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Role of Spore Coat Proteins in the Resistance of *Bacillus subtilis* Spores to *Caenorhabditis elegans* Predation▽

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Bacterial spores are resistant to a wide range of chemical and physical insults that are normally lethal for the vegetative form of the bacterium. While the integrity of the protein coat of the spore is crucial for spore survival in vitro, far less is known about how the coat provides protection in vivo against predation by ecologically relevant hosts. In particular, assays had characterized the in vitro resistance of spores to peptidoglycan-hydrolyzing enzymes like lysozyme that are also important effectors of innate immunity in a wide variety of hosts. Here, we use the bacteriovorous nematode *Caenorhabditis elegans*, a likely predator of *Bacillus* spores in the wild, to characterize the role of the spore coat in an ecologically relevant spore-host interaction. We found that ingested wild-type *Bacillus subtilis* spores were resistant to worm digestion, whereas vegetative forms of the bacterium were efficiently digested by the nematode. Using *B. subtilis* strains carrying mutations in spore coat genes, we observed a correlation between the degree of alteration of the spore coat assembly and the susceptibility to the worm degradation. Surprisingly, we found that the spores that were resistant to lysozyme in vitro can be sensitive to *C. elegans* digestion depending on the extent of the spore coat structure modifications.

Bacilli are soil bacteria that exist in their habitat either as vegetative cells or, more abundantly, as spores (23, 30). The spore is a dormant state that confers in vitro resistance to a broad range of insults that are bactericidal for the vegetative form of bacteria, including extreme temperatures, mechanical stresses, or exposure to chemicals (5). The ability of spores to resist these treatments is at least partially attributed to the spore coat, a proteinaceous layer composed of at least 70 protein species in *Bacillus subtilis* (11). The coat is organized in two distinct layers, an outer coat and an inner coat. The proper assembly of the spore coat requires the expression of at least five morphogenetic proteins (11), including CotE that controls the recruitment and the binding of a large subset of proteins to the coat (36). A Δ*cotE* mutation results in spores that lack the outer coat and frequently display an aberrant inner coat that appears to be disconnected from the outer surface of the forespore (6, 36). Further, the precise timing of CotE expression is critical for the proper recruitment of proteins to the coat during assembly, and delaying CotE expression during sporulation results in spores lacking the outer spore structures (3).

The modifications in the coat structure observed in the Δ*cotE* mutant spores are assumed to be responsible for their sensitivity to peptidoglycan-degrading enzymes, such as hen egg white lysozyme (36). Lysozymes are widely distributed among organisms and are considered effectors of innate immunity in metazoa or as digestive enzymes in unicellular and multicellular eukaryotes (27). In soil, *Bacillus* species encounter different types of predator organisms, including protozoa and bacteriophagous nematodes. Recently, the relevance of in vivo studies in understanding the role of the spore coat structures (15) was demonstrated using the protozoan *Tetrahymena thermophila*. The bacteriovorous nematode *Caenorhabditis elegans* appears to interact with soil bacteria like the endospore-forming *Bacillus cereus* in the wild (9). *C. elegans* expresses and presumably secretes into its intestinal lumen bacteriolytic proteins, including lysozyme-like proteins (19, 20) encoded by numerous genes (>10) in its genome that show homology to either *c*-type, *i*-type, or Ch-type lysozymes (M.-H. Laaberki and J. Dworkin, unpublished data). In the present study, we investigated the fate of *Bacillus subtilis* cells during *C. elegans* predation. We tested the ability of *C. elegans* to ingest and digest vegetative *Bacillus subtilis* cells or spores. In addition, we characterized the role of coat structures in the resistance to *C. elegans* ingestion.

MATERIALS AND METHODS

Care of bacterial strains and *C. elegans* strains. *Bacillus subtilis* strains used in this study (Table 1) were all derivatives of *B. subtilis* PY79 (35). Genetic manipulations of *B. subtilis* were performed by standard methods (4). The *gerD-cwlD* (JDB1486) mutant was obtained by transforming *B. subtilis* PY79 with genomic DNA from strain TB1 (kindly provided by Simon Cutting, University of London, United Kingdom) and by selecting for resistance to neomycin (2.5 μg/ml). The germination deficiency in comparison to the wild-type strain on rich medium of the *gerD-cwlD* mutant was as low (<0.0001%) as described previously (34). The strains ectopically expressing *cotE* at *amyE* under the control of different promoters in a Δ*cotE*::*cat* background were obtained after transformation of the laboratory Δ*cotE* (JDB1323) mutant with the genomic DNA from strains AH2914, AH2915, AH2921, AH2920, and AH2942 to give the strains JDB2122, JDB2124, JDB2127, JDB2125, and JDB2129, respectively. AH strains were kindly provided by A. Henriques (Universidade Nova de Lisboa, Portugal) and are in a different *B. subtilis* background (3). The sensitivity of the AH strains in *C. elegans* was the same as for the PY79 derivative strains (data not shown). The bacteria were maintained in Luria broth liquid media or agar media. *B. subtilis* strains were sporulated in liquid Difco sporulation medium, and spores were purified as described previously (10). Spores were purified using a 33% diatrizoate meglumine–8% diatrizoate sodium solution and washed with cold water until 100% of phase-bright spores were obtained. The spores were stored at 4°C in

