

Unmasking Novel Sporulation Genes in *Bacillus subtilis*

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The *Bacillus subtilis* transcription factor σ^E directs the expression of a regulon of 262 genes, but null mutations in only a small fraction of these genes severely impair sporulation. We have previously reported that mutations in seven σ^E -controlled genes cause a mild (2- to 10-fold) defect in sporulation. In this study, we found that pairwise combinations of some of these seven mutations led to strong synthetic sporulation phenotypes, especially those involving the *ytrHI* operon and *ybaN*. Double mutants of *ybaN* and *ytrH* and of *ybaN* and *ytrI* had >10,000-fold lower sporulation efficiencies than the wild type. Thin-section electron microscopy revealed a block in cortex formation for the *ybaN ytrH* double mutant and coat defects for the *ybaN* single and *ybaN ytrI* double mutants. Sporulating cells of a *ybaN ytrI* double mutant and of a *ybaN ytrHI* triple mutant exhibited a pronounced loss of dipicolinic acid (DPA) between hours 8 and 24 of sporulation, in contrast to the constant levels seen for the wild type. An analysis of the spore cortex peptidoglycans of the *ybaN ytrI* and *ybaN ytrHI* mutants showed striking decreases in the levels of total muramic acid by hour 24 of sporulation. These data, along with the loss of DPA in the mutants, suggest that the developing spores were unstable and that the cortex underwent degradation late in sporulation. The existence of otherwise hidden sporulation pathways indicates that functional redundancy may mask the role of hitherto unrecognized sporulation genes.

A central challenge in the field of microbial development is to identify and characterize the functions of all of the genes that govern morphogenesis during cellular differentiation. An attractive system with which to address this challenge is the process of sporulation in the bacterium *Bacillus subtilis*, in which a growing cell is transformed via a series of well-defined morphological stages into a dormant cell known as a spore (or, more properly, an endospore) (23, 30). Soon after the commitment to sporulate, a two-chamber cell is formed, consisting of a large compartment called the mother cell and a small compartment called the forespore. Initially, the mother cell and the forespore lay side by side, but at an intermediate stage of development the forespore is wholly engulfed by and therefore resides within the mother cell (23). The engulfed forespore is surrounded by two membranes, one derived from the forespore and the other derived from the mother cell membrane that engulfs the forespore. Later in development, a thick layer of peptidoglycan known as the cortex is deposited in the space between the two membranes (24). Distinctive features of the cortex include the modified sugar muramic δ -lactam and a reduced level of muramic acid residues with peptide cross-links to other glycan chains (10). At approximately the same time as cortex formation, a thick protein shell is deposited around the outside of the forespore to create the spore coat (5). Ultimately, the mature spore is released by lysis of the mother cell.

Classical genetic as well as more recent genomic approaches

have made it possible to identify genes that are activated during sporulation on a genome-wide scale. Strikingly, only a small subset of these genes appear to be essential for sporulation, based on analyses of null alleles (7, 8, 13, 26, 35). Most likely, in many cases the apparent lack of a sporulation phenotype is due to the mutations having effects that are undetectable under laboratory conditions but significant in the wild. However, it is also the case that the roles of some genes are largely masked by other genes. In other words, to observe the phenotype of a mutation in a given gene, a second mutation is required. An example of this type of redundancy is provided by the genes *spoIIB* and *spoVG*, in which mutations only mildly impair sporulation on their own but have a severe impact in combination (i.e., they have a synthetic phenotype) (18). Another synthetic effect involves a double mutant of *cwlC* and *cwlH* in which mother cell lysis is strongly blocked, while the corresponding single mutants exhibit little defect (21). A third example involves the activation of the forespore-specific transcription factor σ^F , which is governed by two independent regulatory pathways that are largely redundant (6).

In an effort to determine whether functional redundancy masks the role of other developmentally regulated genes in *B. subtilis*, we focused on genes under the control of the mother-cell-specific regulatory protein σ^E . The σ^E factor directs the transcription of an unusually large regulon, consisting of 262 genes, which are organized into 163 transcription units (excluding genes and operons that are activated under the dual control of σ^E and the DNA-binding protein SpoIIID) (8). We recently created null mutations in 98 newly identified genes and operons in the regulon. Strikingly, in only three cases did the mutation cause a severe defect in sporulation. In seven cases, however, the mutation caused a slight (2- to 10-fold) but reproducible defect in the efficiency of sporulation, as judged

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TABLE 1. Strains and plasmids used for this study

