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13. ABSTRACT (Maximum 200 words)

The spore coat of Bacillus subtilis is comprised of two morphological layers each consisting of several polypeptides. One which was cloned and sequenced is processed from a precursor. A deletion of this gene resulted in some alteration in the structure of the inner coat and of the capacity to respond to a specific spore germinant. When this protein was overproduced from a multicopy plasmid, the spores had a thickened inner coat and germinated poorly. The absence of another spore coat protein had extensive pleiotropic effects on the assembly of the outer spore coat. As a result, the spores were lysozyme-sensitive but they germinated at rates similar to the wild type. Antibody was used to demonstrate that this particular coat protein was synthesized long before the assembly of the spore coat and much earlier than other coat proteins. Despite its hydrophilic properties, it is located in the cell insoluble (membrane) fraction requiring detergent or 8M urea for solubilization. Suppressor strains forming lysozyme-resistant spores were selected from those produced by this deletion strain. The germination properties of these spores were unaltered but novel polypeptides were present in spore coat extracts.

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Function of Bacterial Spore Coat Polypeptides in
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A. Statement of the Problem Studied

The coat of bacterial spores usually consists of two morphological layers which function to protect spores from various solvents and enzymes such as lysozyme. It may also have a direct or indirect role in germination. Little is known, however, about the assembly of the coat, the properties of the polypeptides present in each layer, and the function(s) of each in solvent resistance, germination or assembly. Among the spore formers, Bacillus subtilis has been selected for the study of these problems because of the genetic, biochemical and cloning techniques available. A variety of approaches centering on genetic engineering technology have been used to define the structure and function of particular B. subtilis spore coat proteins.

B. Summary

1. Both the precursor and mature CotT proteins were partially sequenced in order to confirm that a 10.1 kDa precursor initiated with a TTG codon and was processed at an arg-gln bond to produce the 7.8 kDa mature protein. Site directed mutagenesis was used to change the arg to ile (confirmed by sequencing). A heterozygous diploid was constructed and the spores were very similar to the wild type in protein profile and germination responses. Extracts of this diploid contained an excess of CotT precursor detectable in immunoblots whereas little if any was found in the parental strain or in a diploid with two copies of the wild type cotT gene. This modified CotT protein appears not to be processed but its effect in single copy must be determined. Strains containing only a copy of the mutant gene were isolated as chloramphenicol-sensitive revertants of the diploid. The presence of only one copy of the cotT gene was confirmed by Southern hybridization. These revertants accumulated CotT precursor in extracts (Fig. 1) or on spores.
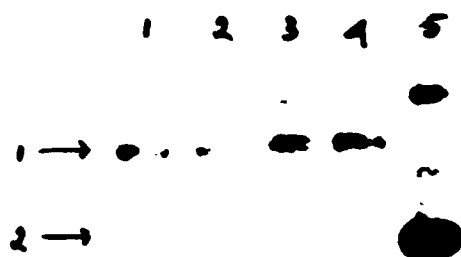
1

Fig. 1. Immunoblot with CotT antibody of spore extracts from

B. subtilis JH642 mutants containing a single copy of

the cotT gene altered at the processing site (lanes 1-4)

and from B. subtilis JH642 (lane 5). Arrow 1 indicates

precursor CotT antigen present in extracts of spores produced

by the mutants and arrow 2 shows the processed CotT antigen in

extracts of spores from the parental strain.

2. As previously mentioned, the CotT protein is over expressed in a gerE strain with most of the precursor in extracts of sporulating cells (Aronson et al. in publication list). Two deletion mutants of cotT were constructed and one contained an intact gene with the presumed RNA polymerase binding site (consistent with nuclease S_1 mapping) but lacked the upstream region. No CotT antigen was produced by this strain and the spores had the same germination defect as a deletion which contained two defective gene copies. Apparently, an upstream region (perhaps the GerE protein binding site) is needed for transcription. We have sent the cotT clone to Dr. R. Losick at Harvard for a further study of regulation (cotT-lacZ fusions). In return, he has sent clones of the cotD and cotE genes (as well as deletion strains) for our analysis of spore properties and coat assembly. We are in communication to be certain that different coat genes are being cloned and studied so that our results will complement each other.

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3. The cotT deletion strains germinated poorly in the presence of the glucose-fructose-asparagine mixture but the same as the wild type in L-alanine (plus inosine) or Penassay broth (Bourne et al. in publication list). The slow germination rate was unchanged when the concentration of germinants was decreased ten fold or increased two fold. Perhaps, the absence of the CotT protein alters the coat structure subtley (little difference from the wild type seen in electron micrograph sections) and thus access to a target for this particular germination mixture.

Increasing the copy number of the cotT gene on a low copy number plasmid resulted in spores which germinated slowly in response to each of three germination systems. These spores have extra inner coat layers and a large amount of precursor antigen was extracted from well washed spores. In fact, there appears to be two CotT precursor proteins present, a major one of 10.1 kDa and a second one of about 8.5-9.0 kDa. Since these precursors can associate tightly with spores, they may be processed after deposition.

4. The cotT gene has been subcloned into an T_7 polymerase expression vector kindly provided by Dr. W. Studier and a 10 kDa band was detected in cell extracts by staining and immunoblotting. The processing protease (probably trypsin-like) is of interest especially since it may be a spore specific protease with other functions related to coat morphogenesis. In vitro, trypsin but not the B. subtilis serine proteases appeared to cleave the CotT precursor to a polypeptide of the correct size (Aronson et al.). A B. subtilis mutant lacking an intracellular trypsin-like activity still processed CotT precursor so a special protease(s) may be involved.

5. A limited number of studies have been done with the cotD gene and its deletion clone kindly provided by Dr. Losick. CotD overproduction (stained band on SDS-PAGE of spore or cell extracts) resulted in some decrease in

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germination for all three germinants but not as marked an effect as overproduction of the CotT protein (on the same low copy number plasmid so gene copy number should be about equal; see Bourne et al.). There were no detectable germination effects due to a deletion of the cotD gene.

6. There was some overproduction of the CotE protein when cloned on a low copy number vector but no noticeable effect on germination nor change in the lysozyme-sensitivity of the spores. Apparently, extensive overproduction of this protein inhibits sporulation (B. Zheng and K. Losick, personal communication) but some overproduction in the low copy number plasmid was evident from stained SDS-PAGE. Since the protein is difficult to detect in stained gels, an antibody was produced by synthesizing a peptide of 20 residues from the sequence at the carboxyl end. This peptide was purified on HPLC and coupled to bovine serum albumen for subcutaneous injection into rabbits. The antibody is specific for the CotE protein and was used in immunoblots to confirm overproduction in the strain containing the clone as well as the absence of CotE antigen in the deletion strain.

CotE antigen appeared early in sporulation (Stage II-III) and was not present in a spoOB mutant. Once produced, the CotE protein was stable since it survived prolonged incubation of sporulating cells in chloramphenicol. Even though it appears to be a hydrophilic protein, CotE antigen was found primarily in the crude insoluble fraction after lysis by sonication (Fig. 2).
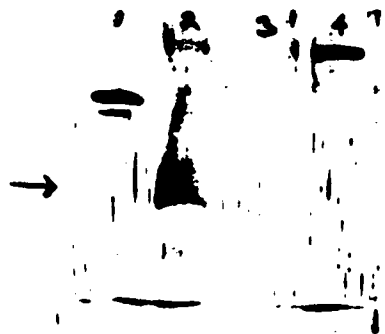
Fig. 2. Immunoblot with CotE antibody of extracts from

sporulating cells (stage II-III, i.e. before the appearance

of phase-white endospores) of the wild type, PY17, (lanes 1

and 2) and the cot E deletion strain (lanes 3 and 4). Lanes

1 and 3 contain soluble fractions prepared by sonication and

centrifugation at 20,000 xg for 10 min. Lanes 2 and 4

contain 6M urea--1% SDS pH 9.5 extracts of the insoluble

fractions. Arrow on left indicates a broad band of CotT

antigen of ca. 12kD. Note also smaller reacting antigen

which may be a degradation product. Sharp reacting band of

higher molecular weight in lanes 1 and 4 is present usually

in the soluble fraction of all cells (even during

exponential growth and in a spoOB mutant).

Endospores were separated from this crude membrane fraction in a Renografin

gradient and both fractions contained CotE antigen whereas CotT antigen was

primarily present in the spore extract. The protein was not solubilized with

low or high (0.5M KCL) ionic strength buffer, with 1% Triton X-100, Brij 35 or

n-octyl-glucoside. Either SDS or 8M urea was needed for solubilization.

