A Bacillus subtilis spore coat polypeptide gene, cotS

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A gene, cotS, encoding a spore coat polypeptide of Bacillus subtilis, was isolated from an EcoRI fragment (54 kb) of the chromosome by using synthetic oligonucleotide probes corresponding to the NH₂-terminal amino acid sequence of Cot40-2 previously purified from the spore coat of B. subtilis. The nucleotide sequence (2603 bp) was determined and sequence analysis suggested the presence of two contiguous ORFs, ORF X and cotS, followed by the 5'-region of an additional ORF, ORF Y, downstream of cotS. The cotS gene is 1053 nucleotides long and encodes a polypeptide of 351 amino acids with a predicted molecular mass of 41 083 Da. The predicted amino acid sequence was in complete agreement with the NH₂-terminal amino acid sequence of Cot40-2. The orFX gene is 1131 nucleotides long and encodes a polypeptide of 377 amino acids with a predicted molecular mass of 42 911 Da. The gene product of cotS was confirmed to be identical to Cot40-2 by SDS-PAGE and immunoblotting from Escherichia coli transformed with a plasmid containing the cots region. Northern hybridization analysis indicated that a transcript of cotS and orFX appeared at about 5 h after the onset of sporulation. The transcriptional start point determined by primer extension analysis suggested that -10 and -35 regions are present upstream of orFX and are very similar to the consensus sequence for the σK-dependent promoter. Terminator-like sequences were not found in the DNA fragment (2603 bp) sequenced in this paper, which suggested that the cots locus may be part of a multicistronic operon. The cots gene is located between dnaB and degQ at about 27° on the genetic map. Insertional mutagenesis of the cots gene by introducing an integrative plasmid resulted in no alteration of growth or sporulation, and had no effect on germination or resistance to chloroform.

Keywords: Bacillus subtilis, spore coat gene, cotS, insertional mutagenesis

INTRODUCTION

Endospore formation in the genus Bacillus is a relatively simple model for cell differentiation that involves sequential changes in the cell physiological state and ultrastructure. The spore coat represents one of the most conspicuous morphological structures formed during sporulation. Spore coat polypeptides are synthesized only in the mother cell compartment beginning at 3-4 h of sporulation (t₃₄), and in a defined temporal order (Cutting et al., 1991; Jenkinson et al., 1981; Sandman et al., 1988; Zheng et al., 1988; Zheng & Losick, 1990). Some of the coat polypeptides have been prepared from B. subtilis spores by solubilization using alkaline and/or detergent treatments, and the genes encoding some of them, designated cotA-D (Donovan et al., 1987), cotE (Zheng et al., 1988), cotF(Cutting et al., 1991), and cotT (Aronson et al., 1989) have been cloned. Additional cot genes, whose products are localized in the insoluble fraction of the B. subtilis spore coat, have recently been cloned and designated cotV-Z (Zhang et al., 1993). The cot genes are controlled by a regulatory cascade (Zheng & Losick, 1990), and their transcription is induced by the successive appearance of the regulatory proteins: σK, SpoIID, σK and GerE. Most cot genes are transcribed by RNA polymerase containing σK (EσK); the cotE gene alone has

Abbreviations: CHEF, contour-clamped homologous electric field; GFAK, 5.6 mM glucose, 5.6 mM fructose, 3.3 mM L-asparagine and 10 mM KCl. The DDBJ/GenBank/EMBL/NCBI accession number for the sequence reported in this paper is D31847.
dual promoters for $\sigma^B$ and $\sigma^N$ dependent RNA polymerase. The cot genes and their products are involved in normal morphogenesis of the spore coat and also in the germination process as components of receptors for germinants or of permeability barriers (Aronson & Fitz-James, 1976; Moir, 1981; Aronson et al., 1989; Bourne et al., 1991).