Strain or plasmid	Genotype	Source or Reference
<i>B. subtilis</i> strains		
PY79	Wild type	34
PS832	Prototrophic revertant of strain 168	D. Popham
JS31	<i>ypjBΔ::spc</i>	This study
JS32	<i>ytrIΔ::spc</i>	This study
JS33	<i>yunBΔ::kan</i>	This study
JS35	<i>ypjBΔ::spc::cat</i> (pDAG32)	This study
JS37	<i>ybaNΔ::tet</i>	This study
JS38	<i>ytvIΔ::tet</i>	This study
JS39	<i>ytrHΔ::spc</i>	This study
JS40	<i>ytrIΔ::erm</i>	This study
JS42	<i>ytrH ytrIΔ::spc</i>	This study
JS43	<i>ybaNΔ::tet ytvIΔ::erm</i>	This study
JS44	<i>ytrIΔ::spc ypjBΔ::cat</i>	This study
JS45	<i>ytrIΔ::spc yhbHΔ::erm</i>	This study
JS46	<i>ytrIΔ::spc ytvIΔ::tet</i>	This study
JS47	<i>ytrIΔ::spc ybaNΔ::tet</i>	This study
JS48	<i>ypjBΔ::spc ytvIΔ::tet</i>	This study
JS49	<i>ypjBΔ::spc yhbHΔ::erm</i>	This study
JS50	<i>ypjBΔ::spc ybaNΔ::tet</i>	This study
JS51	<i>yhbHΔ::spc</i>	This study
JS52	<i>ypjBΔ::spc yunBΔ::kan</i>	This study
JS53	<i>yhbHΔ::spc ytvIΔ::tet</i>	This study
JS54	<i>ytrIΔ::tet ytrHΔ::spc</i>	This study
JS55	<i>yhbHΔ::spc yunBΔ::kan</i>	This study
JS56	<i>ypjBΔ::cat ytrHΔ::spc</i>	This study
JS57	<i>ybaNΔ::tet ytrHΔ::spc</i>	This study
JS58	<i>yhbHΔ::spc ybaNΔ::tet</i>	This study
JS59	<i>yhbHΔ::erm</i>	This study
JS60	<i>yunBΔ::kan ytrHΔ::spc</i>	This study
JS61	<i>yhbHΔ::erm ytrHΔ::spc</i>	This study
JS62	<i>yunBΔ::kan ybaNΔ::tet</i>	This study
JS63	<i>yunBΔ::kan ytvIΔ::tet</i>	This study
JS69	<i>ytrIΔ::spc yunBΔ::kan</i>	This study
JS162	<i>ytrH ytrIΔ::spc amyE::P_{ytrH}-ytrH (cat)</i>	This study
JS163	<i>ytrH ytrIΔ::spc amyE::P_{ytrH}-ytrI (cat)</i>	This study
JS164	<i>ytrH ytrIΔ::spc amyE::ytrHI (cat)</i>	This study
JS176	<i>ybaNΔ::tet ytrIΔ::spc amyE::P_{ytrH}-ytrI (cat)</i>	This study
JS177	<i>ybaNΔ::tet ytrHΔ::spc amyE::P_{ytrH}-ytrI (cat)</i>	This study
JS224	<i>ybaNΔ::tet amyE::P_{ybaN}-gfp-ybaN (cat)</i>	This study
JS225	<i>ytrIΔ::spc amyE::P_{ytrH}-gfp-ytrI (cat)</i>	This study
JS233	<i>ybaNΔ::tet amyE::P_{ybaN}-gfp-ybaN (cat) cotE::erm</i>	This study
JS234	<i>ybaN::tet amyE::P_{ybaN}-gfp-ybaN (cat) spoIVA::erm</i>	This study
PE477	<i>ytrH-gfp</i>	8
DPVB293	[PS832] <i>ytrI::spc</i>	This study
DPVB296	[PS832] <i>ybaN::tet</i>	This study
DPVB297	[PS832] <i>ytrH ytrI::spc</i>	This study
DPVB298	[PS832] <i>ytrI::spc ybaN::tet</i>	This study
DPVB299	[PS832] <i>ytrH ytrI::spc ybaN::tet</i>	This study
<i>E. coli</i> strain		
DH5α	Cloning host	Laboratory stock
Plasmids		
pDG364	Permits recombination into the chromosome at <i>amyE</i>	12
pDAG32	Converts <i>spc</i> to <i>cat</i>	Laboratory stock; similar to that discussed in reference 28
pJS1	pDG364 with <i>ytrH</i>	This study
pJS2	pDG364 with <i>ytrHI</i>	This study
pJS37	pDG364 with P _{ytrH} <i>ytrI</i>	This study
pJS58	pDG364 with P _{ybaN} <i>gfp ybaN</i>	This study
pJS59	pDG364 with P _{ytrH} <i>gfp ytrI</i>	This study
pJS60	pDG364 with <i>gfp ybaN</i>	This study
pJS61	pDG364 with <i>gfp ytrI</i>	This study
pPE56	pCV0119 with <i>ytrH</i>	8, 31
pCV0119	Permits Campbell integration of C-terminal GFP	8

