was prepared at the indicated times from cells grown in DSM, as previously described (25). Primer extension reactions were carried out using primer *ywcE*-356R (Table 2). Sequencing and primer extension reactions were done as described previously (25). Appropriate double-stranded templates were used so that the initiation nucleotide in the mRNA species could be directly determined from the autoradiographs.

Purification of RNA polymerase. The σ^A form of RNA polymerase ($E\sigma^A$) was isolated from exponentially growing cells of the wild-type strain *B. subtilis* MB24 (Table 1) grown in LB medium. The $E\sigma^A$ holoenzyme was purified using a low-pressure affinity chromatography step on a heparin column, followed by anion-exchange fast protein liquid chromatography, as previously described (7, 57, 58).

In vitro transcription. Plasmid pAH103 was cut with either BamHI or HpaI (Fig. 1) and used as a template for in vitro transcription reactions. RNA polymerase (0.04 μ M) and the cut plasmid (5 nM) were preincubated at 37°C for 10 min in 50 μ l (final volume) of a buffer containing 33 mM Tris acetate (pH 7.9), 10 mM magnesium acetate, 0.5 mM dithiothreitol, 0.15 mg bovine serum albumin/ml, and 66 mM potassium acetate. Ribonucleotides were added at the following concentrations: a 500 μ M concentration (each) of ATP, CTP, and GTP (Boehringer Mannheim) and 10 μ Ci of [α -³²P]UTP (800 Ci/mmol; Amersham). The mixture was incubated for 1 min before reinitiation was stopped by the addition of 10 μ g of heparin (Sigma). Five minutes later, unlabeled UTP (Boehringer Mannheim) was added to a final concentration of 500 μ M, and the mixture was incubated for another 5 min. The nucleic acids were then precipitated by the addition of sodium acetate (final concentration, 0.3 M) and ethanol and resuspended in 10 μ l of a sequencing formamide dye. The samples were heated at 95°C for 3 min and then loaded onto a 5% polyacrylamide-7 M urea sequencing gel. Transcripts were localized by autoradiography on a PhosphorImager 445 SI (Molecular Dynamics).

Spore purification and extraction and analysis of spore coat proteins. Spores were purified from 24-h DSM cultures on Renocal gradients as described previously (60). Extraction and analysis of the spore coat proteins and immunoblot analysis of CotG were performed as described previously (60).

Microscopy. Samples for the visualization of fluorescence were prepared as previously described (47). For the visualization of membranes, the membrane dye FM4-64 (Molecular Probes) was added to a final concentration of 10 μ g ml⁻¹ before mounting the cells on agarose-coated slides. All samples were observed with a 63 $\times$ objective lens. Phase-contrast or fluorescence images were acquired with a CoolSNAP HQ Photometrics camera (Roper Scientific, Tucson, Arizona), recorded, and processed for publication using Adobe Photoshop, version 4.0.

Electron microscopy. Spores were purified from DSM cultures of strain MB24 (wild type) and its congenic derivative AH630 (*ywcE::neo*) 24 h after the onset of sporulation, as formerly described (27). The spores were fixed and embedded as described before (27). Electron microscopy analysis and photography were conducted on a Phillips EM301 electron microscope operating at 80 keV.

RESULTS

The *ywcE* gene is expressed during sporulation from a σ^A -type promoter. In a first step towards the functional analysis of the *ywcE* gene, we wanted to determine whether *ywcE* was

