Cloning and Sequencing of a Gene from *Bacillus amyloliquefaciens* that Complements Mutations of the Sporulation Gene *spoIID* in *Bacillus subtilis*

By SHEILA M. TURNER AND JOEL MANDELSTAM*

Microbiology Unit, Department of Biochemistry, University of Oxford, South Parks Road,
Oxford OX1 3QU, UK

(Received 26 March 1986; revised 16 June 1986)

A segment of DNA from *Bacillus amyloliquefaciens*, which complemented a mutant sporulation gene, *spoIID68*, in *Bacillus subtilis*, was cloned into a derivative of the temperate bacteriophage $\phi 105$. The segment of DNA included an entire structural gene and complemented the mutation *spoIID298*, in addition to *spoIID68*, in *B. subtilis*.

The nucleotide sequence of the gene from *B. amyloliquefaciens* was determined and compared with that of the *B. subtilis* gene; 74% homology was found in the coding region. Amino acid primary sequences derived from the nucleotide sequences of the two genes were also compared. The gene from *B. amyloliquefaciens* coded for a protein of 344 amino acid residues, one more than the protein coded by the corresponding gene from *B. subtilis*. Comparison of the primary amino acid sequences of the two genes showed that 78% of the residues were completely conserved and 8% were semi-conserved. Variation, however, was not random, i.e. some segments were much more highly conserved than others. Both proteins had a hydrophobic region at the N-terminus.

INTRODUCTION

The degree of homology of the DNA from different species of *Bacillus* has previously been studied by both interspecific transformation and DNA hybridization (Seki *et al.*, 1975, 1979). Interspecific transformation, however, is known to be extremely inefficient (te Riele & Venema, 1982a). There is evidence that some genes — such as those conferring resistance to some antibiotics, those coding for the ribosomal proteins, RNAs and tRNAs, and those for cell division — are highly conserved (Dubnau *et al.*, 1965a, b; Seki *et al.*, 1975, 1979). Interspecific transformation of sporulation genes *spo0A*, *spo0B*, *spo0F* and *spo0H* with *B. amyloliquefaciens* H strain as donor and *B. subtilis* as recipient has been reported by Hoch & Mathews (1973), although similar attempts with *B. pumilus* as donor were unsuccessful. Neither *B. amyloliquefaciens* nor *B. pumilus* were able to transform auxotrophic mutants of *B. subtilis* to prototrophy (Hoch & Mathews, 1973). It was inferred from these results that sporulation genes were more closely conserved than auxotrophic genes. It has been proposed that differences in sequence homology are major barriers to interspecific transformation (Harris-Warrick & Lederberg, 1978), though base pairing in complexes between heterologous DNA molecules has been observed (te Riele & Venema, 1982a, b, 1984).

Cloning of genes in *B. subtilis* has proved a more useful method than transformation for studying the homology of genes within the genus *Bacillus*. Genes from various species of the genus *Bacillus* have been cloned into *B. subtilis* and found to be functional, for example *leuA* and *ilvC* from *B. amyloliquefaciens* (Yoshimura *et al.*, 1983), neutral protease genes from *B. amyloliquefaciens* (Honjo *et al.*, 1984) and *B. stearothermophilus* (Fujii *et al.*, 1983), *hisH* from *B. licheniformis* (Weinrauch & Dubnau, 1983), and α -amylase genes from *B. amyloliquefaciens* (Palva, 1982; Lehtovaara *et al.*, 1984) and *B. licheniformis* (Joyet *et al.*, 1984).

Sporulation genes cloned from species other than *B. subtilis* have also been found to function in *B. subtilis*, e.g. *spo0H* from *B. licheniformis* (Dubnau *et al.*, 1981) and the spore coat C protein gene from *B. megaterium* (Goldrick & Setlow, 1983). There is also evidence, from studies using monoclonal antibodies, that sporulating cultures of species other than *B. subtilis* recognize proteins similar to the sporulation-induced sigma factor σ^{29} of *B. subtilis* (Trempey & Haldenwang, 1985), which is the product of the sporulation gene *spoIIG* (Trempey *et al.*, 1985).

The purpose of the present study was to clone sporulation genes from several species of *Bacillus*, to test them for complementation of mutated genes in *B. subtilis*, and to compare the degree of conservation of corresponding genes. This paper describes the construction of genomic libraries from seven species: *B. amyloliquefaciens*, *B. cereus*, *B. thuringiensis*, *B. megaterium*, *B. polymyxa*, *B. natto* and *B. globigii*. Complementation for a dozen out of a possible fifty or so known sporulation loci (Young & Mandelstam, 1979) was tested. The cloning vector used for construction of the gene libraries, $\phi 105J9$ (Errington, 1984), has also been used to clone sporulation genes *spoIID* (Lopez-Diaz *et al.*, 1986) and *gerE* (James & Mandelstam, 1985) from *B. subtilis*.

