A Mutant of *Bacillus subtilis* Secreting a DNAase Inhibitor During Sporulation

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(Received 17 November 1981)

Under sporulation conditions, *Bacillus subtilis* mutant SE63 secreted into the medium an inhibitor of the extracellular DNAase that is specifically associated with stage II to III of sporulation. The inhibitor was a heat-labile protein with a molecular weight of 18 000–20 000. A cell-bound, presumably intracellular, inhibitor with the same properties was found in extracts of wild-type cells and of the mutant. The mechanisms by which the mutation, designated din, might cause the secretion of a protein that is normally intracellular are discussed. The mutation was mapped by transduction with phage PBS1 and it lies between met and fru. It was also introduced into a variety of sporulation mutants including some blocked at the earliest known stages of the process. In all of these it caused secretion of the inhibitor. It is concluded that the secretion is therefore not integrally associated with sporulation but is, instead, part of the response of the cells to the nutritional step-down conditions in which spore formation is induced.

**INTRODUCTION**

Sporulation of *Bacillus subtilis* is accompanied by the production of several hydrolytic enzymes (Schaeffer, 1969). Most of these are linked to the process of sporulation and they do not appear in mutants blocked early in the sporulation sequence. These enzymes, e.g. serine protease (Millet, 1970) and alkaline phosphatase (Glenn & Mandelstam, 1971) serve as useful marker events during sporulation (Waites et al., 1970) and they help to determine the order of expression of spo loci (Piggot & Coote, 1976; Young & Mandelstam, 1979). Among them there is an extracellular DNAase (sporulation endonuclease) that is specifically associated with stage II of sporulation (Akriigg & Mandelstam, 1978) and which has been characterized by Akriigg (1978). Since the structural gene for this enzyme is under sporulation control, and thus presumably part of a spo locus, its chromosomal location should define a spo locus with a characterized gene-product. In an attempt to identify this locus mutants with altered DNAase activity were isolated. One of these, designated SE63, sporulated normally but its DNAase activity was substantially reduced. This paper shows that this phenotype is the result neither of a mutation in the structural gene nor of one in a control gene for the DNAase but is caused by a mutation, termed din, that leads to the production of an extracellular inhibitor of sporulation endonuclease.

**METHODS**

*Organisms.* *Bacillus subtilis* 168 (trpC2), which requires tryptophan or indole, and sporulates normally, is referred to as the wild type; other strains were derived from the wild type and are listed in Table 1. Strain SE63, referred to as the mutant, was isolated in this laboratory by Dr A. Akriigg after treatment of the wild type with

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ethyl methanesulphonate (EMS) as described by Coote (1972). It sporulated normally but was characterized by the fact that it produced abnormally small haloes when plated on DNAase detection agar (see below). From transformation and transduction experiments in which the mutation was transferred into several different backgrounds (see Results), this phenotype appeared to be due to a single mutation which is referred to as din (DNAase inhibitor).

**Media.** Lactate/glutamate minimal medium was as described by Piggot (1973). Fructose minimal agar contained, per litre: (NH₄)₂SO₄, 2 g; KH₂PO₄, 14 g; K₂HPO₄, 6 g; tri-sodium citrate (dihydrate), 2·0 mg; MgSO₄·7H₂O, 20 mg; MnCl₂·6H₂O, 20 mg; tryptophan, 10 mg; and agar (Difco, Noble), 17 g. Each plate of agar (20 ml) was supplemented with 0·1 ml fructose solution (10%, w/v) before use. In glucose minimal agar the fructose was replaced by glucose (0·4%, w/v, final concn). Nutrient agar was from Oxoid.