Our approach toward understanding the physiological role of spore coat proteins was to purify the uncharacterized coat components and to identify the genes that encode them. In a previous paper (Abe et al., 1993), we reported the purification of two coat polypeptides (termed Cot40-1 and Cot40-2) each having a molecular mass of about 40 kDa. Determination of the NH$_2$-terminal amino acid sequences showed that they were different from each other and not homologous to sequences of previously described coat polypeptides (Donovan et al., 1987; Zheng et al., 1988; Aronson et al., 1989; Cutting et al., 1991; Zhang et al., 1993). In this paper, we report the cloning and mapping of the cotS gene encoding coat polypeptide Cot40-2. In addition, we examined the effects of insertional mutagenesis of cotS gene on growth, sporulation, ability of the spores to respond to germinants, and spore resistance properties.

**METHODS**

**Bacterial strains, phage, and plasmid.** The cells of *B. subtilis* 60015 (trpC2 metC7) (derived from strain of 168 trpC2, originally provided by E. Freese, NIH, Bethesda, MD, USA) were grown in DS medium (Schaeffer et al., 1965). *Escherichia coli* XL-1 Blue, helper phage VCS-M13 and phagemid vector pBluescriptIISK(+) (Stratagene) were used for sequencing, and *E. coli* was grown in LB medium at 37°C. When necessary, ampicillin or tetracycline were added to final concentration of 50 or 12.5 µg ml$^{-1}$, respectively.

**Preparation and purification of spores.** *B. subtilis* cells were grown in DS medium for 48 h at 37°C, and spores were harvested by centrifugation and purified by washing and lysozyme treatment (100 µg ml$^{-1}$, at 20°C for 20 min), as previously described (Watabe et al., 1981). On the treatment of cell suspension with lysozyme, no change was observed in the spores as monitored by a phase microscope.


**Preparation of DNA.** Chromosomal DNA was prepared from vegetative cells by the method of Saito & Miura (1963). Large- and small-scale plasmid preparations were performed as described by Sambrook et al. (1989).

**Cloning and DNA sequencing of cotS.** Synthetic oligonucleotides were labelled at their 3’ termini with digoxigenin-dUTP and terminal transferase according to the manufacturer’s instructions (Boehringer Mannheim).

**B. subtilis** chromosomal DNA, digested with BstRI, was separated by 0.7% (w/v) agarose gel electrophoresis, denatured and transferred to a positively charged nylon membrane (Boehringer Mannheim). Southern blot analysis was then done with digoxigenin-labelled probes in 2:5 ml hybridization solution per 100 cm$^2$ filter, with overnight incubation at 37°C, and the blotted nylon membrane was washed with tetramethylammonium chloride as described by the manufacturer. The fragments that hybridized on the filter were detected by enzyme immunoassay with luminescence using anti-digoxigenin-POD antibody Fab fragment (Boehringer Mannheim) and enhanced chemiluminescence detection reagents (Amersham) as substrate. In a Southern hybridization experiment, probes A and B hybridized to a 5.4 kb BstRI fragment from digests of *B. subtilis* chromosomal DNA.

For DNA cloning and sequencing, the 5'4 kb BstRI fragment was isolated by 0.7% (w/v) agarose gel electrophoresis and cloned into BstRI and partially digested with pBluescriptIISK(+) phagemid. It was digested again with BstRI and then partially digested with SsaI, and the resulting 2.6 kb fragment was positive on hybridization with probes A and B. This fragment was further digested with appropriate restriction enzymes and subcloned into pBluescriptIISK(+) to construct a series of plasmids containing overlapping fragments ranging in size from about 0.1 to 1.8 kb. Where an appropriate restriction site was not available, deletion with exonuclease III and mung bean nuclease (Deletion Kit, Takara Shuzo) was used. The nucleotide sequence was determined for both strands by the dideoxy chain-termination method (Sanger et al., 1977), using Sequencing High (Chemiluminescent DNA Sequencing Kit, USB) (Tabor & Richardson, 1987) and M13 forward biotin-labelled primer.