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Reference(s) or source
JDB3	Wild-type PY79 strain, prototrophic	35
JDE304	*Escherichia coli* OP50	I. Greenwald
JDB1931	*Listeria innocua*	D. Portnoy
JDB1323	*cotE*::*cat*	14
JDB1380	*safA*::*tet*	14
JDB1371	*spoVID*::*kan*	14
JDB1343	*cotA*::*cat*	14
JDB1345	*cotG*::*erm*	14
JDB1346	*cotH*::*cat*	14
JDB1348	*cotO*::*tet*	14
JDB1381	*yutH*::*tet*	14
JDB1382	*ysxE*::*erm*	14
JDB1582	*amyE*::P_{spoIIQ}-*rfp cat*	This study
JDB1584	*cotE*::*cat*::*tet*	This study
JDB1586	*cotE*::*cat*::*tet amyE*::P_{spoIIQ}-*rfp cat*	This study
JDB1641	*spoVID*::*kan amyE*::P_{spoIIQ}-*rfp cat*	This study
JDB1334	*amyE*::P_{spanc}-*gfp cat*	16
JDB1486	*gerD-cwlD*::*neo*	This study and reference 34
JDB1494	*cotE*::*cat gerD-cwlD*::*neo*	This study
JDB2122	*cotE*::*cat amyE*::P_{gerE}-*cotE neo*	This study and reference 3
JDB2124	*cotE*::*cat amyE*::P_{cotG}-*cotE neo*	This study and reference 3
JDB2127	*cotE*::*cat amyE*::P_{cotEP1}-*cotE neo*	This study and reference 3
JDB2125	*cotE*::*cat amyE*::P_{cotEP2}-*cotE neo*	This study and reference 3
JDB2129	*cotE*::*cat amyE*::$P_{cotEP1P2}$-*cotE neo*	This study and reference 3

double-distilled H_2O and used within a week following the initial growth in Difco sporulation medium. The *C. elegans* N2 strain (a kind gift of Iva Greenwald, Columbia University, New York) and the JM90 strain (kindly provided by Jim McGhee, The University of Calgary, Alberta, Canada) were maintained on nematode growth medium agar (NGM) with *Escherichia coli* OP50 (JDE304) as the food source (33).

Culturing bacteria from worms. *C. elegans* eggs were isolated using a 10% commercial bleach and 1 N NaOH solution followed by three washes with 1× M9 (33). The eggs were allowed to hatch overnight at 20°C in 1× M9 while rocking. The number of larval stage 1 (L1) larvae was scored, and L1 larvae were added to 100-mm NGM plates containing a lawn of *E. coli* OP50 with about 2,000 L1 larvae per plate (33). Development then proceeded until the L4/young adult stage (36 h at 20°C). The larvae were washed four times in a large volume of 1× M9 with 0.01% Tween 20 (M9-T) and incubated for 8 h in M9-T at room temperature while rocking to allow excretion of *E. coli* from the worm intestine. After two washes with M9-T, 400 to 600 worms were added to a 60-mm plate containing nematode minimal medium agar (NMM) (3 g/liter NaCl, 17 g/liter agar, 5 mg/liter cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM KH_2PO_4 [pH 6]) on which 100 μl of spore preparation at 10^9 spores/ml had been spread. The worms were incubated at 20°C for 14 h, and the survival of vegetative cells of *B. subtilis*, *E. coli*, and *Listeria innocua*, was tested with 1-day-old adult worms placed on NGM plates seeded with 100 μl of a culture grown for 6 hours that had 1 CFU in 3 ml of LB at 37°C. The adult worms were incubated for 24 h on the bacteria, harvested, and washed with M9-T, and incubated in M9-T with 30 mM sodium azide to induce paralysis. The surface of the worms was sterilized using 1× M9 with 3% commercial bleach (20 min) and washed twice with M9-T. Since the worms are impermeant to any liquid following paralysis of the pharynx muscles by sodium azide, only the outside of the worms is subjected to sterilization. The worms resumed movement following washings, indicating that sodium hypochlorite had not penetrated their gut. The worms were resuspended in a final volume of 600 μl, and the number of worms in two separate 50-μl aliquots was determined. The outside contamination was estimated by plating 100 μl of this solution. The worms were disrupted by vortexing with high-density 1-mm zirconia beads (BioSpec Products) until completion as determined by microscopy. Dilutions of the worm lysates were made in T-base (24) containing 1 mM $MgSO_4$ and were plated on LB agar to determine the bacterial yield per worm (CFU/worm). The heat resistance of the bacteria in the disrupted worms was determined by exposing the samples to 80°C for 20 min. All experiments were performed a minimum of three times.

Microscopy. To visualize paralyzed worms, agar pads containing melted 2% agar in double-distilled H_2O were prepared on microscope slides. The worms were pipetted from the assay plates with M9-T and 30 mM NaN_3, dropped on the agar pad, and covered with a glass coverslip. Nomarski differential interference contrast (DIC) and fluorescence images were obtained using a Nikon Eclipse 90i microscope with a 100× objective. Pictures were captured with a Hamamatsu ORCA-ER digital camera using Nikon Elements BR software. Green fluorescent protein fluorescence and mCherry fluorescence were detected with EX460-500 BA510-560 and EX530-560 BA590-650 filter sets, respectively (Chroma). The exposure was 500 ms for all fluorescence pictures and 2 ms for DIC pictures.