**DNAase detection agar.** This was based on lactate/glutamate minimal medium but contained methyl green and DNA as described for the agarose plates of Horney & Webster (1971). MOPS (2 g) was added to sporulation salts solution (735 ml) and the solution was adjusted to pH 7·0 with NaOH (2 ml). Sporulation salts solution contained, per litre: FeCl₃, 6H₂O, 0·98 mg; MgCl₂, 6H₂O, 8·3 mg; MnCl₂, 4H₂O, 19·79 mg; NH₄Cl, 535 mg; Na₂SO₄, 160 mg; K₂HPO₄, 68 mg; and NH₄NO₃, 97 mg. The following were then added: MnSO₄ (50 mM), 2 ml; MgSO₄ (1 mM), 10 ml; and agar, 15 g. The mixture was autoclaved at 111 kPa for 30 min, cooled to 55 °C, and the following solutions, also at 55 °C, were added: l-glutamic acid (5%, w/v), 40 ml; CaCl₂ (0-1 M), 10 ml; sodium lactate (35%, w/v), 8 ml; l-alanine (20 mg ml⁻¹), 5 ml; methyl green (1 mg ml⁻¹), 80 ml; and DNA (5 mg ml⁻¹), 100 ml. Before use, the methyl green (BDH) was dissolved in ammonium acetate (0·02 M), the pH being adjusted to 4·3 with glacial acetic acid and the solution extracted three times with an equal volume of chloroform to remove impurities. DNA (Sigma, type III) was prepared for the medium by dissolving it in 10 mM-MOPS, pH 7·0, containing 0·1 M-KCl. After the agar had been poured and allowed to set, its surface was sterilized by UV-irradiation (30 min at 15 cm from a Philips 15 W UV-lamp). DNAase detection plates were inoculated using sterile tooth-picks with bacteria which had been grown on nutrient agar. Growth of colonies and decolorization of the agar required incubation at 42 °C for at least 48 h.

**Growth and sporulation.** The procedure of Sterlini & Mandelstam (1969) was used. Cells were grown at 37 °C in hydrolysed casein medium to a density of about 0·25 mg dry wt ml⁻¹, centrifuged and resuspended in an equal volume of resuspension medium which contained glutamate, inorganic ions and appropriate auxotrophic requirements. Times (h) after resuspension are referred to as t₁, t₂ etc.

**Assay of DNAase activity.** DNAase activity was measured by the release of acid-soluble counts from ³H-labelled DNA under the conditions described by Akrigg (1978). One unit of activity is defined as the amount that releases 1 nmol acid-soluble nucleotides in 20 min (Akrigg & Mandelstam, 1978). The solution of inhibitor to be assayed was mixed with an equal volume of supernatant obtained at t₁ from a sporulating culture of the wild-type and therefore containing an appreciable amount of DNAase. The DNAase activity of the mixture was compared with that of the same supernatant mixed with an equal volume of resuspension medium. One unit of inhibitor is defined as the amount that inhibits one unit of DNAase activity. If necessary, the solution of inhibitor was diluted with resuspension medium so that it did not inhibit all the DNAase activity in the assay mixture.

**Sephadex G-75 chromatography.** A column (bed dimensions 60 cm x 1·7 cm (diameter)) was equilibrated in buffer (0·1 M-Tris/HCl, pH 7·5, containing 50 mM-NaCl) and calibrated with Blue Dextran 2000, ovalbumin

### Table 1. Strains of B. subtilis used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>168</td>
<td>trpC2</td>
</tr>
<tr>
<td>SE63</td>
<td>trpC2 din</td>
</tr>
<tr>
<td>SE63.2</td>
<td>metC3 din</td>
</tr>
<tr>
<td>CU1612</td>
<td>trpC2 metC3 fruB</td>
</tr>
<tr>
<td>ST-3</td>
<td>strB</td>
</tr>
<tr>
<td>69.2</td>
<td>metC3 spoI1A69</td>
</tr>
<tr>
<td>55.1</td>
<td>leu-8 spoI1G55</td>
</tr>
<tr>
<td>485</td>
<td>trpC2 spoO4G85</td>
</tr>
<tr>
<td>34.1</td>
<td>phe-12 rif-2 spoOA34</td>
</tr>
<tr>
<td>38</td>
<td>trpC2 spoNG1.161</td>
</tr>
</tbody>
</table>

See text for descriptions of SE63 and 168. Strain SE63.2 was obtained by transforming strain MB21 (metC3 leu-8) to Leu⁺, using DNA from strain SE63, and obtaining a din derivative by congression. All strains were laboratory stock except for strain CU1612 (R. Korman, Cornell University) and ST-3 (J. Hoch, Scripps Clinic, La Jolla).
DNAase inhibitor during sporulation

DNAase activity in the samples and in the incubation buffer; the times quoted are included only as a rough guide.

