

## ExsY and CotY Are Required for the Correct Assembly of the Exosporium and Spore Coat of *Bacillus cereus*<sup>∇</sup>

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**The exosporium-defective phenotype of a transposon insertion mutant of *Bacillus cereus* implicated ExsY, a homologue of *B. subtilis* cysteine-rich spore coat proteins CotY and CotZ, in assembly of an intact exosporium. Single and double mutants of *B. cereus* lacking ExsY and its paralogue, CotY, were constructed. The *exsY* mutant spores are not surrounded by an intact exosporium, though they often carry attached exosporium fragments. In contrast, the *cotY* mutant spores have an intact exosporium, although its overall shape is altered. The single mutants show altered, but different, spore coat properties. The *exsY* mutant spore coat is permeable to lysozyme, whereas the *cotY* mutant spores are less resistant to several organic solvents than is the case for the wild type. The *exsY cotY* double-mutant spores lack exosporium and have very thin coats that are permeable to lysozyme and are sensitive to chloroform, toluene, and phenol. These spore coat as well as exosporium defects suggest that ExsY and CotY are important to correct formation of both the exosporium and the spore coat in *B. cereus*. Both ExsY and CotY proteins were detected in Western blots of purified wild-type exosporium, in complexes of high molecular weight, and as monomers. Both *exsY* and *cotY* genes are expressed at late stages of sporulation.**

Endospores of the *Bacillus cereus* family, which includes *Bacillus anthracis* and *Bacillus thuringiensis*, are enveloped by a large, balloon-like layer known as the exosporium (11, 19). *B. subtilis*, the paradigm sporeformer, does not have this distinct layer, although the *B. subtilis* spore coat may have a tight-fitting outermost layer that can be visualized after extraction of coat material with urea or mercaptoethanol (23). Scanning electron microscopy has revealed that the exosporium is composed of two layers—a paracrystalline basal layer with hexagonal periodicity and a “hairy nap” outer layer (11). The exosporium is chemically complex and is composed of 53% protein, 20% amino and neutral polysaccharides, 18% lipids, and ~4% ash (17).

Multiple proteins from the exosporium of both *B. cereus* and *B. anthracis* have been identified (22, 24, 31); the majority do not have homologues in *B. subtilis*. For example, the surface layers of *B. cereus*, *B. anthracis*, and *B. thuringiensis* spores all contain glycoproteins with characteristic collagen-like repeat regions (5, 10, 27). Of these, the *B. anthracis* glycoprotein BclA is an essential component of the hairy nap layer of the exosporium (27, 29).

Analysis of genome sequence information from *B. cereus* ATCC 14579 and *B. anthracis* Ames identified a cluster of genes near *bclA* implicated in exosporium production, including the region whose genes were designated *yjbX-exsY-*

*yjcA-yjcB-exsFA-cotY* (31). The ExsY and CotY proteins are homologues (ca. 35% amino acid identity) of the *B. subtilis* coat proteins CotY and CotZ (37). ExsY and CotY proteins have both been detected in purified exosporium from *B. anthracis* endospores by N-terminal sequencing of separated peptides (22). ExsY and CotY are very similar proteins, with ca. 90% amino acid identity for most of their 152- to 156-residue sequence, after a more variable (<50% identical) N-terminal region of ca 20 amino acids. They are cysteine-rich, like their *B. subtilis* homologues, which have been reported to form disulfide-linked multimers (37). This study describes the effects on spore structure and properties of the inactivation of *exsY* and *cotY* genes, singly and in combination.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are listed in Table 1. Oxoid nutrient broth and agar (NB and NA, respectively; Oxoid, Ltd., Basingstoke, United Kingdom) were used for routine culture of *B. cereus*, with antibiotics if appropriate (spectinomycin at 200  $\mu\text{g ml}^{-1}$ , kanamycin at 40  $\mu\text{g ml}^{-1}$ , erythromycin at 1  $\mu\text{g ml}^{-1}$ , and lincomycin at 25  $\mu\text{g ml}^{-1}$ ). *Escherichia coli* strains were grown in Luria broth (LB) or L agar, containing antibiotic (ampicillin at 100  $\mu\text{g ml}^{-1}$ , spectinomycin at 100  $\mu\text{g ml}^{-1}$ , erythromycin at 150  $\mu\text{g ml}^{-1}$ , and kanamycin at 40  $\mu\text{g ml}^{-1}$ ). LB was used to culture *B. cereus* and *E. coli* for conjugation experiments. NBY broth, phage assay broth (30), NBY agar, and phage assay agar (30) were used for *B. cereus* phage transduction. TSB medium (6) was used for preparation of chemically competent *E. coli*.

**Spore preparation.** *B. cereus* strains were incubated in liquid CCY medium (7) at 30°C with shaking until the culture contained >95% free spores, and spores were washed in water, as previously described (31). The final pellet was resuspended in spore resuspension buffer (50 mM Tris-HCl, 0.5 mM EDTA, pH 7.5) and stored at –20°C.

**Proteins from spore culture supernatants.** Spores were pelleted from 35 ml of CCY culture by centrifugation. Sodium deoxycholate (to 200  $\mu\text{g ml}^{-1}$ ) was added to the supernatant, and after 30 min of incubation at 4°C, ice-cold tri-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
<b>Strains</b>		
<i>E. coli</i> DH5 $\alpha$	<i>supE44 <math>\Delta</math>lacU169 (<math>\phi</math>80lacZ<math>\Delta</math>M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Laboratory stock
TG1	<i>supE thi-1 <math>\Delta</math>(lac-proAB) <math>\Delta</math>(mcrB-hsdSM)5 (r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>-</sup>) [F' traD36 proAB lacI<sup>a</sup> lacZ<math>\Delta</math>M15]</i>	Laboratory stock
HB101	<i>supE44 hsdS20(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1</i>	Laboratory stock
BL21(DE3)	<i>F<sup>-</sup> ompT hsdS<sub>B</sub> (r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm (DE3)</i>	Novagen
<i>B. cereus</i>		
ATCC 10876	<i>trp-1 Str<sup>r</sup></i>	7
AM1652	<i>trp-1 Str<sup>r</sup> exsY::spc</i>	This study
AM1653	<i>trp-1 Str<sup>r</sup> cotY::erm</i>	This study
AM1654	<i>trp-1 Str<sup>r</sup> exsY::spc cotY::erm</i>	This study
SJT003	<i>trp-1 Str<sup>r</sup> exsY::Tn917-LTV1</i>	This study
<b>Plasmids</b>		
pUC19	Amp <sup>r</sup>	35
pAT113	Ery <sup>r</sup> Kan <sup>r</sup>	34
pFX113	Spc <sup>r</sup> Kan <sup>r</sup>	3
pUC1318Erm	Ery <sup>r</sup> Amp <sup>r</sup>	33
pUC1318Spc	Spc <sup>r</sup> Amp <sup>r</sup>	33
pEY-Spc	Spc <sup>r</sup> Ery <sup>r</sup> Kan <sup>r</sup> exsY::spc	This study
pCY-Ery	Spc <sup>r</sup> Ery <sup>r</sup> Kan <sup>r</sup> cotY::erm	This study