Spore pulse-chase experiment. A large population of *C. elegans* N2 (L4-young adults) was synchronized, washed, and transferred to a 100-mm NMM agar plate containing a lawn of spores of strain JDB1334 (a PY79 derivative with a spectinomycin resistance gene) as described previously (16). After 2 h of incubation at 20°C, the worms were washed five times with M9-T. A total of 500 to 600 worms were then added to 60-mm NMM plates containing no bacteria ("no food"), Δ*cotE* spores, wild-type spores, or an overnight LB culture of *E. coli* OP50. The number of spectinomycin-resistant CFU per worm was determined as described above.

Measurement of growth rate. About 30 eggs of the *C. elegans* N2 strain were placed on a 60-mm plate containing NMM agar and incubated overnight at 20°C to allow L1 larvae to hatch. An equivalent amount of each bacterial strain (based on optical density) was added to the L1 larvae. Following subsequent incubation at 20°C, the developmental stage was checked every 12 h by microscope observations (4× magnification) until approximately 50% of the animals reached the adult stage. Adults were recognized by the appearance of a mature vulva and by the production, after several hours, of eggs (2). The growth rates were calculated and corresponded to the number of days required for 50% of the population to reach the adulthood at 20°C. *t* tests were performed to compare the three sets of growth rate measurements.

RESULTS

Sensitivity of *B. subtilis* to digestion by *C. elegans*. We tested the resistance of vegetative *B. subtilis* cells to *C. elegans* digestion by feeding a population of young adult *C. elegans* N2 worms (about 600 for each sample) with vegetative cells of wild-type *B. subtilis* (JDB3). We determined the number of surviving bacteria per worm and recovered only 16 ± 1 CFU per worm (mean ± standard deviation), which indicates that few viable bacteria were recovered from the nematode intestines (Fig. 1). We compared this recovery with that obtained following incubation of the worms with cultures of *Listeria*

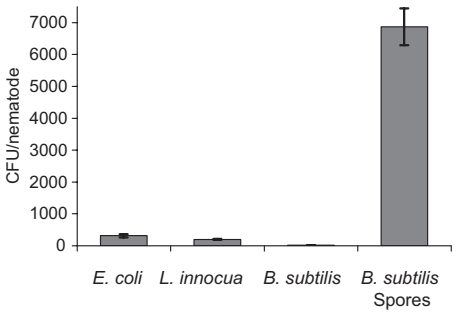

FIG. 1. Survival of *B. subtilis* spores in the *C. elegans* intestine. Adult *C. elegans* worms were fed with either *B. subtilis* PY79 vegetative cells or spores or *Listeria innocua* or *Escherichia coli* OP50 cells. After mechanical disruption of the nematodes (~600 worms per sample), the surviving bacteria were determined after growth on LB agar. The values represent the mean CFU/worm ± standard deviation (error bars) for three experiments.