A data bank search revealed some homology over a stretch of ca. 65 amino

acids in the middle of the protein to an acidic cytokeratin intermediate

filament protein from bovine bladder urothelium. There are 17 identical and 29

similar residues within this region which is believed to form an a helix in the cytokeratin important for the protein interactions required for filament formation. The homology is intriguing because (1) intermediate filament proteins have not been found in prokaryotes and because (2) one of the phenotypic effects of a cotE deletion is the production of very small condensed germinated spores (see below) perhaps due to the absence of some critical spore structural component.

Germination studies have been done with the cotE deletion and there was a short lag (5-10 minutes) in L-alanine (plus inosine and KCL; Fig. 3),
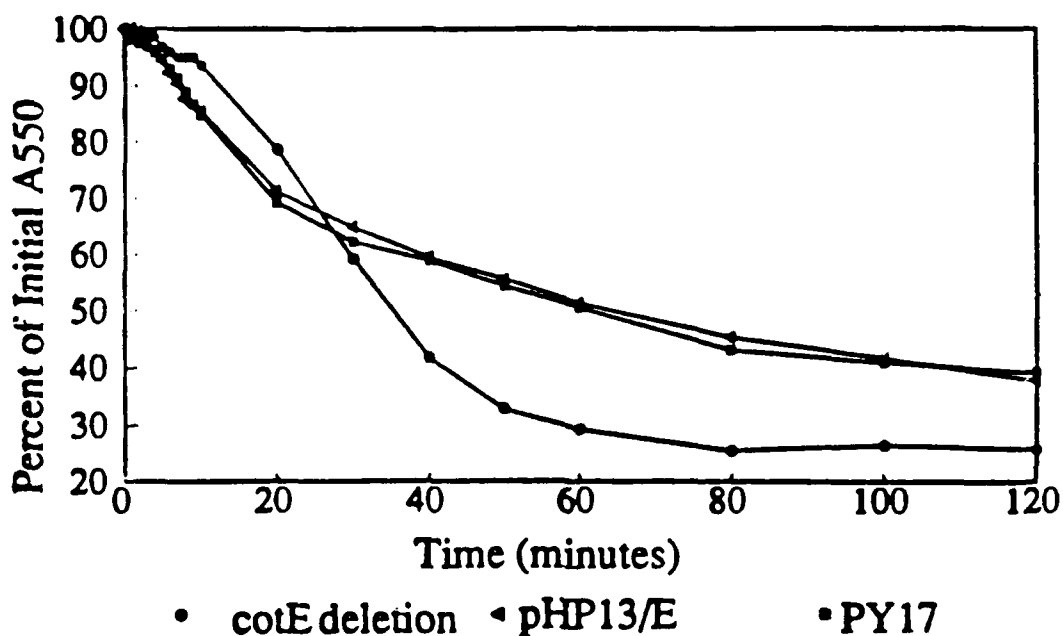

Fig. 3. Germination of spores of the wild type (PY17), the cotE deletion and a strain with the cotE gene on a low copy number plasmid (pHP13/E). Spores were suspended to an A_{550} value of 1.0, and heat activated at 80°C for 20 min. Germination was in 0.05M Tris-0.1M KCL, pH 7.6 plus 1mM each of L-alanine and inosine.

but not in Penassay broth. The lag was found with or without heat activation and even when the L-alanine concentration was increased tenfold (to 10mM) but not if KCL were omitted from the germination mixture. Germination proceeded after the lag perhaps more rapidly than in the control and always to a much lower optical density (i.e. 80% decrease versus about 50-60% in the control; Fig. 3). There was little lysis of these germinated spores as determined by viability assays before and after germination for 120 min. The germinated cotE spores were very condensed in appearance in the phase microscope which could account for the extensive decrease in light scattering. There are obviously a lot of interesting properties of this cotE deletion strain in relation to coat structure, assembly and germination. CotE antibody was sent to Dr. Losick for electron microscope studies of the localization of this antigen.

Lysozyme-resistant spores produced by the cotE deletion strain were isolated from B. subtilis Marburg containing the deletion. The presence of the deletion was confirmed by transforming the marker (chloramphenicol[R]) to B. subtilis JH642 and by Southern hybridization. Two suppressor strains have been studied and both were as solvent-resistant (n-octanol, chloroform) and the spores germinated the same as the original deletion strain. There were one or two novel polypeptides of ca. 25 kDa in extracts of spores or spore coats from the suppressor strains but most of the other high molecular weight outer spore coat proteins were still absent. Both suppressors appear to be linked to the aroD gene but more extensive mapping is needed.

One other interesting property of the spores formed in the cotE deletion strain is the presence of CotT antigen primarily in the precursor form (Fig. 4).
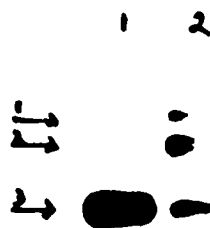
Fig. 4. Immunoblot with CotT antibody of spore extracts
from the wild type strain PY17 (lane 1) and the CotE
deletion strain (lane 2). The processed CotT antigen is
prevalent in the wild type (arrow 3) whereas the precursor
forms of CotT (arrows 1 and 2) are present in spores lacking
outer coat due to the cotE deletion.

This protein is probably present in the inner coat but apparently is not
processed in the absence of outer coat. A possibly functional relation between
processing and outer coat assembly will be studied in the strain with the CotT
processing mutation.

Two other coat genes are being cloned. In one case, a coat polypeptide
was transferred to a PVDF membrane for sequencing. In the other, the coat
insoluble fraction (i.e. 20-30% of the protein remaining after extensive
extraction of purified spore coats) was digested with formic acid and the
peptides were resolved on HPLC. One pure peptide was sequenced and mixed
probes prepared. Southern transfers of restriction enzyme digests of B.
subtilis JH642 DNA were hybridized with these probes. Some HindIII fragments
have been subcloned and will be sequenced using the probes as primers.

C. PUBLICATIONS

Aronson, A. I., Song, H-Y. and N. Bourne (1989) Gene structure and
precursor processing of a novel Bacillus subtilis spore coat protein.
Molec. Microbiol. 3:437-444.

Bourne, N., Huang, T-S. and A. I. Aronson (1990) Properties of Bacillus
subtilis spores with alteration in spore coat structure. In Genetics
and Biotechnology of Bacilli. A. T. Ganesan and J. A. Hoch (eds.) in press.

Bourne, N., FitzJames, P. C. and A. I. Aronson. Germination defects in
Bacillus subtilis spores altered in spore coat protein composition.
J. Bacteriol., under revision.

Abstract

Bourne, N. and A. I. Aronson. Properties of Bacillus subtilis spores with
alterations in spore coat structure. Fifth International Conference
on Genetics and Biotechnology of Bacilli. July 9-12, 1989, Pacific
Grove, California.

D. Participating Scientific Personnel

Dr. N. Bourne, Postdoctoral Associate	1987-1989
Mr. J. Zhang, Graduate Assistant	1988-present
Dr. L. Ekanayake, Postdoctoral Associate (part time)	1990

Gene structure and precursor processing of a novel *Bacillus subtilis* spore coat protein

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Summary

The gene for an unusual 8 kD *Bacillus subtilis* spore coat polypeptide has been cloned and sequenced. It contains high percentages of proline, glycine and tyrosine, lacks thirteen amino acids, and is present as the carboxyl two-thirds of an open reading frame encoding a 12 kD polypeptide. Two presumptive precursors which could be converted to the 8 kD antigen by incubation with trypsin were found in extracts of cells or spores of a strain containing multiple copies of this gene. Large amounts of these coat antigens were also present in extracts of a germination-defective mutant which is altered in spore coat structure. There was little 8 kD coat protein in the mutant, however, implying that processing is dependent on proper coat assembly. This gene was mapped to the *metA* region of the *B. subtilis* chromosome, a unique location as is true for other spore coat genes. Transcription and translation occurred late in sporulation (stage V) and the upstream region contained sequences similar to those found in other spore coat genes.

Introduction

The proteinaceous coat layers of the bacterial spore (exclusive of the exosporium) are believed to have a protective function and to play some role in germination (Aronson and Fitz-James, 1976; Kutima and Foegeding, 1987). A cryptic cortex lytic enzyme (Brown *et al.*, 1982; Foster and Johnstone, 1987) or proteases involved in the activation of this enzyme (Boschwitz *et al.*, 1985) may be associated with an integral part of the coat. Alternatively, the coat may simply function as a barrier or conduit for germination factors reaching their target at the inner spore membrane (Skomurski *et al.*, 1983).