chloroacetic acid was added to 6% (wt/vol) to precipitate proteins. After incubation on ice for 60 min, samples were centrifuged at  $2,500 \times g$  at 4°C for 45 min. Pellets were resuspended in 0.5 ml spore resuspension buffer (50 mM Tris-HCl, 0.5 mM EDTA, pH 7.5), and 20  $\mu$ l was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Synchronous sporulation.** A synchronized sporulation method, based on the resuspension method of Sterlini and Mandelstam (26) and modified as described in reference 1, was used. Cells were subjected to an amino acid downshift, and the appearance of phase-bright prespores was monitored by phase-contrast microscopy. Duplicate 0.5-ml samples were taken at 30-min intervals throughout sporulation and centrifuged at  $20,000 \times g$  for 5 min, and the cell pellets were stored at -20°C.

**Bacterial transformation.** *E. coli* was transformed by electroporation (8) or by chemical methods (6). Plasmids were introduced into *B. cereus* by electroporation (2) or by conjugation (32).

**Enrichment for exosporium mutants.** Exosporium mutants were isolated from Tn917-LTV1 transposon insertion libraries (4, 7) following enrichment for reduced surface hydrophobicity (15) and screening for an exosporium defect by phase-contrast microscopy, as described by Bailey-Smith et al. (1).

**Electron microscopy.** Spore sections were prepared as described previously (16). Sections were placed on Formvar-coated copper grids and examined under a CM100 transmission electron microscope operating at an acceleration voltage of 100 kV. Micrographs were recorded using a Gatan Multiscan 794 1k  $\times$  1k charge-coupled device camera. For imaging of whole spores, suspensions were diluted to 10 mg dry weight per ml, and 3  $\mu$ l of spore suspension was deposited on a carbon-coated copper/palladium grid for 2 min, washed twice in distilled water, washed rapidly in 1% uranyl formate negative stain, and negatively stained for 30 s, blotted, and air dried.

**Measurement of heat resistance.** Spore dilutions in water were incubated at 90°C for 10, 20, 30, and 40 min before plating on NA at 30°C. Counts were compared to those for unheated control samples.

**Lysozyme resistance of spores.** Spores were diluted in phosphate-buffered saline at 37°C to an optical density at 600 nm (OD<sub>600</sub>) of ~2. Following addition of lysozyme (to 250  $\mu$ g ml<sup>-1</sup>), the OD<sub>550</sub> was measured every 2 minutes, after shaking, in a temperature-controlled Wallac Victor<sup>2</sup> 1420 Multilabel counter.

**Chemical resistance of spores.** Spores were resuspended in water to an OD<sub>600</sub> of ~1. For ethanol resistance, suspensions were diluted in 9 volumes of ethanol at room temperature for 10 min before further dilution and plating. For the water-immiscible solvents chloroform and toluene, 100  $\mu$ l of solvent was added to 900  $\mu$ l of spore suspension, vortexed for 1 min, and left to settle at room temperature for 9 min before sampling of the aqueous layer. To test phenol resistance, 100  $\mu$ l of 5% (wt/vol) neutralized phenol was added to 900  $\mu$ l of the spore suspension, vortexed for 1 min, and left at room temperature for 9 min before sampling. Dilutions of all treated cultures were in 10 mM potassium

phosphate buffer, pH 7.4, containing 1 mM MgSO<sub>4</sub> and 50 mM KCl, and aliquots were plated on NA and incubated at 30°C overnight. Viable counts were compared to those for untreated samples.

**Insertional inactivation of the *B. cereus* exsY and cotY genes.** The suicide vector pAT113 (Kan<sup>r</sup> Ery<sup>r</sup>) (34) and its derivative, pFX113 (Kan<sup>r</sup> Spc<sup>r</sup>) (3), can be introduced from *E. coli* by conjugation and have been used successfully for gene knockout by allelic exchange in both *B. cereus* (20) and *B. anthracis* (27). Primers EyF1 (CCCATACACCTGTACACCAATAACAGC) and EyR1 (GTCCACA TAATAAATCATTAAAGGATATG) were used with Expand HiFi polymerase (Roche) to generate a 1.1-kb PCR product that contained the *B. cereus* exsY gene and flanking DNA. The fragment was gel purified and blunt cloned into HindII-digested pUC19 (35). The resulting construct (pUCEXS) was linearized with NcoI, which cuts in the exsY open reading frame (ORF) after the 24th bp, and was end filled with VENT polymerase (Roche). A 1.2-kb fragment from pUC1318Spc (33), containing a spectinomycin resistance cassette (18), was blunt cloned into the NcoI-digested pUCEXS to generate pUCEXS-Spc. pUCEXS-Spc was digested with HindIII to release the fragment containing the disrupted exsY ORF, which was gel purified and cloned into HindIII-digested pAT113 (34), giving rise to pEY-Spc. This plasmid was introduced into *E. coli* HB101 carrying the mobilizing plasmid pRK212.1 and thence to *B. cereus* ATCC 10876 by filter mating (32). Spectinomycin-resistant but kanamycin-sensitive *B. cereus* potential exconjugant colonies were screened by colony PCR for presence of both expected junction fragments to confirm the presence of the exsY::spc allele. The region, including flanking DNA, was sequenced in one such isolate (AM1652) to check that no additional point mutations had been introduced.

The cotY gene was inactivated in a similar fashion. Primers CyF1 (GGAAG AATTAATGGAGTCTGCAACTCGTTTCACCG) and CyR1 (CCATGCTTA ACTCATTATTAGTTTTAAATAG) were used to generate a PCR fragment of 1,228 bp containing the cotY ORF and flanking DNA. The gel-purified fragment was blunt cloned into HindII-digested pUC19 (pUCCOTY). The resulting construct was digested with MfeI/SpeI to remove the portion of cotY between bases 9 and 232 of the ORF, and Vent polymerase was used to generate blunt ends. A 1.2-kb erythromycin resistance cassette, obtained by SmaI digestion of pUC1318Erm (33), was blunt cloned into the digested pUCCOTY vector to give rise to pUCCOTY-Erm. This vector was digested with HindIII/EcoRI to release the disrupted cotY gene fragment, which was cloned into HindIII/EcoRI-digested pFX113 (3) to give pCY-Erm. This was transferred to the donor *E. coli* strain and thence into *B. cereus* ATCC 10876 by conjugation in a filter mating. An erythromycin-resistant, kanamycin-sensitive exconjugant (AM1653) with the appropriate cotY::erm insertion was confirmed by colony PCR, and the flanking regions were checked for the absence of additional mutations by DNA sequencing.