The degree of conservation between the *B. amyloliquefaciens* and *B. subtilis* *spoIID* genes was studied by restriction analysis and by DNA sequencing. The gene from *B. subtilis* is a monocistronic operon, approximately 1.2 kb in length, coding for a protein of 343 amino acid residues with a strongly hydrophobic N-terminal region (Lopez-Diaz *et al.*, 1986). The present paper shows that the corresponding gene from *B. amyloliquefaciens* is very similar.

METHODS

Bacterial strains, phages and plasmids. These are listed in Table 1.

Bacillus strains were routinely maintained on nutrient agar (Oxoid). All Spo⁺ strains sporulated readily on this medium. *B. amyloliquefaciens*, however, did not sporulate on this medium or on any of the other media tested, e.g. Schaeffer's agar (Schaeffer *et al.*, 1965), soil extract agar (Fred & Waksman, 1928), lactate-glutamate minimal agar (LGMA) or resuspension medium (Sterlini & Mandelstam, 1969). *Bacillus* strains were tested for prototrophy by streaking onto LGMA plates incubated both at 30 °C and at 37 °C. All the species of *Bacillus* listed in Table 1 grew prototrophically on LGMA. *B. globigii* and *B. thuringiensis* grew more readily on this medium at 30 °C than at 37 °C. All cultures grew well on nutrient agar at 37 °C, and all except *B. amyloliquefaciens* produced spores.

Buffers. Ligation buffer was 50 mM-Tris/HCl pH 7.4, 10 mM-MgCl₂, 10 mM-DTT, 1 mM-spermidine, 1 mM-ATP, 0.1 mg bovine serum albumin (BSA) ml⁻¹. TE buffer was 10 mM-Tris/HCl, 1 mM-EDTA. Klenow reaction buffer (final concentration) was 10 mM-Tris pH 8.0, 5 mM-MgCl₂. TBE buffer was 90 mM-Tris/HCl pH 8.3, 90 mM-boric acid, 2.5 mM-EDTA. Agarose gel loading buffer was 10 mM-Tris/HCl pH 7.4, 1 mM-EDTA, 20% (w/v) Ficoll, 0.05% bromophenol blue.

Restriction endonuclease digestions. Restriction enzymes were obtained from Amersham or BRL and used as recommended by the suppliers.

Transformations. Recipient cells of *B. subtilis* MY2000.68 blocked at stage II were transformed as described by Jenkinson (1983) using *B. subtilis* 168 chromosomal DNA, *B. amyloliquefaciens* chromosomal DNA, plasmid pS1 DNA and phage $\phi 105S1$ DNA digested with *Bcl*I. The final concentration of transforming DNA used was approximately 1 μ g ml⁻¹. Transforming mixtures were spread onto Schaeffer's agar, and treated with chloroform after 18 h incubation at 37 °C. Spo⁺ colonies were identified by the brown colour associated with spore production. The colonies were checked microscopically for the presence of spores.

Preparation of DNA. Chromosomal DNA from *B. amyloliquefaciens* was prepared by the method of Marmur (1961). Plasmid DNA was extracted from *E. coli* by the alkaline lysis method (Maniatis *et al.*, 1982). Phage DNA (M13, $\phi 105J9$ and recombinants of both) was prepared as described in the Amersham booklet *M13 Cloning and Sequencing Handbook*.

Construction of genomic libraries. Chromosomal DNA from various species of bacilli (*B. amyloliquefaciens*, *B. cereus*, *B. thuringiensis*, *B. megaterium*, *B. polymyxa*, *B. natto* and *B. globigii*) was digested with *Bcl*I. The phage vector $\phi 105J9$ (Errington, 1984) was cleaved at a unique site in the polylinker with *Bam*HI, and the chromosomal DNA (250 ng) fragments were ligated into the vector (500 ng). The resulting recombinant phage preparation was used to transfect protoplasts of CU267 and the resulting pool of recombinant phages was harvested as described by Errington (1984).