DNAase inhibitors could then be located as fluorescent bands in a dark background by transilluminating the gel with UV light.

Molecular weights of DNAases and DNAase inhibitors were estimated as described for SDS-PAGE (above) by adapting to permit the detection of DNAase inhibitors. Gels and samples were prepared and electrophoresed as described above for SDS–PAGE except that DNA (Sigma, type III) was incorporated at a concentration of 100 μg ml⁻¹ into stacking and running gels before polymerization. After electrophoresis, gels were either stained with Coomassie Blue (Jenkinson et al., 1981) or treated for detecting DNAase or DNAase inhibitors. For the latter, the gels were washed once in 1 M NaCl (10 ml), resuspended in pellet buffer (1.5 ml 50 mm Tris/HCl, pH 8.8, containing 1 mM-EDTA and 1 mM-phenylmethyl sulphonyl fluoride (PMSF), to inhibit metallo- and serine proteases), and passed through a French pressure cell at a pressure of 83 MPa. The broken cells were centrifuged at 12000 g for 1 h at 4 °C for 1 h at 4 °C and the precipitate resuspended in about 1 ml of the supernatant; this was centrifuged (Beckman, microfuge B) for 30 s. The pellet was resuspended in pellet buffer (90 μl) and 20% (w/v) SDS (20% w/w) was added to a final concentration of 2% (w/v).

Preparation of extracellular proteins. Portions (100 ml) of sporulating cultures (1 litre) were centrifuged at tᵣ. The supernatants were passed through a glass fibre filter (Whatman, AF/F), and trichloroacetic acid (50%, w/v) was added to 5% (w/v). The mixture was allowed to stand at 4 °C for 16 h, centrifuged at 16000 g for 1 h at 4 °C and the precipitate resuspended in about 1 ml of the supernatant; this was centrifuged (Beckman, microfuge B) for 30 s. The pellet was resuspended in pellet buffer (90 μl) and 20% (w/v) SDS (10 μl).

Genetic mapping. Transduction with bacteriophage PBS1 and transformation were carried out as described by Piggot (1973).

Protein estimations. The Lowry method was used, with bovine serum albumin as standard.

Non-denaturing (native) PAGE. The method of Davis (1964) as described by Porter (1981) was used.

Sporulation endonuclease. DNAase was purified from sporulating cultures of the wild type according to the method of Akrigg (1978) except that the step involving isoelectric focusing was omitted.

RESULTS

Reduced sporulation endonuclease activity of strain SE63

Strain SE63 grown on DNAase detection agar produced smaller haloes than colonies of the wild-type (Fig. 1) and it was on the basis of this phenotype that the mutant was initially isolated. DNAase assays of supernatant obtained from sporulating cultures of both strains also showed that the mutant produced much less DNAase activity than the wild-type (Fig. 2). The maximum amount (approx. 10% of that produced by the wild type), was found at tᵣ, i.e. 2 h earlier than in cultures of strain 168.
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Fig. 1. Use of DNAase detection agar to show reduced sporulation endonuclease activity by strain SE63. Colonies, produced by stab inocula, of strain 168 (right) and strain SE63 (left) were grown on DNAase detection agar. The haloes shown were obtained after incubation for 48 h.