To construct the  $\Delta$ exsY  $\Delta$ cotY double mutant AM1654, a CP51ts phage lysate (36) propagated on AM1653 (cotY::erm) was used to transduce AM1652 (exsY::spc) as described previously (7). Transductants were selected on LB agar

containing erythromycin (1  $\mu\text{g ml}^{-1}$ ) and lincomycin (25  $\mu\text{g ml}^{-1}$ ). As the *cotY* and *exsY* genes are only 2.5 kb apart, there was significant (ca. 60%) cotransduction of the wild-type *exsY* allele present in the donor. Transductants that had retained the *exsY::spc* allele were purified, and the presence of both mutant alleles was confirmed by PCR as well as by antibiotic resistance.

**Overexpression of ExsY.** The *exsY* ORF was amplified using primers MJ74 (CGCGGGATCCGATGAGTTGTAACGAAAATAAACAC) and MJ75 (TTT TCTCGAGGATAGTAACGTCGCGTAAGC), digested with BamHI/XhoI, and ligated into BamHI/XhoI-digested pET41b (Novagen). The resulting plasmid construct, pET-EXSY, was introduced into *E. coli* BL21(DE3), and ExsY, tagged at the N terminus with a glutathione *S*-transferase (GST) domain, was overexpressed and purified using the Novagen GST · Resin system. All buffers were supplemented with 200 mM dithiothreitol (DTT) to prevent aggregation of the overexpressed protein by disulfide bond formation.

**Production of ExsY and CotY antipeptide antibodies.** Peptides were synthesized by Arthur Moir, University of Sheffield, based on the N-terminal sequences of CotY (EDHQHECDFNCV; residues 7 to 18) and ExsY (ENKHHGSSHCV; residues 5 to 15), in each case polymerized on the eight available amino groups of a C-terminal polylysine tree. Polyclonal antibodies against the GST-ExsY fusion protein and the two synthetic peptides were raised in rabbits by the Antibody Resource Centre, University of Sheffield, by subcutaneous injection using Freund's adjuvant.

**Exosporium extraction and protein separation.** Exosporium was released from spores by treatment in a French pressure cell: extraction, purification, and washes were all performed as previously described (31). Protein concentrations were measured by an adaptation of the Lowry method (21), using bovine serum albumin as the protein standard. Proteins were analyzed by boiling samples in an equal volume of Tricine gel sample buffer (3 M Tris-HCl, pH 8.45, 12% glycerol, 4% SDS, 0.1% Coomassie blue G), and separating the proteins by SDS-PAGE on precast 16% Tris-Tricine gels (Novex). Gels were stained with SYPRO Ruby protein gel stain (Molecular Probes).

**Glycoprotein staining.** Exosporium samples were reduced with 200 mM DTT at 4°C for 14 h prior to SDS-PAGE on Novex 10 to 20% Tris-glycine gradient gels (Novex). Proteins were then blotted onto nitrocellulose membrane (Amersham) using 10 mM CAPS (3-[cyclohexylamino-1 propanesulfonic acid]) transfer buffer. Glycoprotein staining was performed using a glycoprotein ECL enhanced chemiluminescence detection kit (Amersham).

**Western blotting.** Exosporium samples were reduced with 200 mM DTT and separated by SDS-PAGE as described above for glycoprotein staining. Proteins were then blotted onto Hybond-P polyvinylidene difluoride membrane (Amersham). Extra lanes with molecular weight markers (Mark 12; Invitrogen) were removed and stained with Coomassie blue for comparison. The concentrations of primary antibody used were 1/500 for the ExsY-GST antibody and 1/100 for the antipeptide antibodies. A 1/5,000 dilution of secondary anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (anti-mouse horseradish peroxidase-conjugated immunoglobulin G for monoclonal antibody CR1) was used in the ECL-Plus Western blotting detection system (Amersham), with Hyperfilm ECL (Amersham).

**Nucleotide sequence accession number.** The *B. cereus* ATCC 10876 sequences were submitted to GenBank under accession no. AY121973 (*exsY*) and AY186996 (*cotY*).

## RESULTS

**Isolation of a transposon insertion *exsY* mutant.** Spores from libraries of *B. cereus* ATCC 10876 carrying a chromosomal copy of Tn917-LTV1 (7) were subjected to a hexadecane/aqueous-phase partitioning system (1) to enrich for spores that had altered surface hydrophobicity. One of the mutants obtained, SJT003, produced spores that were not surrounded by exosporium but retained a coat layer. Generalized transduction with the phage CP51ts showed 100% linkage between the transposon insertion and the exosporium defect. The transposon Tn917-LTV1 is designed to allow recovery of flanking sequences. SJT003 chromosomal DNA was digested with EcoRI, and the excised plasmid was recovered in *E. coli* (4). Sequencing of the transposon-chromosome junction revealed that the transposon was inserted into the promoter region of *exsY*, encoding a homologue of the *B. subtilis* CotY and CotZ

outer spore coat proteins. The ExsY protein had already been identified and named in our laboratory as a formic acid-solubilized component of *B. cereus* ATCC 10876 exosporium (S. J. Todd, unpublished observation).

**Mutant construction.** The transposon insertion was located in the intergenic region between two divergently transcribed genes, 34 bp upstream from the start codon of *exsY* and 91 bp upstream of *yjcA*. It was therefore possible, though unlikely, that the insertion would also affect *yjcA* expression. A directed mutagenesis strategy was therefore adopted to generate null mutants AM1652 ( $\Delta exsY$ ), which carries an *exsY::spc* allele, and AM1653 ( $\Delta cotY$ ), which carries a *cotY::erm* allele. Inactivation of the *cotY* gene is not likely to affect expression of the *exsFA* gene 175 bases downstream, because the latter has its own promoter, as indicated by complementation studies (28). A double mutant, AM1654, was constructed using generalized transduction with phage CP51ts (36) to cross the *cotY::erm* mutation into the *exsY::spc* mutant strain.