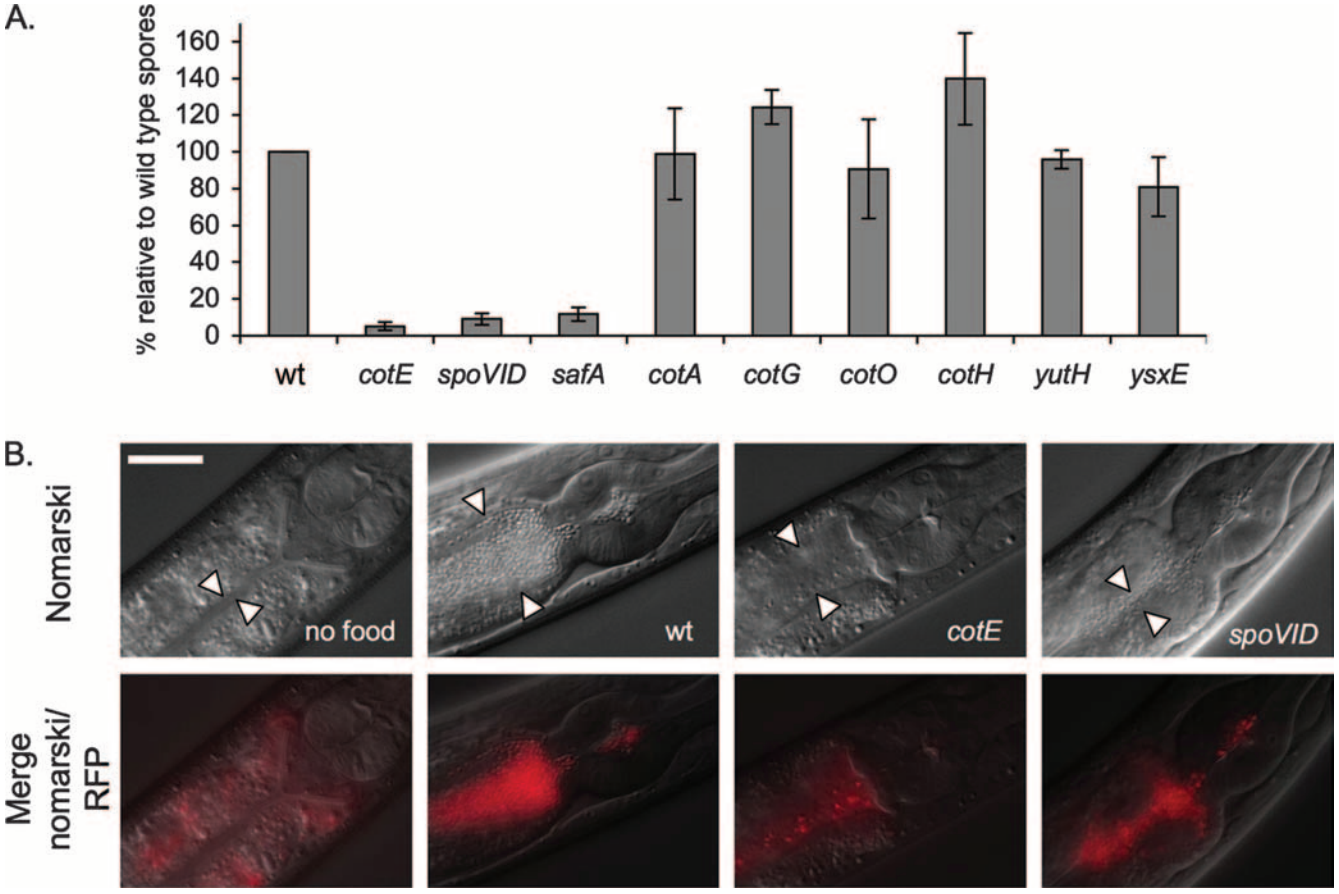

FIG. 2. Role of the coat morphogenetic proteins in the resistance of spores to *C. elegans* digestion. (A) Measurement of the survival of *B. subtilis* wild-type and coat mutant spores in *C. elegans*. L4-young adults were fed with wild-type spores (wt) or *cotE*, *safA*, *spoVID*, *cotA*, *cotG*, *cotO*, *yutH*, and *ysxE* mutant spores. Survival of mutant spores is represented relative to the survival of wild-type spores. The experiment was performed three times in duplicate, and error bars indicate the standard deviations. (B) Survival of *B. subtilis* spores within the *C. elegans* intestine. Merged Nomarski and red fluorescence and Nomarski photomicrographs of the upper intestine of *C. elegans* L3 larvae are shown. The worms were maintained without food ("no food") or were fed with spores carrying the P_{spoIIQ}-mCherry reporter in a wild-type background, Δ*cotE* P_{spoIIQ}-mCherry spores. and Δ*spoVID* P_{spoIIQ}-mCherry spores. Note the autofluorescence of the intestinal granules on the merged Nomarski and red fluorescence images. The white arrowheads indicate the borders of the intestinal lumen. RFP, red fluorescent protein. Bar = 0.025 mm.

innocua (JDB1931), another nonpathogenic gram-positive bacterium, or a gram-negative bacterium, *Escherichia coli* OP50 (JDE304). In both cases, the number of recovered bacteria was at least 10-fold greater than for *B. subtilis* vegetative cells with 311 ± 48 CFU/worm obtained for *E. coli* and 199 ± 15 CFU obtained for *L. innocua* (Fig. 1). Vegetative cells of *B. subtilis* were detected at less than one CFU per worm in worms at earlier stages (larval stages 1 to 3) of development (data not shown). Although we cannot rule out the possibility that vegetative cells of *B. subtilis* are less taken up by the nematode, this result suggests that vegetative *B. subtilis* cells were highly sensitive to digestion by the nematode.

In contrast with vegetative *B. subtilis* cells, large numbers of bacteria were easily recovered from the worms when *C. elegans* were fed wild-type *B. subtilis* spores (6,869 ± 575 CFU/worm [Fig. 1]). We confirmed that the bacteria recovered were spores and not vegetative cells by demonstrating their ability to survive high heat treatment (80°C, 20 min). Although there are numerous differences between spores and vegetative cells that could account for the increased survival of spores following ingestion by *C. elegans*, we focused on the possible protective role of the spore coat. We examined three strains carrying mutations in coat morphogenetic proteins (*cotE*, *safA*, and *spoVID*) necessary for lysozyme resistance (15). In addition, we examined the correlation between the in vitro chemical sensitivity of spores and their survival following ingestion by *C. elegans* by testing strains carrying mutations in the *cotG*, *cotH*, *cotO*, *yutH*, and *ysxE* genes that exhibit increased sensitivity to various reagents. We also tested the spores of the *cotA* mutant that do not exhibit any sensitivity in vitro using standard assays (28). CotH, CotO, and CotG are required for the recruitment of a small subset of proteins to the coat and are considered minor morphogenetic proteins (21, 29, 37). While *cotO* spores, unlike *cotG* mutant spores, have been reported to be sensitive in vitro to lysozyme (21), we found using the same lysozyme assay (24) that *cotO* mutant spores were as resistant as wild-type spores were (data not shown). The *cotH*, *yutH*, and *ysxE* mutant spores are slightly sensitive to hypochlorite, and the *yutH* and *ysxE* spores were also partially digested by *T. thermophila* (15).

