***Bacillus subtilis* Extracellular Nuclease Production Associated with the *spoOH* Sporulation Locus**

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A number of nuclease-deficient mutants of *Bacillus subtilis* were isolated and found to be concurrently asporogenous. The nuclease-deficient phenotype appeared to be associated with the *spoOH* sporulation locus. Spontaneously occurring sporogenous revertants concomitantly recovered the ability to produce extracellular nuclease activity. The position of the *ncl* mutation was determined using transformation and PBS1 transduction and found to map in the same site as *spoOH*. The map order of *ncl* and markers in the vicinity was determined to be *purA*–*cysA*–*ncl*(*spoOH*)–*strA*.

INTRODUCTION

Under certain conditions cells of *Bacillus subtilis* undergo a process of rapid cell differentiation, culminating in the development of a complex intracellular structure, the bacterial endospore. This process appears to involve the activation of genes, or groups of genes, in a strict temporal sequence. This is truly a process of cell differentiation since it involves a sequence of profound morphological alterations accompanied by biochemical changes and by the synthesis of qualitatively new proteins.

Since sporulation proceeds by a primary dependent sequence of events, various genetic blocks introduced by mutations stop spore formation at various stages. Stage O mutants remain similar to vegetative cells, showing no specific morphological signs of sporulation (Balassa, 1971). These types of mutants have been the subject of extensive genetic analysis (Hoch & Mathews, 1973; Hoch *et al.*, 1978; Piggot & Coote, 1976). Each locus may have its own control elements and so be considered an operon (Piggot, 1973). It has been suggested that the products of the stage O loci may be involved in transcription and translation (Hoch *et al.*, 1978). However, the function and products of many of these loci, including the *spoOH* locus, have remained unknown.

The studies presented in this paper identify a biochemical marker associated with the *spoOH* sporulation locus. This genetic locus is involved in the elaboration of four extracellular nucleases. These enzymes are exonucleolytic in nature and are elaborated during the earliest stages of sporulation (Slinker & Burke, 1979, 1981).

METHODS

Bacterial strains. All bacterial strains used in this work were derivatives of *B. subtilis* 168 and are described in Table 1.

Mutagenesis. Nuclease-deficient mutants were obtained after mutagenesis of purified *B. subtilis* 168 spores with ethyl methanesulphonate (Ito & Spizizen, 1971). Mutagenized spores were plated on minimal agar with tryptophan (50 µg ml⁻¹) and incubated at 37 °C for 3 d. Colonies were then transferred to DNAase detection agar plates and incubated at 43 °C for 2–3 d.

Revertant selection. Sporogenous revertants of strain JH651 were obtained by growing the strain in Penassay Broth for 5 h at 37 °C and 300 rev. min⁻¹. The cells were collected by centrifugation at 10000 g for 15 min at

Table 1. *Bacillus subtilis* strains

Strain	Genotype	Origin
168	<i>trpC2</i>	J. Spizizen
SR22	<i>trpC2 spoOA12</i>	J. Spizizen
IS16	<i>trpC2 pheA1 spoOB136</i>	BGSC*
IS19	<i>trpC2 pheA1 spoOF221</i>	BGSC
IS20	<i>trpC2 spoOH4</i>	BGSC
IS22	<i>trpC2 rjm-2 spoOH17</i>	BGSC
JH651	<i>trpC2 pheA1 spoOH81</i>	J. Hoch
IS27	<i>metC3 tal-1 spoOJ87</i>	BGSC
IS28	<i>trpC2 spoOK14</i>	BGSC
SCR2011	<i>cysA14 strA</i>	J. Ito
SCR2020	<i>leuA8 metB5 purA16</i>	J. Ito
ASB164	<i>trpC2 ncl-1</i>	This laboratory
ASB170	<i>trpC2 ncl-2</i>	This laboratory
ASB184	<i>trpC2 ncl-3</i>	This laboratory

* BGSC, Bacillus Genetic Stock Center.