The nature and heterogeneity of the proteins comprising the spore coat vary from a rather simple array of low molecular weight polypeptides in *Bacillus cereus* (Aronson and Fitz-James, 1976), *Bacillus megaterium* (Stewart

and Ellar, 1982), and perhaps clostridia (Tsuzuki and Ando, 1985) to a rather heterogeneous group of polypeptides in *B. subtilis* (Goldman and Tipper 1978; Pandey and Aronson, 1979; Jenkinson *et al.*, 1981). The appearance in freeze-etched electron micrographs of the surface layers of the latter spores also differs considerably from the others (Holt and Leadbetter, 1969; Aronson and Fitz-James, 1976). In the case of *B. subtilis*, the extractable proteins vary in size from about 65 kD to 7 kD with a preponderance of 4–5 species of 7–12 kD. In fact, some of the larger, less prevalent proteins found in SDS-PAGE may be contaminants or superficial components since their presence varies with the medium used for spore formation, with the age of the spores and perhaps with the methods used to prepare spore coats or to extract spore coat proteins (Jenkinson *et al.*, 1981; Sastry *et al.*, 1983; Feng and Aronson, 1986).

In addition, about 30% of the protein in *B. subtilis* spore coats is not soluble under a variety of conditions and is probably composed of cross-linked polypeptides, in part antigenically similar to some of those found in the soluble coat fraction (Pandey and Aronson, 1979; Feng and Aronson, 1986). The nature of the cross-linking is not known (Pandey and Aronson, 1979; Goldman and Tipper, 1981).

To date, efforts to isolate mutants defective in spore coat assembly or structure in *B. subtilis* have not been very fruitful. There is only a single germination defective mutant, GerE (Moir *et al.*, 1979; Moir, 1981), altered in spore coat structure. This gene appears to encode a DNA-binding protein (Holland *et al.*, 1987) and this mutant is pleiotropically altered in coat protein composition (Jenkinson and Lord, 1983; Feng and Aronson, 1986). In addition, there are a few other less well-defined presumptive coat mutants (SpoVI, A, B, C) (Jenkinson, 1981; 1983; James and Mandelstam, 1985).

The complexity of *B. subtilis* spore coat polypeptides poses an interesting problem regarding assembly of a protective structure which may be directly involved in germination. As a start in dissecting this complex process, several spore coat genes have been cloned and their regulation as well as their functions have been examined (Donovan *et al.*, 1987; Sandman *et al.*, 1988). Here we report the cloning and analysis of the gene for one of the more prevalent and unusual low molecular weight spore coat proteins (Goldman and Tipper, 1978). It appears that
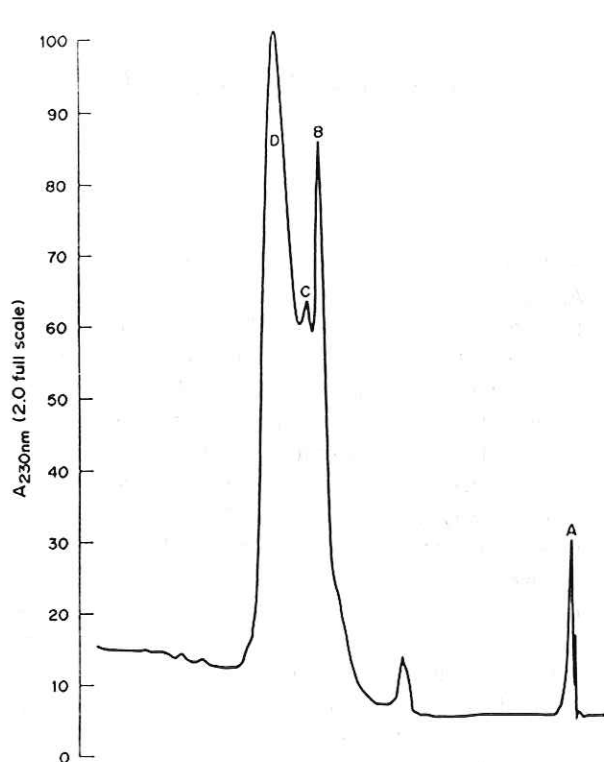
Fig. 1. HPLC elution profile of the Na$_2$SO$_4$ precipitate of a spore coat extract. The precipitate was dissolved in 20% n-propanol-0.1% TFA and loaded onto an RP-P column. Elution was performed with a linear gradient of 20–100% n-propanol-0.1% TFA. Peaks B–D (eluting at about 40% n-propanol) were collected manually, pooled from several runs, lyophilized and rechromatographed. Initially, peak D was sequenced in order to prepare an oligonucleotide probe. Subsequently 20–30 residues of purified peaks B and C were sequenced and found to be identical to peak D.

the mature coat protein is processed from a precursor, perhaps on the spore surface.

Results

Isolation of a spore coat protein and cloning of the gene

One of the major, low molecular weight spore coat proteins was salted out from a spore coat extract with 0.1 M Na$_2$SO$_4$ (Goldman and Tipper, 1978). Following extensive washing with distilled water, the precipitate was dissolved in 20% n-propanol-0.1% TFA and fractionated on a reversed phase column (Fig. 1). Peaks B–D were collected manually, lyophilized and refractionated in the same system as in Fig. 1. Initially, purified peak D was lyophilized and sequenced: NH$_2$ gln-pro-tyr-tyr-tyr-pro-arg-pro-arg-pro-pro-phe-tyr-pro-pro-tyr-tyr-tyr-pro-arg. A mixed deoxyoligonucleotide encoding the underlined amino acids (the pro-tyr-tyr-tyr-pro portion is repeated)

was synthesized: CA^A_GCCXUA^U_CUA^U_CUA^U_CCC. It was later found that the sequence of the first twenty amino acids for peaks B and C were the same as for peak D (unpublished results). There is no evidence for multiple genes based on Southern hybridization of the cloned 1.2 kb *Hind*III fragment (see below) to total *B. subtilis* DNA digested with *Hind*III, *Eco*RI or *Bam*HI (unpublished results). It is assumed, therefore, that the three closely eluting peaks in Fig. 1 reflect the same polypeptide bound to some other components, but this possibility has not been explored any further.

The purified oligonucleotide was labelled and hybridized to pools of plasmids containing fragments from a partial *Sau*3A digest or to Southern blots of a *Hind*III digest of *B. subtilis* DNA, as described in the *Experimental procedures*. In both cases, a 1.2 kb *Hind*III fragment was isolated and sequenced (Fig. 2). Only 759 nucleotides are shown, since this region includes the major open reading frame (ORF) encoding a polypeptide of 107 amino acids (13 kD) including the sequence of peak D (the gene encoding this ORF is designated *cotT*). The only other ORFs greater than 40 amino acids long extensively overlapped *cotT*. The sequence of peak D starts about one third of the way from the amino end of the ORF (thick arrow in Fig. 2) and would encode a polypeptide of 7.8 kD. S1 nuclease mapping using either a *Hind*III-*Hha*I end-labelled fragment or primer extension as described in *Experimental procedures* placed the start of transcription as indicated by the thin arrow in Fig. 2. In addition, there are sequences (underlined) in this region which are similar to the presumed RNA polymerase binding sites for other spore coat genes (Zheng *et al.*, 1987, Abstract of the Fourth International Conference on Genetics and Biotechnology of Bacilli) but the closest potential ribosome binding site is 25–27 nucleotides from the ATG codon. Since the optimum spacing is about nine nucleotides (Stormo, 1986), secondary structure is probably important for translation of this mRNA. Data presented below are consistent with the presence of one or more precursors of the 7.8 kD spore coat protein and thus the translation of the larger ORF.

The amino acid composition of the 7.8 kD processed product is very similar to that of the Na$_2$SO$_4$ precipitate from spore coats and is virtually identical to that of peak D (Table 1). This polypeptide is very unusual because of the absence of thirteen amino acids and the very high content of glycine, tyrosine and proline. There are several repeats of pro-tyr-tyr-tyr-pro or pro-tyr-tyr-pro as well as pro-arg (or pro-arg-pro-arg) and all of the eleven gly residues interspersed with tyr are confined to the carboxyl end.