Complementation of asporogenous B. subtilis mutants. Portions (300 μ l) of 5 ml cultures of *B. subtilis* sporulation mutants grown in brain heart infusion broth (BHIB) were infected with 200 μ l of the mixed pool of recombinant phages while still in exponential phase. Each 500 μ l of the mixture of cells and phage was then spread on Schaeffer's agar and Spo⁺ colonies were selected as described above. The colonies were checked microscopically

Table 1. *Bacterial strains, phages and plasmids*

Strain	Genotype or description	Source or reference
<i>B. subtilis</i>		
MY2000.68	<i>spoIID68 lys-1 pyrD1</i>	M. Yudkin, Oxford Univ., UK
298.2	<i>spoIID298 phe-12</i>	Laboratory stock
CU267	<i>trpC2 ilvB2 leuB16</i>	S. A. Zahler, Cornell Univ., NY, USA
168	<i>trpC2; Spo⁺</i>	Laboratory stock
<i>B. amyloliquefaciens</i>	Prototrophic; Spo ⁻ *	Torrey strain 10785
<i>B. cereus</i>	Prototrophic; Spo ⁺	Laboratory strain
<i>B. thuringiensis</i>		
<i>B. megaterium</i> K.M.		
<i>B. polymyxa</i>		
<i>B. natto</i>	Prototrophic; Spo ⁺	K. McQuillen, Cambridge Univ., UK
<i>B. globigii</i>		
<i>E. coli</i>		
JM103	$\Delta(lac-pro)$ <i>thi strA supE endA sbcB15 hsdR4</i> F' <i>traD36 proAB lacI^q Z</i> ΔM15	Messing <i>et al.</i> (1981)
JM107	$\Delta(lac-pro)$ <i>endA1 gyrA96 thi-1 hsdR17 supE44</i> <i>relA1</i> F' <i>traD36 proAB⁺ lacI^q Z</i> ΔM15	Yanisch-Perron <i>et al.</i> (1985)
Bacteriophages		
φ105J9	Cloning vector	Errington (1984)
φ105LD2	<i>spoIID⁺</i> (<i>B. subtilis</i>), 2 kb <i>Mbo</i> I insertion in φ105J9	Lopez-Diaz <i>et al.</i> (1986)
φ105S1	<i>spoIID⁺</i> (<i>B. amyloliquefaciens</i>), 3.8 kb <i>Bcl</i> I insertion in φ105J9	This paper
M13mp10, 11	Sequencing vector	Messing (1983)
Plasmids		
pUC18	Amp ^R	Norrandar <i>et al.</i> (1983)
pS1	pUC18 + 3.8 kb insert from φ105S1	
pS2	pUC18 + 1.6 kb <i>Eco</i> RI fragment of pS1	
pS3	pUC18 + 0.4 kb <i>Eco</i> RI fragment of pS1	

* Unable to cultivate spores under conditions provided (see Methods).

for the presence of spores. The auxotrophic requirements of the recipient cells for lysine and uracil were checked using lactate-glutamate minimal agar supplemented with the appropriate nutritional requirements. Sporulating colonies were tested for the presence of transducing phage as described below.

Preparation and testing of phage lysates. Small scale lysates were made from 5 ml bacterial cultures in BHIB. Cultures were grown with shaking at 37 °C to a density of 0.085 mg dry wt bacteria ml⁻¹, 12 µl mitomycin C (Sigma; 200 µg ml⁻¹) was added and the cultures were incubated for a further 25 min. Each culture was centrifuged and the cells were resuspended in 5 ml of fresh warm BHIB and incubated for 2–3 h, until they had lysed. Cells and debris were pelleted by centrifugation and the supernatant was sterilized by passage through a 0.45 µm Millipore filter. The phage lysate thus obtained was stored at 4 °C.

Large scale lysates were prepared in the same manner from 200 ml culture, using 0.5 ml mitomycin C (200 µg ml⁻¹) to induce the prophage. Phage was then purified as described by Jenkinson & Mandelstam (1983).

Lysates were tested for transducing ability by spotting 10 µl drops on lawns of recipient cells growing on Schaeffer's agar. Where the phage conferred the ability to sporulate on recipient cells a patch of Spo⁺ cells could be observed amidst a lawn of Spo⁻ cells.

DNA hybridization. Plasmid pUC18, with the entire 3.8 kb chromosomal DNA fragment from *B. amyloliquefaciens* inserted into its polylinker (see Results), was digested with *Hind*III and *Eco*RI. The digests were run on agarose gels and transferred to nitrocellulose (Anderman, East Molesey, Surrey, UK) by the method of Southern (1975).