TABLE 2. Ingestion of *cotE* spores by *C. elegans*[a]

Time after transfer to plate	No. of spectinomycin-resistant CFU/worm (% of CFU/worm before transfer)			
	OP50	*cotE*	wt	Without food
20 min	12	17	17	ND[b]
40 min	7	5	10	79
3 h	0	0	2	49

[a] L4 larvae were fed spectinomycin-resistant *B. subtilis* spores and transferred to agar plates containing either a lawn of *E. coli* OP50, a lawn of Δ*cotE* spores, a lawn of wild-type spores (wt), or without any source of food (without food). The number of spectinomycin-resistant CFU per worm was determined before transfer and at 20 min, 40 min, and 3 h following the transfer to each plate. The results are represented as a percentage of the number of CFU per worm before the transfer. The data shown are from one experiment but are representative of two independent experiments.

[b] ND, not done.

TABLE 3. Growth rate of *C. elegans* on *B. subtilis* spores[a]

Strain	Mean no. of days to adulthood (±SD)[b]
E. coli OP50	2.6 (±0.11)
B. subtilis	
Vegetative cells	2.6 (±0.17)
Spores	5.3 A (±0.29)
Δ*cotE* mutant spores	4.3 AC (±0.43)
gerD-cwlD mutant spores	8.5 B (±0.71)
Δ*cotE gerD-cwlD* mutant spores	6.3 BC (±0.32)

[a] *C. elegans* L1 larvae were fed with either *E. coli* OP50 strain, *B. subtilis* PY79 wild-type strain vegetative cells or spores, *cotE* mutant spores, *gerD-cwlD* mutant spores, or *cotE gerD-cwlD* spores.

[b] The number of days for *C. elegans* to reach adulthood at 20°C is indicated. The values are the means from three independent experiments. Values with the same letter (A, B, or C) are significantly different ($P < 0.05$).

We compared the survival of these mutant spores with wild-type spores by determining the number of recovered bacteria per worm after 16 h of incubation at 20°C with L4-young adult *C. elegans*. In this assay, there was no significant difference between the values obtained for the wild-type spores and for the spores of the *cotA* (JDB1343), *cotG* (JDB1345), *cotO* (JDB1348), *cotH* (JDB1346), *yutH* (JDB1381), and *ysxE* (JDB1382) strains (98% ± 25%, 125% ± 9%, 91% ± 27%, 139% ± 25%, 96% ± 5%, and 81% ± 16% as a percentage of the wild-type spores, respectively [Fig. 2A]). In contrast, between 10- and 20-fold-less *cotE* (JDB1323), *spoVID* (JDB1371), and *safA* (JDB1380) spores were recovered from the worm intestine (5% ± 2%, 9% ± 3%, and 12% ± 4% as a percentage of wild-type spores, respectively [Fig. 2A]). There was no significant difference observed between the recovery of *cotE* spores and either *spoVID* or *safA* spores.

In the course of the experiment, we observed that the densities of the spores on the agar plates for the *cotE*, *spoVID*, and *safA* strains were lower in comparison to the plates containing the wild-type spores, consistent with the digestion of the *cotE*, *spoVID*, and *safA* spores by the nematode (data not shown). We confirmed that the number of spores of *cotE*, *safA*, and *spoVID* mutants were indeed reduced in the worm intestine by monitoring the fate of spores containing red fluorescent protein from the wild-type, *cotE*, *spoVID*, and *safA* strains in the gut (Fig. 2B, *cotE* and *spoVID*) (*safA* strain not shown). These observations indicated that the overall structure of wild-type spores is maintained in the worm intestine and that wild-type spores accumulated in the worm intestine (Fig. 2B). In contrast, fluorescence associated with the *cotE*, *safA*, and *spoVID* spores was considerably reduced in the worm intestine (Fig. 2B and data not shown). Differential interference contrast microscopy of nematodes fed with spores of the *cotA*, *cotO*, *cotG*, *yutH*, and *ysxE* mutants confirmed that the spores of these strains accumulate in the intestine of worms as in the case of wild-type spores (data not shown). Thus, only the major alterations in coat formation that are seen in mutants of the major morphogenetic proteins like CotE, SafA, and SpoVID render spores sensitive to digestion by the worm.

Analysis of the ingestion and digestion of *cotE* mutant spores. We examined the possibility that the lower recovery of Δ*cotE* spores was due to decreased ingestion by the nematodes by measuring the kinetics of spore ingestion and excretion. We fed nematodes with spectinomycin-resistant (Spec^r) spores of *B. subtilis* strain JDB1334 that has a spectinomycin resistance cassette inserted in a locus (*amyE*) that does not affect sporulation. We transferred these worms to agar plates containing either no bacteria or a lawn with either *E. coli* OP50, wild-type *B. subtilis*, or Δ*cotE* mutant spores. We assumed that ingestion of new bacteria would chase the Spec^r spores out of the intestine, and therefore, we monitored the kinetics of excretion of the Spec^r spores from the nematode intestine. After 20 min, less than 20% of the Spec^r spores were recovered from the worms shifted to plates containing either *E. coli*, Δ*cotE* spores, or wild-type spores (Table 2). After 40 min, less than 10% of the Spec^r spores remained in the intestine (Table 2). In contrast, the number of wild-type spores decreased at a slower rate in the absence of food, with almost 50% of the Spec^r spores detected in the intestine of the nematodes 3 h after the transfer (Table 2). After 3 h on *E. coli*, wild-type or Δ*cotE* spores, few (2%) of the Spec^r spores were detected in the worms (Table 2). Given that the Δ*cotE* spores were as efficient as the wild-type spores at chasing the initial spores out of the worm intestine, we concluded that the Δ*cotE* spores seemed to be as efficiently ingested as wild-type spores or *E. coli* and so the low CFU/worm observed (Fig. 2A) was likely due to the sensitivity of the spores to the nematode digestive process.