**Insertional mutagenesis.** The cotS gene was inactivated by introducing a 2.4 kb ampicillin-chloramphenicol resistance gene cassette (amp$^R$, cm$^R$) from plasmid pCX18 (2.4 kb) (Sato et al., 1994; Yamada, 1989) as shown in Fig. 2. The PstI–HindIII segment (302 bp), corresponding to a central region of cotS, was isolated and cloned in the PstI–HindIII sites of plasmid pCX18 to yield plasmid pCX18S. Competent cells of *B. subtilis* 60015, prepared as previously described (Anagnostopoulos & Spizizen, 1961), were transformed with the plasmid, and the cotS insertion mutant, BS-1, was obtained by integration of the plasmid into the chromosome by single reciprocal recombination (Campbell-like) between *B. subtilis* DNA sequences in pCX18S and the corresponding homologous sequences on the chromosome. The single recombination event was confirmed by restriction enzyme digestion of chromosomal DNA and Southern hybridization to appropriate probes (data not shown).

**RNA preparation and Northern analysis.** *B. subtilis* cells, grown on a DS plate at 37°C for 12 h, were suspended in a small amount of DS medium, and added to 100 ml DS medium to an initial OD$_{600}$ ~ 0.1 and incubated at 37°C with shaking. Samples (25 ml) were harvested at the end of the exponential phase (t$_0$) and during sporulation, and RNA was prepared by the method of Igo & Losick (1986). The RNA (20 µg) was analysed by size fractionation through a 1.5% (w/v) agarose gel containing 2.2 M formaldehyde, transferred to a positively charged nylon membrane (Boehringer Mannheim), and then hybridized to the probe DNAs (Northern probes for orfX and cotS, Fig. 1), which were directly labelled with the horseradish peroxidase according to the procedure for direct nucleic acid labelling and detection systems provided by Amersham.

**Mapping the 5’ terminus of cotS mRNA.** Primer extension analysis was performed as previously described (Sambrook et al., 1989). The synthesized 18-mer oligonucleotide primers, Pr.1 (5’-GAATGGGACTGCCCTAAG-3’), Pr.2 (5’-CTCTT-
CGAACGGATGGGA-3'), and Pr. 3 (5'-GCTTTTTTGGTG-ACACTC-3'), were complementary to 72-89 bp upstream and 38-55 bp downstream of the putative translational start point of orfX, and to −2-16 bp downstream of the putative translational start point of cotS, respectively (Fig. 2). The primers labelled at their 5′ terminus with biotin were purchased from Funakoshi. Total RNA (100 μg) extracted from sporulating cells at different stages was hybridized with 1 pmol primer at 30 °C overnight and used for the assay. Extension reactions were done with Rous-associated virus 2 reverse transcriptase (Takara Shuzo). The lengths of the primer-extended products were determined by comparison with sequencing ladders generated by using the same primer and the single-stranded DNA template containing orfX and cotS.

Mapping of cotS. The location of the cotS gene on the chromosome was determined as described by Itaya & Tanaka (1991). B. subtilis 168 trpC2 chromosomal DNA, digested with NotI or SfiI, was loaded into the well of an agarose gel (10 x 10 cm) placed in a contour-clamped homologous electric field (CHEF) gel apparatus. Running conditions were as follows: 1 % (w/v) agarose, TBE [45 mM Tris/borate (pH 8.0), 45 mM boric acid, 1 mM EDTA] solution (14 °C), 30 s pulse time, 3 V cm⁻¹, 30 h running time. After running the CHEF electrophoresis, the gel was stained with ethidium bromide and run. DNA in the CHEF gels was fractionated by SDS-12.5% (w/v) PAGE as described previously (Abe et al., 1993).

Preparation of lysates from E. coli harbouring the cotS gene. The phagemid pBluescriptIIISK(+) harbouring the cotS DNA was introduced into E. coli XL1-Blue by transformation. The cells were grown in LB medium containing 50 μg ampicillin ml⁻¹ and 1 mM IPTG to OD₆₀₀ ~ 1.0 to induce the expression of the cotS gene. The cells (10 ml) were pelleted and suspended into 20 μl of 62.5 mM Tris/HCl buffer (pH 6.8) containing 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol, and were treated in boiling water for 3 min. The cell lysate was obtained by centrifugation at 12000 r.p.m. for 10 min at room temperature.