Fig. 2. Sporulation endonuclease activity in supernatants from sporulating cultures of strain 168 (□) and SE63 (○). Cultures (50 ml) of both strains were grown and induced to sporulate. Samples (2 ml) were removed from the cultures at hourly intervals and centrifuged. The supernatants were assayed for DNAase activity. A portion (1 ml) of each sample from the culture of strain SE63 was heated at 70 °C for 10 min and also assayed for DNAase activity to produce the third curve (●).

Evidence that strain SE63 produces an inhibitor of sporulation endonuclease

It was known from the work of A. Akrigg (personal communication) that sporulation endonuclease is resistant to heating at 70 °C. Accordingly, samples of supernatant from sporulating cultures of strain SE63 were heated at 70 °C for 10 min. When these were assayed for DNAase they were found to contain wild-type quantities of activity (Fig. 2). A likely interpretation of this observation was that strain SE63 secreted a heat-labile inhibitor of sporulation endonuclease and that the heat treatment had destroyed the inhibitory activity and so revealed wild-type amounts of sporulation endonuclease activity.
DNAase inhibitor during sporulation

Fig. 3. Use of DNAase detection agar to show the secretion of DNAase inhibitor by strain SE63. Eight stab inocula of strain 168 (central column), strain SE63 (position a) and strain 69.2 (position b) were grown on DNAase detection agar. The effect of inhibitor was clearly seen after incubation for 48 h. This technique was also used for demonstrating the production of inhibitor by spo din strains (see text).

Table 2. Construction of spo din double mutants

In all cases, except for SES38 where donor DNA was used, the double mutants were obtained by transforming the recipient with a fully-grown culture of the donor strain (Ephrati-Elizur, 1968). Selection was on lactate/glutamate minimal medium containing the appropriate supplements. Asporogenous derivatives of the selected recombinants were obtained by congression and could be recognized by the reduced pigmentation of their colonies.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Donor</th>
<th>Recipient</th>
<th>Selected marker</th>
</tr>
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<tbody>
<tr>
<td>SES38 (spoONG1.16I din)</td>
<td>38 (spoONG1.16I trp)</td>
<td>SE63.2 (met din)</td>
<td>met&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>SES34 (spoOA din rif trp)</td>
<td>34.1 (phe rif spoOA)</td>
<td>SE63 (trp din)</td>
<td>rif</td>
</tr>
<tr>
<td>SES485 (spoOG din)</td>
<td>485 (spoOG trp)</td>
<td>SE63.2 (met din)</td>
<td>met&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>SES55 (spoIG din)</td>
<td>55.1 (spoIG leu)</td>
<td>SE63 (trp din)</td>
<td>trp&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>SES69 (spoIA din)</td>
<td>69.2 (spoIA met)</td>
<td>SE63 (trp din)</td>
<td>trp&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

To see if such an inhibitor could be detected on DNAase detection agar, colonies of strain SE63 and 69.2 (spoIIA) were grown on a DNAase detection plate close to colonies of strain 168. The decolorization that was caused by the secreted sporulation endonuclease of the wild-type colonies was inhibited by strain SE63 but not by strain 69.2 (Fig. 3). The latter strain was used as a control since it is blocked early in sporulation and does not produce sporulation endonuclease (Akrigg & Mandelstam, 1978).

DNAase inhibitor was also detected when supernatant, obtained at t<sub>4</sub> from sporulating cultures of strain SE63, was assayed for inhibitory activity by mixing it with DNAase solutions (see Methods). There was, however, considerable day-to-day variation in the amounts of inhibitor produced by sporulating cultures of the mutant. For example, values of 10, 40 and 116 units ml<sup>-1</sup> were measured in the supernatants of three different cultures. Nevertheless, in each case, heating the supernatant at 70 °C for 10 min destroyed the inhibitory activity and showed that the supernatants contained normal (i.e. wild-type) amounts of enzyme.