4 °C and resuspended in 1/20 the volume of minimal salts. Revertants were selected using a modification of the procedure of Hoch (1971). Samples were plated on sporulation plates containing the basal salts medium of Donnellan *et al.* (1964) supplemented with 0.1% (w/v) glutamate, 0.1% (w/v) casein hydrolysate, and 50 µg phenylalanine ml⁻¹ in glass Petri dishes. After 20 h incubation at 37 °C, the lawn of growth was killed by exposure to 2 ml of chloroform pipetted into the lid of the inverted Petri dish. After the chloroform treatment, the plates were incubated at 37 °C for 5 d. This incubation allowed the spores formed by revertants to germinate, divide and form discrete colonies.

Transformation and transduction. Transformation experiments were carried out by the procedure of Anagnostopoulos & Spizizen (1961). The DNA concentration was 0.02 µg per ml of competent culture, and the cells were exposed to DNA for 30 min. DNA was prepared by the method described previously (Burke & Spizizen, 1977). PBS1-mediated transduction was performed according to the method of Hoch *et al.* (1967) as modified by Young *et al.* (1969). Cells were grown in a motility medium consisting of minimal salts (Burke & McCammon, 1978) supplemented with 0.5% (w/v) yeast extract (Difco), 0.1% (w/v) casein hydrolysate (Nutritional Biochemicals Corp.), 0.5% (w/v) glucose, 5 mM-MgSO₄ and 50 µg per ml of the auxotrophic requirements of the strain. Antibiotic selection, including overlay and expression time, was as described by Harford & Sueoka (1970).

Nuclease assay. The procedure used for the quantitative assay of exonuclease activity was essentially that of Burke & Spizizen (1977). The standard reaction mixture consisted of 0.1 M-Tris/HCl (pH 8.0), 10 mM-CaCl₂ and approximately 6.5 µg [³H]DNA ml⁻¹ (specific activity: 10 157 c.p.m. per µg) in a total volume of 0.29 ml. Following preincubation of the reaction mixture for at least 10 min at 37 °C, the reaction was initiated by the addition of a 10 µl portion of the sample to be tested. After a 30 min incubation at 37 °C, 0.4 ml of 7% (w/v) perchloric acid and 0.4 ml salmon sperm DNA solution (5 mg ml⁻¹) were added. The solution was mixed, and then placed on ice for 30 min. The sample was centrifuged at 12 800 g for 2 min at room temperature, and 0.2 ml of the resultant supernatant fluid was transferred to a scintillation vial containing 5 ml of Aquasol (New England Nuclear). Radioactivity was measured using a Packard Tri-Carb 3320 liquid scintillation counter. A unit of DNAase activity is defined as the amount of enzyme catalysing the formation of 10 nmol of acid-soluble nucleotides at 37 °C in 30 min.

DNAase detection agar. Agar plates for extracellular DNAase detection were prepared by adding 10 ml salmon sperm DNA solution (5 mg ml⁻¹) and 4 ml acridine orange (2 mg ml⁻¹) to 400 ml of molten AK agar no. 2 medium supplemented with 1.6 g of agar. The mixture was incubated at 50 °C for 1 h prior to pouring.

Sporulation media. Nutrient broth sporulation medium was essentially the same as that of Schaeffer's medium as modified by Leighton & Doi (1971). The medium consisted of Difco nutrient broth (16 mg ml⁻¹), 25 mM-KCl, 2 mM-CaCl₂, 10 µM-FeSO₄, 10 µM-MnSO₄, 0.1 mM-MgSO₄ and 5 mM-glucose. The minimal salts sporulation medium of Donnellan *et al.* (1964) was also utilized in several experiments.

Sporulation test. Cells were grown in nutrient broth sporulation medium for 18 h at 37 °C at 250 rev. min⁻¹ in a controlled environment incubator shaker (New Brunswick Scientific). For maximal aeration, the culture volume never exceeded 10% of the total flask volume. Viable cells were estimated by plating on tryptose blood agar base (TBAB, Difco). The heat-resistant colony-forming units were determined by first heating the culture for 10 min at 80 °C and then plating on TBAB.