transcribed during sporulation in DSM. We used an oligonucleotide complementary to the *ywcE* transcribed strand to prime cDNA synthesis of total RNA prepared during growth and sporulation of a wild-type strain in DSM. The results in Fig. 2A show that a single primer extension product was detected from the onset of sporulation (or time zero) until at least hour 6 after the initiation of the process. The presumed initiation nucleotide was an "A" located 76 bp upstream of the first nucleotide in the *ywcE* start codon (Fig. 1A and B). An inspection of the sequence upstream of this position did not reveal sequences similar to those known to be utilized by any of the four compartment-specific σ factors that control sporulation (21). Instead, the putative -10 sequence was identical to the consensus for σ^A -type promoters (TATAAT) (Fig. 1A and B). In contrast, the predicted -35 sequence (GTATCA), located 17 bp upstream, did not conform as well to the consensus (TTGACA) (21). However, note that the -10 region was preceded by the TG dinucleotide characteristic of extended -10 promoters (22). The results suggest that *ywcE* is transcribed during sporulation from a σ^A -type promoter with an extended -10 region. Because no transcript was detected during the exponential growth phase (Fig. 2A, lane -1), the results also suggest that the *ywcE* promoter is repressed during the exponential phase of growth.

We then tested whether purified σ^A -containing RNA polymerase could direct transcription from the *ywcE* promoter in vitro. The RNA polymerase σ^A holoenzyme was purified from exponentially growing cells harvested at late exponential phase in LB medium (7, 57, 58). For in vitro transcription reactions, RNA polymerase was incubated with plasmid pAH103 cut with either HpaI, which cuts within the *ywcE* coding sequence, or BamHI, which cuts downstream of *ywcE* (Fig. 1). We found a runoff product of about 285 nucleotides (nt) with HpaI-cut pAH103 (Fig. 2B, lane a), consistent with the transcription initiation site determined in vivo (Fig. 2A). However, we found two products, of about 347 and 390 nt, with BamHI-cut pAH103 (Fig. 2B, lane b). The 347-nt product is consistent with transcription initiating at the +1 position found in vivo but stopping at the putative transcriptional terminator located between the *ywcE* and *qoxD* genes (Fig. 1A). The 390-nt species has the expected size for a runoff product reaching the end of the BamHI-linearized template. To determine whether the transcription initiation nucleotide was the same in vivo and in vitro, the RNA generated in vitro with HpaI-cut pAH103 was subjected to a primer extension reaction with the same oligonucleotide primer used for the in vivo experiment depicted in Fig. 2A. The results in Fig. 2C show that transcription in vitro was initiated from an "A" nucleotide 76 bp upstream from the first base of the *ywcE* start codon. From these results, we infer that the transcription of *ywcE* in vitro with purified σ^A -containing RNA polymerase and in vivo during sporulation takes place from the same σ^A -recognized promoter. We further infer that transcription from this promoter is repressed during vegetative growth and that this repression is relieved at the onset of sporulation.

The *ywcE* gene is repressed by AbrB. The dependence of *ywcE* expression on loci known to control the initiation of sporulation was examined by transferring a *ywcE-lacZ* fusion (see Materials and Methods) into a series of congenic mutants and by monitoring β -galactosidase production throughout growth and sporulation. In agreement with the primer exten-

FIG. 2. Transcriptional analysis of the *ywcE* gene. Panel A shows an autoradiograph of *ywcE*-specific primer extension products after electrophoretic resolution in denaturing polyacrylamide gels. An oligonucleotide complementary to the 5' end of the nontranscribed strand of *ywcE* was used to direct the cDNA synthesis of total RNA purified from DSM cultures of a wild-type strain (MB24) at the indicated times (in hours) before or after time zero, defined as the onset of sporulation. Panel B is an autoradiograph of runoff transcription assays, in which HpaI-linearized (a) or BamHI-linearized (b) pAH103 (see Materials and Methods) was used as the template. The sizes of the runoff products (in nucleotides) are shown on the left. Panel C shows an autoradiograph of the *ywcE*-specific primer extension product generated using the RNA produced in vitro with purified RNA polymerase and HpaI-linearized pAH103 after resolution in denaturing acrylamide gels. For panels A and C, the oligonucleotide *ywcE*-356R (Table 2) was used to prime RNA synthesis. The same oligonucleotide used for the primer extension experiments was also used in parallel to prime sequencing reactions (labeled G, A, T, and C) from pAH103 so that the initiating nucleotide in the nontranscribed strand (+1) could be determined by direct inspection of the sequencing ladder.

