**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(spo0H)-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 DNAse 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

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perchloric acid and phenylalanine ml⁻¹ in glass Petri dishes. After 20 h incubation at Donnellan procedure of Hoch 4 discrete colonies. never exceeded controlled environment incubator shaker (New Brunswick Scientific). For maximal aeration, the culture volume resultant supernatant fluid was transferred to a scintillation vial containing placed on ice for 30 min. The sample was centrifuged at 12 800 g for 10 min at 4 °C and then plating on TBAB.

Table 1. *Bacillus subtilis* strains

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* 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 result 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 mM-Feso₄, 10 mM-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.
**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
sporulation deficiency, a series of strains with well characterized stage 0 mutations were also examined for nuclease production. With one exception, all of the stage 0 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 strains 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

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

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<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Sporulation (%)*</th>
<th>Nuclease activity (units ml⁻¹)</th>
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* Percentage sporulation is expressed as 100 × (no. of heat-resistant spores ml⁻¹)/(no. of viable cells ml⁻¹).
† Represents revertant strain of JH651.
B. subtilis nuclease and the spoOH locus

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

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<th>Donor</th>
<th>Recipient</th>
<th>Selected marker</th>
<th>Recombinant classes*</th>
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* 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 0 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 – cotransfer) x 100. The arrow heads point to selected markers.

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

<table>
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<tr>
<th>Donor</th>
<th>Recipient</th>
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* 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.
B. subtilis nuclease and the spoOH locus

(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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