Fig. 1

Fig. 2

Fig. 1. DNAase detection plate inoculated with a series of stage O sporulation mutants. The strains were incubated at 43 °C for 48 h. The dark areas around the colonies indicate extracellular nuclease activity. The arrow indicates the position of 168 (wild-type) at the top of the plate; SR22 (*spoOA12*), IS16 (*spoOB136*), IS19 (*spoOF221*), JH651 (*spoOH81*), IS27 (*spoOJ87*) and IS28 (*spoOK14*) are positioned in a clockwise array from 168.

Fig. 2. DNAase detection plate inoculated with a series of *spoOH* and *ncl* mutants. The arrow indicates the position of 168 (wild-type) at the top of the plate; IS20 (*spoOH4*), IS22 (*spoOH17*), JH651 (*spoOH81*), ASB164 (*ncl-1*), ASB170 (*ncl-2*) and ASB184 (*ncl-3*) are positioned in a clockwise array from 168.

RESULTS

Isolation of nuclease-deficient mutants

In an effort to isolate mutants deficient in exonuclease production, spores of *B. subtilis* 168 were mutagenized with ethyl methanesulphonate. Isolated colonies from the mutagenized culture were transferred to DNA agar plates containing DNA and acridine orange in a sporulation agar. Following incubation at 43 °C for 48 h, the plates were examined under short-wave ultraviolet light. Dark, non-fluorescing areas were observed around the control colonies indicating extracellular exonuclease activity. Colonies which were deficient in nuclease production were detected by the absence of DNA hydrolysis in the vicinity of the colony. More than 30 nuclease-deficient mutants were isolated as the result of screening approximately 2500 colonies in this manner.

Two distinct mutant types were recognized among these nuclease-deficient strains. Approximately 25% of the mutants produced greatly reduced, although detectable, amounts of nuclease activity as observed on DNAase detection plates. All of these strains exhibited typical *Spo*⁺ colony morphologies on AK sporulation medium, and refractile spores were observed upon microscopic examination. However, no attempts were made to determine whether these mutants were truly sporogenous or oligosporogenous; and, because of their variable behaviour on DNAase detection plates, further studies with these mutants were not pursued.

The remaining mutants appeared to be completely deficient in nuclease production on the DNAase detection medium. Mutants of this type were designated *ncl* and were found to be asporogenous. Three of these *ncl* mutants were chosen for further study.

In order to determine whether the nuclease deficiency in these mutants was related to the

Table 2. *Sporulation and nuclease activities of spoOH revertants*

Strain	Phenotype	Sporulation (%) [*]	Nuclease activity (units ml ⁻¹)
168	Spo ⁺	65.3	21.5
JH651	Spo ⁻	<10 ⁻⁶	0.3
651-R2†	Spo ⁺	61.6	6.7
651-R4†	Spo ⁺	64.7	21.2
651-R5†	Spo ⁺	64.1	20.6

^{*} Percentage sporulation is expressed as $100 \times (\text{no. of heat-resistant spores ml}^{-1})/(\text{no. of viable cells ml}^{-1})$.

† Represents revertant strain of JH651.

sporulation deficiency, a series of strains with well characterized stage O mutations were also examined for nuclease production. With one exception, all of the stage O mutants tested were able to synthesize nuclease (Fig. 1). The exception was a strain mutated in the *spoOH* locus of the *B. subtilis* chromosome. Next, a series of well defined *spoOH* mutants were examined to determine if this nuclease deficiency was a general property of *spoOH* mutants. All of the *spoOH* mutants tested were deficient in nuclease production (Fig. 2).

A number of spontaneously occurring sporogenous revertants of the nuclease-deficient strains were examined for their ability to produce nuclease using DNAase detection agar. All of the Spo⁺ revertants of JH651 (6/6), ASB164 (74/74), ASB170 (48/48) and ASB184 (89/89) demonstrated nuclease production on the detection plates. Three of the spontaneously occurring Spo⁺ revertants of JH651 (*spoOH81 ncl-81*) were studied further in order to quantify the sporulation frequencies and extracellular nuclease levels. It was determined that these revertants had regained the ability to sporulate simultaneously with the ability to synthesize extracellular nuclease (Table 2).