Production of inhibitor by double mutant (din spo) strains

To determine whether the production of inhibitor by strain SE63 is a sporulation-specific event, double mutants were constructed that were blocked at stage 0 or at stage II of
sporulation and which also carried the din gene. These strains and the method of their construction are summarized in Table 2. The double mutants and their parental asporogenous strains were grown in casein hydrolysate and transferred to resuspension medium as already described. Samples of culture supernatant were assayed for DNAase inhibitor (see Methods). In each case the double mutant began to produce inhibitor between \( t_2 \) and \( t_4 \) while the parental Spo\(^-\) strain produced negligible amounts of inhibitor. The results for one pair of cultures (containing the spoIIA mutation) are shown in Fig. 4.

The double mutants were also tested for inhibitor production on DNAase detection plates. Colonies were grown as shown in Fig. 3 with the double mutant in the positions labelled (a) and its parental asporogenous strain in those labelled (b). A pattern of decolorization similar to that shown in Fig. 3 was observed with each of the double mutants.

These results showed that inhibitor production by Din\(^-\) strains occurred at about \( t_2 \) and that this was not dependent on the successful completion of stage II or even of stage O of sporulation since it did not require the expression of any of the following spo loci: spo38 (early stage O), spoOA, spoOG, spoIIG or spoIIA.

**Partial purification of the DNAase inhibitor from strain SES69**

The first steps in this were similar to those in the purification of sporulation endonuclease from the wild type (Akrigg, 1978). One of the double mutants, strain SES69, was chosen since it produced inhibitor alone without the added complication of sporulation endonuclease. A 11 culture of strain SES69 was grown in casein hydrolysate medium and the cells were transferred to resuspension medium. At \( t_4 \) the culture was centrifuged for 15 min at 5000 g. Subsequent steps were carried out at 4 °C. The supernatant was passed through a glass-fibre filter (Whatman GF/F), dialysed against 10 l buffer A [50 mM-Tris/HCl, pH 8-1, containing PMSF (0-03%, w/v)] for 16 h, and then stirred for 2 h with 45 g (wet wt) of ion-exchange resin (Whatman DE52) which had been equilibrated with buffer A. The resin was collected by filtration (Whatman, no. 1 filter) and stirred with buffer A (100 ml) containing NaCl (0-4 M) for 30 min. The slurry was filtered (Whatman no. 1 filter) and the filtrate (100 ml) was dialysed for 4 h against buffer A (4 l) before it was loaded on to an ion-exchange column (7 cm x 1-7 cm internal diameter) of DE52 which had been equilibrated with buffer A. A linear salt gradient (100 ml, 0-0-4 m-NaCl in buffer A) was passed through the column and 3 ml fractions were collected and assayed for the DNAase inhibitor. A peak of inhibitory activity was eluted at a salt concentration of about 0-3 M. The fractions containing inhibitor were
DNAase inhibitor during sporulation

Fig. 5. Estimation of molecular weight of inhibitor by gel filtration. Inhibitor was passed down a column of Sephadex G-75 which had been calibrated with three marker proteins (see Methods). Values of $V_v/V_t$ were calculated from the elution volumes ($V_v$) of the marker proteins and the total bed volume ($V_t$) (Andrews, 1964) and plotted against molecular weight as shown. The elution volume for the inhibitor indicated that its molecular weight was about 20 000. The numbers in parentheses indicate the molecular weights of the marker proteins.

pooled and reduced in volume from 30 ml to 1 ml by ultrafiltration (Amicon, UM2 filter) and dialysis against solid polyethylene glycol 6000 (Fisons). The final preparation was dialysed against buffer (0.1 M Tris/HCl, pH 7.5 containing 3 mM PMSF) and contained about 0.75 mg protein.

**Molecular weight determination and sensitivity of inhibitor to pronase**

A portion (15 µl) of the preparation described above was mixed with 3 µl buffer (50 mM-Tris/HCl, pH 7.5). An identical portion of the same preparation was mixed with 3 µl of the same buffer containing 12 µg protease (Sigma type VI). Both mixtures were incubated at 37 °C for 30 min and subjected to native PAGE, after which the polyacrylamide gel was treated for detection of the inhibitor of sporulation endonuclease (Porter, 1981). The preparation to which protease had been added showed no detectable inhibitory activity, while the preparation containing no protease produced a clear zone of inhibition.