sion analysis, the expression of *ywcE-lacZ* was not detected above background levels during vegetative growth but was induced around the onset of sporulation (Fig. 3A). Peak levels of β -galactosidase synthesis were reached between hours 1 and 2. The expression of *ywcE-lacZ* was independent of the expression of *spo0H* (Fig. 3A) but required the expression of *spo0A* from the isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *Pspac* promoter (Fig. 3B). Spo0A could be directly or indirectly involved in *ywcE* transcription. For example, Spo0A represses transcription of the *abrB* gene, which encodes the transition-state regulator AbrB, a repressor of several genes important for the initiation of sporulation (45, 61). The results in Fig. 3C show that mutation of the *abrB* gene leads to an increased expression of *ywcE-lacZ* during growth and at the onset of sporulation and that *spo0A* was no longer required for *ywcE-lacZ* expression in cells with an *abrB* mutation. These results suggest that AbrB represses transcription of the *ywcE* gene during growth and the initial stages of sporulation and that at the onset of sporulation, this repression is relieved in a Spo0A-dependent manner. In agreement with the results of the in vitro transcription studies described above, these results further suggest that Spo0A is not directly involved in the utilization of the *ywcE* promoter.

A YwcE-GFP fusion localizes to the cell and spore membranes. To investigate the localization of YwcE, we constructed a strain (AH3279) expressing a translational C-terminal YwcE-GFP fusion from the *ywcE* promoter at the nonessential *amyE* locus. Complementation of the *ywcE* phenotype (described below) by ectopic expression of *ywcE-gfp* showed that the GFP fusion protein was functional. The localization of YwcE-GFP was then investigated throughout sporulation by fluorescence microscopy. Samples of cultures grown in DSM were collected, and the cells were stained with FM4-64 to visualize the septal and engulfment membranes. In agreement with our analysis of *ywcE* transcription, GFP fluorescence was detected from hour 1 of sporulation onwards and was localized to the cell membrane (Fig. 4). At the onset of sporulation, the cell division protein FtsZ assembles into rings at two potential division sites near each cell pole of the sporangium (37). Only one of these two potential division sites is used to form the sporulation septum, while the other is aborted. At hour 2 of sporulation, many cells showed an asymmetric septum, and at this time, fluorescence from the YwcE-GFP fusion protein was found to colocalize with the polar septum, seen as a straight line across the short axis of the cell (Fig. 4). At later stages, the septal membranes migrate towards

FIG. 3. Analysis of *ywcE-lacZ* expression. The production of β -galactosidase from a *ywcE-lacZ* transcriptional fusion was monitored during growth and throughout sporulation in DSM cultures of a wild-type strain and congenic derivatives bearing mutations in loci known to control entry into sporulation. Panel A shows the expression of *ywcE-lacZ* in a wild-type background (AH3382) (●) and in a strain bearing an IPTG-inducible *spo0H* allele (AH3385) in the absence (△) or presence (○) of 1 mM IPTG. (B) Effects of an IPTG-inducible allele of *spo0A* on the expression of *ywcE-lacZ*. The strains used are as follows: AH3382 (wild-type background) (●), AH3384 ($\Delta spo0A::P_{spac-spo0A}$; no inducer) (○), and AH3384 ($\Delta spo0A::P_{spac-spo0A}$; 1 mM IPTG) (△). (C) Role of *abrB* on the expression of *ywcE-lacZ* with strains AH3351 ($\Delta abrB::neo$) (△), AH3352 ($\Delta abrB::neo \Delta spo0A::P_{spac-spo0A}$; no inducer) (▲), and AH3384 ($\Delta spo0A::P_{spac-spo0A}$; no inducer) (○). For all panels, endogenous levels of activity were determined with strain MB24 (no fusion) (■). The β -galactosidase activity is shown in Miller units. Time zero indicates the initiation of the stationary phase of growth, defined as the onset of sporulation.

