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 (5.4 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 σ^K -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 270–275° 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 (t_{3-4}), 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: σ^E , SpoIIID, σ^K and GerE. Most *cot* genes are transcribed by RNA polymerase containing σ^K ($E\sigma^K$); 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.

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dual promoters for σ^E and σ^K 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 NH₂-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* XL1-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 with a phase microscope.

Synthetic oligonucleotides. Oligonucleotides were synthesized in an Applied Biosystems DNA Synthesizer (model 380 A). As probes for Cot40-2, short and mixed oligonucleotides (17 bases long), corresponding to all possible codon combinations for residues 3 through 8 and 20 through 25, respectively, were designed on the basis of the NH₂-terminal amino acid sequence of Cot40-2. The synthetic oligonucleotides were as follows: 5'-C-A-A/G-A-A-A/G-G-A-A/G-C-A-T/C-G-A-A/G-G-A-3' for probe A, and 5'-T-T-T/C-C-A-T/C-A-T-T/C-A-G-A-T/C-C-A-T/C-G-T-3' for probe B.

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-ddUTP and terminal transferase according to the manufacturer's instructions (Boehringer Mannheim).

B. subtilis chromosomal DNA, digested with *EcoRI*, 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 cm² 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 *EcoRI* fragment from digests of *B. subtilis* chromosomal DNA.

For DNA cloning and sequencing, the 5.4 kb *EcoRI* fragment was isolated by 0.7% (w/v) agarose gel electrophoresis and cloned into pBluescriptIISK(+) phagemid. It was digested again with *EcoRI* and then partially digested with *SacI*, 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 OD₆₀₀ ~ 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 as described by 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'-GAATGGGACTGCCCTAAA-3'), Pr. 2 (5'-CTCCT-

Fig. 1. Restriction map of the cloned *EcoRI* fragment harbouring the *cotS* gene of *B. subtilis*. The upper part of the figure shows a restriction map of the 5.4 kb *EcoRI* chromosomal fragment, and the hatched arrows in the middle part indicate the position of ORFs for *orfX*, *cotS* and *orfY*. Open boxes indicate the probes for *orfX* and *cotS* in the Northern hybridization in Fig. 4 (a). Horizontal arrows in the expanded section represent the direction and extent of sequencing. E, *EcoRI*; H, *HindIII*; Sa, *SacI*; P, *PstI*; X, *XhoI*; Su, *Sau3AI*; A, *AluI*.

Fig. 2. Schematic representation of the integration of pCX185 by homologous recombination. Boxes indicate the *cotS* and *orfX* coding regions, solid lines indicate the chromosome sequence and thin lines indicate the plasmid sequence. The figure is not to scale.

CGAACCGATGGGA-3'), and Pr.3 (5'-GCTCTTTTGGT-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 × 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 (1 µg ml⁻¹) and photographed. DNA in the CHEF gels was transferred onto a nylon membrane (Nytran 13N, Schleicher & Schuell) by capillary blotting for 15–17 h (Sambrook *et al.*, 1989). A non-radioactive labelled nucleotide, digoxigenin-11-dUTP, was used for preparing DNA probes. The random primer labelling technique, pre-hybridization and hybridization procedures and colour development were according to the protocol of Boehringer Mannheim.

Preparation of lysates from *E. coli* harbouring the *cotS* gene. The phagemid pBluescriptIISK(+) 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 OD₆₅₀ ~ 1.0 to induce the expression of the *cotS* gene. The cells (1.0 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. BPB/glycerol buffer [0.05% bromophenol 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 polyvinylidene difluoride 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 OD₆₁₀ ~ 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 *Eco*RI fragment and also to a 2.6 kb *Eco*RI-*Sac*I 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 *Eco*RI-*Sac*I 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 *Eco*RI-*Sac*I 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 (t_0) and at hourly intervals (t_{1-10}) during sporulation. The 0.76 kb *Pst*I-*Sac*I and 0.47 kb *Alu*I fragments (Fig. 1) were used as specific probes for *cotS* and *orfX*, respectively. The *Pst*I-*Sac*I 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 *Alu*I fragment was used as probe (data not shown). Positive bands were also found in the wells of the samples prepared from t_0 to t_6 , 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 t_5 sample is unknown. The synthesis of mRNA for *cot* genes and that of most coat polypeptides starts around t_3 to t_4 during sporulation (Setlow, 1993). According to these results, the synthesis of mRNA for coat polypeptides should be completed by t_{10} , so the positive fragment observed in the t_{10} 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 t_5 or t_6 but not from that of cells at t_0 or t_4 . 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 σ^K . 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 σ^K -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 *Sac*I 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