SDS-PAGE and immunoblotting. The cell lysate was mixed with 0.1 vol. BBP/glycerol buffer [0.05% bromphenol blue, 70% (v/v) glycerol and 62.5 mM Tris/HCl buffer, pH 6.8], and then fractionated by SDS-12.5% (w/v) PAGE as described previously (Abe et al., 1993). Gels were stained in 0.25% (w/v) Coomassie Brilliant Blue (R250)/50% (v/v) methanol/10% (v/v) acetic acid for 20-40 min.

For immunoblotting, proteins were transferred onto a polyvinylidifluoride membrane (Immobilon, 0.45 μm pore size, Millipore), and detected using rabbit IgG against the spore coat protein fraction as the first antibody, and goat (anti-rabbit IgG) IgG–horseradish peroxidase conjugate as the second antibody (BioRad) (Abe et al., 1993).

Germination. Purified spores were heat activated at 65 °C for 15 min and suspended in 0.05 M Tris/HCl, pH 7.5, to OD₆₀₀ ~ 0.8. Either (i) 10 mM L-alanine, (ii)10 mM glucose plus 10 mM KCl, or (iii) 5.6 mM glucose, 5.6 mM fructose, 3.3 mM l-asparagine, and 10 mM KCl (GFAK) were then added. Germination was monitored by measurement of the decrease in OD₆₁₀ of the spore suspensions at 37 °C for up to 90 min.

Spore resistance assays. Cells were grown in DS medium at 37 °C for at least 12 h after exponential growth and spore resistance was assayed as follows: the cultures were heated at 80 °C for 30 min or were treated with 10% (v/v) chloroform at room temperature for 10 min, and viable cells were determined as previously described (Nicholson & Setlow, 1990).
RESULTS

Cloning and sequencing of the Cot40-2-encoding gene, cotS

The strategy for identifying and cloning the structural gene for this spore coat polypeptide was to use synthetic oligonucleotides as probes that were designed on the basis of the partial NH₂-terminal amino acid sequence. Two different probes (A and B) for Cot40-2 hybridized to a 5.4 kb EcoRI fragment and also to a 2.6 kb EcoRI-SacI fragment (data not shown), so the latter was cloned and sequenced (see Methods).

The restriction map of the 5.4 kb insert is shown in Fig. 1. The restriction sites were used to construct a series of plasmids containing various parts of the Cot40-2-encoding region. The 2.6 kb EcoRI-SacI fragment was positive on Southern blot analysis with probes A and B, and the sequence of this region was determined; a total of 2603 bp was sequenced, revealing the presence of two contiguous full-length ORFs and part of an additional ORF (Fig. 3).

The predicted amino acid sequence from one of the ORFs was found to be in complete agreement with the results of the partial NH₂-terminal amino acid sequence of Cot40-2 for the first 30 residues (Abe et al., 1993). The ninth amino acid residue Glu (E) was erroneously recorded and should be replaced by Gln (Q). This ORF was designated cots; the cots sequence is 1053 nucleotides long, beginning with a GUG start codon at position 1388 and ending with a UGA stop codon at position 2441. Alternative start codons GUG, UUG and AUU are frequently used in prokaryote genes (Parker, 1989; Sorokin et al., 1993). The cots gene encodes a polypeptide of 351 amino acids with predicted molecular mass of 41083 Da.

The other complete ORF, designated orfX, is 1131 nucleotides long, beginning with an AUG start codon at position 240 and ending with a UAG stop codon at position 1371. orfX potentially encodes a polypeptide of 377 amino acids with predicted molecular mass of 42911 Da. While the biological function of the product remains unclear, the predicted amino acid sequence of orfX is partially homologous (18.8%) to the product of the Salmonella typhimurium gene rfaK (Fig. 4), that is believed to encode N-acetylglucosamine transferase (Maclachlan et al., 1991). This enzyme is involved in lipopolysaccharide synthesis in S. typhimurium and is associated with the membrane.