A single transcript found only in late sporulating cultures hybridized to the 1.2 kb *Hind*III fragment (Fig. 3). The extrapolated size based on ribosomal RNAs and the glutamine synthetase transcript was approximately 10^5 Daltons.

```
      750        740        730        720        710        700        690
ATGAAACCAT TTGATTCTTT TTGCCAACAA CAAAAACTCC TTCTTTCGTA GCCATTTCGA TTTACTCTTC

      680        670        660        650        640        630        620
ATCAGTTGGA ACTGTATCTA TTTAGAAATG TTTTTGTAAC ATTGTAGTAA CATTTATTTC ACATTTGTAA

      610        600      ↓590        580        570        560        550
TACTATGAGA TTTAGAAGTT CATGTCAATC CTTTTTAAGG AATTTCATCA TAAAAAAAAC CGAATTTC
```

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                                   522                                  492
ATG AAT GTA CAT ACA CCC AAC TTA AGC ATC AGG AAT ATG GTA AAA GGA ATA AAA AAA GCT
Met Asn Val His Thr Pro Asn Leu Ser Ile Arg Asn Met Val Lys Gly Ile Lys Lys Ala

                                   462                                  432
AGG GAG GTT TTC CTC TTG GAT TAC CCT TTG AAT GAA CAG TCA TTT GAA CAA ATT ACC CCT
Arg Glu Val Phe Leu Leu Asp Tyr Pro Leu Asn Glu Gln Ser Phe Glu Gln Ile Thr Pro

         ↓                         402                                  372
TAT GAT GAA AGA CAG CCT TAT TAT TAT CCG CGT CCG AGA CCG CCA TTT TAT CCG CCT TAT
Tyr Asp Glu Arg Gln Pro Tyr Tyr Tyr Pro Arg Pro Arg Pro Pro Phe Tyr Pro Pro Tyr

                                   342                                  312
TAT TAT CCA AGA CCG TAT TAT CCG TTC TAC CCG TTT TAT CCG CGC CCG CCT TAT TAC TAC
Tyr Tyr Pro Arg Pro Tyr Tyr Pro Phe Tyr Pro Phe Tyr Pro Arg Pro Pro Tyr Tyr Tyr
                                             Hhaf
                                   282                                  252
CCG CGC CCG CGA CCG CCT TAC TAC CCT TGG TAC GGT TAC GGC GGA GGT TAT GGC GGA GGA
Pro Arg Pro Arg Pro Pro Tyr Tyr Pro Trp Tyr Gly Tyr Gly Gly Gly Tyr Gly Gly Gly

                            220        210        200        190
TAT GGG GGA GGT TAC GGT TAC TAG  TCGCCACA TATCAAAATG GGGCCAGTCC AGCTCCCCAT
Tyr Gly Gly Gly Tyr Gly Tyr End
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```
      180        170        160        150        140        130        120
TTTTTTGTTT TAAAAATGAC AATATCATGA CAAATATGGG AAATAATTGT GATTCTGTCC CCCTCTTGTT

      110        100         90         80         70         60         50
ACGATAACAA AAGCATGCAT TTTGGGGAGC GTCTATTTTT GGAGAATACA AGAAATTCTG CAGCCATATC