**Spore morphology is altered in *exsY* and *cotY* mutants.** Spores of the AM1652 ( $\Delta exsY$ ), AM1653 ( $\Delta cotY$ ), and AM1654 ( $\Delta exsY \Delta cotY$ ) mutants were compared to those of the wild type, using electron microscopy to assess any effect on gross spore morphology. Samples were viewed as both whole spores and embedded thin sections. The  $\Delta exsY$  mutant whole spores (Fig. 1B) lack an intact exosporium layer: some fragments of exosporium are attached, and some are free in the spore preparation. Electron microscopy of thin sections of AM1652 ( $\Delta exsY$ ) confirmed the absence of an exosporium layer (Fig. 1F). The spore coat did not stain evenly, and there were occasional lumps on the coat surface. Darkly staining particulate material was also present in these preparations.

In contrast, the AM1653 ( $\Delta cotY$ ) mutant spore has an intact exosporium that fully encloses the spore (Fig. 1C), although the exosporium appears to make a tighter fit around the poles of the spore compared with the loose "balloon-like" exosporium of the wild type. Thin sections of these spores look like wild type (Fig. 1G).

Spore preparations of AM1654 ( $\Delta exsY \Delta cotY$ ) contained fragmented exosporium, most of which was not attached to spores (Fig. 1D). Spore sections (Fig. 1H) revealed exosporium-free spores, all with some degree of spore coat defect. Large fragments of coat or exosporium material were present in the preparation.

Spores of the  $\Delta exsY$  mutant and, more dramatically, of the double mutant were prone to aggregation in suspension, as reported for *B. subtilis* coat-defective or chemically extracted spores (12, 37).

**Hydrophobicity and resistance properties of *exsY* and *cotY* mutants.** Electron microscopy shows that the spore surface layers of the single mutants are clearly different (Fig. 1). Despite this, the individual  $\Delta exsY$  and  $\Delta cotY$  mutations had only small effects on the partition of spores into hexadecane (Table 2). In contrast, the spores of the double mutant were much less efficiently partitioned into hexadecane, suggesting a large reduction in surface hydrophobicity.

Spore coats contribute to the resistance of *B. cereus* spores to organic solvents (14). Spores of all three mutants were tested for resistance to chloroform, toluene, ethanol, and phenol (Table 2). The  $\Delta exsY$  mutation had no effect on resistance,

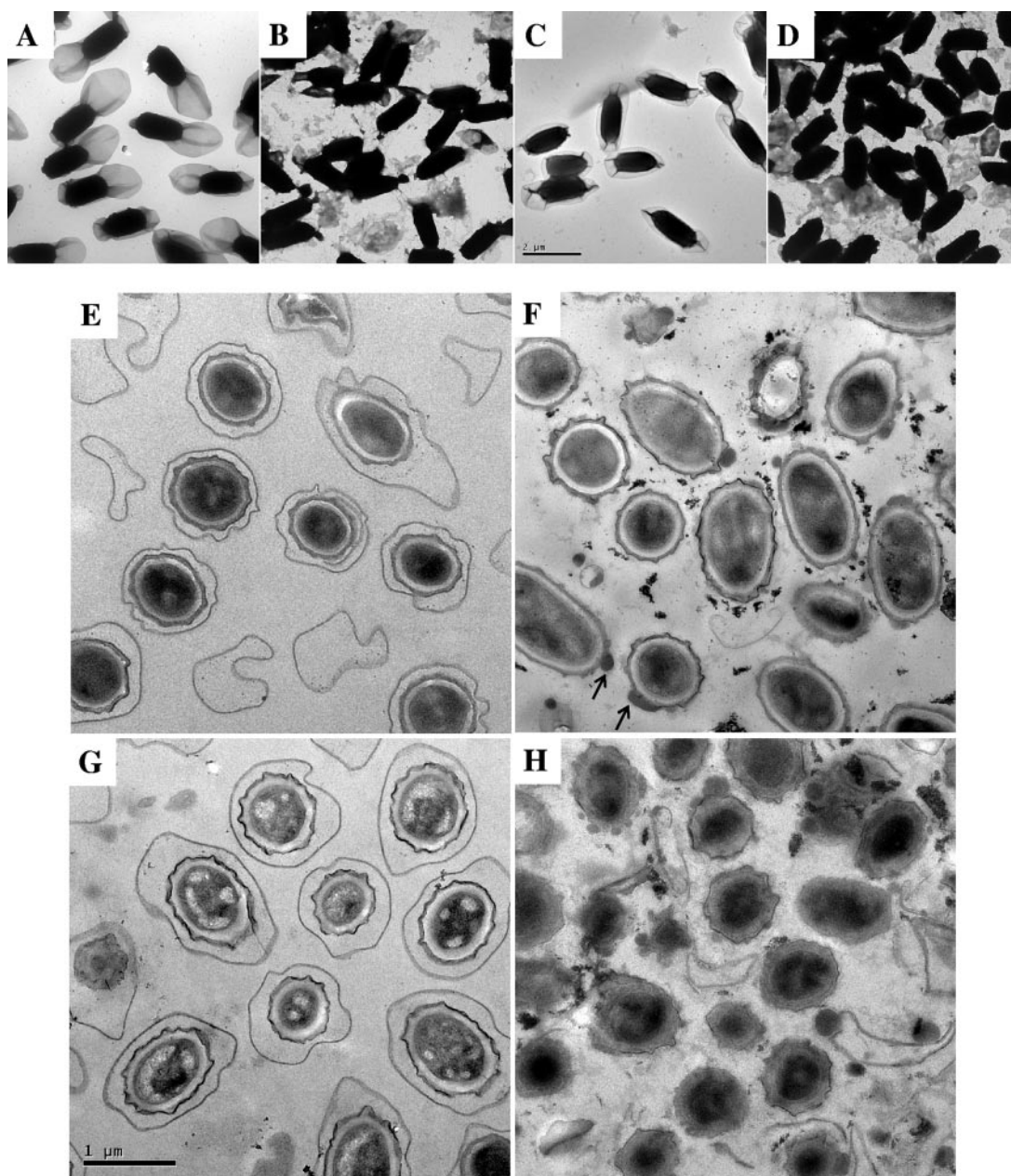


FIG. 1. Electron micrographs showing fields of whole spores of *B. cereus* (A to D) and of thin sections (E to H). Images are at the same size for all of panels A to D (scale bar on panel C) and at higher magnification for panels E to H (scale bar on panel G). Panels A and E show wild-type *B. cereus* ATCC 10876, B and F show AM1652 ( $\Delta$ *exsY*), C and G show AM1653 ( $\Delta$ *cotY*), and D and H show AM1654 ( $\Delta$ *exsY*  $\Delta$ *cotY*). Arrows on panel F point to lumps of material on spore coats of AM1652.

whereas the  $\Delta$ *cotY* mutation affected resistance to phenol and, more dramatically, to toluene. Spores of the  $\Delta$ *exsY*  $\Delta$ *cotY* double mutant were much more sensitive to all four solvents.