To estimate the molecular weight of the inhibitor, a portion (0.75 ml) of the inhibitor preparation from strain SES69 was analysed by Sepadex G-75 chromatography (see Methods). A single peak of inhibitory activity was eluted from the column, and from the elution volume the molecular weight of the inhibitor was estimated at 20 000 (Fig. 5).

An independent estimate of the molecular weight was made by pooling the active fractions eluted from the column of Sephadex and reducing their combined volume from 12 ml to 0.5 ml by dialysis against solid polyethylene glycol 6000 (Fisons). Two portions (75 µl each) of the resulting preparation were subjected to native PAGE. After electrophoresis, the lanes (12 × 1.5 × 0.15 cm) containing the two samples were excised from the slab gel with a razor blade. That portion of each lane known from previous experiments to contain the inhibitor was then cut into 15 slices (0.2 × 1.5 × 0.15 cm each). The slices from one lane were tested for their ability to inhibit DNAase activity on methyl green/DNA agarose (Porter, 1981). The corresponding pieces of gel from the other lane were subjected to SDS–PAGE as described in Methods. The resulting slab-gels were stained by the method of Oakley et al. (1980). For each piece of gel that inhibited sporulation endonuclease when tested on methyl green/DNA agarose, the corresponding piece, analysed by SDS–PAGE, was found to contain a single protein. Its molecular weight was estimated at 18 000.
Fig. 6. Analysis of cellular protein by DNA/SDS–PAGE. Samples of total cellular protein from strain 168 (lanes a and c) and from strain SE63 (lanes b, d and e) were subjected to DNA/SDS–PAGE (see Methods). Each lane contained 120 μg protein. After electrophoresis, lanes a, b and e were treated for the detection of DNAase activity (see Methods); lanes c and d were treated for the detection of DNAase inhibitor. Lane e was incubated at 30 °C for a shorter period (approx. 1·5 h) so that the individual bands of DNAase activity could be resolved. The arrows indicate the positions of the proteins of the sizes shown (in kilodaltons: K).

Analysis of cellular proteins by DNA/SDS–PAGE

At this stage there seemed to be two possible explanations for the din mutation: (a) that it had altered one of the extracellular proteins normally produced after resuspension in such a way that it acted as an inhibitor of DNAase; (b) that wild-type cells normally contained an intracellular inhibitor of DNAase and that the mutation had caused this to become extracellular.

To test the latter possibility, intracellular proteins from broken cells of the wild type and of the mutant were analysed by DNA/SDS–PAGE. Treatment of the gel for detecting DNAase inhibitors showed that both strains do indeed possess an intracellular DNAase inhibitor of molecular weight 18 000 (18K) (Fig. 6, lane c). However, the fluorescent band indicating inhibitory activity was both wider and more intense in the mutant (Fig. 6, lane d). Additional differences between the two strains were noted when the gel was treated for detection of DNAase activity (Fig. 6, lanes a and b). The major band of DNAase activity at mol. wt 17 000 (17K) was more intense in the proteins from the mutant. Also DNAase activity in the mol. wt 14 000–15 000 region, which on further analysis was shown to consist of three bands (Fig. 6, lane e), was detected in the lane of proteins from the mutant but was absent from the wild-type extract. In a similar experiment (not illustrated) the DNAase having the lowest molecular weight (14 000) was shown to co-migrate with the band of activity produced by
Analysis of total extracellular protein by DNA/SDS–PAGE. Samples of extracellular protein from sporulating cultures of wild-type 168 (lanes a, c and e) and strain SE63 (lanes b, d and f) were prepared as described in Methods and portions (20 μl per lane) were subjected to DNA/SDS–PAGE. After electrophoresis, lanes a and b were stained with Coomassie Blue, lanes c and d were treated for the detection of DNAase activity and lanes e and f were treated for the detection of DNAase inhibitor activity.