A part of the 5' region of an additional ORF, termed orfY, was found just downstream of the cots sequence and 53 amino acids were predicted (Fig. 3). orfY has a potential ribosome-binding site with optimal distances from the first codon as well as orfX and cots. Sequences homologous to the predicted orfY gene product were not found in recent database searches. No terminator-like sequences such as rho-independent terminators or stem-loop structures were found in the 2.6 kb EcoRI-SacI fragment reported here, which suggests that the cots locus may be part of a multicistronic operon. Determination of the whole sequence of the putative operon is in progress.

Northern analysis of cotS mRNA

To determine the size and approximate time of appearance of the RNA product, the in vivo transcript of cotS and orfX was detected by Northern blot analysis with total RNA of B. subtilis 60015 harvested at the onset (t₀) and at hourly intervals (t₁-t₁₀) during sporulation. The 0.76 kb PstI-SacI and 0.47 kb AluI fragments (Fig. 1) were used as specific probes for cots and orfX, respectively. The PstI-SacI probe detected the transcript of an approximately 2.9 kb fragment that appeared about 5 h after the onset of sporulation (Fig. 5a). The same result was obtained when the 0.47 kb AluI fragment was used as probe (data not shown). Positive bands were also found in the wells of the samples prepared from t₁ to t₆, suggesting that there may have been some denatured DNA in the samples. The nature of an additional RNA species that migrated faster than 2.9 kb in the t₆ sample is unknown. The synthesis of mRNA for cots genes and that of most coat polypeptides starts around t₃ to t₄ during sporulation (Setlow, 1993). According to these results, the synthesis of mRNA for coat polypeptides should be completed by t₁₀, so the positive fragment observed in the t₁₀ sample is quite questionable.

Analysis of the 5' terminus of cotS mRNA

To determine the transcriptional start point(s), we performed extension analysis with primers Pr.1, Pr.2 and Pr.3. Fig. 5(b) shows that primer Pr.2 generated an extension product from RNA isolated from cells at t₃ or t₄ but not from that of cells at t₀ or t₁. From the electrophoretic mobility of the extension product relative to that of a Pr.2-generated sequencing ladder (Fig. 5b), we infer that the 5' terminus of mRNA for orfX and cotS maps to the base pair 204 in the sequence of Fig. 3, i.e. 36 bp upstream of orfX. This inference was strongly supported from the primer extension analysis in which other primers, Pr.1 or Pr.3, complementing either the upstream part of orfX or the downstream part of the cots, respectively, failed to detect any extension products (data not shown).

The sequence preceding the 5' terminus of orfX is aligned with several promoters recognized by σ⁷. The nucleotide sequence of the −35 (AC) and −10 (CgTAtcaTg) regions in the upstream sequence of orfX is similar to the consensus sequence for the −35 (AC) and −10 (CATA----TA) sequences for σ⁷-controlled promoters (Fig. 6).

Analysis of an E. coli lysate

The above results indicated that a transcript containing both orfX and cotS is co-expressed during a late stage of sporulation. To identify the products of these genes, we prepared a 1.7 kb SacI fragment containing about the 3'-half of the orfX sequence, the full-length cots region and the 5' region of orfY (Fig. 1). This DNA was inserted in-frame into the multicloning site of vector pBluescriptII(SK+). The expression of the inserted region is controlled by the lacZ promoter and is inducible.
Fig. 3. Nucleotide sequence of 2603 bp of the cloned EcoRI-SacI fragment and deduced amino acid sequence. A putative promoter region is indicated at -35 and -10. SD, ribosome-binding sites preceding each ORF; rr, a possible initiation site, determined by primer extension (shown in Fig. 5). Nucleotide sequences complementary to the synthetic oligonucleotides (Pr.1, Pr.2 and Pr.3) used in primer extension are underlined. Asterisks indicate stop codons.