The coats of wild-type spores are impermeable to lysozyme, which would otherwise be able to access and degrade the peptidoglycan cortex, leading to a decrease in OD and phase darkening. Spores of the  $\Delta$ *cotY* mutant, like wild-type spores, were resistant to lysozyme, but spores of the  $\Delta$ *exsY* mutant and  $\Delta$ *exsY*  $\Delta$ *cotY* double mutant were sensitive to lysozyme, with suspensions decreasing in OD between 30 and 60 min after exposure (data not shown). For compari-

TABLE 2. Spore hydrophobicity and resistance to organic solvents

<i>B. cereus</i> strain	Partition into hexadecane (%)	Spore resistance/recovery (%) <sup>a</sup>			
		Ethanol	Chloroform	Toluene	Phenol
ATCC 10876 (wild type)	88 ± 2	79 ± 3	42 ± 1	63 ± 5	85 ± 3
AM1652 ( $\Delta$ <i>exsY</i> )	80 ± 2	94 ± 15	46 ± 7	69 ± 16	85 ± 13
AM1653 ( $\Delta$ <i>cotY</i> )	82 ± 2	55 ± 1	35 ± 4	25 ± 4	55 ± 3
AM1654 ( $\Delta$ <i>exsY</i> $\Delta$ <i>cotY</i> )	22 ± 2	25 ± 5	6 ± 5	2 ± 1	7 ± 3

<sup>a</sup> Colony counts are expressed relative to untreated controls as mean ± standard deviation.

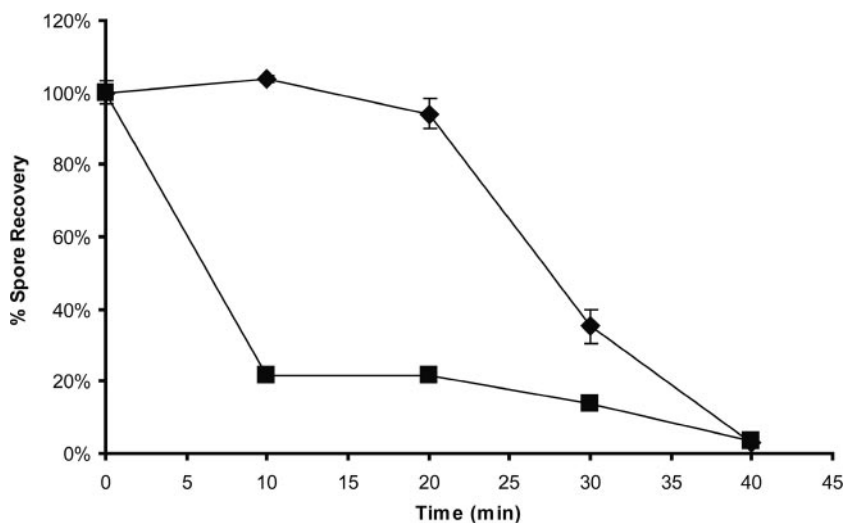


FIG. 2. Heat resistance of dormant spores. Spore dilutions in water were incubated at 90°C for up to 40 min. Survivors were enumerated by plating on NA. Counts are expressed as a proportion of unheated controls. ♦, wild type; ■, AM1654 ( $\Delta exsY \Delta cotY$ ). Error bars show 1 standard deviation.

son, a complete coat permeability defect, such as that found in an *exsA* mutant (1), results in a rapid and synchronous fall in OD within 3 min of exposure to lysozyme. The delayed and less synchronous decrease in OD implies that a partial barrier to passage of large molecules still remains in the  $\Delta exsY$  single and double mutants. To test whether the exosporium is required for lysozyme resistance, wild-type spores were passed twice through the French press under the standard conditions used to release exosporium from the spore surface (31). Staining and microscopy of these French-pressed spores showed that their exosporium was no longer intact, although many remnants remained spore associated. Tests showed that these spores were as resistant to lysozyme as wild-type spores, so penetration through the exosporium is not sufficient to allow lysozyme access to the cortex. This suggests that the reduced resistance in the  $\Delta exsY$  mutant spores is not directly due to absence of the exosporium but that coat layers must also be affected in this mutant, consistent with the observed properties of the spore coat in thin section.

Single- and double-mutant spores showed the same resistance as wild-type spores to wet heat at 80°C, but the double mutant showed a defect in resistance to wet heat at 90°C (Fig. 2). Most of the double-mutant spores (80%) were not recoverable after 10 min, and the remainder were gradually killed over the next 30 min. This may reflect the heterogeneity in the properties of spores within the preparation.

#### Exosporium protein profiles of *exsY* and *cotY* mutants.

Exosporium was purified from water-washed spore preparations of the wild type and single mutants. It was collected following French press treatment of AM1652 ( $\Delta exsY$ ) spores, but the yield was low (ca. one-fifth that of the wild type). We did not attempt to purify exosporium from spore preparations of the double mutant AM1654 as the large amount of loose material evident in the electron micrograph sections of this spore preparation is likely to include coat as well as exosporium material.

Samples of washed exosporium preparations from *B. cereus* ATCC 10876, AM1652 ( $\Delta exsY$ ), and AM1653 ( $\Delta cotY$ ) were analyzed using SDS-PAGE on a 16% Tris-Tricine gel (Fig. 3). Both mutants showed some differences from wild type in exosporium protein composition. The AM1652 ( $\Delta exsY$ ) profile was deficient in many of the protein bands observed in the wild-type exosporium, but two prominent bands at ~70 kDa and ~200 kDa were retained. The AM1653 ( $\Delta cotY$ ) profile was more similar to the wild type, as might be expected given that a complete exosporium is present in this mutant, but there were some minor differ-

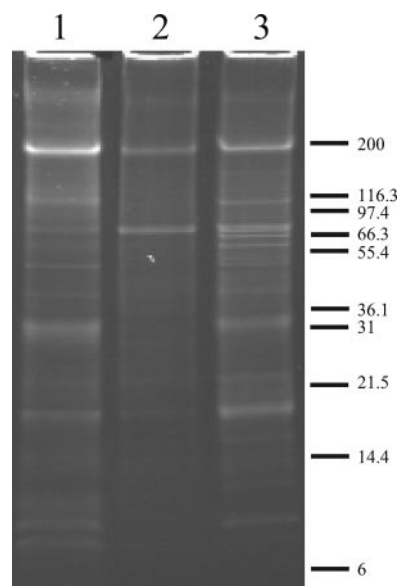


FIG. 3. Protein profiles of washed exosporium of *B. cereus* ATCC 10876 wild-type and mutant strains. Proteins were separated on a 16% SDS-Tris-Tricine gel and stained with SYPRO Ruby. Lane 1, wild type; lane 2, AM1652 ( $\Delta exsY$ ); lane 3, AM1653 ( $\Delta cotY$ ).