Given that Δ*cotE* spores were more sensitive to worm digestion, we hypothesized that they would be more easily used as a food source by *C. elegans* than wild-type spores. We examined this possibility by comparing the development of L1 larvae at 20°C on either metabolically active bacteria (*E. coli* OP50 and vegetative *B. subtilis* cells) or on dormant wild-type and Δ*cotE* spores. The worms reached adulthood on either *E. coli* strain OP50 or *B. subtilis* vegetative cells in less than 3 days (Table 3), which was similar to the results reported previously (2) and confirming both the sensitivity of vegetative *B. subtilis* to worm ingestion and that these bacteria are indeed taken up by the nematode. Consistent with our hypothesis, worms developed faster on Δ*cotE* spores than on wild-type spores (5.3 and 4.3 days, respectively [Table 3]; $P < 0.05$). Surprisingly, however, the worms developed on wild-type spores that are presumably resistant to *C. elegans* digestion, albeit more slowly than on vegetative cells (5.3 and 2.6 days, respectively [Table 3]). In fact, nematodes grown on wild-type spores manifested the hallmarks of development under caloric restriction described for

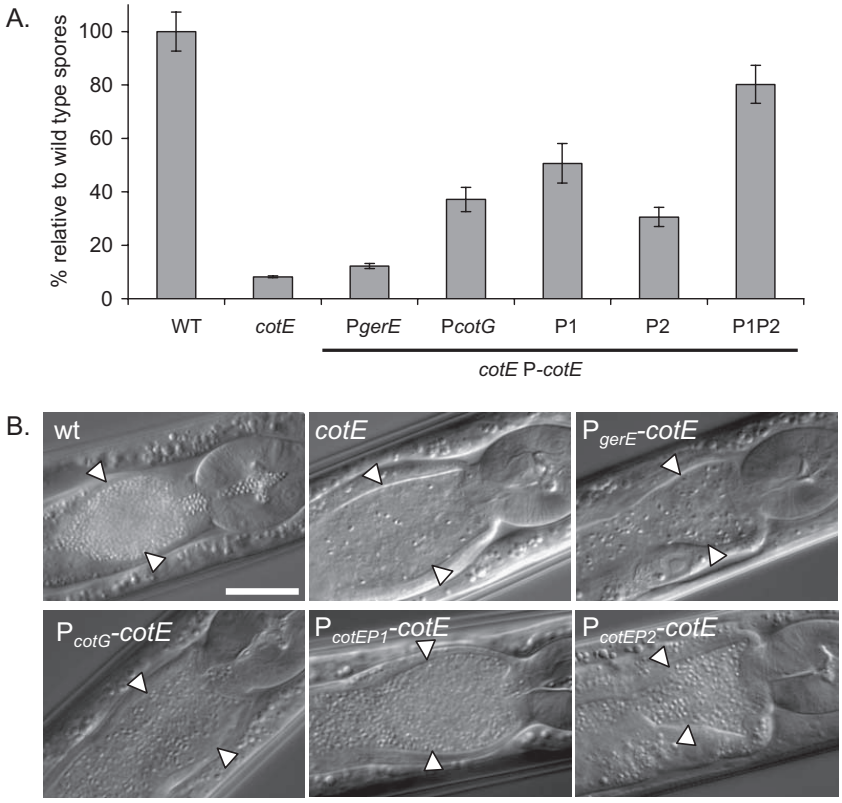

FIG. 3. Role of outer coat formation in the resistance of spores to *C. elegans*. The sensitivity of *B. subtilis* wild-type spores and various *cotE* mutant spores in *C. elegans* was measured. (A) L4 larvae were fed with wild-type spores (WT), Δ*cotE* mutant spores, or spores of a Δ*cotE* mutant expressing *cotE* from five promoters at the *amyE* locus: P_{gerE}, P_{cotG}, P_{cotEP1} (P1), P_{cotEP2} (P2), $P_{cotEP1P2}$ (P1P2). Data are represented in comparison to the number of CFU/worm obtained for the wild-type spores. The experiment was performed four times in duplicate, and a representative experiment is shown (standard deviations are indicated by error bars). (B) Nomarski pictures of the upper intestine of N2 L4 larvae fed with wild-type (wt), Δ*cotE*, Δ*cotE* P_{gerE}-*cotE*, Δ*cotE* P_{cotG}-*cotE*, Δ*cotE* P_{cotEP1}-*cotE*, and Δ*cotE* P_{cotEP2}-*cotE* spores. Worms fed with the Δ*cotE* $P_{cotEP1P2}$-*cotE* spores appeared similar to worms fed with the wild-type spores or Δ*cotE* P_{cotEP1}-*cotE* spores. The white arrowheads indicate the borders of the intestinal lumen. Bar = 0.025 mm.