The radioactive probe used for hybridization consisted of φ105J9 with a 1.6 kb *Eco*RI–*Sal*I insert of *B. subtilis* DNA, comprising the *spoIID* locus, designated φ105LD2 (Lopez-Diaz *et al.*, 1986). The probe was nick-translated and labelled with ³²P (Amersham kit). Filter preparation and autoradiography were as described by Maniatis *et al.* (1982).

DNA electrophoresis. Digested DNA was mixed with loading buffer, heated at 65 °C for 10 min, separated on 0.7% agarose gels (Sigma type II) in TBE buffer and visualized by ethidium bromide fluorescence.

To remove a particular fragment for ligation, the band was cut out of a low melting point (0.6%, w/v) agarose gel (BRL). The agarose was melted by heating at 65 °C for 5 min with an equal volume of TE buffer. The DNA was extracted with phenol, and precipitated overnight at -20 °C in 2 vols ethanol and 0.1 vol. 2.5 M-ammonium acetate. The DNA was collected by centrifugation in a Beckman microfuge for 10 min at 4 °C, washed once in 80% (v/v) ethanol, vacuum dried and resuspended in TE buffer.

DNA sequencing. Fragments of DNA from plasmid pS2 and pS3 were sequenced by the dideoxy chain termination method of Sanger *et al.* (1977). All procedures, including cloning in M13 phage, selection of recombinants in *E. coli* JM103 and JM107, the sequencing reaction, and the running of buffer gradient gels, were done as described in the Amersham booklet *M13 Cloning and Sequencing Handbook*.

Subcloning fragments of chromosomal DNA. The original 3.8 kb fragment of chromosomal DNA was removed from the polylinker of ϕ 105J9 with *Xba*I and *Pst*I. A sample of the fragment (100 ng) was then ligated into the corresponding unique restriction sites in the polylinker of pUC18 (20 ng), and the plasmid was transformed into *E. coli* JM103 as described by Amersham in the *M13 Cloning and Sequencing Handbook*.

Comparison of DNA sequences. Comparison of DNA sequences from *B. subtilis* and *B. amyloliquefaciens* was done by eye.

Comparison of amino acid sequences. This was done by eye and by using the computer program HYDROPLLOT described by Kyte & Doolittle (1982) and modified by R. Staden (personal communication).

RESULTS AND DISCUSSION

Genomic libraries

When genomic libraries constructed from seven species of *Bacillus* (*B. amyloliquefaciens*, *B. cereus*, *B. thuringiensis*, *B. megaterium*, *B. polymyxa*, *B. natto* and *B. globigii*) were tested for their ability to complement a variety of sporulation genes (*spoIIAA*, *spoIIAC*, *spoIIB*, *spoIIC*, *spoIID*, *spoIIE*, *spoIIG*, *spoIIIA*, *spoIIIB*, *spoIVA*, *spoIVC*, *spoIVF*, *spoVA*) in *B. subtilis*, only one positive result was obtained. The library made using *B. amyloliquefaciens* chromosomal DNA contained a recombinant phage able to complement a defective *spoIID* gene in *B. subtilis*.

B. amyloliquefaciens is taxonomically one of the most similar species to *B. subtilis* (Gibson & Gordon, 1974; Seki *et al.*, 1978). It is worth noting that although the library of the strain of *B. amyloliquefaciens* used gave a positive result, the strain did not sporulate under any of the conditions used.

Isolation of recombinant phage ϕ 105S1

Competent cells of *B. subtilis* MY2000.68 *spoIID68* were transformed with chromosomal DNA from *B. amyloliquefaciens*; no Spo⁺ colonies were observed. The same *B. subtilis* mutant was infected with the pool of recombinant phages made using ϕ 105J9; approximately 50 Spo⁺ colonies per ml of infected culture were observed. In these colonies, where the mutant gene had been complemented by the phage, spores were produced at the same incidence as in wild-type Spo⁺ strains.

Eight of the Spo⁺ colonies were sub-cultured on nutrient agar. All eight cultures retained their Spo⁺ phenotype on repeated sub-culture. The auxotrophic markers of these isolates were checked and all the isolates were found to require both uracil and lysine, as expected. Each of the small scale lysates prepared from these isolates gave rise to a transducing lysate able to confer the ability to sporulate on further cultures of MY2000.68. Lysates were checked, before testing, to see that there was no bacterial contamination.

A single colony was selected from the first sub-culture of one of the eight isolates. This strain was used for all subsequent work. The transducing lysate obtained from this culture not only complemented *spoIID68* but also complemented *spoIID298* (Lopez-Diaz *et al.*, 1986). The phage was designated ϕ 105S1.