by the production of heat-resistant spores. We wondered whether the impact on sporulation of some of these seven genes is partially masked by redundancy with one or more other genes under the control of σ^E . As a test of this idea, we created strains bearing mutations in pairs of the seven genes. Here we report that certain pairwise combinations of mutations resulted in striking synthetic phenotypes in which cortex and coat formation were severely impaired.

MATERIALS AND METHODS

Strain construction. The *B. subtilis* strains used for this study are listed in Table 1. Standard techniques were used for strain construction (11). All deletion strains were generated by the technique of long-flanking-homology PCR (33). Chromosomal DNAs obtained from these strains were analyzed by PCR to confirm the integration of the resistance cassette at the expected locus. The sequences of the primers used for the constructions are available upon request. To obtain strains that were doubly mutant, we transformed chromosomal DNA from one mutant strain into competent cells containing a replacement of a different gene with a second antibiotic cassette. The marker for JS31 was switched from spectinomycin to chloramphenicol by using plasmid pDAG32. The chromosomal DNAs of the mutants were transformed into the PS832 background for peptidoglycan analysis experiments.

gfp fusion genes were generated by in-frame fusions to appropriate coding sequences. 3' *gfp* fusions were obtained by a Campbell-type (single reciprocal) recombination of plasmids generated by cloning XhoI- and BamHI-digested PCR fragments of the gene of interest into pCV0119, which carries *gfp* (8, 31). 5' *gfp* fusions were constructed by placing the appropriate promoter in front of *gfp*, which in turn was fused in frame with the corresponding gene. The *gfp* fragment used for the 5' fusion constructs was amplified by PCR from pKL47 (16) and digested with HindIII and XhoI. The PCR fragments for the open reading frames were digested with XhoI and BamHI and were ligated to *gfp* and pDG364 digested with HindIII and BamHI to create pJS60 and pJS61. pJS60 and pJS61 and the promoter PCR fragments were digested with EcoRI and HindIII and ligated to obtain pJS58 and pJS59. All plasmid constructions were performed in *Escherichia coli* DH5 α by standard methods.

Sporulation efficiency. One colony was inoculated into 5 ml of Difco sporulation (DS) medium (27). After growth for 30 h at 37°C, the number of spores was determined by heat killing for 10 min at 80°C.

Phase-contrast and fluorescence microscopy. For phase-contrast microscopic observations, one colony from each mutant was inoculated into 5 ml of DS medium at 37°C overnight. A 0.3-ml sample of each culture was centrifuged briefly and resuspended in 10 μ l of 1 \times phosphate-buffered saline. For fluorescence microscopic observations, strains were grown in hydrolyzed casein growth medium at 37°C and induced to sporulate by the resuspension method (11, 29). At the appropriate times, 0.3-ml aliquots were centrifuged and resuspended in 10 μ l of 1 \times phosphate-buffered saline supplemented with the membrane stain FM4-64 (see Fig. 2) or FM1-43 (for engulfment experiments [not shown]) (Molecular Probes) at a final concentration of 1.5 μ g ml⁻¹. Three-microliter samples of concentrated cell suspensions were placed on microscope slides and immobilized with poly-L-lysine-treated coverslips.

Electron microscopy. Cultures were induced to sporulate by exhaustion in DS medium at 37°C and samples were fixed after 24 h of sporulation. Thin-section electron microscopy was performed as previously described by Catalano et al. (3).

Spore peptidoglycan analysis. Analyses of spore peptidoglycan synthesis and structure were performed in the *B. subtilis* strain PS832 background to maximize sporulation synchronization and peptidoglycan recovery. Mutations were introduced into the PS832 background as previously described (1) and sporulated by nutrient exhaustion in 2 \times SG medium (15) in the absence of antibiotics. Alkaline phosphatase and dipicolinic acid (DPA) accumulation were assayed as previously described (20). Total muramic acid accumulation was determined by an amino acid-sugar analysis, and the forespore peptidoglycan structure was quantified after muramidase digestion and high-performance liquid chromatography separation of the muropeptides as previously described (19).