C. elegans mutants that have an eating defect (1). Specifically, we observed that *C. elegans* fed on spores were smaller and thinner than the nematode fed on vegetative cells of *B. subtilis* or *E. coli* OP50 and that adult hermaphrodites generally contained fewer eggs (data not shown). This observation indicated that growth on spores compared to metabolically active bacteria led to limited nutrient availability and suggested that spores become sensitive to the worm's digestion.

The ability of worms to develop on spores, albeit more slowly than on vegetative cells, may indicate that ingested spores had germinated and were therefore sensitive to digestion. We therefore investigated the role of spore germination on nematode growth rate by incubating L1 nematodes with spores of *B. subtilis* that are defective for germination due to an insertion in the *gerD-cwlD* genes (strain JDB1486) (34). The *gerD-cwlD* spores are indeed highly defective in germination, with less than 0.0002% of the spores germinating on rich medium in comparison to wild-type spores (not shown) (34). The growth of *C. elegans* was slower in the presence of *gerD-cwlD* spores (8.5 days [Table 3]) than on either wild-type or Δ*cotE* spores (5.3 and 4.3 days, respectively [Table 3]), suggesting that the germination of *B. subtilis* spores and their subsequent sensitivity to digestion permitted the growth of *C. elegans*. We excluded the possibility that the nematode could develop on NMM agar by demonstrating that L1 larvae incubated on a plate without any source of food (spores or metabolically active bacteria) did not grow in the course of the experiment (data not shown). Finally, Δ*cotE gerD-cwlD* spores (JDB1494) supported a growth rate (6.3 days [Table 3]) intermediate to those of the Δ*cotE* and *gerD-cwlD* spores (4.3 and 8.5 days, respectively [Table 3]). This result indicates that part of the growth rate on Δ*cotE* spores is due to germination of the spores during the incubation, similarly to the wild-type spores, but also that the Δ*cotE gerD-cwlD* spores are a better source of nutrient than the *gerD-cwlD* spores, indicating that both Δ*cotE* mutant spores are digested due to their increased sensitivity to digestion.

Role of the spore coat structures in sensitivity to *C. elegans* digestion. The sensitivity of Δ*cotE* spores to *C. elegans* digestion prompted us to study how coat protein composition afforded protection during the digestive process. This composition can be affected by modulating the timing of CotE protein expressed ectopically in a Δ*cotE* mutant background (3). Early expression of CotE from the *amyE* locus is observed when it is under the control of its own promoters (P_{cotEP1}, P_{cotEP2}, or $P_{cotEP1P2}$), whereas late expression is observed when it is under

the control of the *gerE* and *cotG* promoters (P_{gerE} and P_{cotG}) (3). Expression of *cotE* under P_{gerE} or P_{cotG} and, to a lesser extent under the P_{cotEP2} promoter, led to a similar, but not identical, defect in overall protein composition and the structure of the coat to that observed in a Δ*cotE* null mutant strain (3). However, coat composition and structure were more similar to those in the wild-type spores in strains expressing *cotE* under P_{cotEP1} or $P_{cotEP1P2}$ (3).

While these experiments indicated that early expression of *cotE* from either P_{cotEP1}, P_{cotEP2}, or $P_{cotEP1P2}$ is required for the proper assembly of the outer coat, spores derived from these strains were as resistant to lysozyme as the wild-type spores were (3). Thus, these strains uncoupled, for the first time, lysozyme resistance observed in vitro and outer coat assembly (3) and allowed us to test the distinct role of each of these phenotypes in sensitivity to digestion by *C. elegans*. Surprisingly, the spores of all the strains tested except for the Δ*cotE* $P_{cotEP1P2}$-*cotE* strain (JDB2129) were susceptible to ingestion by worms (Fig. 3). Using the measurements of spores per worm and further supported by data obtained in growth rate experiments and microscopy observations, we can distinguish three classes of mutants. The first class comprises Δ*cotE* P_{gerE}-*cotE* spores (JDB2122) recovered at a level comparable to that obtained for the Δ*cotE* spores (12% ± 1%,and 8% ± 0.3% as a percentage of wild-type spores, respectively [Fig. 3A]). These spores supported the growth of worms as well as Δ*cotE* spores did (data not shown) and are not distinguishable from Δ*cotE* spores in the intestine (Fig. 3B). The second class comprises the Δ*cotE* P_{cotG}-*cotE* (JDB2124) and Δ*cotE* P_{cotEP2}-*cotE* (JDB2125) spores which were recovered at fourfold- and threefold-higher levels, respectively, than the level of Δ*cotE* spores (37% ± 4% and 31% ± 4%, respectively [Fig. 3A]). These spores supported nematode growth at a rate similar to the Δ*cotE* spores (data not shown) and are clearly seen in the worm intestine (Fig. 3B). The third class of mutants, including the Δ*cotE* P_{cotEP1}-*cotE* (JDB2127) and Δ*cotE* $P_{cotEP1P2}$-*cotE* (JDB2129) spores, was recovered at 6- and 10-fold-higher levels than the level of Δ*cotE* spores (51% ± 7%, 80% ± 7%, and 8% ± 0.3%, respectively [Fig. 3A]) and supported the growth of the worms as well as wild-type spores did (data not shown) and accumulated in the intestine (Fig. 3B). Thus, the viability of these three classes of mutant spores following ingestion by the worm correlates with their respective defect in coat protein composition and structural modification of the coat.