Hybridization and restriction analysis

A preliminary restriction map of the insert in phage ϕ 105S1 was constructed, and it was found that there were no sites for *Pst*I or *Xba*I other than those in the polylinker. The insert was removed with these two enzymes and subcloned into the corresponding unique sites in the

Fig. 1. At the top of the figure the restriction maps of pieces of chromosomal DNA containing the *spoIID* genes of *B. subtilis* and *B. amyloliquefaciens* are given. *B. amyloliquefaciens* DNA was cloned from a *Bcl*I end digest of chromosomal DNA into phage vector ϕ 10519. *B. subtilis* DNA was originally isolated from a 4.0 kbp fragment from an *Eco*RI end digest of chromosomal DNA (Lopez-Diaz *et al.*, 1986). The portion shown here was the part subsequently shown to include the *spoIID* gene. Phage ϕ 10519 (Lopez-Diaz *et al.*, 1986) was hybridized to *Eco*RI and *Hind*III fragments from pS1; the 2.1 kb *Hind*III fragment (a) and the 1.6 kb *Eco*RI fragment (c) hybridized strongly, while the 0.4 kb *Eco*RI fragment (b) hybridized less strongly. At the bottom of the figure the fragments of the *spoIID* locus that were sequenced are shown, together with a restriction map. The length of DNA from the left hand *Eco*RI site to the second *Acc*I site (which coincides with the termination codon) was sequenced in both directions. The length of DNA downstream from this was sequenced mainly in one direction only. The arrows indicate the start point, extent and direction of sequencing of particular subclones. Single headed arrows denote clones sequenced in one direction only, double headed arrows denote those sequenced in both directions. The hatched box indicates the position of the single open reading frame. Restriction endonucleases are abbreviated as follows: A, *Acc*I; B, *Bcl*I; E, *Eco*RI; F, *Hinf*I; H, *Hind*III; L, *Alu*I; N, *Nco*I; P, *Pvu*II; Q, *Hae*III; R, *Rsa*I; S, *Sal*I; T, *Taq*I; U, *Sau*3A; V, *Ava*I.

It was hoped that hybridization of a radioactively labelled probe consisting of the *B. subtilis* *spoIID* gene to the *B. amyloliquefaciens* DNA would reveal a section of homologous DNA. Plasmid pS1 was digested with *Hind*III and *Eco*RI, blotted to nitrocellulose as described by Southern (1975) and hybridized to ϕ 105LD2 (Lopez-Diaz *et al.*, 1986) labelled with 32 P. The probe hybridized strongly to a 1.6 kb *Eco*RI fragment and to a 2.1 kb *Hind*III fragment of the insert, and less strongly to a 0.4 kb *Eco*RI fragment (Fig. 1). It was known that the coding region

of the *spoIID* gene of *B. subtilis* was approximately 1.0 kb in length so it was possible that the gene from *B. amyloliquefaciens* could be entirely contained within the 1.6 kb *EcoRI* fragment (Fig. 1). This fragment was subcloned into pUC18 as a preliminary step to DNA sequencing. The plasmid was designated pS2. The 0.4 kb *EcoRI* fragment was also cloned into pUC18, and the plasmid was designated pS3.

DNA sequencing

The nucleotide sequence of the *B. amyloliquefaciens spoIID* gene, determined using the dideoxy chain termination method of Sanger *et al.* (1977), is shown in Fig. 2. As expected, the whole of the gene lay entirely within the 1.6 kbp *EcoRI* fragment contained in pS2. The fragments sequenced are shown in Fig. 1. The structural gene and short stretches on either side were sequenced in both directions. A further portion downstream from the termination codon was sequenced in one direction only, as was a portion of the DNA upstream of the first *EcoRI* site. Because our main interest was in the portion of DNA containing the coding region, we made no further effort to determine either the upstream or the downstream sequence with complete accuracy. The degree of homology decreased as distance from the coding region increased. The homology found in the 0.4 kb *EcoRI* upstream fragment would account for the observed hybridization to ϕ 105LD2.

The DNA sequence of the *B. amyloliquefaciens* gene was compared with the sequence from the *B. subtilis* gene. It was possible to align the two sequences and to identify the initiation codon, the coding region of the gene, the termination codon, the probable ribosome binding site and the σ^{29} recognition site (see Fig. 2). Only one large open reading frame was found, as in *B. subtilis*. The protein encoded by the gene is almost exactly the same size in both species, 343 amino acid residues in *B. subtilis* and 344 in *B. amyloliquefaciens*.