the proximal cell pole, during the process of engulfment. YwcE-GFP remained associated with the septal membranes during engulfment (Fig. 4). Once the engulfment process was complete, the signal from the lipophilic dye FM4-64 was lost (53), but the fusion protein remained associated with the spore membranes, completely encircling the developing spore (Fig. 4). YwcE-GFP remained associated with the spore during the subsequent stages of spore maturation when refractile spores developed and also after release from the mother cell (data not shown). In addition, the results show that YwcE-GFP remains associated with the mother cell membrane throughout sporulation. Since YwcE-GFP complemented the DPA phenotype of a *ywcE* mutant (see below) and since the pattern of YwcE-GFP localization in cells containing only the *ywcE-gfp* allele (not shown) did not differ in any discernible way from that described above for cells containing both the *ywcE-gfp* and wild-type alleles of *ywcE*, it seems likely that the localization of the fusion protein reflects that of untagged YwcE.

It is not known whether YwcE-GFP localizes to both the inner and outer membranes of the spore. However, in agreement with the temporal profile of *ywcE-lacZ* expression (Fig. 3), fluorescence resulting from GFP accumulation in a strain (AH3279) expressing a transcriptional fusion of the *ywcE* promoter to *gfp* was also detected from hour 1 (T1) after the onset of sporulation in cells that had not yet formed a prespore (not shown). The fusion protein YwcE-GFP also accumulated from T1 in cells that did not show any signs of asymmetric division, as assessed by staining with the membrane dye FM4-64 (Fig. 4). Since YwcE production is induced prior to the asymmetric division of the sporangium and decorates both the mother cell and the prespore membranes, we infer that YwcE is likely to be localized in both the inner and outer prespore membranes.

The *ywcE* gene is required for proper spore morphogenesis.

We examined whether *ywcE* expression was required for proper spore morphogenesis. In preliminary experiments, we observed spores purified from 24-h cultures of the *ywcE* mutant grown in DSM by phase-contrast microscopy. We found that almost 50% of the *ywcE* spores were phase dark, whereas <0.1% phase-dark spores were found for the wild-type strain (Fig. 5).

Wild-type spores as well as *ywcE* spores were also observed by transmission electron microscopy. Wild-type spores exhibited a well-developed cortex layer and a coat that was closely apposed to it; the coat presented the normal pattern of structural differentiation into an inner lamellar layer and a thick, electron-dense, striated outer coat (Fig. 5). In contrast, *ywcE* spores showed several features that deviated from the wild-type pattern. The spore cores of *ywcE* mutant spores in the electron micrographs exhibited a punctate pattern, presumably due to the ribosomes (Fig. 5). The inner spore coat was adjacent to the cortex and did not appear reduced or otherwise compromised, but the outer coat formed a thin layer, essentially lacking the striated pattern of wild-type spores, and it did not adhere to the underlying inner coat (Fig. 5). Because the electron micrographs of *ywcE* spores revealed a reduced outer coat layer, we analyzed the coat polypeptide composition of *ywcE* spores by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). We observed only a few differences between the coat protein profiles of wild-type and *ywcE* mutant spores. We found that CotG (36 kDa) and CotB (64 kDa), both of which are outer coat proteins (50, 60),

FIG. 4. Localization of a functional YwcE-GFP fusion protein. Strain AH3279 was induced to sporulate in DSM, and samples were collected throughout sporulation. Cells were stained with the vital membrane stain FM4-64 (second column) and observed by fluorescence microscopy as described in Materials and Methods. Phase-contrast images are shown in the first column, the localization of YwcE-GFP is shown in the third column, and merges between the FM4-64 and GFP images are shown in the fourth column. Time zero (T0) represents the onset of sporulation, and the numbers for the other time points (shown on the left) indicate the time (in hours) after T0. Bars, 2 μ m.

appeared to be more extractable from *ywcE* spores. This result is consistent with the observation that CotG is also more easily extractable (presumably less cross-linked) from *sodA* mutant spores. Moreover, mutations in *sodA* or *cotG* cause a structural alteration of the spore outer coat that is reminiscent of the phenotype described here for *ywcE* spores (27). The only other differences noted between the protein patterns of wild-type and *ywcE* spores were two minor unidentified proteins that were missing from *ywcE* spores. We also investigated the pres-

ence of SafA among the collection of spore coat polypeptides that could be extracted from the coats of *ywcE* spores by immunoblot analysis. However, we detected no difference in the patterns of assembly of SafA between wild-type spores and spores of the *ywcE* mutant (data not shown).