DISCUSSION

Bacillus species are abundant soil bacteria (~10^6 organisms/gram [18]) and likely serve as a source of nutrients for the bacteriovorous nematode *C. elegans* (9). Indeed, *B. subtilis* vegetative cells are efficiently digested by the worm (Fig. 1) (7), consistent with the longer life span observed for *C. elegans* grown on vegetative *Bacillus subtilis* compared to the life span for *C. elegans* grown on *E. coli* OP50 (8). In contrast, we observed that wild-type *B. subtilis* spores were highly resistant to worm digestion (Fig. 1) as would be predicted from their resistance to phagocytosis by the protozoan *T. thermophila* and by their survival in the mammalian gastrointestinal tract (11, 15, 34). In a previous study, we found that other *Bacillus* species (*Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*) are also highly sensitive to the worm ingestion and that *B. anthracis* spores are fully resistant to the worm digestive process (16). In *B. subtilis*, the proper recruitment and assembly of the spore coat are crucial for the spore resistance properties, and in particular, mutations in coat morphogenetic proteins (CotE, SpoVID, and SafA) result in lysozyme sensitivity (25, 26, 36). Our finding that spores carrying mutations in the genes encoding these proteins were sensitive to digestion (Fig. 2A) is consistent with the role of the overall structure and composition of the coat in providing resistance to nematode predation. In contrast, spores carrying mutations in minor morphogenetic proteins, (CotG, CotO, and CotH) exhibited little or no effect on coat structure (21, 29) and were as resistant as the wild-type spores were (Fig. 2A).

The precise expression of *cotE* and the subsequent proper assembly of the *cotE*-dependent proteins in the coat appear to be crucial steps in achieving resistance to digestion (Fig. 3). Increased defects in coat structure, particularly the outer coat, led to increased digestion by the worm (Fig. 3). However, even Δ*cotE* P_{cotEP2}-*cotE* spores that have a coat structure and composition similar to those wild-type spores and in which only a few protein species were found missing by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (3) are still somewhat sensitive to digestion. Thus, either the defect in this small subset of proteins is sufficient to enable the digestion of the spore, or the structure or composition of the coat is more affected in this strain than previously explored. Whatever the ultimate explanation, this result clearly highlights the usefulness of the *C. elegans* model for uncovering subtle coat protein mutant phenotypes.

In contrast to previous experiments using *T. thermophila* (15), we did not observe a correlation between in vitro lysozyme sensitivity and *C. elegans* digestibility. Indeed, the *yutH* and *ysxE* spores that are sensitive both to lysozyme and to digestion by *T. thermophila* (15) were as resistant to *C. elegans* digestion as the wild-type spores were. One explanation could be that these two studies used nonisogenic *B. subtilis* 168 strains. This discrepancy could also be explained by a longer time of residency of the spores in the protozoan digestive vacuole compared to the intestine of a L4 worm (~1 h) (Table 2). Longer persistence in the *T. thermophila* vacuoles would result in longer exposure to digestive enzymes and thus increased degradation of the *yutH* and *ysxE* spores.

Δ*cotE* mutant spores are degraded during incubation with a high concentration of purified lysozyme (36) and are also readily digested by *C. elegans* (Fig. 2A and B). However, spores produced by cells in which *cotE* expression was delayed were resistant to lysozyme (3) but sensitive to digestion (Fig. 3). Thus, the timing of expression of *cotE* appeared critical for the survival of the spore in the worm gut and may indicate more subtle defects in spore coat assembly in these *cotE* expression mutants.

The *C. elegans* digestive process has been suggested to involve both mechanical disruption mediated by a pharyngeal grinder and enzymatic digestion of the ingested microorganisms in the intestine (19). However, since Δ*cotE* spores do not exhibit increased survival in mutant worms with a defective grinder (M.-H. Laaberki and J. Dworkin, unpublished data), they, and presumably other defective spores, are likely to be degraded by enzymes secreted into the intestinal lumen. This

result is consistent with a previous study showing that wild-type and *cotE* spores exhibit essentially identical resistance to mechanical disruption (12). *C. elegans* expresses and potentially secretes into its digestive system several enzymes that could degrade sensitive spores, including 10 lysozyme genes (*lys-1* to *lys-10*) and 5 insect-lysozyme genes (*ilys-1* to *ilys-5*) (19, 31), and 5 of the *lys* genes and 2 of the *ilys* genes are expressed in intestinal cells (13, 17, 20, 32). In addition, numerous peptidases and lipases are expressed and also presumably secreted in the intestine of the nematode (20). A synergistic activity of these enzymes could be responsible not only for the degradation of Δ*cotE* spores but also for the degradation of the ectopically expressed *cotE* spores that are lysozyme resistant in vitro (3). An understanding of the enzymatic basis for digestion of spore coat mutants as well as vegetative *Bacillus* cells in *C. elegans* will be greatly facilitated by the genetic tools available in this organism. For example, RNA interference could be used to reduce the expression of potential candidate bacteriolytic enzymes (17, 22), and then the effect of these manipulations on bacterial survival could be determined using the assays we have described. Finally, this knowledge will help us refine our understanding of the role of the spore coat structures in resistance to an ecologically relevant predator.

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