A gene (*sleC*) encoding a spore-cortex-lytic enzyme from *Clostridium perfringens* S40 spores; cloning, sequence analysis and molecular characterization

Shigeru Miyata, Ryuichi Moriyama, Nobuko Miyahara and Shio Makino

Author for correspondence: Shio Makino. Tel: +81 52 789 4132. Fax: +81 52 789 4012.

Department of Applied Biological Sciences, School of Agricultural Sciences, Nagoya University, Nagoya, Chikusa-ku, Aichi 464-01, Japan

Antiserum was raised against a 31 kDa spore-cortex-lytic enzyme, which is released during germination of *Clostridium perfringens* S40 spores. Western blotting of dormant spore and vegetative cell fractions separated by SDS-PAGE indicated that the 31 kDa enzyme is spore-specific and that the enzyme in the dormant spore exists as a 36 kDa protein which has no cortex-lytic activity. A gene encoding the 31 kDa enzyme, *sleC*, was cloned into *Escherichia coli* using a synthetic oligonucleotide as a hybridization probe and the nucleotide sequence of the entire gene was determined. The N-terminal amino acid sequence of the 36 kDa protein was found in this reading frame, confirming that the 36 kDa protein is a pro-form of the 31 kDa enzyme. The deduced amino acid sequence indicated that the 31 kDa enzyme is produced as a precursor, comprising three portions; an N-terminal prepro-sequence (114 amino acid residues), a pro-sequence (35 amino acid residues) and a mature enzyme (289 amino acid residues). It is suggested that the 36 kDa pro-enzyme is non-covalently attached to the exterior of the cortex layer, and that the pro-form is processed to release the active enzyme during germination.

Keywords: *Clostridium perfringens*, germination, spore-cortex-lytic enzyme, precursor

INTRODUCTION

Bacterial spore germination, defined as the irreversible loss of spore properties, is induced by specific germinants and proceeds through a set of sequential steps. Once the initial trigger reaction has been activated, germination continues in the absence of the inducer. The latter stage of germination involves decomposition of the spore peptidoglycan by a spore-cortex-lytic enzyme(s) (Foster & Johnstone, 1990; Moir & Smith, 1990).

A number of spore-cortex-lytic enzymes have been extracted or isolated from spores of different organisms (Ando, 1979; Brown & Cuhel, 1975; Brown *et al.*, 1978; Foster & Johnstone, 1987; Gombas & Labbe, 1981, 1985; Gould *et al.*, 1966; Makino *et al.*, 1994; Miyata *et al.*, 1995; Tang & Labbe, 1987). Among them, a 29 kDa

enzyme from *Bacillus megaterium* KM spores (an N-acetylmuramoyl-L-alanine amidase; Foster & Johnstone, 1987), a 24 kDa enzyme from *B. cereus* IFO 13597 spores (an amidase or a peptidase; Makino *et al.*, 1994) and a 31 kDa enzyme from *Clostridium perfringens* S40 spores (an amidase; Miyata *et al.*, 1995) require *in situ* stressed spore cortex for hydrolytic activity, but have minimal activity on isolated peptidoglycan substrates and vegetative cells. These enzymes have been implicated in the germination response. Although cortex hydrolysis may constitute a key event in spore germination (Foster & Johnstone, 1990), germination-specific spore-cortex-lytic enzymes have been poorly characterized at a molecular level. In this study, we have cloned a gene, named *sleC*, encoding the 31 kDa spore-cortex-lytic enzyme from *C. perfringens* S40 spore, which is referred to as SCLE. We report that SCLE is probably synthesized as a prepro-form and exists as an inactive pro-enzyme in the dormant spore, which is processed to release the active enzyme during germination. In addition, the location of pro-SCLE in the dormant spore and some structural features of SCLE are discussed. To our knowledge, this is the first time that the

Abbreviation: SCLE, 31 kDa spore-cortex-lytic enzyme of *Clostridium perfringens* S40.

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sequence of a spore-cortex-lytic enzyme has been described.

METHODS

Bacterial strains, plasmid and phage. Spores and vegetative cells of *C. perfringens* S40 were used as the source of SCLE and chromosomal DNA, respectively. Culture and spore germination of the organism were done as described previously (Miyata *et al.*, 1995). *Escherichia coli* XL1-Blue (Stratagene) was used as a host for screening the library. Plasmid pBluescript II SK+ (Stratagene) and phage M13mp19 were used as cloning vectors, and helper phage M13KO7 was used in preparing single-stranded DNA for sequencing. *E. coli* was routinely grown at 37 °C in 2 × YT medium with ampicillin added to 100 µg ml⁻¹ for plasmid-carrying strains (Sambrook *et al.*, 1989).