Because we found that expression of the σ^K -controlled *gerE* gene (9) occurred at the normal time and level in sporulating cells of the *ywcE* mutant (data not shown), the structural features seen for the *ywcE* mutant spores did not seem to arise

FIG. 5. Structural alterations of *ywcE* mutant spores. Spores produced by MB24 (A and C) and by the *ywcE* insertional mutant AH630 (B, D, E, and F) were purified from 24-h DSM cultures of both strains and observed by phase-contrast microscopy (A and B) or transmission electron microscopy (C to F). The arrowheads in panel A point to phase-bright spores, whereas in panel B the arrowheads point to phase-dark spores. In panels C to F, black arrowheads point to the outer coat layer, and white arrowheads point to the inner coat structure. Cx, cortex; Cr, spore core. Bars, 1 μ m in panels A and B and 0.2 μ m in panels C to F.

from delayed sporulation. Therefore, the absence of the *ywcE* gene results in spores with structural deficiencies in the core and the outer coat layers. In spite of these alterations, *ywcE* spores showed wild-type levels of resistance to heat and lysozyme treatment (data not shown).

YwcE is required for normal DPA accumulation. The presence of phase-dark spores in cultures of the *ywcE* mutant could be due to increased levels of core water. DPA-free spores show higher levels of core water and are less heat resistant than wild-type spores (42, 43). Since we found no impairment in the expression of at least one σ^K -governed gene (data not shown) and since the dipicolinate synthetase (SpoVFAB) is expressed under σ^K control (11), we assumed that the mutant cells were capable of producing DPA but could accumulate it in reduced amounts. We measured the levels of DPA in both the culture supernatants and developing spores of both the wild-type and *ywcE* mutant strains. DPA started to accumulate in wild-type sporulating cells between hours 4 and 5 of sporulation, and there was a marked increase from hour 6 onwards (Fig. 6). At least until hour 9, DPA remained undetectable in the super-

natant of a wild-type culture. DPA accumulation also began between hours 4 and 5 of sporulation in the mutant, and it also increased from hour 6 onwards (Fig. 6). However, DPA accumulation in the mutant was slower than that in the wild type, and the peak level of DPA (at hour 16) in the mutant was about 40% lower than that in the wild type (Fig. 6). Also, in contrast to the case for the wild type, DPA accumulated in the culture medium in parallel with its accumulation in *ywcE* spores. These results indicate that the mutant produces DPA but loses about 40% of it to the medium. As a consequence, the levels of DPA in *ywcE* mutant spores are reduced relative to those in the wild type. The effect on DPA accumulation caused by the *ywcE* mutation was complemented by ectopic expression of *ywcE-gfp* (data not shown).

The *ywcE* gene is involved in spore germination. Hydrolysis of the spore cortex, which takes place during germination, results from the activity of two cortex-lytic enzymes, SleB and CwlJ, which have redundant roles (1, 6, 8). It is not clear what activates SleB (reviewed in reference 52). However, since wild-

FIG. 6. The *ywcE* mutation affects DPA accumulation. The graph shows DPA accumulation in sporulating cells and culture supernatants of various strains throughout sporulation in DSM. The strains used were as follows: MB24, wild-type cells (filled squares) and supernatant (open squares); and AH630, *ywcE::neo* cells (filled circles) and supernatant (open circles).