As the *B. amyloliquefaciens* gene was fully functional in *B. subtilis* it was apparent that the 'foreign' gene was able to use the transcriptional and translational machinery of the *B. subtilis* host cell. This is not surprising in view of the strong similarity between the two genes.

Cases in which promoters from other organisms function in *B. subtilis* are well documented: e.g., the promoters of the subtilisin gene (Wells *et al.*, 1983) and the α -amylase gene (Palva, 1982) from *B. amyloliquefaciens*, the penicillinase gene (Gray & Chang, 1981) and a thermostable α -amylase gene (Ortlepp *et al.*, 1983) from *B. licheniformis*, and the β -lactamase I gene from *B. cereus* (Wang *et al.*, 1985).

The nucleotide sequence of the region of DNA upstream of the codon initiating translation is highly conserved between the two species, although *B. amyloliquefaciens* has an insertion of 11 base pairs at one position (*a*, Fig. 2) and of a further base pair (*b*, Fig. 2) just before the initiation of translation. In the protein-coding region, *B. amyloliquefaciens* has an extra triplet (*c*, Fig. 2), which codes for the extra amino acid already mentioned. There is an 84% homology between the two nucleotide sequences in the region between nucleotide 1 and the initiation of translation codon (Fig. 2).

The six base pair sequence, CATATT, located 60 nucleotides before the start of translation shows homology to the -10 region recognized by σ^{29} (Johnson *et al.*, 1983). Lopez-Diaz *et al.* (1986) found exactly the same sequence in the *B. subtilis spoIID* gene. The proposed ribosome binding site, AGGAGG (Shine & Dalgarno 1974), was also the same in the *B. amyloliquefaciens* and *B. subtilis spoIID* genes. The additional base just before the initiation codon of the *B. amyloliquefaciens spoIID* gene means that there is a 10 nucleotide space between the Shine Dalgarno sequence and the initiation codon, rather than the nine found in the *B. subtilis spoIID* gene. Both species use the codon ATG for the initiation of translation. Overall homology between the coding regions of the genes from the two species is 74%. As Fig. 2 shows, differences are not randomly distributed throughout the length of the DNA.

Since a change in just one nucleotide can create or destroy a restriction site, the observed differences in sequence have given rise to very dissimilar restriction maps, in spite of the high degree of homology of the genes (Fig. 1).

Fig. 4. Hydroplots of *spoIID* proteins from *B. subtilis* and *B. amyloliquefaciens*. Primary protein sequences were inferred from the nucleotide sequences. Hydrophobic areas are displayed above the central lines, hydrophilic areas below. Span length = 11, output length = 3.

The same termination codon, TAG, is found in both nucleotide sequences. In the *B. subtilis* sequence, a putative transcriptional termination stem and loop structure has been identified after the termination codon (Lopez-Diaz *et al.*, 1986). A similar function is proposed for an analogous region in the *B. amyloliquefaciens* sequence (Fig. 2), although this proposed stem and loop structure is smaller by six base pairs than that for *B. subtilis*.

In conclusion it can be said that the genes from the two species are very similar both in respect of nucleotide sequence and inferred primary protein structure. Secondary protein structure and possible functions of the proteins are being investigated.

We would like to thank Dr J. Errington for advice and helpful discussions. This work was supported by grants from Elf Aquitaine (UK) and the Science and Engineering Research Council.

REFERENCES

- DAYHOFF, M. O., SCHWARTZ, R. M. & ORCUTT, B. C. (1978). A model of evolutionary change in proteins. In *Atlas of Protein Sequence and Structure*, vol. 5, supplement 3, pp. 345–352. Edited by M. O. Dayhoff. Washington, DC: National Biomedical Research Foundation.
- DUBNAU, D., SMITH, I., MORELL, P. & MARMUR, J. (1965a). Gene conservation in *Bacillus* species. I. Conserved genetic and nucleic acid base sequence homologies. *Proceedings of the National Academy of Sciences of the United States of America* **54**, 491–498.
- DUBNAU, D., SMITH, I. & MARMUR, J. (1965b). Gene conservation in *Bacillus* species. II. The location of genes concerned with the synthesis of ribosomal components and soluble RNA. *Proceedings of the National Academy of Sciences of the United States of America* **54**, 724–730.
- DUBNAU, E., RAMAKRISHNA, N., CABANE, K. & SMITH, I. (1981). Cloning of an early sporulation gene in *Bacillus subtilis*. *Journal of Bacteriology* **147**, 622–632.
- ERRINGTON, J. (1984). Efficient *Bacillus subtilis* cloning system using bacteriophage vector ϕ 105J9. *Journal of General Microbiology* **130**, 2615–2628.