RESULTS AND DISCUSSION

Synergy between mutations in σ^E -controlled genes. Twenty-one strains were created that were doubly mutant for all possi-

TABLE 2. Synergistic effects of pairs of mutations on sporulation efficiency

Genotype	Predicted sporulation efficiency ^b	Actual sporulation efficiency ^c	Synergistic effect ^d
WT ^a		1.0	
<i>yunB</i>		0.5	
<i>ytrI</i>		0.29	
<i>yhbH</i>		0.25	
<i>ypjB</i>		0.25	
<i>ytrI</i>		0.14	
<i>ybaN</i>		0.12	
<i>ytrH</i>		0.11	
<i>yhbH ytrI</i>	0.07	0.15	0.5
<i>yhbH yunB</i>	0.13	0.19	0.7
<i>ytrH ytrI</i>	0.02	0.05	0.4
<i>ypjB yunB</i>	0.13	0.12	1
<i>yhbH ytrH</i>	0.03	0.03	1
<i>ypjB yhbH</i>	0.06	0.04	1.5
<i>yunB ytrI</i>	0.15	0.13	1.2
<i>yunB ybaN</i>	0.06	0.02	3
<i>yunB ytrH</i>	0.06	0.02	3
<i>ypjB ytrH</i>	0.03	0.01	3
<i>ytrI yhbH</i>	0.07	0.018	4
<i>yhbH ybaN</i>	0.03	0.005	6
<i>ytrI ytrI</i>	0.04	0.003	13
<i>ytrI ytrH</i>	0.03	0.002	15
<i>ytrI yunB</i>	0.07	0.004	18
<i>ytrI ytrI</i>	0.04	0.002	20
<i>ypjB ytrI</i>	0.07	0.003	23
<i>ypjB ybaN</i>	0.03	0.001	30
<i>ybaN ytrI</i>	0.04	0.0008	50
<i>ybaN ytrH</i>	0.01	0.00006	167
<i>ytrI ybaN</i>	0.02	0.00003	667
<i>ytrH ytrI ybaN</i>	0.006	0.00006	100

^a WT (wild type) is strain PY79.

^b The product of the sporulation efficiencies observed for the corresponding single mutants of the two indicated genes.

^c Ratio of heat-resistant spores produced by the indicated mutant, after 30 h of culturing at 37°C, to those produced by the wild type. Values reported are the average of at least three experiments.

^d The predicted sporulation efficiency divided by the observed sporulation efficiency.

ble pairs of the seven null mutations (Table 2). We reasoned that pairs of mutations for genes with redundant functions would act synergistically to impair sporulation. Synergy was defined as a sporulation efficiency that was ≥ 10 -fold lower for a double mutant than that predicted by the simple product of the sporulation efficiencies of the two corresponding single mutants (6). By this criterion, 9 of the 21 pairs of mutations exhibited a synergistic effect (Table 2). For two cases (both involving *ybaN*), the impairment caused by two mutations was >2 (mutations of *ybaN* and *ytrH*) and almost 3 (mutations of *ybaN* and *ytrI*) orders of magnitude more severe than that predicted from the product of the effects of the two single mutations. In both cases, the double mutants exhibited a $>10,000$ -fold defect in sporulation compared to the wild type. A triple mutant of all three genes exhibited a similarly severe defect in sporulation. Henceforth, we restrict our analysis to the interaction of *ybaN* with *ytrH* and *ytrI*.

Because *ytrH* is directly upstream of and in an operon with *ytrI*, it was possible that the strong sporulation defect of the *ybaN ytrH* double mutant was due to a polar effect. Therefore, we inserted a copy of *ytrI* that had been joined to the promoter for the operon into the chromosome at the *amyE* locus (re-