       40         30         20         10
TGAAACAAAA TACATAAAAG CTTGCATGCC TGCAGGTCGA CTCTAAGGA
```

Fig. 2. Nucleotide sequence of 759 bases of the 1.2 kb HindIII fragment and the amino acid sequence of the open reading frame (ORF). The start site of transcription based on S1 nuclease mapping is indicated by a thin arrow. Possible binding sites for RNA polymerase based on comparable sequences in other spore coat genes are underlined. A thick arrow indicates the start of the sequenced spore coat protein and inverted arrows designate a potential termination loop. The sequence used for preparing an oligonucleotide for S1-nuclease mapping is indicated by a broken overline. The location of the HhaI site is also marked. These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number X13740.

Mapping of the cotT gene

The cotT gene was mapped by integration into the B. subtilis chromosome via plasmid pDE194 and selection for Cm[R]. The Cm[R] marker was mapped by transduction employing the strains of Dedonder et al. (1977) and by transformation using the strains listed in Experimental procedures (Table 2). The gene mapped to the metA, C, D region of the chromosome but the extent of cotransformation with metA was only 9%. This location is distinct from other spore coat genes mapped to date (Donovan et al., 1987), each of which has its unique site (Fig. 4).

Possible precursor antigens: overproduction and processing

In extracts of late sporulating cells, a possible precursor as well as the 7.8 kD coat antigen were detected (Fig. 5). The larger antigen was about 13 kD in size (based on RNase and cytochrome c standards), the size expected from the ORF (Fig. 2). Cells containing multiple copies of pT5A produced much larger quantities of the 13 kD antigen as well as a somewhat smaller 'precursor'. Spore extracts

Table 1. Amino acid composition of spore coat protein (mol. %).

		Cloned gene		
	Coat extract[a]	Total ORF	Carboxyl portion[b]	Peak D[c]
Ala	0.96	0.9	0.0	0.0
Arg	13.00	8.3	10.0	10.0
Asn	1.25	3.7	0.0	0.0
Asp				
Cys	0.01	0.0	0.0	0.0
Gln	2.18	2.8	1.8	2.0
Glu		3.7	0.0	0.0
Gly	19.11	11.1	17.8	17.0
His	0.18	0.9	0.0	0.0
Ile	0.45	2.8	0.0	0.0
Leu	0.89	3.7	0.0	0.0
Lys	0.52	2.8	0.0	0.0
Met	0.01	1.9	0.0	0.0
Phe	6.00	4.6	5.0	5.0
Pro	24.20	20.4	30.0	29.0
Ser	0.99	1.9	0.0	0.0
Thr	0.38	1.9	0.0	0.0
Trp	–	0.9	1.8	2.0
Tyr	32.20	22.2	35.0	35.0
Val	0.57	2.8	0.0	0.0

a. From Goldman and Tipper (1978).
b. Start based on protein sequencing (see Fig. 2).
c. See Fig. 1.

Fig. 3. Northern blot (Thomas, 1980) of RNA prepared from *B. subtilis* JH642 during exponential growth (lane 1), when most of the cells had formed phase-white endospores (lane 2), and 1.5 h later (lane 3). Hybridization with nick-translated 1.2 kb *Hin*dIII fragment (first three lanes) or with an internal fragment from the glutamine synthetase structural gene, E11 (Strauch et al., 1988) to RNA from exponential cells (lane 4). Marks to the right of lane 4 indicate positions of rRNAs.

These larger antigens were not removed by extensive washing of the spores (repeating the protocol in *Experimental procedures*) and resulted in spores which responded slowly (3–5 times) to the germinants L-alanine plus adenosine or Penassay broth (unpublished results). Pretreatment of the antibody with HPLC purified spore coat protein (peak D in Fig. 1) coupled to Sepharose 4B resulted in no reaction with all three antigenic species (Fig. 6, lane 8).

It is known that a particular germination mutant, GerE (Moir, 1981) has pleiotropic alterations of the spore coat, including the absence of this 7.8 kD spore coat protein (Jenkinson and Lord, 1983; Feng and Aronson, 1986). The cloned gene on plasmid pT5A was introduced into a *gerE* strain, AA9-1, but the spores still germinated slowly and were lysozyme-sensitive. Surprisingly, the *gerE* strain overproduced *cotT* precursor antigen even without extra gene copies (Fig. 6). Despite the excess precursor, there was less 7.8 kD antigen in cell or spore extracts of the *gerE* strain than in the wild type.

On the basis of the *cotT* ORF, the arg-gln sequence is the apparent site of cleavage for producing the 7.8 kD spore coat protein (see the thick arrow in Fig. 2). This peptide bond should be susceptible to trypsin, so cell and spore extracts were dialysed against 0.02 M Tris-HCl, pH 8.0 and incubated with trypsin (Worthington, TRL-3 at 1:50). There was a decrease in the presumed precursor antigens and an increase in one of 7.8 kD (Fig. 7). Neither the quantitation of the conversion nor the characterization of the product has been carried out.
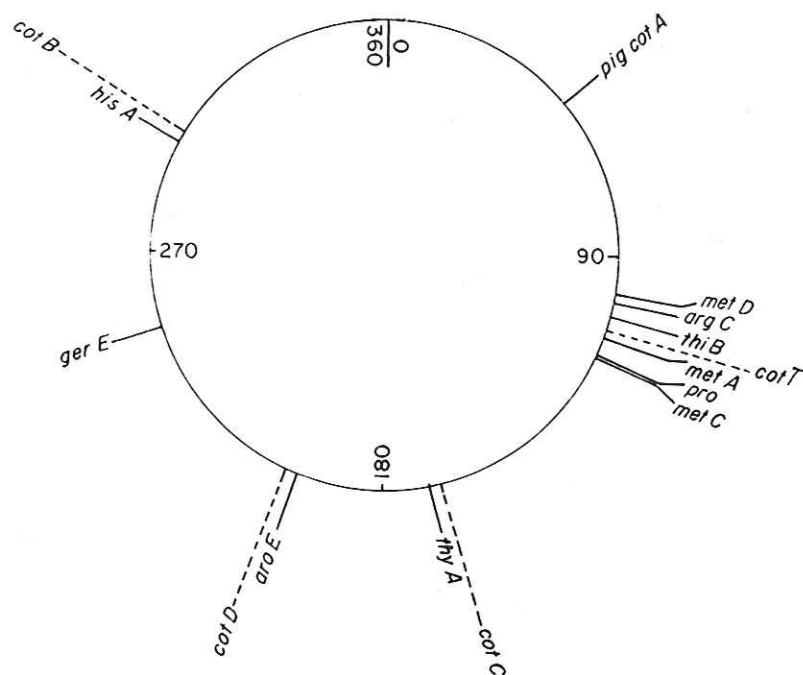
contained large amounts of the 7.8 kD antigen and surprisingly, the two 'precursor' antigens were present on spores from the strain containing multiple copies of pT5A.

Fig. 4. Approximate location of spore coat genes (*cotA–D, cotT*) on the *B. subtilis* chromosome (Piggot and Hoch, 1985). Location of *cotA–D* from Donovan et al. (1987); for *cotT*, the data in Table 2 were used.

Table 2. Mapping of the cotT gene.

Recipient		
Strain[a]	Genotype	% Contransduction
1A5	glyB133	0
	metC3	51
1A39	argC4	65
1A84	metD1	54
1A125	metA29	96
	argC4	82
1A652	proAB	39
		% Contransformation
1A125	metA29	9.2
	argC4	1.0

a. Strain designations are from the Bacillus Genetic Stock Center. There was no cotransduction with the following markers: cysA14, purA26, glyB133, ilvA1, pyrD1, thyA,B, gltA-292, aroD120, lys-1, thr5, hisA, aro-1, metB, cysC, purB.

Discussion

cotT is unique among the spore coat genes which have been analysed (Donovan et al., 1987; Sandman et al., 1988) in its amino acid composition and the apparent presence of one or more precursors. There is some evidence for cotT antigen in the insoluble fraction (Feng
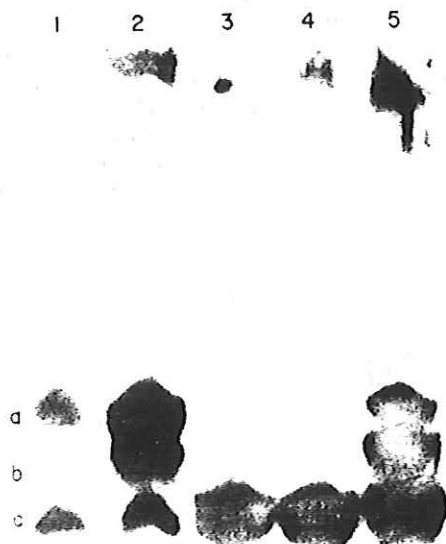

Fig. 5. Immunoblot of extracts from a late sporulating culture (greater than 60% with phase-bright endospores) or washed spores of B. subtilis JH642, containing the cloning vehicle pHP13 (lanes 1 and 4); cells or spores containing pT5A (lanes 2 and 5) or washed spores of B. subtilis JH642 (lane 3). The immunoblot was treated with rabbit antibody to the Na2SO4 precipitate from a spore coat extract plus anti-rabbit alkaline phosphatase conjugate, as described in Experimental procedures. Bands labelled (a) and (b) refer to potential precursors; band (c) is the 7.8 kD spore coat protein.
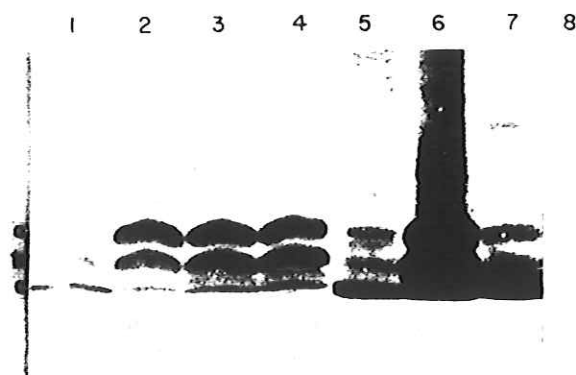
and Aronson, 1986) perhaps in the form of dityrosine or isopeptide cross-linked monomers. The glycine repeats at the carboxyl terminus may be indicative of multiple chain interactions, as found in silk fibroin or collagen, and the presence of multiple peaks in HPLC (Fig. 1) is suggestive of other alterations such as, for example, components binding to this polypeptide or secondary modifications like those found in collagens (Eyre, 1980).

The evidence for precursors is based on the presence of an ORF encoding a polypeptide larger than the one isolated from spore coats, the presence of larger antigens in extracts from cells and spores, the accumulation of these larger antigens in extracts of the GerE mutant, and preliminary evidence for the conversion by trypsin of these larger antigens to one of 7.8 kD. In fact, the association of these potential precursors with repeatedly washed spores (roughly the same quantity as extracted from unwashed spores) implies that processing may occur on the spore surface. The inability of the GerE mutant to process the precursor may mean that proper coat assembly is required. The gerE locus appears to encode a DNA-binding protein (Holland et al., 1987) and at least one other coat protein is overproduced in a gerE strain (Sandman et al., 1988). It therefore appears to be a negative regulator of at least two spore coat genes, so overproduction of two or more coat proteins may result in altered assembly and hence the extensive pleiotropic defects in the spore coat of the gerE mutant (Jenkinson and Lord, 1983; Feng and Aronson, 1986).