- FRED, E. B. & WAKSMAN, S. A. (1928). *Laboratory Manual of General Microbiology*. New York: McGraw Hill Book Company.
- FUJII, M., TAKAGI, M., IMANAKA, T. & AIBA, S. (1983). Molecular cloning of a thermostable neutral protease gene from *Bacillus stearothermophilus* in a vector-plasmid and its expression in *Bacillus stearothermophilus* and *Bacillus subtilis*. *Journal of Bacteriology* **154**, 831–837.
- GIBSON, T. & GORDON, R. E. (1974). Genus I. *Bacillus* Cohn. 1872, 174. In *Bergey's Manual of Determinative Bacteriology*, 8th edn, pp. 529–550. Baltimore: Williams & Wilkins.
- GOLDRICK, S. & SETLOW, P. (1983). Expression of a *Bacillus megaterium* sporulation-specific gene during sporulation of *Bacillus subtilis*. *Journal of Bacteriology* **155**, 1459–1462.
- GRAY, O. & CHANG, S. (1981). Molecular cloning and expression of *Bacillus licheniformis* β -lactamase gene in *Escherichia coli* and *Bacillus subtilis*. *Journal of Bacteriology* **145**, 422–428.
- HARRIS-WARRICK, R. M. & LEDERBERG, J. (1978). Interspecies transformation in *Bacillus*: sequence heterology as the major barrier. *Journal of Bacteriology* **133**, 1237–1245.
- HOCH, J. A. & MATHEWS, J. (1973). Chromosomal location of pleiotrophic negative sporulation mutations in *Bacillus subtilis*. *Genetics* **73**, 215–228.
- HONJO, M., MANABE, K., SHIMADA, H., MITA, I., NAKAYAMA, A. & FURUTANI, Y. (1984). Cloning and expression of the gene for neutral protease of *Bacillus amyloliquefaciens* in *Bacillus subtilis*. *Journal of Biotechnology* **1**, 265–277.
- JAMES, W. & MANDELSTAM, J. (1985). Protease production during sporulation of germination mutants of *Bacillus subtilis* and the cloning of a functional *gerE* gene. *Journal of General Microbiology* **131**, 2421–2430.
- JENKINSON, H. F. (1983). Altered arrangement of proteins in the spore coat of a germination mutant of *Bacillus subtilis*. *Journal of General Microbiology* **129**, 1945–1958.
- JENKINSON, H. F. & MANDELSTAM, J. (1983). Cloning of the *Bacillus subtilis* *lys* and *spoIIIB* genes in phage ϕ 105. *Journal of General Microbiology* **129**, 2229–2240.
- JOHNSON, W. C., MORAN, C. P., JR & LOSICK, R. (1983). Two RNA polymerase sigma factors from *Bacillus subtilis* discriminate between overlapping promoters for a developmentally regulated gene. *Nature, London* **302**, 800–804.
- JOYET, P., GUERINEAU, M. & HESLOT, H. (1984). Cloning of a thermostable α -amylase gene from *Bacillus licheniformis* and its expression in *Escherichia coli* and *Bacillus subtilis*. *FEMS Microbiology Letters* **21**, 353–358.
- KYTE, J. & DOOLITTLE, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157**, 105–132.
- LEHTOVAARA, P., ULMANEN, I. & PALVA, I. (1984). *In vivo* transcription initiation and termination sites of an α -amylase gene from *Bacillus amyloliquefaciens* cloned in *Bacillus subtilis*. *Gene* **30**, 11–16.
- LOPEZ-DIAZ, I., CLARKE, S. & MANDELSTAM, J. (1986). *spoIID* operon of *Bacillus subtilis*: cloning and sequence. *Journal of General Microbiology* **132**, 341–354.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *Journal of Molecular Biology* **3**, 208–218.
- MESSING, J. (1983). New M13 vectors for cloning. *Methods in Enzymology* **101**, 20–78.
- MESSING, J., CREA, R. & SEEBURG, P. H. (1981). A system for shotgun DNA sequencing. *Nucleic Acids Research* **9**, 309–321.
- NORRANDER, J., KEMPE, T. & MESSING, J. (1983). Improved M13 vectors using oligonucleotide-directed mutagenesis. *Gene* **26**, 101–106.
- ORTLEPP, S. A., OLLINGTON, J. F. & MCCONNELL, D. J. (1983). Molecular cloning in *Bacillus subtilis* of a *Bacillus licheniformis* gene encoding a thermostable α -amylase. *Gene* **23**, 267–276.
- PALVA, I. (1982). Molecular cloning of the α -amylase gene from *Bacillus amyloliquefaciens* and its expression in *Bacillus subtilis*. *Gene* **19**, 81–87.
- PALVA, I., PETTERSSON, R. F., KALKKINEN, N., LEHTOVAARA, P., SARVAS, M., SÖDERLUND, H., TAKKINEN, K. & KÄÄRIÄINEN, L. (1981). Nucleotide sequence of the promoter and NH₂-terminal signal peptide region of the α -amylase gene from *Bacillus amyloliquefaciens*. *Gene* **15**, 43–51.
- PERLMAN, D. & HALVORSON, H. O. (1983). A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *Journal of Molecular Biology* **167**, 391–409.
- TE RIELE, H. P. & VENEMA, G. (1982a). Molecular fate of heterologous bacterial DNA in competent *Bacillus subtilis*. I. Processing of *B. pumilus* and *B. licheniformis* DNA in *B. subtilis*. *Genetics* **101**, 179–188.
- TE RIELE, H. P. & VENEMA, G. (1982b). Molecular fate of heterologous bacterial DNA in competent *Bacillus subtilis*. II. Unstable association of heterologous DNA with the recipient chromosome. *Genetics* **102**, 329–340.
- TE RIELE, H. P. & VENEMA, G. (1984). Molecular fate of heterologous bacterial DNA in competent *Bacillus subtilis*: further characterization of unstable association between donor and recipient DNA and the involvement of the cellular membrane. *Molecular and General Genetics* **195**, 200–208.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SCHAEFFER, P., IONESCO, H., RYTER, A. & BALASSA, G. (1965). La sporulation de *Bacillus subtilis*: étude génétique et physiologique. *Colloques internationaux du Centre national de la recherche scientifique* **124**, 553–563.
- SEKI, T., OSHIMA, T. & OSHIMA, Y. (1975). Taxonomic study of *Bacillus* by deoxyribonucleic acid hybridization and interspecific transformation. *International Journal of Systematic Bacteriology* **25**, 258–270.
- SEKI, T., CHUNG, W. C., MIKANI, H. & OSHIMA, Y. (1978). Deoxyribonucleic acid homology and taxonomy of the genus *Bacillus*. *International Journal of Systematic Bacteriology* **28**, 182–189.