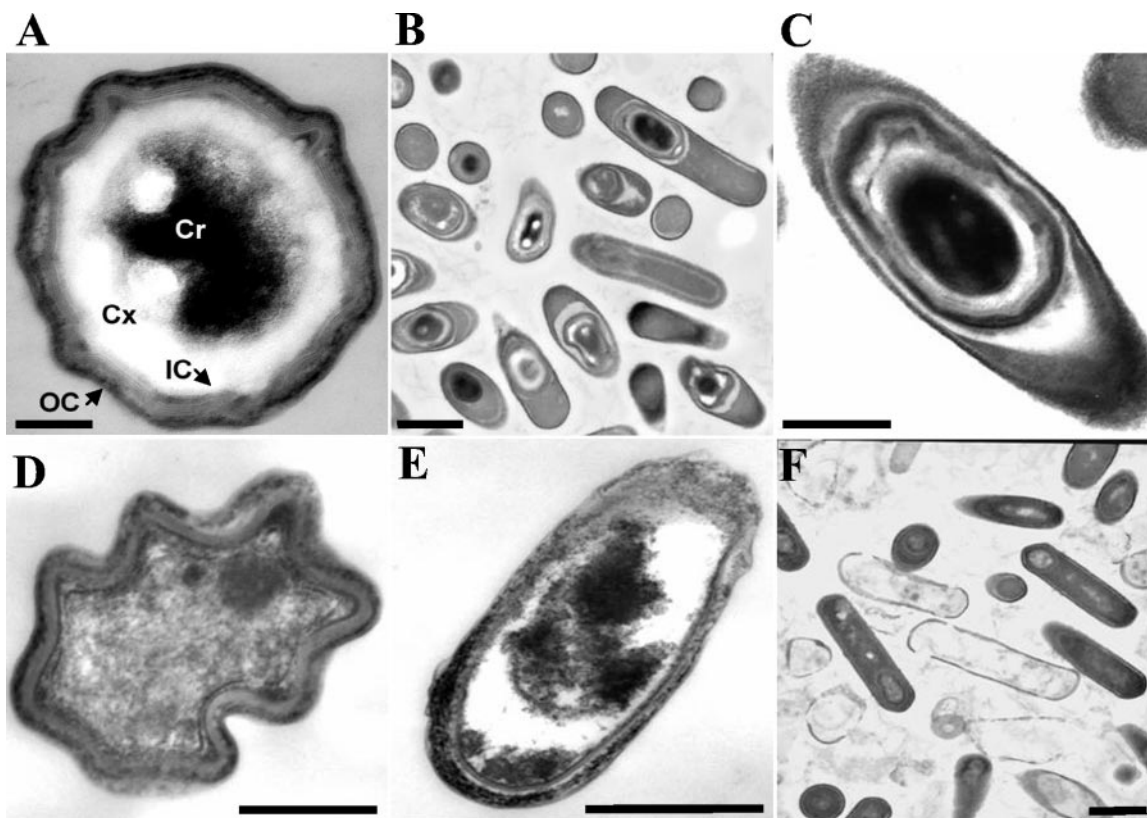


FIG. 1. Thin-section electron micrographs of mutant spores. Cells were fixed after 24 h of sporulation. (A) PY79 (wild type); (B) JS32 (*ytrI::Spc*); (C) JS42 (*ytrH ytrI::Spc*); (D) JS37 (*ybaN::Tet*); (E) JS47 (*ybaN::Tet ytrI::Spc*); (F) JS57 (*ybaN::Tet ytrH::Spc*). OC, outer coat; IC, inner coat; Cx, cortex; Cr, core. Bars, 0.125 μm (A), 1 μm (B and F), 0.5 μm (C and E), and 0.25 μm (D).

sulting in strains JS176 and JS177) to test for its capacity to complement the sporulation defects of the *ybaN ytrH* and *ybaN ytrI* double mutants. The results showed that the presence of *ytrI* at *amyE* substantially alleviated the sporulation defect of the *ybaN ytrI* double mutant but not that of the *ybaN ytrH* double mutant. This finding indicates that the severe sporulation defect of the *ybaN ytrH* double mutant was not due to a polar effect, at least not exclusively so. Also, the mild sporulation defect of a deletion of the entire *ytrHI* operon was alleviated by the presence of a copy of the entire operon at *amyE* (in strain JS164) but not by the presence of *ytrI* at *amyE*. We concluded that both *ytrH* and *ytrI* contribute to proper sporulation and interact genetically with *ybaN*.