by the addition of IPTG. Plasmids pSB or pSA, in which the 1.7 kb SacI fragment was inserted in forward or reverse direction, respectively, were transformed into E. coli, and the cell lysates were analysed by SDS-PAGE. When expression of the inserted region was induced by the addition of IPTG during exponential growth, no significant cell lysis was observed (data not shown), so overproduction of the Cots protein is not toxic for E. coli. As shown in Fig. 7, E. coli cells harbouring plasmid pSB strongly produced two additional polypeptides with molecular mass of about 40 and 25 kDa in the presence of IPTG (Fig. 7, lane 8), while cells harbouring pSA (reverse direction) did not make any additional gene products (Fig. 7, lane 7). To identify the newly produced polypeptides in an E. coli cell lysate, the products were analysed by immunoblotting. The 40 kDa polypeptide alone reacted with anti-spore-coat-protein antibodies (data not shown), and its size coincides with the cotS gene.
components of the spore coat are located at widely

We estimated the size (23 kDa) predicted for the gene product of orfX. Whether this polypeptide is identical to part of the orfX product or whether orfX is expressed at the protein level in B. subtilis is not known.

**Map position of cotS**

The position of cotS on the chromosome was determined by Southern hybridization using NotI- or SfiI-digested B. subtilis 168 trpC2 genomic DNA fragments and the DNA fragments containing cotS as described by Itaya & Tanaka (1991). As shown in Fig. 5, the EcoRI fragment (5.4 kb) was hybridized with NotI fragment 27N (60 kb) and SfiI fragment MS (143 kb) (lanes 2 and 3), and the same results were obtained using the SacI fragment (1.8 kb) (lanes 5 and 6). The physical map analysis using NotI and SfiI fragments confirmed that the genomic map of B. subtilis 168 trpC2 is identical to that of our strain (60015). The positions of the cotB and cotD genes previously reported. The position of the cotS gene is at about 270–275° on the genetic map of Henner & Hoch (1980), and its location is distinct from those of cotB (290°) or cotD (200°). The cotV, -W, -X, -Y and -Z genes are clustered and map at 107° between thiB and metA on the chromosome (Zheng et al., 1993). From our results and the previous reports, it is interesting that the genes so far identified as encoding components of the spore coat are located at widely scattered sites on the chromosome as are the spo genes.

**Mutation in cotS**

We examined the effects of mutation in the cotS gene on growth, sporulation, germination, and spore resistance.
were as resistant to chloroform insertion in the central region of the mutant of strain BS-1, carrying a Campbell-like plasmid, pSB or pSA, containing the 1.7 kb Sad fragment with the -10 and -35 regions relative to the transcriptional start sites (underlined). Minor adjustments were made to bring the most highly conserved blocks of sequence at around the -10 position into register. The promoter sequence information was taken from the following references: $\sigma^s$, Kunkel et al., (1988); $\sigma^A$ and $\sigma^D$, Sandman et al., (1988); $\sigma^B$ and $\sigma^C$, Donovan et al., (1987); $\sigma^E$, Zheng, Losick (1990); $\sigma^E$, Cutting et al., (1989); and spoVJ $\sigma^E$, Foulger & Errington (1991).

**Fig. 6.** Comparison of $\sigma^K$-dependent promoter sequences. The upstream regions of promoters known to be used by $\sigma^K$ are aligned with respect to conserved nucleotides (capital letters) in the -10 and -35 regions relative to the transcriptional start sites (underlined). Minor adjustments were made to bring the most highly conserved blocks of sequence at around the -10 position into register. The promoter sequence information was taken from the following references: $\sigma^s$, Kunkel et al., (1988); $\sigma^A$ and $\sigma^D$, Sandman et al., (1988); $\sigma^B$ and $\sigma^C$, Donovan et al., (1987); $\sigma^E$, Zheng & Losick (1990); $\sigma^E$, Cutting et al., (1989); and spoVJ $\sigma^E$, Foulger & Errington (1991).