- SEKI, T., TSUNEKAWA, H., NAKAMURA, K., YOSHIMURA, K. & OSHIMA, Y. (1979). Conserved genes in *Bacillus subtilis* and related species. *Journal of Fermentation Technology* **57**, 488–504.
- SHINE, J. & DALGARNO, L. (1974). The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 1342–1346.
- SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.
- STERLINI, J. M. & MANDELSTAM, J. (1969). Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. *Biochemical Journal* **113**, 29–37.
- TREMPY, J. E. & HALDENWANG, W. G. (1985). σ^{29} -like protein is a common sporulation-specific element in bacteria of the genus *Bacillus*. *Journal of Bacteriology* **164**, 1356–1358.
- TREMPY, J. E., BONAMY, C., SZULMAJSTER, J. & HALDENWANG, W. G. (1985). *Bacillus subtilis* sigma factor σ^{29} is the product of the sporulation-essential gene *spoIIG*. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 4189–4192.
- WANG, W., MEZES, P. S. F., YANG, Y. Q., BLACHER, R. W. & LAMPEN, J. O. (1985). Cloning and sequencing of the β -lactamase I gene of *Bacillus cereus* 5/B and its expression in *Bacillus subtilis*. *Journal of Bacteriology* **163**, 487–492.
- WEINRAUCH, Y. & DUBNAU, D. (1983). Plasmid marker rescue transformation in *Bacillus subtilis*. *Journal of Bacteriology* **154**, 1077–1087.
- WELLS, J. A., FERRARI, E., HENNER, D. J., ESTELL, D. A. & CHEN, E. Y. (1983). Cloning, sequencing and secretion of *Bacillus amyloliquefaciens* subtilisin in *Bacillus subtilis*. *Nucleic Acids Research* **11**, 7911–7925.
- YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
- YOSHIMURA, K., IKENAKA, Y., MURAI, M., TANABE, M., SEKI, T. & OSHIMA, Y. (1983). Construction of a *Bacillus subtilis* cloning vehicle with heterologous DNA sequence. *Gene* **24**, 255–263.
- YOUNG, M. & MANDELSTAM, J. (1979). Early events during bacterial endospore formation. *Advances in Microbial Physiology* **20**, 103–162.