Mapping of *ncl* mutations

The location of the *spoOH* locus is well established (Hoch & Mathews, 1973; Piggot, 1973; Piggot & Coote, 1976) and is situated between *cysA14* and *strA* on the genetic map. Since preliminary experiments with the DNAase detection agar indicated that the nuclease gene might be associated with the *spoOH* locus, mapping experiments were concentrated in the region of this sporulation locus.

PBS1 transductions localized the *ncl* mutations between the *purA* and *strA* loci. The *ncl* mutations were found to weakly cotransduce with *purA16* at frequencies of between 4 and 10%. Three-factor crosses indicated that the *ncl* markers were all closely linked to the *cysA14* locus and were tentatively located on the side of the *cysA14* locus proximal to *strA* (Table 3). However, the high frequency of cotransduction of the *ncl* mutations with *cysA14* made accurate location difficult. Unequivocal ordering of the *ncl* mutations relative to the *cysA14* locus was not possible from these data. Transformation crosses were undertaken to clarify these results. The mapping data obtained for the *spoOH81* locus and the *ncl* mutations in the three-factor transformation crosses are consistent with the transduction data (Table 4). Reciprocal transformation crosses into the poorly competent *spoOH* and *ncl* mutants failed to provide useful information. Inasmuch as Piggot (1973) has positioned the *spoOH* locus just to the right of *cysA14*, the sequence *cysA14-ncl-strA* is assumed.

In all of the experiments described above, inheritance of the nuclease deficiency was completely correlated to the acquisition of the Spo⁻ phenotype. This result further supports the supposition that the production of nuclease is an integral function of the *spoOH* locus. From the data presented here, it is not possible to determine whether the *spoOH* locus contains the structural gene for the nuclease or whether the nuclease deficiency is a pleiotropic effect of mutation of the *spoOH* locus. Because the *spoOH* and *ncl* strains were poorly competent, mutual transformation crosses between strains carrying the *spoOH* or *ncl*

Table 3. Three-factor crosses by PBSI transduction for mapping nuclease deficiency mutations

Donor	Recipient	Selected marker	Recombinant classes*			No. of recombinants	Marker order
<i>spoOH81</i>	<i>cysA14 strA</i>	Cys ⁺	<i>cysA14</i>	<i>spoOH81</i>	<i>strA</i>	10	<i>cysA14-spoOH81-strA</i>
			1	1	1	238	
			1	0	1	14	
			1	0	0	0	
<i>ncl-1</i>	<i>cysA14 strA</i>	Cys ⁺	<i>cysA14</i>	<i>ncl-1</i>	<i>strA</i>	13	<i>cysA14-ncl-1-strA</i>
			1	1	1	191	
			1	0	1	12	
			1	0	0	0	
<i>ncl-2</i>	<i>cysA14 strA</i>	Cys ⁺	<i>cysA14</i>	<i>ncl-2</i>	<i>strA</i>	5	<i>cysA14-ncl-2-strA</i>
			1	1	1	198	
			1	0	1	10	
			1	0	0	0	
<i>ncl-3</i>	<i>cysA14 strA</i>	Cys ⁺	<i>cysA14</i>	<i>ncl-3</i>	<i>strA</i>	12	<i>cysA14-ncl-3-strA</i>
			1	1	1	112	
			1	0	1	10	
			1	0	0	0	
<i>strA cysA14</i>	<i>spoOH81</i>	Str ^R	<i>strA</i>	<i>spoOH81</i>	<i>cysA14</i>	155	<i>cysA14-spoOH81-strA</i>
			1	1	1	2	
			1	0	1	0	
			1	0	0	29	
<i>strA cysA14</i>	<i>ncl-1</i>	Str ^R	<i>strA</i>	<i>ncl-1</i>	<i>cysA14</i>	152	<i>cysA14-ncl-1-strA</i>
			1	1	1	2	
			1	0	1	0	
			1	0	0	28	
<i>strA cysA14</i>	<i>ncl-2</i>	Str ^R	<i>strA</i>	<i>ncl-2</i>	<i>cysA14</i>	144	<i>cysA14-ncl-2-strA</i>
			1	1	1	9	
			1	0	1	0	
			1	0	0	28	
<i>strA cysA14</i>	<i>ncl-3</i>	Str ^R	<i>strA</i>	<i>ncl-3</i>	<i>cysA14</i>	125	<i>cysA14-ncl-3-strA</i>
			1	1	1	3	
			1	0	1	0	
			1	0	0	22	

* 1 and 0 refer to donor and recipient markers, respectively.

mutations did not contribute to the understanding of the relationship between these mutations.