To investigate the nature of the sporulation defects caused by single and double mutants of *ybaN*, *ytrH*, and *ytrI*, we performed phase-contrast and fluorescence microscopy. Phase-contrast microscopy revealed that the *ybaN* and *ytrI* single mutants and the *ytrHI* operon mutant were all capable of producing phase-bright spores, but at efficiencies of only about 10 to 20% that of the wild type. In contrast, the *ybaN ytrH* and *ybaN ytrI* double mutants produced <1% phase-bright spores. As spores develop, they first appear dark gray by phase-contrast microscopy (phase dark), but as the cortex is deposited the spores then begin to refract light and appear bright white (phase bright), which indicates spore maturation. In the case of the *ybaN ytrI* double mutant (but rarely in the case of the *ybaN ytrH* mutant), some immature spores could be detected that

appeared phase dark and were generally not released from the sporangium. When examined by fluorescence microscopy after treatment with the vital membrane stain FM1-43, none of the mutants, including the two double mutants, exhibited a conspicuous defect in asymmetric division or in engulfment. In toto, these results indicate that the strong block in sporulation that occurs in the absence of *ybaN* and either *ytrH* or *ytrI* takes place after engulfment.

Mutant spore morphology. We examined the morphology of the mutant spores by a thin-section electron microscopic analysis of cells after 24 h of sporulation. The *ytrI* and *ytrHI* operon mutants produced free, mature spores that resembled those of the wild type. However, in contrast to the wild type, the mutants exhibited many sporangia in which spores were still sequestered in the mother cell (Fig. 1B and C). These sporangia were at various stages of development between stage IV, the point at which the cortex is first detectable but the coat is not yet present, and full maturity.

Sporulating cells of the *ybaN* mutant exhibited several defects. First, similar to the observations with the *ytrI* and *ytrHI* mutants, even after 24 h of sporulation a significant number of sporangia had failed to release their prespores, many of which had not yet assembled coats. Unlike the *ytrI* and *ytrHI* mutants, however, *ybaN* mutant spores had defective outer coats. In contrast to wild-type spores, whose outer coats are composed of one or more uniformly dark-staining layers (Fig. 1A), *ybaN* mutant spore coats were thin and appeared mottled. Most

TABLE 3. Analysis of spore peptidoglycans

Strain	% of muramic acid with indicated side chains ^a								% of muramic acid with cross-links		% Total muramic acid	
	δ -Lactam		Ala		TriP		TP		T_8	T_{24}	T_8	T_{24}
	T_8^c	T_{24}^c	T_8	T_{24}	T_8	T_{24}	T_8	T_{24}				
WT ^b	45.9	48.3	25.9	26.8	2.8	2.1	25.4	22.9	3.0	2.7	70.3	71.9
<i>yrI</i>	46.2	46.8	26.9	27.5	3.8	3.5	23.1	22.1	3.1	2.7	45.3	17.8
<i>ybaN</i>	41.2	42.4	18.4	16.7	6.1	7.4	34.2	33.5	3.1	3.2	30.5	2.7
<i>yrHI</i>	44.2	45.8	25.3	26.7	4.9	4.2	25.5	23.2	3.4	2.9	44.8	18.4
<i>ybaN</i> , <i>yrI</i>	40.4	35.5	20.6	20.4	7.7	16.5	31.4	27.6	3.2	3.9	26.2	0.9
<i>ybaN</i> , <i>yrHI</i>	41.1	35.3	19.0	16.3	7.0	16.2	32.9	32.2	3.1	3.5	21.6	0.4

^a Abbreviations: TriP, tripeptide; TP, tetrapeptide.

^b WT, wild type (PS832).

^c T_8 and T_{24} , 8 and 24 h after sporulation, respectively.

strikingly, however, a significant number of released *ybaN* mutant spores possessed ridges that were much more deeply folded than those of wild-type spores (Fig. 1D). This could have been due to defects in either the coat or the cortex. Finally, the *ybaN yrI* double mutant resembled the *ybaN* single mutant, producing immature spores with defective coats (Fig. 1E). Many of the *ybaN yrH* double mutant cells appeared to have lysed during sporulation, and the remainder were blocked at the stage of forespore engulfment prior to cortex synthesis (Fig. 1F). These data corroborate the sporulation blocks seen with phase-contrast and fluorescence microscopy.

Spore peptidoglycan analysis. To investigate whether the mutants were defective in cortex formation, we analyzed muramic acid accumulation and peptidoglycan structures during sporulation. Before performing this analysis, we confirmed that the time course of sporulation for the mutants was similar to that for the wild type by measuring the onset of the accumulation of alkaline phosphatase, a marker for early sporulation, and DPA, a marker of late sporulation. All of the mutants produced alkaline phosphatase with the same timing and abundance as the wild type. All of the mutants also accumulated DPA to wild-type levels after 4 to 7 h of sporulation, but from 8 to 24 h, the level of DPA in the mutants decreased whereas that of the wild type remained approximately constant. The loss of DPA was most severe for the *ybaN yrHI* triple and *ybaN yrI* double mutants, which exhibited a sixfold decrease in DPA levels relative to the wild type at 24 h, a finding that might indicate that the mutants produced unstable spores that were unable to retain their cytoplasmic contents.