**Fig. 7.** SDS-PAGE analysis of cloned $\textit{cotS}$ products in E. coli. The plasmids, pSB or pSA, containing the 1.7 kb SacI fragment with about half of the orfX sequence, the full-length cotS region and the 5' region of orfY (Fig. 1) in forward or reverse directions, were transformed into E. coli, and the products induced with (+) or without (-) IPTG in the cell were subjected to electrophoresis. Samples were fractionated by SDS-12.5% (w/v) PAGE and stained with Coomassie Blue. Lanes: 1 and 2, E. coli cell lysate without plasmid (X); 3 and 4, E. coli cell lysate with pBluescriptISK(+) (W); 5 and 6, E. coli cell lysate with pSA (A); 7 and 8, E. coli cell lysate with pSB (B); 9, spore coat proteins (S) extracted by SDS-DTT solution (1); 10, size marker. The numbers on the right indicate the apparent molecular masses (kDa) of the marker proteins.

The mutant of strain BS-1, carrying a Campbell-like insertion in the central region of $\textit{cotS}$, was indistinguishable from the wild-type for growth (data not shown) and spore production (Table 1). The spores of the mutant were as resistant to chloroform as the wild-type (Table 1), and also resistant to lysozyme treatment (250 $\mu$g ml$^{-1}$, 37 °C, 10 min) (data not shown). In addition, the response to various germinants was the same as that of the wild-type (Fig. 9), the mutant spores germinated immediately on incubation with L-alanine and more slowly with GFAK, but with glucose the mutant did not germinate as well as the wild-type.

**DISCUSSION**

The spore coat reveals a unique morphology in dormant spores of Bacillus and consists of polypeptides of different size, whose synthesis and assembly are precisely regulated by spo genes coordinately with other biological events taking place during sporulation. Coat polypeptide synthesis begins at $t_3$ and some of them are deposited on the surface of the forespore to form the coat layer, while others are synthesized as precursors and converted to mature forms during sporulation. One example is CoTg: it is first synthesized as a 14 kDa precursor and processed to the 12.5 kDa form (Aronson et al., 1989). CoTg is thought to be a morphogenic protein required for outer coat assembly. CoTg mutant spores are lysozyme sensitive and germinate slowly as compared with wild-type spores (Zheng et al., 1988). In this work, we isolated and sequenced a new cot gene, termed cotS, the contiguous ORF X and part of ORF Y. The product of cotS was confirmed as Cot40-2 by comparison of the amino acid sequence and antigenic activity against anti-spore-coat-protein antibodies. The predicted amino acid sequences of cotS and orfX are not homologous to those of the known cot genes and coat polypeptides previously reported (Donovan et al., 1987; Zheng et al., 1988; Aronson et al., 1989; Cutting et al., 1991; Zhang et al., 1993). There is no significant homology in the analysis of the hydrophathy profile of cotS and orfX (data not shown). The product of cotS was shown to be one of the coat components as mentioned above, but we have no evidence that the products of orfX and orfY are also coat components.

Analysis of Northern blots suggested that cotS and orfX are simultaneously transcribed into mRNA during the late stages of sporulation. The synthesis of mRNA coincides with the time of synthesis of spore coat proteins (Zheng & Losick, 1990) and morphogenesis of spore coat structure observed by electron microscopy (Setlow, 1993). Since the estimated size of mRNA from gel electrophoresis was greater than the sum of the nucleotides of cotS (1053 bp) and orfX (1131 bp), and no termination signal was found between cotS and orfY, these results suggest that cotS and orfX may be transcribed simultaneously with orfY. It has been reported that cot genes are controlled both temporally and compartmentally by RNA polymerase containing mother-cell-specific $\sigma^K$ (Cutting et al., 1989; Sandman et al., 1988; Zheng & Losick, 1990). The time-course experiments of coat protein mRNA show that transcripts of cotE, cotA, cotB and cotC appear 4, 6, 7 and 7 h after the start of sporulation, respectively, and many cot genes, except cotE, are members of the so-called $\sigma^K$-controlled genes (Sandman et al., 1988; Cutting et al., 1989; Zheng & Losick, 1990). From the...
results of Northern blot analysis and comparison of $\sigma^K$-dependent promoter sequences, the putative cotS operon seems to have a $\sigma^K$-controlled promoter. To address this question directly, we are now preparing cotS–lacZ fusions to test $\sigma^K$-dependence of the gene fusion expression. The cotV–Z genes, encoding polypeptides present in the insoluble fraction of the spore coat of B. subtilis, were recently cloned. These genes are clustered on the chromosome and three possible $-10$ sequences for $\sigma^K$-like promoters and potential stem-loop structures of transcriptional terminators were found in a 2.8 kb DNA region (Zhang et al., 1993). As shown in Fig. 3, a terminator-like sequence was not found in the 2.6 kb EcoRI–SacI frag-