Preparations of coatless spores and spore extracts. Coatless spores were prepared by treatment of spores with 50 mM DTT in 0.1 M H₃BO₃, pH 10.0, at 40 °C for 1 h, according to the method of Ando & Tsuzuki (1984). Dormant and coatless spores (75 mg packed spores) were disrupted at 0–4 °C with a bead-beater (Edmund Buhler) in a vessel containing 200 µl 0.15 M KCl, 50 mM potassium phosphate, pH 7.0, and 100 mg glass beads (diam. 0.1 mm). The suspensions were centrifuged (14000 g, 10 min, 4 °C). Spore-cortex-lytic activity of the supernatant was assayed using coatless spores as substrate, according to the method described previously (Makino *et al.*, 1994). One unit of activity was defined as a decrease in OD₆₀₀ of 0.10 min⁻¹ of coatless spore suspensions in a cell of 1 mm light path at 32 °C. Protein in the supernatant was examined by SDS-PAGE and Western blotting. To determine whether SCLE-related protein(s) bind strongly to spore integuments, disrupted dormant and coatless spores were extracted with 1% (w/v) SDS plus 2 M urea, pH 8.0, containing 2% (v/v) 2-mercaptoethanol at 95 °C for 5 min, and protein in the SDS/urea extracts was also analysed.

Preparation of antibody and immunoprecipitation. SCLE was purified as described previously (Miyata *et al.*, 1995). A mouse was intraperitoneally injected with 20 µg of purified SCLE emulsified in Freund's complete adjuvant. After 3 weeks, the mouse was injected with the same amount of protein in Freund's incomplete adjuvant. A second boost was performed 5 weeks later, and the mouse was bled 10 d after the final injection. The blood was brought to 0.15 M NaCl, 5 mM sodium phosphate, 0.1% NaN₃, pH 7.0, via the addition of a concentrated buffer and then stored at 4 °C for 20 h. After centrifugation (14000 g, 5 min, 4 °C), antiserum was stored at –20 °C.

For immunoprecipitations, the antiserum (30 µl), or non-immune control serum of the immunized mouse (30 µl), was incubated with 60 µl protein A-agarose (Pharmacia) at 4 °C for 16 h. After centrifugation (5000 g, 5 min, 4 °C), the gel was washed with a 1.5 M glycine-NaOH buffer, pH 7.0, containing 3 M NaCl, and then equilibrated with 0.15 M KCl, 40 mM potassium phosphate, pH 7.0. Germination exudate (100 µl), which has coatless-spore-lytic activity of 1.44 units (Miyata *et al.*, 1995), was mixed with serum-treated protein A-agarose gels. The mixture was incubated at 4 °C for 16 h. After centrifugation (6000 g, 5 min, 4 °C), the coatless-spore-lytic activity of the supernatant was examined.

Oligonucleotide synthesis. Based on the N-terminal amino acid sequence of SCLE (Miyata *et al.*, 1995) and the reported codon usage of *C. perfringens* (Rood & Cole, 1991), an oligonucleotide, 5' T(C)TA(T)CCA(T)GAG(A)CCA(T)GTA-

(T)GTA(T)CC 3', was synthesized. A ³²P-labelled form of the nucleotide was used as a probe for Southern and colony hybridization.

Preparation of DNA. *C. perfringens* chromosomal DNA was prepared as described by Wilson (1989). Plasmid DNA was prepared from *E. coli* by the conventional alkaline-SDS method (Birnboim & Doly, 1979). Phage DNA was prepared as described by Sambrook *et al.* (1989).

Genomic Southern analysis. Southern hybridization was carried out by the method of Southern (1975) with some modifications. Chromosomal DNA was digested with appropriate restriction enzymes. After agarose gel electrophoresis, fragments were transferred to a nylon membrane. The membrane was hybridized at 40 °C with a ³²P-labelled oligonucleotide probe in 4 × SSC containing 0.2% Ficol 400 (Pharmacia), 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.1% SDS, and 0.0005% denatured salmon sperm DNA. After hybridization, the membrane was washed with 4 × SSC containing 0.1% SDS at 50 °C.