type but not *cwlJ* spores are induced to germinate by the addition of exogenous Ca^{2+} -DPA, it seems that this compound directly or indirectly activates CwlJ (42–44). Since *ywcE* spores accumulated reduced levels of DPA, we reasoned that the germination of *ywcE* spores would be less dependent on CwlJ than on SleB. To test this idea, we examined the nutrient-induced germination of spores from strains mutated singly or doubly in *cwlJ*, *sleB*, and/or *ywcE*. *ywcE* spores germinated at a slightly lower rate than wild-type spores in response to L-

alanine and attained a slightly lower final level of germination (Fig. 7). In agreement with previous observations (1, 6, 8), *sleB* and *cwlJ* spores were somewhat affected in both their initial rates of germination with L-alanine and their final levels of germination, and the double mutant was severely affected (Fig. 7). The introduction of a *ywcE* mutation into *sleB* cells did not greatly affect the phenotype of the *sleB* single mutant. However, spores of the *cwlJ ywcE* double mutant were much more affected in their response to the germinant L-alanine than were spores of either single mutant. Thus, in contrast to our expectations, these results suggest that *ywcE* affects germination independently of CwlJ function. The *ywcE* mutation had no effect on spore germination in response to a mixture of asparagine, glucose, fructose, and potassium and did not change the germination phenotype of *sleB* or *cwlJ* spores in response to this mix.

DISCUSSION

The *ywcE* gene is expressed at the onset of sporulation from a σ^A -type promoter. We base this conclusion primarily on the consensus -10 motif in the *ywcE* promoter and on the observation that σ^A RNA polymerase utilized the *ywcE* promoter in vitro. The -35 region of the *ywcE* promoter deviated significantly from the consensus for σ^A -type promoters. However, the *ywcE* promoter appears to have an extended -10 region, which would presumably compensate for a weaker interaction of the polymerase with the -35 promoter element (22). Transcription of the *ywcE* gene is repressed during growth by the transition-state regulator AbrB (29, 54), and Spo0A-dependent repression of *abrB* at the onset of sporulation results in the activation of *ywcE* transcription in predilethelial cells.

ywcE mutant spores were found to accumulate reduced levels of DPA, part of which was lost to the medium. DPA-free

FIG. 7. The *ywcE* mutation affects spore germination. The graphs show the change in optical density associated with germination in response to L-alanine of spores produced by the following strains: for panel A, MB24 (wild type) (open squares), AH630 (*ywcE::neo*) (filled circles), AH3443 ($\Delta cwlJ::tet$) (open triangles), and AH3445 ($\Delta ywcE::neo \Delta cwlJ::tet$) (open diamonds); for panel B, MB24 (wild type) (open squares), AH3442 ($\Delta sleB::sp$) (filled circles), AH3444 ($\Delta ywcE::neo \Delta sleB::sp$) (open triangles), and AH3433 ($\Delta sleB::sp \Delta cwlJ::tet$) (open diamonds). Germination was monitored for 60 min, and samples were collected every 5 or 10 min for absorbance readings at 580 nm.

spores show reduced heat resistance (43), but *ywcE* spores were heat resistant. We infer that the decrease in DPA accumulation is not sufficient to affect the development of spore heat resistance. The reduced accumulation of DPA in the mutant is intriguing. One possibility is that YwcE is required to facilitate the localization to the cortex region of a factor (or factors) required for normal formation of the spore cortex and that in its absence the altered cortex structure is not able to promote the normal uptake or retention of DPA in the spore core. We speculate that the factors postulated to be controlled by YwcE could be proteins that are involved in both cortex and coat assembly because *ywcE* spores also show a defective coat structure. There are several proteins that associate with the coat layers that appear to have roles in spore cortex synthesis or degradation during spore germination. For instance, CwlJ, one of the two major cortex-lytic enzymes, and YaaH, which shares sequence similarity with CwlJ and other cortex-lytic enzymes, are both coat components (2, 34). It is not currently known whether the assembly or function of these or other proteins is controlled by YwcE. CwlJ and SleB have redundant roles in cortex hydrolysis during spore germination (1, 6, 8). CwlJ can be activated by either endogenous DPA, released from the spore core during the initial stages of germination, or exogenous DPA. However, a mutation in the *ywcE* gene exacerbated the germination phenotype of a *cwlJ* mutant specifically in response to L-alanine. It is not known what triggers SleB activity, although it has been suggested that the enzyme could only be active on peptidoglycan with a higher degree of stress than that found in dormant spores (52). The expression of *ywcE* was found to be important for germination with L-alanine in the absence of CwlJ, suggesting that *ywcE* may be required for the localization or function of SleB or that *ywcE* somehow affects the structure of the peptidoglycan that serves as a substrate for SleB. However, we cannot exclude the possibility that other alterations in the spore morphology of the *ywcE* mutant, such as the abnormal coat structure, affect SleB function.