It is also possible that one or both of the presumptive precursors per se is an integral part of the spore coat, although excess precursor accumulation on the spores led to altered germination rates. Extracts of wild-type spores contained little, if any, precursor (Figs 5 and 6) so if they were present, the insoluble fraction would be the most likely place.

All six of the cloned spore coat genes map at unique sites (Fig. 4) but they appear to share common upstream sequences for the binding of a unique form of RNA polymerase and perhaps for other regulatory factors such as the gerE protein. They are all transcribed late in sporulation, coincident with the appearance of the spore coat on the endospore, i.e. stage V of sporulation, so the apparent coordinate regulation is probably partly the result of these upstream sequences. The genes for the spore acid-soluble proteins are also unlinked but are coordinately regulated (Connors et al., 1986).

Several of the spore coat proteins appear to be dispensable at least on the basis of the criteria used (i.e. heat and lysozyme resistance and germination rate of the spores) with the exception of cotD (Donovan et al., 1987). Disruption of a more recently characterized locus, cotE, did result in extensive alterations of the structure of the spore coat (Sandman et al., 1988). Whether or not alterations in cotT

Fig. 6. Immunoblot of extracts (lanes 1–4) of sporulating cells (as in Fig. 5) or washed spores (lanes 5–7) treated as described in the legend to Fig. 5. Lanes 1 and 5: extract or spores of *B. subtilis* JH642/pHP13; lanes 2 and 6: extract or spores of *B. subtilis* JH642/pT5A; lanes 3 and 7: extract or spores of *B. subtilis* AA9-1/pHP13; lane 4: extract of *B. subtilis* AA9-1/pT5A; lane 8: as in lane 2 but treated with antibody preadsorbed with purified 7.8kD protein bound to sepharose. Bands labelled (a) and (b) represent potential precursor antigens; band (c) represents the 7.8 kD spore coat protein.

result in phenotypic changes in spores remains to be determined.

Experimental procedures

Bacterial strains

The wild-type strain was *B. subtilis* JH642 (*trp*, *phe*), obtained from Dr J. Hoch. Strain AA9-1 (*gerE*, *leu*) was described by Feng and Aronson (1986). A series of strains for mapping (Dedonder *et al.*, 1977) and designated 1A3-1A10, was obtained from the Bacillus Genetic Stock Center. Strains 1A39 (*argC4*), 1A55 (*aroI*, *metB5*), 1A79 (*cysC1*), 1A84 (*glyB133*, *metD*), 1A25 (*metA29*, *argC4*), 1A156 (*purB*) and 1A652 (*proAB*) were obtained from the same source.

Isolation of a spore coat protein

Spores of *B. subtilis* JH642 were prepared from cells grown in a nutrient sporulation medium (NSM; Schaeffer *et al.*, 1963). Spore purification, preparation of coats and extraction were as previously described (Pandey and Aronson, 1979). One of the soluble spore coat proteins was salted out with Na_2SO_4 (Goldman and Tipper, 1978), washed 4–5 times with distilled water, and lyophilized. The material was dissolved in 20% n-propanol-0.1% trifluoroacetic acid (TFA) and fractionated by HPLC on a 4.6 × 250 mm reverse phase RP-P (SynChrom Inc.) column employing a linear gradient of 20–100% n-propanol-0.1% TFA in a Waters system. The effluent was monitored at 230 nm and peak fractions collected manually and then lyophilized. Amino acid sequencing was done on a Model 420 gas phase sequenator and deoxyoligonucleotides were synthesized in an Applied Biosystems Model 380A DNA synthesizer.

A mixed oligonucleotide was labelled by incubation with γ-[^{32}P]-ATP and polynucleotide kinase (Maniatis *et al.*, 1982) and used as a probe to screen eight pools of plasmids containing partial *Sau*3A digests of *B. subtilis* DNA (Hasnain and Thomas, 1986). The one reactive pool was then transformed into *E. coli* HB101 and colonies on filters screened with the probe. Simultaneously, *B. subtilis* JH642 DNA digested with various restriction enzymes was resolved on a 0.8% agarose gel and Southern blots prepared. A region of *c*. 1 kb from a *Hin*dIII digest reacting with the probe was electroeluted from an agarose gel and, following phenol extraction and precipitation with two volumes of ethanol, was cloned into the *Hin*dIII-digested shuttle vector, pLP1201 (Ostroff and Pène, 1984). Following transformation of *E. coli* HB101 and plating on L-agar plus 20 μg ml⁻¹ ampicillin, colonies were screened for tetracycline sensitivity. About 500 colonies were streaked onto Millipore HA filters (0.45 μ) on L-agar plus ampicillin (20 μg ml⁻¹), lysed and screened with the probe. Both screenings yielded an identical 1.2 kb *Hin*dIII insert which was sequenced in both strands by the dideoxy procedure (Sanger *et al.*, 1977) after cloning into m13mp18 and m13mp19 and then deleting with exonuclease III (Henikoff, 1984). Sequences of certain regions were also confirmed by primer extension of synthetic deoxyoligonucleotides.

Since no *B. subtilis* transformants were obtained with the pLP1201 clone, the 1.2 kb *Hin*dIII fragment was cloned into another shuttle vector, pHP13 (Haima *et al.*, 1987). The clone containing the *Hin*dIII fragment in pHP13 is designated pT5A.

Mapping

The 1.2 kb *Hin*dIII fragment was isolated as described above and cloned into pDE194 (Ebbole and Zalkin, 1987), an integration vehicle which replicates in *E. coli* (ampicillin resistance) but not in *B. subtilis* (expresses chloramphenicol resistance, Cm^R, when integrated). Transformants (Anagnostopoulos and Spizizen, 1961) of *B. subtilis* JH642 were selected on NSM plus 5 μg ml⁻¹ chloramphenicol. The Cm^R marker was then mapped by PBS1 transduction (Hoch *et al.*, 1967) employing the strains previously
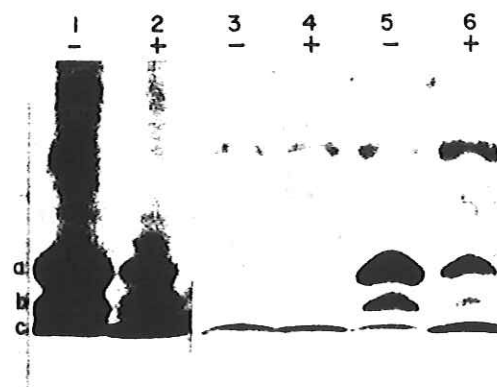

Fig. 7. Immunoblot (as in Fig. 5) of extracts of sporulating cells of *B. subtilis* JH642/pT5A (lanes 1 and 2) and washed spores of *B. subtilis* JH642/pT5A (lanes 5 and 6) or of *B. subtilis* JH642/pHP13 (lanes 3 and 4). Extracts (50 μg of protein) were dialysed for 12 h at 4°C versus 1 l of 0.01 M Tris, pH 8.0 and then incubated at 37°C for 1 h with (+) or without (−) 1 μg of trypsin. Bands are labelled (a), (b) and (c), as in Figs 5 and 6.

listed and subsequently by transformation. In both cases, selection was for Cm^r on NSM agar plates. Various prototrophic markers were then scored on a minimal glucose medium (Spizizen, 1958) appropriately supplemented in order to determine linkage.

RNA fractionation and S1 mapping

Total RNA was prepared from cells grown in NSM to various stages of sporulation (approximated by the appearance of cells in the phase microscope, i.e. by the percentage of dull, phase-white or bright endospores). 100 ml portions were harvested by centrifugation at 8000 rpm for 8 min in a Sorvall GSA rotor. The cells were washed twice by suspension in 20 ml each of 0.05 M Na acetate-0.1 M NaCl-1 mM EDTA, pH 5.5. A final suspension in 2 ml of this buffer was supplemented with sodium dodecyl sulphate (SDS) to 1%. After pouring of the suspension into a French pressure cell, 0.7 ml of a purified bentonite suspension (Fraenkel-Conrat et al., 1961) at 32 mg ml^{-1} was added. The cells were extruded at 9000 psi directly into an equal volume of phenol saturated with 0.03 M Tris, pH 7.6. Following extraction, the aqueous phase was precipitated with two volumes of ethanol and some DNA was removed by winding onto a sterile glass rod. After incubation at −20°C for 20 min, the precipitate was collected and dissolved in 1 ml 0.01 M Tris-2 mM MgCl, pH 7.6, and ribonuclease-free deoxyribonuclease I (BRL) was added to 5 µg ml^{-1}. Following incubation at 37°C for 40 min, the phenol extraction was repeated twice and the RNA collected by ethanol precipitation (after addition of Na acetate to 0.3 M). The final precipitate was dried, dissolved in distilled water and the concentration determined by measuring the adsorption at 260, 280 and 320 nm. Portions were frozen in sterile tubes at −70°C.