[illegible]

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; $\rightarrow$, 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

<i>orfX</i>	1	MKIALIATEKLPVPSVRGGAIQIYLEAVAPLIAKKHEVTVFSIKDPNLADREKVDGV
<i>rfaK</i>	1	IMIKKIIFTVTPIFSIPRGAAAVETWIIYQVAKRLSIPNAIA--CIKNAGYPEYNKINDN
<i>orfX</i>	58	HYVHLDEDRYEEAVGAELKK-SRFDLVHVCNRPQWPKLKKQAPDAVFILSVHNEMFAYD
<i>rfaK</i>	59	CDIHY--IGFSKVYKRLFKQWTRLDPLPYSQRILNIRDKVTTQEDSVIVIHNSMKLYRQI
<i>orfX</i>	117	KISQAEGEICIDSVAQI-VTVSDYIGQTI-TSRFPSARSKTKTVYSGVDLKYTHPRWTNE
<i>rfaK</i>	117	RERNPNAKLVMHMHNAFEPELPDNDAKIIVPSQFLKAFYEERLPAAAVSIVPNGFCAETY
<i>orfX</i>	175	GQRAREEMRSELGL-HGKKIVLFVGRLSKVKGPHILLQALPDIEEHPDVMVMVFIGSKWF
<i>rfaK</i>	177	KRNPDQNLRRQLNIAEDATVLLYAGRISPKGILLLLQAFKQLRTLRSNIKLVVVGDPYA
<i>orfX</i>	234	G-DNELNNYVKHLHTLGAMQKDHVTFIQFVKPKDIPRLYTMSDVFVCSSQWQEPLARVHY
<i>rfaK</i>	237	SRKGEKAEYQKKVLDAAKEIGTDOCTMAGGQSPDQMHNFYHIADLVIVPSQVEEAFCMVAV
<i>orfX</i>	293	EAMAAGLPIITSNRGGNPEVIEEGKNGYIHDFFENPKQYAEIRINDLLSSSEKRERLGKYS
<i>rfaK</i>	297	EAMAAGKAVLASKGGISEFVLDGITGYHLAEPMSDSSIINDINRALADKERHQIAEK-A
<i>orfX</i>	353	RREAESNFGWQRVAENLLSVYEKNR
<i>rfaK</i>	356	KSLVFSKYSWENVAQRFEEMKNWFDK

Fig. 4. Alignment of predicted amino acid sequences of *orfX* and *S. typhimurium rfaK*. Identical and similar amino acids are indicated by asterisks and dots, respectively.

product predicted from DNA sequencing. The newly detected 25 kDa polypeptide, which did not react with anti-spore-coat-protein antibodies (data not shown), is about 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. 8(b), the *EcoRI* fragment (5.4 kb) was hybridized with *NotI* fragment 27N (60 kb) and *SfiI* fragment MS (143 kb) (lane 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 *trpC2 metC7*) (Itaya, 1993). Based on the physical map of the *B. subtilis* chromosome (full length 4165 kb) constructed by Itaya & Tanaka (1991), the *cotS* gene is located at 3119–3179 kb, which maps between *dnaB* and *degQ* (Fig. 8c). The location of the *cotS* gene is again shown in Fig. 8(d) by comparison with the other *cot* genes previously reported. The position of *cotS* 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.

Fig. 5. Northern analysis of *cotS* mRNA (a) and determination of the transcriptional start site by primer extension analysis (b). (a) Each lane corresponds to RNA (20 µg) from sporulating cells at t_0 to t_{10} , respectively. Northern hybridization was performed with the labelled probe DNA (0.76 kb *PstI*–*SacI*, see Fig. 3) as described in Methods. The position and estimated size of the *cotS* mRNA is indicated by an arrowhead on the right. The positions of the 23S rRNA (2.9 kb) and 16S rRNA (1.6 kb) in the *B. subtilis* *rrnB* operon (Green *et al.*, 1985) are indicated. (b) RNA was hybridized with biotin-5'-end-labelled primer Pr.2, which is complementary to 38–55 bp downstream of the putative translational start point of *orfX* (Fig. 3). Primer-extended products generated by primer Pr.2 were analysed as described in Methods. Each lane corresponds to RNA (100 µg) from sporulating cells at t_0 , t_4 , t_5 and t_6 , respectively. The positions of the track labelled T/C and G/A are dideoxynucleotide sequencing ladders generated from cloned *EcoRI* fragment (5.4 kb) using Pr.2 as a primer. The position of the 5' end of the extension product is indicated by an arrowhead on the sequence.