Next, cells were collected from hours 2 to 8 and at hour 24 of sporulation and were analyzed for their peptidoglycan abundance and composition. The muramic acid contents and spore peptidoglycan structures of the mutants were similar to those of the wild type from hours 2 to 6 of sporulation, at which time the wild type had produced about 50% of its total spore peptidoglycan content (data not shown). From hours 6 to 8 of each sporulation, the mutant strains exhibited defects in spore peptidoglycan accumulation. The *yrI* and *yrHI* mutants accumulated >60%, the *ybaN* mutant accumulated >40%, and the *ybaN yrI* double mutant and the *ybaN yrHI* triple mutant accumulated <40% of the amount of muramic acid accumulated by the wild type. In each case, the structure of the spore peptidoglycan was similar to that of the wild type, except for slight decreases in the percentages of muramic acid in the

lactam form and with single L-Ala side chains and slight increases in the percentages of muramic acid residues with tripeptide side chains (Table 3). These structural changes are consistent with a decreased synthesis or degradation of spore peptidoglycan, leaving a larger percentage of the spore peptidoglycan as the germ cell wall (19). After 24 h, the *yrI* and *yrHI* mutants exhibited ~25% and the *ybaN* mutant exhibited 4% of the amount of muramic acid accumulated by the wild type. The most striking results were observed at 24 h for the *ybaN yrI* double mutant and the *ybaN yrHI* triple mutant, which exhibited <2% of the amount of muramic acid accumulated by the wild type. In toto, the results shown in Table 3 suggest that the double mutants do make a spore cortex with a relatively normal structure but that the developing spores are unstable and the cortex is degraded late in sporulation.

Subcellular localization of YbaN. The effects of *yrH*, *yrI*, and *ybaN* mutations on cortex stability raised the question of whether any of the three gene products are localized in the mother cell membrane that surrounds the engulfed forespore. Indeed, the predicted amino acid sequences of all three gene products are consistent with the idea that they are membrane proteins. To investigate the subcellular localization of the proteins, we attempted to create functional, in-frame fusions of the genes to *gfp*. We found that fusions of *gfp* to the 5' or 3' end of *yrI* or to the 3' end of *yrH* were nonfunctional (data not shown). However, a fusion of *gfp* to the 5' end of *ybaN* was functional. We initially detected fluorescence from this fusion after 2 h of sporulation, and it ultimately appeared as a ring around the forespore (Fig. 2). Since YbaN is synthesized only in the mother cell (as a result of σ^E -dependent expression of this gene), we inferred that YbaN is associated with, or very close to, the outer forespore membrane. This localization was not dependent on SpoIVA or CotE, which are morphogenetic proteins that are required for the assembly of the spore coat around the outer forespore membrane (5). Since a *spoIVA* mutation results in the release of the entire coat from the forespore (22), we took the lack of dependence of YbaN assembly on *spoIVA* to indicate that YbaN is not a coat protein. There are several other SpoIVA-independent, outer forespore-associated proteins that affect spore formation, including SpoVM (17, 25, 32), YabP, and YabQ (2, 31). The expression of *ybaN* and *yrHI* is under the negative control of SpoIID, which is the case for most integral membrane proteins under σ^E control for

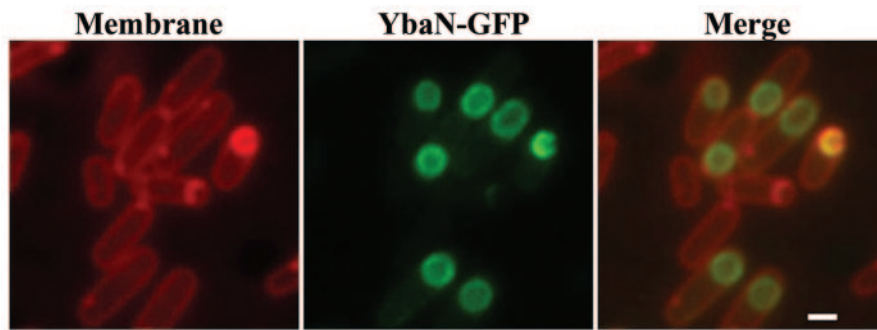


FIG. 2. Subcellular localization of GFP-YbaN. Cells were collected after 3 h of sporulation at 37°C, treated with the vital membrane stain FM4-64, and observed by fluorescence microscopy. Bar, 1 μ m.

which the subcellular localization has been determined (7). A gel shift analysis has confirmed that SpoIIID binds to the promoter of *ybaN* (7). We concluded that YbaN is located in the outer forespore membrane, from which it may participate in the formation or stability of the spore cortex.