Fig. 7. Analysis of total extracellular protein by DNA/SDS–PAGE. Samples of extracellular protein from sporulating cultures of wild-type 168 (lanes a, c and e) and strain SE63 (lanes b, d and f) were prepared as described in Methods and portions (20 μl per lane) were subjected to DNA/SDS–PAGE. After electrophoresis, lanes a and b were stained with Coomassie Blue, lanes c and d were treated for the detection of DNAase activity and lanes e and f were treated for the detection of DNAase inhibitor activity.

purified sporulation endonuclease. [This estimate by DNA/SDS–PAGE of 14000 for the molecular weight of sporulation endonuclease agrees fairly well with the estimate by gel filtration (Akrigg, 1978) of 12000.]

Analysis of extracellular proteins by DNA/SDS–PAGE

Although it seemed likely that the extracellular inhibitor produced by din strains did indeed derive from the cellular form of the inhibitor present in the wild type, the mechanism of its release into the medium was not clear. One possibility was that there was a generalized, i.e. indiscriminate, release of cellular protein by din strains; the other was that the din mutation had specifically caused secretion of the inhibitor.

To test the possibility that the mutant was 'leaking' proteins into the medium, extracellular protein from the wild type and from the mutant was prepared and analysed by DNA/SDS–PAGE (see Methods). When the gel was stained with Coomassie Blue both the pattern and intensity of the protein bands produced by the two strains were very similar (Fig. 7, lanes a and b). Also, when the gel was treated for DNAase activity, both strains were shown to produce similar quantities of an enzyme of molecular weight 14000 (14K) (Fig. 7, lanes c and d). However, as expected from the earlier experiments, the DNAase inhibitor was found only in the lane containing protein from strain SE63 (Fig. 7, lanes e and f). It therefore appeared that the din mutation had caused a specific release of DNAase inhibitor from cells.
Table 3. Three-factor transduction crosses

Fru⁺ recombinants were selected on fructose minimal agar supplemented with methionine (20 μg ml⁻¹). Met⁺ recombinants were selected on lactate/glutamate minimal plates. Transductants were scored for din on DNAase detection plates, met on lactate/glutamate minimal plates, and str on glucose minimal plates containing streptomycin (1 mg ml⁻¹). Linkages are expressed as percentage cotransduction.

<table>
<thead>
<tr>
<th>Cross no.</th>
<th>Donor (III)</th>
<th>Recipient (OOO)</th>
<th>Selected marker</th>
<th>No. of colonies in recombinant classes</th>
<th>Suggested order</th>
<th>Linkages (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 SE63</td>
<td>CU1612</td>
<td>fru⁺ din met⁺</td>
<td>fru⁺</td>
<td>30 0 64 56</td>
<td>din to fru, 80</td>
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<td></td>
<td></td>
<td>fru din⁺ met</td>
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<td>met to fru, 37</td>
<td></td>
</tr>
<tr>
<td>2 ST-3</td>
<td>SE63.2</td>
<td>met⁺ din str⁺</td>
<td>met⁺</td>
<td>85 1 22 44</td>
<td>met din str</td>
<td>din to met, 43</td>
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</tr>
</tbody>
</table>

Fig. 8. Genetic map (not to scale) of the chromosomal region containing din. Distances are expressed as 100 — percentage cotransduction; those shown below the chromosome were determined from the crosses described in Table 3 and their subscripts refer to the cross number; those shown above the chromosome are taken from the literature and their subscripts refer to (a) Staal & Hoch (1972), (b) Gay et al. (1973) and (c) Hranueli et al. (1974).

Specificity of DNAase inhibitor

When preparations of the inhibitor were tested against bovine DNAase on methyl green/DNA agarose plates (Porter, 1981) no inhibition was observed.