DISCUSSION

Mutants deficient in extracellular nuclease production were isolated from mutagenized *B. subtilis* 168 spores. Examination of a number of stage O sporulation mutants indicated that the extracellular enzyme production was in some way associated with the *spoOH* locus.

Spontaneous reversion to the Spo⁺ phenotype resulted in concomitant recovery of exonuclease production. Genetic mapping of three of the *ncl* mutants isolated in this study, in addition to the well characterized *spoOH81* strain, suggested the following arrangement of loci on the chromosome: *purA-cysA-ncl(spoOH)-strA* (Fig. 3).

Fig. 3. Location of the *ncl-81* (*spoOH81*) mutation on the *B. subtilis* genetic map. Distances between markers are expressed as percentage of recombination according to the convention: % recombination = $(1 - \text{cotransfer}) \times 100$. The arrow heads point to selected markers.

Table 4. Three-factor transformation crosses for mapping nuclease deficiency mutation

Donor	Recipient	Selected marker	Recombination classes*			No. of recombinants	Marker order
<i>spoOH81</i>	<i>cysA14 strA</i>	Cys ⁺	<i>cysA14</i>	<i>spoOH81</i>	<i>strA</i>	97	<i>cysA14-spoOH81-strA</i>
			1	1	1	102	
			1	0	1	14	
			1	0	0	44	
			1	0	0	27	
<i>ncl-1</i>	<i>cysA14 strA</i>	Cys ⁺	<i>cysA14</i>	<i>ncl-1</i>	<i>strA</i>	129	<i>cysA14-ncl-1-strA</i>
			1	1	0	21	
			1	0	1	59	
			1	0	0	31	
<i>ncl-2</i>	<i>cysA14 strA</i>	Cys ⁺	<i>cysA14</i>	<i>ncl-2</i>	<i>strA</i>	148	<i>cysA14-ncl-2-strA</i>
			1	1	0	12	
			1	0	1	48	
			1	0	0	45	
<i>ncl-3</i>	<i>cysA14 strA</i>	Cys ⁺	<i>cysA14</i>	<i>ncl-3</i>	<i>strA</i>	120	<i>cysA14-ncl-3-strA</i>
			1	1	0	21	
			1	0	1	44	
			1	0	0		

* 1 and 0 refer to donor and recipient markers, respectively.

At present no conclusive evidence is available to indicate that the *ncl* mutations are mutations in the structural gene of the nuclease. It is possible that the failure to produce extracellular nucleases early in sporulation results from a pleiotropic effect of a mutation in the *spoOH* locus. Indeed, mutations of the *spoOH* locus preclude the synthesis of all four nuclease forms. However, it is interesting to note that the *Ncl*⁻ phenotype, unlike many other sporulation properties, appears to be associated with only one stage O locus. Further, of the approximately 2500 colonies examined in screening *ncl* mutants, not one nuclease-deficient, sporogenous mutant was observed.

Akrigg & Mandelstam (1978) have demonstrated that during sporulation *B. subtilis* 168 also produces a manganese-stimulated endodeoxyribonuclease that is released into the medium. The production of this nuclease is associated with events late in stage II of sporulation. The nucleases associated with *spoOH* function are exonucleolytic in nature and preferentially degrade single strand DNA (Slinker & Burke, 1979). These *ncl* controlled nucleases are thought to be the nucleases described by Kerr *et al.* (1965) and Kanamori *et al.*

(1973). Although these enzymes have ribonuclease capabilities, they do not represent the major form of ribonuclease associated with the early stages of sporulation.

The data obtained in this study are consistent with the supposition that the production of the extracellular exonuclease activity observed early in sporulation is determined in some way by genetic information located in the *spoOH* locus. This DNAase production provides an easily measurable biochemical property characteristic of the *spoOH* locus.

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