Mutation in *cotS*

We examined the effects of mutation in the *cotS* gene on growth, sporulation, germination, and spore resistance.

Consensus	-35 AC	-17bp -	-10 CATA---TA
<i>cotS</i>	cccattccgcACaagttggtt-gttcagcCgTatcaTgattctaact		
<i>sigK</i>	cggtagacagACagacagcctcccggtcaCATAcatTtacataataggg		
<i>cotA</i>	atTTTTgttaACcatcacgtccttattgtCATaacTatagtaccaat		
<i>cotB</i>	ttgaattagttCaacaaataaagtgcacgTatataatgcagtatgt		
<i>cotC</i>	aactgtccaaCcgcaaatc-tactcgcCgTataaTAAagcgtagta		
<i>cotD</i>	ttgcatcagaACatgtacccttatttttCATAactTAGtattgtaat		
<i>cotE</i> P ₂	ataaaatgcACactagacaaatgccagCATAagaTAacacgaagaa		
<i>gerE</i>	tgtaaacgtcACctcctgcgccttcttCATAatgaTAtctcgactat		
<i>spoVJ</i> P ₂	ggcggttGtcACaatcgccatcc-gcttgaATAtcaTAtagagagaac		

Fig. 6. Comparison of σ^K -dependent promoter sequences. The upstream regions of promoters known to be used by σ^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: σ^K , Kunkel *et al.* (1988); *cotA* and *-D*, Sandman *et al.* (1988); *cotB* and *-C*, Donovan *et al.* (1987); *cotE* P₂, Zheng & Losick (1990); *gerE*, Cutting *et al.* (1989); and *spoVJ* P₂, Foulger & Errington (1991).

Fig. 7. SDS-PAGE analysis of cloned *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 pBluescriptIIISK (+) (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 *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 $\mu\text{g ml}^{-1}$, 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 CotT: it is first synthesized as a 14 kDa precursor and processed to the 12.5 kDa form (Aronson *et al.*, 1989). CotE is thought to be a morphogenic protein required for outer coat assembly. CotE 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 hydropathy 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 σ^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 σ^K -controlled genes (Sandman *et al.*, 1988; Cutting *et al.*, 1989; Zheng & Losick, 1990). From the

Fig. 8. Location of *cotS* on the *B. subtilis* chromosome. Chromosomal DNA from *B. subtilis* 168 *trpC2* was digested with *Not*I or *Sfi*I and was fractionated on agarose gel placed in a CHEF gel apparatus as described in Methods. (a) and (b) Lanes: 1 and 7, λ DNA oligomers and *Hind*III digest of λ DNA; 2 and 5, *Not*I fragments of chromosomal DNA; 3 and 6, *Sfi*I fragments of chromosomal DNA; 4, λ DNA digested with *Hind*III. (a) Ethidium bromide-stained gel. (b) Detection of Southern hybridization bands. Labelled *Eco*RI fragment (5.4 kb) and *Sac*I 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). (c) A portion of the physical map of the *B. subtilis* chromosome region from 3000 to 3400 kb. (d) Location of the *cotS* gene on the *B. subtilis* chromosome. The map positions are taken from the following references: *cotA* to *cotD*, Donovan et al. (1987); *cotE*, Zheng et al. (1988); *cotF*, Cutting et al. (1991); *cotT*, Aronson et al. (1989); *dnaB* and *degQ*, Amjad et al. (1991); and *cotS*, this study.

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

Strain	Sporulation		Chloroform resistance† (%)
	Spores ml ⁻¹ *	Percentage†	
60015	8.0 × 10 ⁷	45.2	52.5
BS-1	4.1 × 10 ⁸	72.5	46.8

* 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) × 100.

‡ Chloroform resistance: percentage ratio of c.f.u. after treatment to c.f.u. viable cells.

results of Northern blot analysis and comparison of σ^K -dependent promoter sequences, the putative *cotS* operon seems to have a σ^K -controlled promoter. To address this question directly, we are now preparing *cotS-lacZ* fusions to test σ^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 σ^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 *Eco*RI-*Sac*I frag-

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 (○), 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 σ^K , 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 preparation of the *orfX* mutant and *cotS-orfX* double mutant and in the analysis of possible alterations in structure and location with ferritin-labelled antibody to pure Cot40-2.

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