Genetic mapping of the din gene

A preliminary mapping by A. Akrigg (personal communication) had indicated that the din gene might lie between the markers metC and pyrA. This was confirmed, and three-factor crosses were carried out using two other markers in this region to place the gene more precisely. These show that din lies to the right of metC and to the left of fruB (Table 3). The frequencies of the various recombinant classes were used to obtain a rough estimate of linkage value (see Fig. 8).
DISCUSSION

From the results presented it is apparent that the reduced extracellular DNAase activity of strain SE63 is caused by the secretion of a heat-labile DNAase inhibitor. The inhibitor has been partially purified and shown to be a protein with a molecular weight estimated at 20000 by Sephadex G-75 chromatography and 18000 by SDS–PAGE. A number of DNA-binding proteins with molecular weights in the range 9000–25000 have been described by Chestukhin et al. (1979). These were all shown to be positively charged and thermostable and to inhibit several activities including ATP-dependent DNAase and pancreatic DNAase I. The specificity of the inhibitor described here has not been extensively studied, but it does not inhibit bovine pancreatic DNAase I. This, and its heat-sensitivity show that it is not one of those described by Chestukhin et al. (1979).

The gel illustrated in Fig. 6 shows that both the wild-type and the mutant contain an intracellular, or perhaps periplasmic, DNAase inhibitor with a molecular weight of 18000. It thus appears likely that the din mutation in some way causes this protein to be released from the cell. In addition, analysis of extracellular proteins (Fig. 7) shows that the release of the inhibitor by din strains is not part of an indiscriminate release of cellular proteins. These results suggest that the din mutation is either in a gene involved in the secretory mechanism of the cell or in the structural gene for the inhibitor itself. Perhaps this has become fused to a 'signal sequence' (see Davis & Tai, 1980; Bassford & Beckwith, 1979; Emr et al., 1978) so that the inhibitor becomes recognized as a protein to be secreted.

Several other differences between the wild-type and strain SE63 are revealed by DNA/SDS–PAGE and they show that the din mutation is either pleiotropic or that it has not been separated from other mutations. Nevertheless, the defining characteristic of din, the production of the extracellular inhibitor, is found whenever the din mutation is transferred into a different genetic background. This was done both in the course of mapping the mutation and in the construction of a variety of din spo double mutants. In all these experiments din behaves as a single mutation, but it is still possible that the additional phenotypic properties – the quantitative differences in intracellular DNAase and inhibitor and the two additional bands of DNAase – result from very closely linked mutations.

Alternatively, and more probably, the additional bands of DNAase activity in the mutant may be precursors of the extracellular endonuclease whose signal sequences have not been fully removed and hence have not been secreted. It is consistent with this interpretation that their molecular weights are similar to, or slightly higher than, that of the extracellular DNAase. In fact, it has been shown that in Escherichia coli, precursors of the periplasmic maltose-binding protein accumulate in the cytoplasm when mutations are introduced into the signal sequence (Bassford & Beckwith, 1979). Other pleiotropic mutations affecting exoenzyme production have been recorded. Thus, the pap mutations, which affect the production of several exoenzymes of B. subtilis, are single mutations which were shown to revert in a single step (Yoneda et al., 1973). It is unfortunate that there is no obvious way of selecting for reversion of the din mutation.

Finally, we wish to consider whether secretion of the inhibitor is linked to sporulation and might therefore serve as a useful marker event. The inhibitor begins to be released at a substantial rate after t5 (Fig. 4). The kinetics of its appearance raised the possibility that its production might be specifically associated with stage II of sporulation. However, the din mutation causes the inhibitor to be secreted even by strains blocked at the earliest stages of sporulation. It is therefore much more likely that its production, like that of some other exo-proteins, e.g. α-amylase, is a response of the cells to the nutritional shift-down that is used to induce sporulation (Mandelstam, 1976).

We are indebted to Dr Alan Akrigg who isolated mutant SE63 and advised us on the preparation of DNAase detection plates, etc. We also thank various members of the Microbiology Unit, in particular Dr Howard Jenkinson, for helpful discussions and suggestions. A.C.G.P. gratefully acknowledges the receipt of a Research Studentship (S.R.C.). This work was supported by the Science Research Council.