The RNA was fractionated for Northern blots as described by Thomas (1980). S1 nuclease mapping (Burke, 1984) was done by labelling either an 898 bp HindIII-HhaI fragment of the insert with polynucleotide kinase plus γ-[P^{32}]-ATP or by oligonucleotide primer extension (dashed overlined region, GATGCTTAAGTTGGGTGTATG in Fig. 2) employing the 1.2 kb HindIII fragment as template. Klenow fragment and α-[P^{32}]-dCTP (Maniatis et al., 1982).

Immunoblotting

For immunoblotting (Towbin et al., 1979) extracts of cells grown in NSM (plus or minus 5 µg ml^{-1} chloramphenicol) were prepared by centrifuging 10–30 ml portions in a Sorvall SS-34 rotor at 8000 rpm for 10 min. The pellets were washed once in 5 ml 0.03 M Tris, pH 7.6, suspended in 50 µl Tris plus 40 µg lysozyme, and incubated at 37°C for 10 min. After addition of SDS to 1.0%, and phenyl methyl sulfonyl fluoride (PMSF) to 5 mM, the suspensions were placed in a boiling water bath for 3 min. The tubes were cooled, centrifuged in an Eppendorf for 5 min and the supernatants collected. The pellets were suspended in 30 µl UDS (Sastry et al., 1983) plus 5 mM PMSF and incubated at 37°C for 30 min. Following centrifugation in an Eppendorf for 7–8 min, the supernatants were pooled and portions removed for Folin protein determinations. Equal quantities of protein in the extracts were fractionated on 20% SDS-PAGE. After transfer to nitrocellulose, treatment was with rabbit antibody to the Na_2SO_4 precipitate of a spore coat extract plus anti-rabbit alkaline phosphatase conjugate (Promega). Control treatments included pre-immune serum and antibody absorbed with the HPLC purified protein conjugated to cyanogen bromide activated Sepharose 4B (Sigma).

Spores were harvested after 24–36 h incubation by centrifugation in a Sorvall SS34 rotor at 12000 × g for 10 min. They were washed with 10 ml portions of 1 M KCl and distilled water (twice), suspended in 10 ml 0.03 M Tris-HCl, pH 7.6 plus 50 µg ml^{-1} lysozyme, and incubated at 37°C for 1 h. They were then washed with 1 M KCl, distilled water, 0.05% SDS, and again with distilled water (three times). Intact spores were counted in a Petroff-Hauser chamber and then extracted as described by Donovan et al. (1987).

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PROPERTIES OF *BACILLUS SUBTILIS* SPORES WITH ALTERATIONS IN SPORE COAT STRUCTURE

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I. INTRODUCTION

The proteinaceous coat of *Bacillus* spores is believed to be important in both resistance to adverse environmental conditions and in the ability to respond to germinants (Aronson and Fitz-James, 1976; Dion and Mandelstam, 1980). The composition of this structure differs considerably among members of the genus. In *Bacillus subtilis* it consists of a group of 10-20 extractable polypeptides which may vary in size from 7 kd to 65 kd (Goldman and Tipper, 1978; Pandey and Aronson, 1979; Jenkinson *et al.*, 1981), plus an insoluble proteinaceous fraction which may comprise up to 30% of the total spore coat protein (Pandey and Aronson, 1979). In contrast, the spore coats of some other members of the genus such as *Bacillus cereus* and *Bacillus megaterium* contain only a few major low molecular weight species (Aronson and Fitz-James, 1976; Stewart and Ellar, 1982).

The reason for the heterogeneity of the *B. subtilis* spore coat polypeptides and the mechanism by which they are assembled into the finished structure are unknown. As a preliminary step in the elucidation of their functions, individual spore coat genes have been cloned and analyzed (Donovan *et al.*, 1987; Zheng *et al.*, 1988; Aronson *et al.*, 1989). Many of the polypeptides are small, with unusual compositions which frequently include regions of repeating amino acid sequences. So far, only the absence of the CotE protein (among the 5-6 deleted) has resulted in extensive alteration of the coat structure (Zheng *et al.*, 1988).

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CotT is of interest because it is a prevalent low molecular weight spore coat polypeptide which may also be a component of the coat insoluble fraction (Feng and Aronson, 1986). It is also the only coat protein identified which is processed from a precursor (Aronson *et al.*, 1989).

The transcription and translation of the *cotT* gene have been further investigated and are described here. In addition the effects of extra copies and deletions of this gene and one encoding another low molecular weight protein, CotD (Donovan *et al.*, 1987), on spore coat structure, resistance properties of the spore and the ability of spores to respond to a variety of germinants have been examined. There are effects of overexpression on spore germination and a specific germination defect due to the absence of the CotT protein.

II. TRANSCRIPTION AND TRANSLATION OF THE *cotT* GENE

The sequence of the complete open reading frame of the *cotT* gene is shown in Fig. 1. The original transcription start site (Aronson *et al.*, 1989) as indicated by S1 nuclease mapping (Burke, 1984) is shown by the thin arrow at nt 593. A second major transcription start site was established by reverse transcriptase mapping (Wu *et al.*, 1989) and is shown by the large open arrow at nt 505. Possible sites for the binding of RNA polymerase are represented by the broken overline while the putative ribosome binding site is underlined. The *leu* residue shown in bold face is the predicted translation start site. The large solid arrow represents the beginning of the sequenced CotT protein extracted from spores (*i.e.* NH$_2$ Gln-Pro-Tyr, etc.).

Evidence to support translation initiation at the *leu* residue was provided by studies with strain *B. subtilis* 642/T5A (Aronson *et al.*, 1989) which carries the *cotT* gene on the low copy number vector pHP13 (Haima *et al.*, 1987). There were two presumptive precursors present in immunoblots of extracts from late sporulating cultures (Aronson *et al.*, 1989) and in spore coat extracts (unpublished results). The larger was electroblotted onto a polyvinylidene difluouride membrane (Matsudaira, 1987), excised and subjected to protein sequencing. The amino terminal region matched that predicted from the *cotT* DNA sequence starting at the TTG encoded *leu* residue. The predicted size of this precursor is 10.1 kd, but its electrophoretic mobility is similar to that of lysozyme (14.4 kd) possibly due to its unusual amino acid composition.
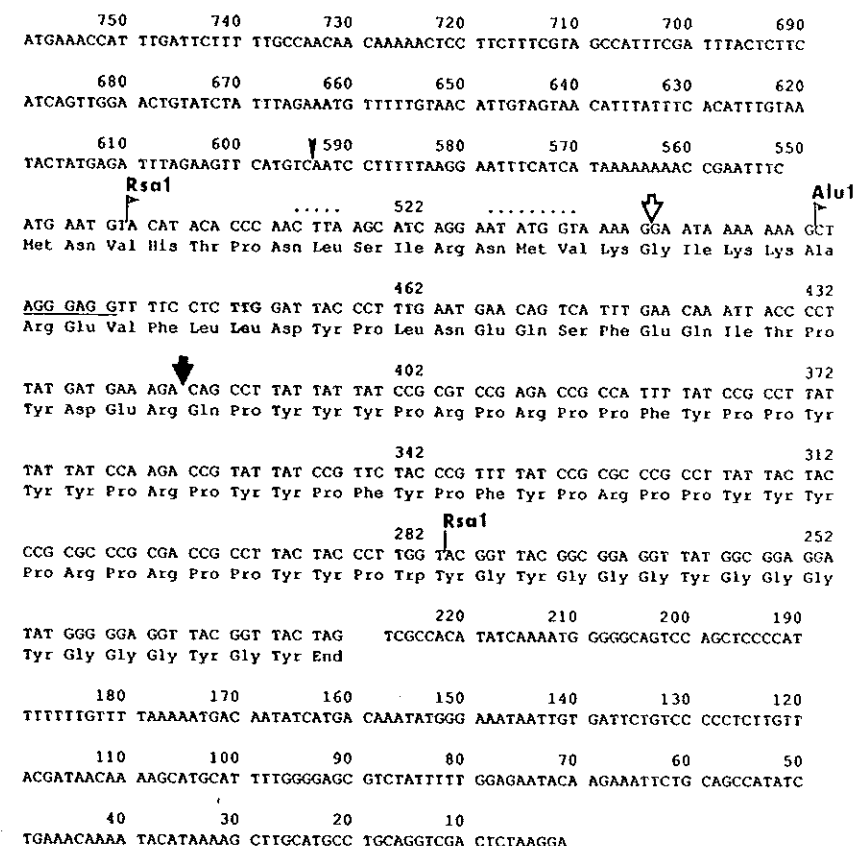
Figure 1. Nucleotide sequence of the open reading frame of the *cotT* gene as modified from Aronson *et al.* (1989). See text for details.

Further evidence that the precursor is processed at the *arg-gln* site (shown by the thick arrow in Fig. 1) to produce the 7.8 kd mature spore coat protein was provided by site-directed mutagenesis (Kunkel, 1985) of R191. The altered gene was integrated into the chromosome of *B. subtilis* 642 creating a partial diploid (*B. subtilis* 642/PD633) with one wild-type and one altered gene copy. The large CotT precursor accumulated as determined by immunoblots and SDS/PAGE of stained gels of spore extracts (unpublished results). This accumulation of precursor was not found in a strain containing two wild-type *cotT* genes (see Section IV).

III. CONSTRUCTION AND PROPERTIES OF *cotT* DELETIONS