Protein homology. To look for clues about the functions of *ytrH*, *ytrI*, and *ybaN*, we searched the databases for proteins with significant similarities to the inferred products of these genes. All three genes have orthologs in other endospore-forming bacteria (8), but YtrH and YtrI exhibited little similarity to the products of other genes in these species. Interestingly, orthologs for *ytrH* and *ytrI* were found only in the *Bacillus* genus and not in *Clostridium*. In all of the currently published genomes for endospore-forming bacteria, both genes are always present in tandem, suggesting that they may be functionally related. We also noted that the inferred stop codon (TGA) for the *ytrH* open reading frame in *B. subtilis* overlaps with the inferred start codon (ATG) for *ytrI* (the overlapping sequence being ATGA), which suggests that translation of the two open reading frames is coupled. If so, this might indicate that the levels of synthesis of YtrH and YtrI are closely coordinated and perhaps that the two proteins interact with each other.

Whereas YtrH and YtrI are not significantly similar to other proteins in the databases, YbaN is paralogous to a known polysaccharide deacetylase in *B. subtilis*, the *pdaA* gene product. *pdaA* is a developmentally regulated gene that is transcribed in the forespore under the control of σ^G (9) and is required for the production of essentially all muramic δ -lactam, which is unique to the spore cortex and serves as a specificity determinant for autolytic enzymes that perform cortex degradation during spore germination (10). The similarity of YbaN to a polysaccharide deacetylase, as well as its sequestration to the outer forespore membrane, suggests that it has some role in spore cortex formation. However, the subtle phenotype of a *ybaN* mutation indicates a function distinct from that of *pdaA*.

Nomenclature. In toto, the results of this study indicate that when they are present individually, mutations of *ybaN* and of the *ytrHI* operon impair sporulation at a late stage (VI) (22) of morphogenesis. In other words, the mutations did not block cortex or coat synthesis but did affect the stability of the former and the structure of the latter in the case of the *ybaN* mutation. On this basis, and following the nomenclature of Piggot and Coote (22), the most recent compilation of *spo* genes (23), and a recent assignment of a *spoVIF* gene

(14), we propose the designations *spoVIE* for *ybaN* and *spoVIGA* and *spoVIGB* for *ytrH* and *ytrI*, respectively. Of course, as we have seen, pairwise combinations of mutations in *ybaN* (*spoVIE*) with mutations in either *ytrH* (*spoVIGA*) or *ytrI* (*spoVIGB*) severely impaired the production of heat-resistant spores and hence exhibited a block in sporulation at an earlier stage than stage VI.

Conclusions. The σ^E factor directs the transcription of an especially large regulon, but mutations in relatively few (<20%) members of the regulon cause a severe defect in sporulation. The principal contribution of the present work is the demonstration that some genes in which a mutation causes only a very mild defect in development do in fact play critical roles in sporulation but that these roles are normally masked by redundancy with other genes. Thus, certain pairwise combinations of mutations revealed strong synthetic defects in sporulation. We presume that in these cases the genes in question function in separate pathways that contribute independently to a common step in morphogenesis. Thus, for example, we infer that *ybaN* (*spoVIE*) on the one hand and *ytrH* (*spoVIGA*) and *ytrI* (*spoVIGB*) on the other hand contribute independently to some common aspect of cortex formation or stability. It is conceivable that the functions of other genes in the σ^E regulon are also masked by redundancy. A test of this will require the large-scale creation of all possible double mutants for all members of the regulon for which a mutant phenotype was not observed. We note that describing a gene as redundant does not necessarily imply that it is dispensable but rather that laboratory assays are insufficient to detect its role. This is particularly likely in the case of the cortex, a complex structure that is essential to spore survival. Even a small increase in its protective ability (as may hypothetically be contributed by YbaN [SpoVIE]) is likely to be readily selected in the environment. Complex synergistic interactions between sporulation genes are not restricted to those involved in cortex formation. For example, analyses of strains bearing multiple coat protein gene mutations revealed synergistic interactions that would not be predicted from the corresponding single gene mutations (4). Most likely, more novel functions for sporulation genes, both those that were recently identified and those that have been previously characterized, remain to be revealed by the creation of multiply mutant strains.

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