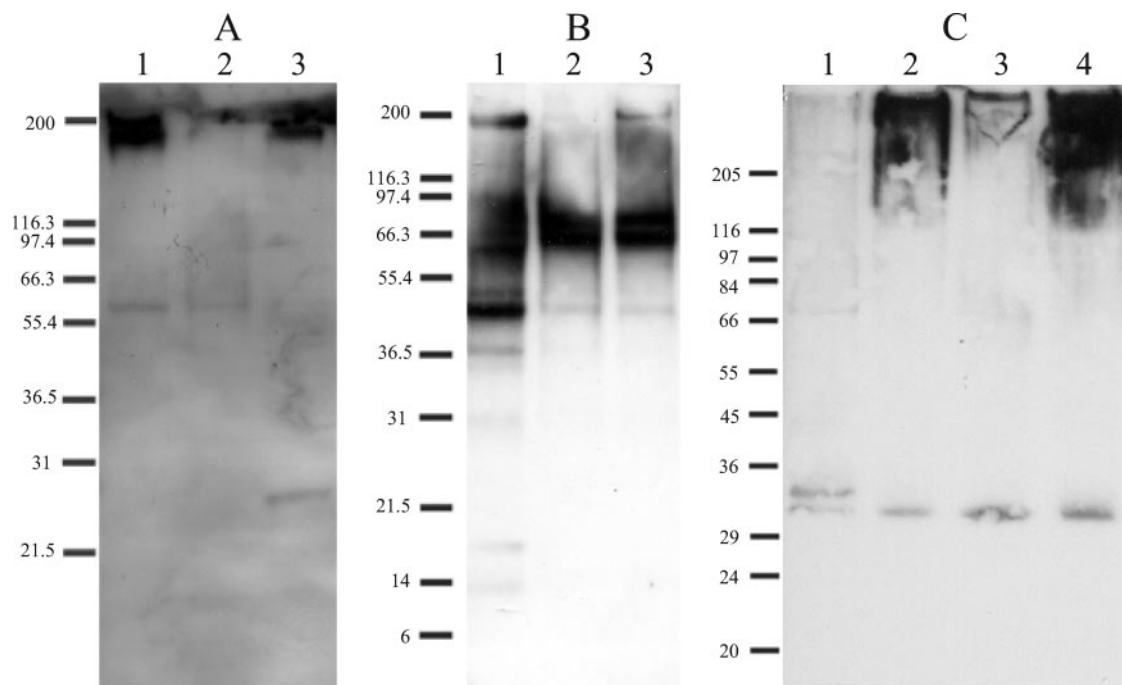


FIG. 4. Detection of BclA and other glycoproteins in washed exosporium following SDS-PAGE on 10 to 20% gradient Tris-glycine gels. (A) Western blot of proteins from washed exosporium probed with CR1, an anti-BclA monoclonal antibody. (B) Total glycoprotein profile of the same exosporium samples. (C) Proteins concentrated from spore culture supernatants, again probed with CR1 antibody to detect BclA. Lane 1, *B. cereus* ATCC 10876 (wild type); lane 2, AM1652 ( $\Delta exsY$ ); lane 3, AM1653 ( $\Delta cotY$ ). In panel C; lane 4 shows AM1654 ( $\Delta exsY \Delta cotY$ ).

ences, including several extra bands in the 40- to 60-kDa region and the absence or reduction of several wild-type protein bands in the 6- to 14-kDa range.

**Glycoproteins in *exsY* and *cotY* mutants.** ExsY and CotY have been found in protein complexes with other exosporium proteins, including the glycoprotein BclA (22). CR1, a monoclonal antibody that detects the BclA glycoprotein, was provided by Caroline Redmond (DSTL, Porton Down, United Kingdom). CR1 was used as the primary antibody in Western blots of DTT-treated exosporium samples from *B. cereus* ATCC 10876  $\Delta exsY$  and  $\Delta cotY$  strains (Fig. 4A). In the wild-type exosporium, the CR1 antibody detected a broad (possibly double) band of 200 kDa, which is the expected position of glycosylated BclA in *B. cereus* (1, 27). The 200-kDa BclA band was severely reduced in the  $\Delta exsY$  sample but retained in the  $\Delta cotY$  exosporium. Minor cross-reacting bands were seen at 60 kDa in the wild type and the  $\Delta exsY$  mutant and at 27 kDa in the  $\Delta cotY$  mutant. These are unlikely to be BclA, as the deglycosylated BclA protein from our wild-type strain of *B. cereus* has been reported at 40 kDa (1).

There are also differences in the overall glycoprotein profiles of exosporium of the wild type and mutants (Fig. 4B), as detected by the Amersham ECL glycoprotein kit, in which metaperiodate-oxidized carbohydrates are biotinylated and then detected after binding of horseradish peroxidase-linked streptavidin. Wild-type exosporium contained strongly labeled bands at  $\sim$ 200 kDa and 40 kDa as well as a smear between 60 and 70 kDa. Several fainter bands were also apparent. In both *ExsY* exosporium and *CotY* exosporium, the 200-kDa and 40-kDa bands were much reduced, but the diffuse doublet band in the 60- to 70-kDa region was predominant. The pres-

ence of additional glycoprotein bands seen in Fig. 4B, compared to the pattern for BclA in Fig. 4A, confirms previous observations that BclA is not the only glycoprotein in *B. cereus* ATCC 10876 exosporium (1). It is also clear that not all the glycoproteins are equally depleted in the exosporium layer of the mutant spores.

**BclA is not assembled completely in the double  $\Delta exsY \Delta cotY$  mutant.** Spore culture supernatants were precipitated at the end of sporulation in CCY medium, following mother cell lysis, and the SDS-PAGE-separated proteins were probed in Western blots with the anti-BclA antibody CR1 (Fig. 4C). There was no significant residual free BclA in wild-type culture supernatant: only two faint cross-reacting bands at around 33 and 34 kDa were detected, which are not likely to represent a BclA monomer, as discussed above for minor bands in Fig. 4A. There was, however, a very strongly stained smear of very-high-molecular-mass material, extending from the top of the gel down to the 200-kDa region, detected in the culture supernatants from the  $\Delta exsY$  mutant and the  $\Delta exsY \Delta cotY$  mutant. This suggests that BclA is produced by these two mutants, but that much of it is misassembled and released into the supernatant on mother cell lysis.