The *Alu*1-*Rsa*1 fragment (nt 280-493) shown in Fig. 1 was cloned into the integrative vector pDE194 (Ebole and Zalkin, 1987) and introduced into *B. subtilis* 642 by transformation. Recombination within this cloned region and the chromosomal *cotT* gene resulted in integration of the vector into the chromosome and production of two incomplete gene copies (Fig. 2). The first of these contains the promoter region (A) and part of the protein coding region, but lacks the carboxyl portion of the protein (B). The second copy, although intact, lacks the promoter region (A). This construct (NB200) was confirmed by Southern hybridization (Southern, 1975) and there was no detectable CotT protein in SDS/PAGE of spore coat extracts, nor in immunoblots of extracts from spores or late sporulating cells (unpublished results). Electron micrographs of spore sections of strain NB200 differed from the wild-type in that the outer spore coat was less electron-dense than the comparable layer from wild-type spores (P. C. Fitz-James, personal communication). Despite these differences, NB200 spores were as resistant to heat, lysozyme and octanol as wild-type spores. Similarly their response to the germinants L-alanine (plus 1 mM inosine) at a variety of concentrations between 0.1 mM and 10 mM and to Penassay broth were the same as the wild-type. NB200 spores responded slowly, however, to a germination mixture consisting of 0.1 M KCl, 5.6 mM glucose, 5.6 mM fructose and 3.3 mM L-asparagine (Wax and Freese, 1968; Fig. 3). There was no change in the germination of NB200 or the wild-type spores by lowering the glucose or fructose concentration tenfold.

Another *cotT* deletion was constructed by integration of the 263 bp *Rsa*1 gene fragment (nt 280-543 in Fig. 1) as in Fig. 2. The resulting strain (NB2) contained one complete copy of *cotT* including the presumptive promoter but little if any CotT protein was found in spore coat extracts, or in extracts from sporulating cells. Perhaps the intact gene copy lacks upstream regions important for *cotT* transcription. As anticipated, the germination of NB2 spores like those of NB200 was slower than the wild-type but only in the glucose, fructose and L-asparagine mixture.
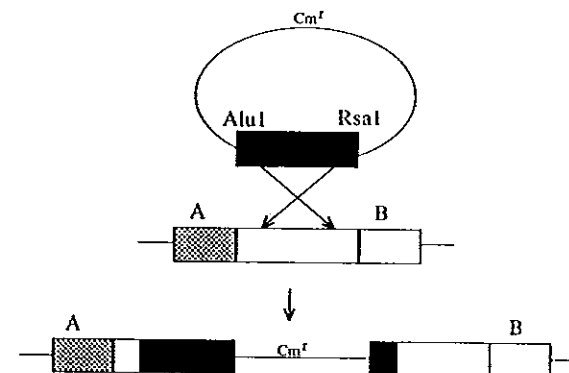
Figure 2. Construction of the *cotT* deletion strain NB200 by integration into the chromosomal gene copy of a vector containing an internal *cotT Alu*1-*Rsa*1 fragment. A is the promoter region and B is the portion of the gene encoding the carboxyl end of the CotT protein.
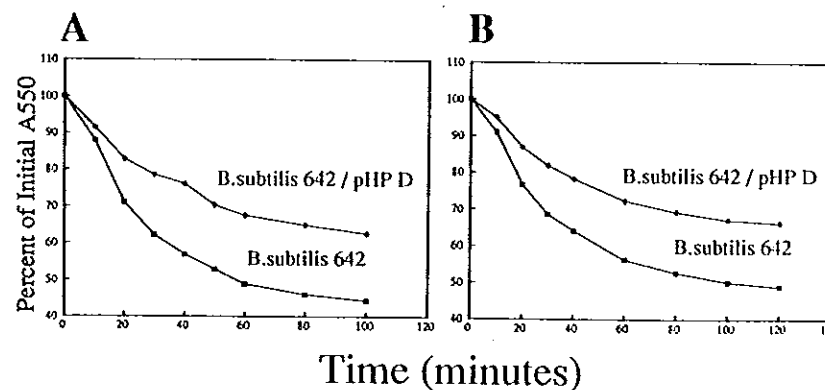

Time (minutes)

Figure 3. Germination of *B. subtilis* 642 and NB200 spores in 0.05 M Tris pH 7.6 containing 0.1 M KCl and 5.6 mM glucose, 5.6 mM fructose and 3.3 mM L-asparagine at 37°C both without (A) and with (B) heat activation at 80°C for 20 min.

IV. OVERPRODUCTION OF CotT

Spores of *B. subtilis* 642/T5A in which the *cotT* gene is present on the vector pHP13 at 5-6 copies per genome responded slowly to L-alanine plus inosine (Fig. 4A), the glucose, fructose and L-asparagine mixture (Fig. 4B) as well as Penassay broth (as in Fig. 4A) even when heat activated prior to germination. As previously described (Aronson *et al.*, 1989), extra CotT precursor proteins can be extracted from these spores and they appear to have a thickened inner spore coat (P. C. Fitz-James, personal communication), so altered spore coat structure and composition could account for the germination defect.

B. subtilis 642/PD1 was constructed by integration of the entire wild-type *cotT* gene into the chromosome resulting in a strain with two functional chromosomal gene copies (confirmed by Southern hybridization and increased *cotT* mRNA content). Spores of this strain germinated as well as the wild-type in response to all of the germinants tested and there was no excessive accumulation of CotT precursors in immunoblots or in SDS/PAGE of protein extracts from purified spore coats (unpublished results). Apparently, more than two gene copies are needed for the excessive accumulation of CotT protein in the spore coat with the resulting germination defect.

V. STUDIES WITH *cotD* MUTANTS

The clone of *cotD* and a deletion strain (BZ109; Donovan *et al.*, 1987) were generously provided by B. Zheng and R. Losick. When the intact gene was transferred to the vector pHP13 and transformed into *B. subtilis* 642, there was an increased amount of protein of the size of CotD in SDS/PAGE of spore extracts (unpublished results). Germination of this overproducing strain was slower than the wild-type in L-alanine plus inosine (Fig. 5A), the glucose, fructose and L-asparagine mixture (Fig. 5B) and Penassay broth (as in Fig. 5A) with or without heat activation but in no case was the impairment as great as for the CotT-overproducing strain (Fig. 4). Strain BZ109 was constructed by the insertional inactivation of the *cotD* gene (Donovan *et al.*, 1987) but in contrast to the *cotT* deletion (Fig. 3) there was no discernible effect on germination.
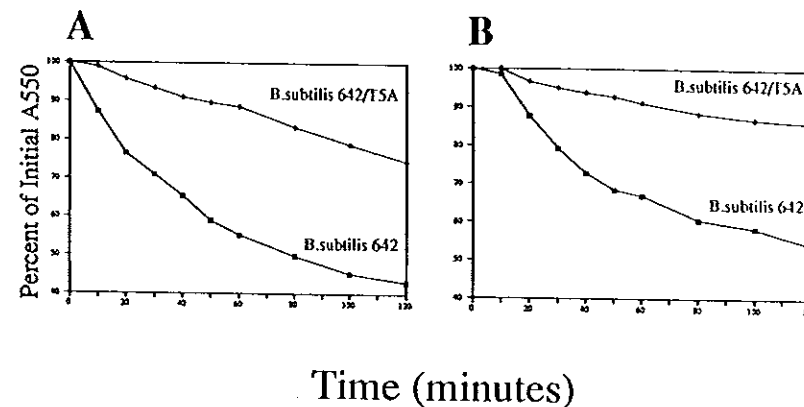
Figure 4. Germination of *B. subtilis* 642 and *B. subtilis* 642/T5A in 0.05 M Tris pH 7.6 containing 0.1 M KCl and (A) 1 mM inosine plus 1 mM L-alanine, or, (B) 5.6 mM glucose, 5.6 mM fructose and 3.3 mM L-asparagine. Spores were heat activated at 80°C for 20 min.
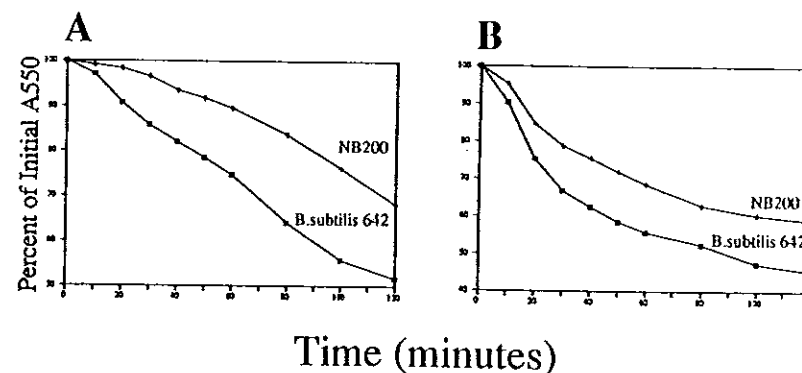

Figure 5. Germination of *B. subtilis* 642 and *B. subtilis* 642/pHP D spores as in Figures 4A and 4B, but without heat activation.

VI. SUMMARY

A major transcription initiation site for the *cotT* gene has been determined. The predicted translation product has an N-terminal *leu* and a molecular weight of 10.1 kd, whereas the mature spore coat protein is 7.8 kd. Processing is at the *arg-gln* bond and an R191 mutation resulted in accumulation of CotT precursor protein indicating that a trypsin-like protease may be involved. There was no effect on spore properties or germination in the *cotT* heterozygote but the effect of this recessive mutation by itself must be determined.

The amino acid sequence of the amino terminal portion of the larger CotT precursor protein is in agreement with the predicted amino acid sequence from the *cotT* gene. In addition to the 10.1 kd precursor there may be a second, somewhat smaller precursor in sporulating cells and in spore coats of the strain which over produces CotT protein.

Deletion of the *cotT* gene resulted in a thinning of the outer spore coat and an impaired germination response, but only to a mixture containing glucose, fructose and L-asparagine. Altering the concentration of glucose or fructose did not affect the germination rate implying that the CotT protein is not directly involved. This protein is probably not a receptor for specific germinants but more likely, its function in spore coat structure is important for the accessibility of this particular germination mixture to its site of action.

Another *cotT* deletion strain lacked only a region upstream of the promoter but there was no CotT antigen synthesis implying that a transcriptional regulatory region is within the deleted portion.

Germination of a *cotT* diploid strain did not differ from the wild-type in any system tested. Overexpression of the gene on a low copy number vector at 5-6 copies per genome however, resulted in spores which were slow to respond to all germinants. This inhibition probably results from the deposition of excess CotT precursor, perhaps in the inner spore coat as is suggested by electronmicrographs. The correct stoichiometry of coat proteins is probably important for normal coat assembly and thus germination.

Response of a mutant lacking another coat protein (CotD) was indistinguishable from the wild-type for all germinants tested. Excess synthesis of this protein resulted in slower germination than the wild-type but not to the same extent as was caused by CotT protein production.

ACKNOWLEDGEMENTS

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