The *B. subtilis ywcE* gene encodes a protein with features of the class I holins, of which the S protein from phage λ is a prototype member (18). YwcE is an 83-residue protein with three transmembrane domains and a highly charged C-terminal tail. Moreover, YwcE has a dual start motif, which plays a role in the regulation of class I or class II holins (3, 18, 59). However, YwcE differs from the λ S protein in two ways. First, the residue between the second and third methionines in YwcE is acidic (aspartate), whereas in the S protein the two methionines are separated by a basic (lysine in λ S) residue (Fig. 1C). However, the phage P1 holin LydA also contains an aspartate near its N terminus, and the holin Hol118 from the *Listeria monocytogenes* phage A118 contains an acidic residue between its two start codons (59). Second, YwcE is likely to have its N terminus on the outside of the membrane and its C terminus in the cytoplasm, whereas for λ S the N terminus is predicted to face the cytoplasm (18). We base our prediction for the membrane topology of YwcE on the observation that the fusion of the C-terminal region of YwcE to GFP results in a functional protein (at least when the accumulation of DPA is assessed as a functional criterion [see below]) which decorates both the mother cell and prespore membranes (Fig. 5). GFP does not fold properly when attached to periplasmic domains of inner membrane proteins in *E. coli* and hence has been used to

discriminate between cytoplasmic and periplasmic domains (12). Because YwcE has only three predicted transmembrane domains, the only way for the C-terminally fused GFP moiety to remain in the cytoplasm is by assuming an N terminus-out, C terminus-in topology (Fig. 1C). We suspect that the different topology of YwcE from that of the λ S protein may be related to the particular arrangement of the prespore membranes, but since the exact role of YwcE in sporulation is currently unknown, this assumption remains to be tested. It is not known whether YwcE functions as a holin. We have constructed a fusion of the IPTG-dependent *Pspac* promoter to the *ywcE* gene but found no IPTG-dependent cell lysis during growth or sporulation (not shown). In addition, we found no delay in lysis of the mother cell during the late stages of sporulation for the *ywcE* mutant (not shown). The activity of holins is usually inhibited by the presence of a homologous antiholin (59). In the λ system, the antiholin, which inhibits holin activity, is the longer product of the S gene (S107), whereas the shorter protein (S105), which is the protein that initiates at the second methionine of the dual start motif, is the holin (reviewed in reference 59). However, there are reports of holins and their cognate antiholins being encoded by different chromosomal loci. For example, the *lrgA* gene of *Staphylococcus aureus* functions in a manner analogous to an antiholin, presumably controlling the activity of a holin encoded by the *cidA* gene (19, 48). Because of the similarity between holins and antiholins, we cannot exclude the possibility that the *ywcE* gene encodes an antiholin for which the cognate holin is unknown. If *ywcE* is an antiholin, then the phenotypes reported herein are due to uncontrolled activity of an as yet unknown holin. Alternatively, it is possible that the structural similarity of YwcE to holins and their antiholins is fortuitous and that the role of YwcE in sporulation is unrelated to a pore-forming capacity.

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