**Detection of the ExsY and CotY proteins in exosporium.** Antibody raised against the ExsY-GST fusion protein is expected to detect both ExsY and CotY, given the high degree of amino acid identity between the two proteins. Synthetic peptide antibodies EY-N and CY-N were therefore raised against the N-terminal regions that are unique to ExsY and CotY, respectively, with the aim of distinguishing between these two proteins on Western blots. The EY-N antibody detected the 49-kDa GST-ExsY fusion protein in a Western blot of partially

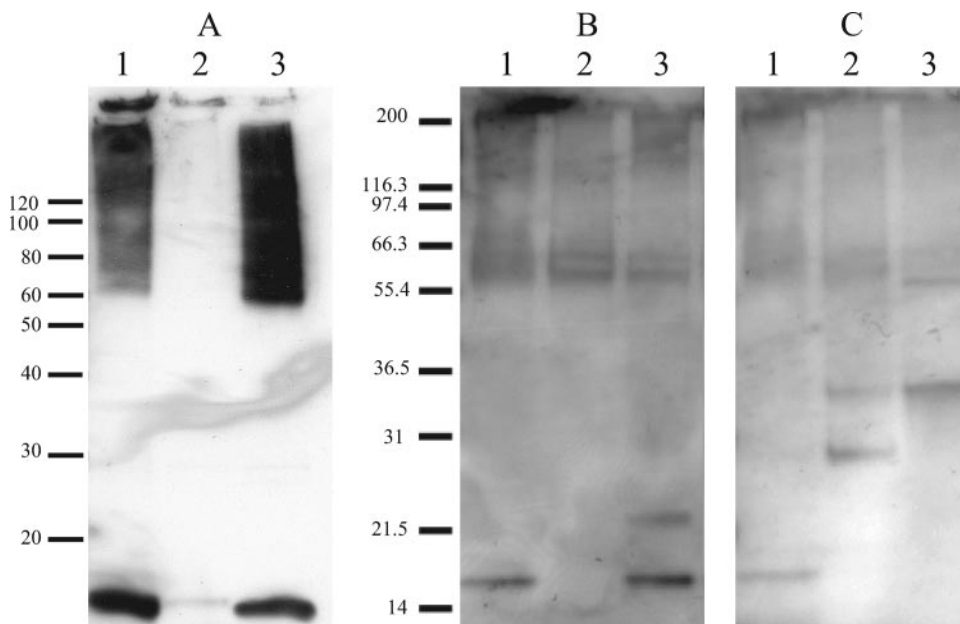


FIG. 5. Detection of ExsY and CotY in exosporium fractions. Western blots are shown with primary polyclonal antibodies as follows: (A) EY-GST (raised against purified ExsY/GST, which detects both ExsY and CotY), (B) EY-N (raised against an ExsY-specific peptide), and (C) CY-N (raised against a CotY-specific peptide). Lane 1, *B. cereus* ATCC 10876 (wild type); lane 2, AM1652 ( $\Delta$ exsY); lane 3, AM1653 ( $\Delta$ cotY). ExsY and CotY monomers migrate at 16 kDa.

purified protein, as expected. The CY-N antibody did not detect this protein on the same blot, confirming its ability to distinguish the two homologues (13).

All three antibodies were used in Western blots against SDS-PAGE-separated proteins of washed, DTT-treated wild-type,  $\Delta$ exsY mutant, and  $\Delta$ cotY mutant exosporium (Fig. 5). When probed with anti-ExsY-GST (Fig. 5A), which would detect both proteins, bands were detected in wild-type exosporium as a large-molecular-mass smear from 60 to 200 kDa, as well as a 16-kDa monomeric form. The profile was essentially unchanged in the  $\Delta$ cotY mutant, where both the large and 16-kDa bands must represent ExsY protein, in the absence of CotY. Therefore, ExsY is definitely present in the exosporium. These bands were massively reduced in intensity in the  $\Delta$ exsY mutant, suggesting either that only a little CotY is present in exosporium or that the absence of ExsY reduces the assembly of CotY into the exosporium. An 8-kDa band (not shown) was sometimes seen in exosporium of the wild type and CotY mutants and may represent a proteolysed fragment, as there are also reports of smaller protein bands containing ExsY- and CotY-derived sequences in exosporium preparations of *B. anthracis* (22).

Western blots of exosporium proteins were probed with CY-N antipeptide antibody and then stripped and reprobed with EY-N antipeptide antibody. The EY-N antibody (Fig. 5B) detected a 16-kDa band, representing ExsY monomer, in wild-type exosporium and in a  $\Delta$ cotY mutant, but not in a  $\Delta$ exsY mutant. Because of the apparently low level of CotY in the  $\Delta$ exsY mutant exosporium, we have no perfect control to test the discriminative ability of the anti-ExsY (EY-N) antibody. We can be confident, however, about the ability of the anti-CotY (CY-N) antibody to discriminate between the two homologues. For example, CY-N (Fig. 5C) did not detect a 16-

kDa monomer in the  $\Delta$ cotY mutant exosporium, where ExsY is still present at normal levels. As this antibody does detect a 16-kDa band in wild-type exosporium, but does not detect ExsY monomer or GST-ExsY, we can deduce that this 16-kDa band in wild-type exosporium is CotY protein. Other bands detected by the antipeptide antibodies in the 25- to 35-kDa range on the Western blots were variably present or absent in different wild-type exosporium preparations and are therefore likely to reflect nonspecific binding.

**Synthesis of ExsY and CotY protein.** Cultures of *B. cereus* ATCC 10876 at 37°C were subjected to a resuspension sporulation regime in which sporulation is synchronously induced by an amino acid downshift, classically used for monitoring sporulation stages in *B. subtilis* (26). Phase-gray immature prespores become visible within mother cells, increasing from 7% at 4 h after resuspension to 80% at 5 h. Mature spores were released from mother cells from around 10 h. ExsY protein (16 kDa) was detected with EY-N antibody in extracts of sporulating wild-type cells from 5 h onwards (Fig. 6A). This strong 16-kDa signal was absent in the  $\Delta$ exsY mutant (Fig. 6C), although a faint cross-reacting band at the same position, possibly CotY, did appear very late in sporulation. The minor band at ~60 kDa was detected in wild-type and  $\Delta$ exsY mutant extracts, so it is not ExsY related (data not shown). The membrane from Fig. 6A was stripped and reprobed (Fig. 6C) with CY-N antibody, which detected CotY monomer (16 kDa) in the wild-type sporulating cells after 6 h.