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**Table 1. Effect of null mutation in cotS on sporulation and spore resistance**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sporulation</th>
<th>Chloroform resistance; $\dagger$ (%)</th>
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<tr>
<td></td>
<td>Spores ml$^{-1}$</td>
<td>Percentage $\dagger$</td>
</tr>
<tr>
<td>60015</td>
<td>$8.0 \times 10^7$</td>
<td>45:2</td>
</tr>
<tr>
<td>BS-1</td>
<td>$4.1 \times 10^8$</td>
<td>72:5</td>
</tr>
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* Spores per ml culture: number of c.f.u. after heat treatment (80 °C, 30 min).
† Percentage sporulation: (spores ml$^{-1}$/viable cells ml$^{-1}$) x 100.
‡ Chloroform resistance: percentage ratio of c.f.u. after treatment to c.f.u. viable cells.

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**Fig. 8.** Location of cotS on the B. subtilis chromosome. Chromosomal DNA from B. subtilis 168 trpC2 was digested with NotI or SfiI and was fractionated on agarose gel placed in a CHEF gel apparatus as described in Methods. (a) and (b) Lanes: 1 and 7, $\lambda$DNA oligomers and HindIII digest of $\lambda$DNA; 2 and 5, NotI fragments of chromosomal DNA; 3 and 6, SfiI fragments of chromosomal DNA; 4, $\lambda$DNA digested with HindIII. (a) Ethidium bromide-stained gel. (b) Detection of Southern hybridization bands. Labelled EcoRI fragment (5.4 kb) and SacI fragment (1.7 kb) (both shown in Fig. 1) were used as probes for lanes 2 and 3, and 5 and 6, respectively, and labelled DNA oligomers and labelled DNA were co-hybridized. A non-radioactive detection method for Southern analysis was used (see Methods).

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**Fig. 9.** Germination of B. subtilis 60015 (a) and BS-1 (b) spores. Spores were heat activated at 65 °C for 15 min, and germinated by 10 mM L-alanine (O), 10 mM glucose plus 10 mM KCl ( ), and GFAK ( ). Germination (%) was calculated as described by Nicholson & Setlow (1990).
ment. From the sequence data and the Northern blot analysis, the cotS locus may have a promoter that is recognized by σR, and may be part of a multicistronic operon consisting of orfX, cotS and orfY.

Since the physiological function of these gene products in *B. subtilis* is unknown, we constructed an insertional mutant of the cotS gene using an integrative plasmid. The mutant cells grew and sporulated normally, which suggests that the gene is either a null mutant or apparently not essential for growth and spore formation. The spores produced by this null mutant responded to germinants and were as resistant to heat, chloroform and lysozyme as the wild-type spores. The CotE protein is of interest both because it is involved in lysozyme resistance and because it responds to L-alanine germination (Zheng et al., 1988). From these results, some biological functions of the cotS gene or its product are expected in morphogenesis of spores. While the present work did not provide information about any essential function of the gene in the development in *B. subtilis*, progress is being made in the analysis of possible alterations in structure and location with ferritin-labelled antibody to pure Cot40-2.

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