The time of synthesis of ExsY and CotY proteins in the developing spores would correspond to late stage IV of sporulation, when expression is under the control of the mother cell sigma factor, sigma K (9). Reasonable matches to the (rather similar) consensus sequences for sigma K and sigma E pro-

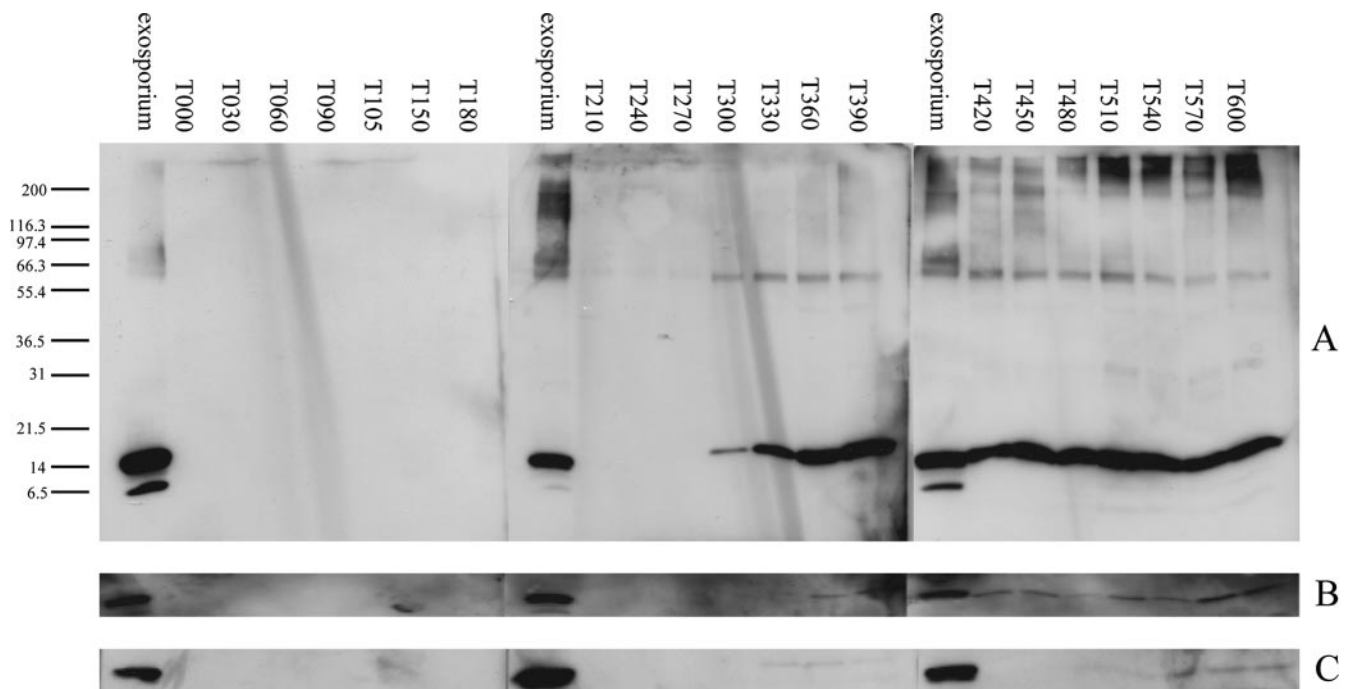


FIG. 6. Synthesis of ExsY and CotY in sporulating cells. The times of synthesis of ExsY and CotY were determined during synchronous sporulation. Samples were taken at intervals, and proteins were separated by SDS-PAGE on 10 to 20% Tris-glycine gradient gels and probed by Western blotting with EY-N antibody (raised against an ExsY peptide) and CY-N antibody (raised against a CotY peptide). Blots represent the following: (A) *B. cereus* ATCC 10876 wild type, with antibody EY-N; and (B), same as panel A, but probed with antibody CY-N. The blot shown in panel C represents a culture of AM1652 ( $\Delta exsY$ ) probed with antibody EY-N as a control for the blot in panel A. Size markers (in kDa) are shown. For conciseness, the blots in panels B and C only show the relevant region for the protein monomers. T000 is the time of resuspension to initiate sporulation, and the numbers thereafter are in minutes.

moters can be found upstream of each gene, with better similarity to K for *exsY* and E for *cotY* (13).

## DISCUSSION

These experiments demonstrate a role for both ExsY and CotY proteins in *B. cereus* exosporium. They may be present in this layer in disulfide-cross-linked forms that can be reduced to monomers by incubation in high concentrations of dithiothreitol, as was the case for the *B. subtilis* proteins (37). The intensity and continuous nature of the remaining smear of 60- to 200-kDa material that contains CotY/ExsY epitopes in exosporium protein profiles suggest that they may also be cross-linked in a less-reversible fashion to other proteins in the exosporium.

Although little is known about the proteins involved in assembly of the exosporium layer, data from this study and several others (1, 14, 25, 28) are gradually elucidating dependencies between proteins that contribute to exosporium formation and integrity. BclA, ExsFA, ExsY, and CotY have all been detected in large >200-kDa aggregates in *B. anthracis* spores (22). Correct assembly of the BclA-containing hairy nap in *B. anthracis* is dependent upon the presence of ExsFA (also designated as BxpB) and its homologue, ExsFB (25, 28). Assembly of ExsY and CotY in *B. cereus* is not ExsFA dependent because both proteins are still present in the exosporium of an  $\Delta exsFA$  mutant (data not shown). The BclA levels may be lower in *exsY* mutant exosporium, but preparations of purified

exosporium fragments from  $\Delta exsY$  or  $\Delta cotY$  single-mutant strains both yielded crystalline fragments with an intact hairy nap (D. A. Ball and P. Bullough, personal communication). *B. cereus* ATCC 10876 has additional major exosporium glycoproteins, including ExsJ (31), that may also contribute to nap formation.

In comparison to *B. subtilis cotY cotZ* double mutants (37), double mutants lacking both *B. cereus* paralogues ExsY and CotY cause a more severe spore defect. The loss of a complete exosporium in the *exsY* mutant, but not the *cotY* mutant, led us to name these genes and their encoded proteins for the two layers, respectively, and as it is now established in the literature (31), we have not changed their names. Although they are closely related homologues, ExsY and CotY have somewhat different roles in spore coat and exosporium formation in *Bacillus cereus*. Both have roles in normal coat assembly, as indicated by the consequences for lysozyme or solvent resistance if either is absent. We therefore assume that both proteins are present in wild-type spore coats, but it has not been possible to generate coat fractions that are entirely exosporium free to prove this definitively.

The role of ExsA in the assembly of both the spore coat and exosporium has already indicated that the assembly of these layers is linked (1). We have now demonstrated two further exosporium and likely spore coat proteins, ExsY and CotY, that are also involved in assembly of both layers. This reinforces the relationship between spore coat and exosporium assembly in the *B. cereus* group of species.



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