Gene library of *C. perfringens* chromosomal DNA in *E. coli*. Genomic DNA from *C. perfringens* S40 was digested with *Hind*III. After agarose gel electrophoresis, fragments in the size range 1.7–2.5 kb were purified using a GeneClean II kit (Bio101). These were ligated into pBluescript II SK+ that had been digested with *Hind*III and dephosphorylated with calf intestine alkaline phosphatase using a ligation kit (T4 DNA ligase, Takara Shuzo), and transformed into *E. coli* XL1-Blue according to the method of Hanahan (1983). The ampicillin-resistant (Ap^r) colonies were selected on LB agar plates containing 100 µg ampicillin ml⁻¹, and used as the gene library.

Selection of *E. coli* harbouring *sleC*. The Ap^r transformants were transferred onto sheets of nylon membrane, followed by incubation at 37 °C for 5 h on LB agar plates containing ampicillin (100 µg ml⁻¹). Colonies on the membrane were screened by hybridization with a ³²P-labelled oligonucleotide probe. A positive colony with pCP22H (pBluescript II plasmid containing a 2277 bp *Hind*III fragment) was isolated.

Nucleotide sequencing and analysis. Nucleotide sequencing was performed by the dideoxynucleotide chain termination method of Sanger *et al.* (1977), using an ALF DNA sequencer (Pharmacia) with an AutoRead sequencing kit (T7 DNA polymerase, Pharmacia). Two sets of nested deletion clones were generated from both sides of *sleC* in the 2277 bp insert of pCP22H with a Kiro-sequence deletion kit (Takara Shuzo), to allow sequencing of the entire fragment in both directions. One set of deletions, which were deleted from the *Eco*RV site (downstream of *sleC* in pCH22H), were subcloned into M13mp19 to obtain the single-stranded templates for sequencing. For another set of deletions, which were deleted from the *Sal*I site (upstream of *sleC* in pCP22H), single- or double-stranded templates were used for sequencing. The nucleotide and amino acid sequence analysis, and sequence comparison with DNAs and proteins registered in various databases (GenBank, EMBL, PIR and Swiss-Prot) were performed with MACDNASIS software (Hitachi Software Engineering).

Analytical methods. Protein concentrations were measured according to the method of Lowry, using bovine serum albumin as standard. SDS-PAGE was carried out on 13% (w/v) slab gels using a Laemmli buffer system (Laemmli, 1970) at a constant current of 20 mA. Proteins were silver-stained (Oakley *et al.*, 1980). Immunoblotting was carried out by the method of Towbin *et al.* (1979). Sequencing of N-terminal amino acids was

carried out on an Applied Biosystems 477A/120A sequence analyser.

RESULTS

Extraction and N-terminal amino acid sequence of a 36 kDa protein which cross-reacts with the antiserum raised against SCLE

Spores were incubated in 100 µg chloramphenicol ml⁻¹, an inhibitor of protein synthesis, for 30 min at 30 °C before initiating germination. The germinants were then added to the suspension. After 20 min, when germination was almost complete, the activity of SCLE released into the exudate was measured. The release did occur in the presence of chloramphenicol, suggesting that the enzyme is not synthesized *de novo* during germination. The amount of SCLE released in the presence of chloramphenicol

was identical ($\pm 5\%$) to that observed without chloramphenicol.

Treatment of germination exudate with antiserum raised against SCLE and pre-immune serum as described in Methods, resulted in $> 98\%$ and $< 3\%$ losses in enzyme activity, respectively. On this basis, the antiserum was used to detect SCLE-related proteins in spore and vegetative cell fractions separated by SDS-PAGE. As shown by Western blotting (Fig. 1), the antiserum recognized two components in the germination exudate; an active 31 kDa enzyme and a 36 kDa protein. When dormant spores were disrupted in, and extracted with, 0.15 M KCl, 50 mM potassium phosphate, pH 7.0, the extract contained only the 36 kDa protein as the component which cross-reacted with the antiserum. The extract had no activity to lyse coatless spores. No 31 kDa cross-reactive material was found in SDS/urea extracts of

Fig. 1. Detection of SCLE-related proteins in dormant spores and vegetative cells. Spore germination and partial purification of SCLE with a SP-Sephadex C-25 column were done as described previously (Miyata *et al.*, 1995). Dormant spores and vegetative cells were disrupted and extracted as described in Methods. The germination exudate and the extracts were electrophoresed in the presence of SDS, and then silver-stained (S) and Western-blotted (W). Partially purified SCLE was also electrophoresed to show the migration position of SCLE. Approximately 100 µg protein was loaded on the gel. Lanes: 1, partially purified SCLE; 2, germination exudate; 3, extract made from dormant spores; 4, extract made from vegetative cells. Prior to electrophoresis, coatless-spore-lytic activity of the samples was examined: +, positive hydrolytic activity; –, no hydrolytic activity. Molecular mass was determined using standard proteins: rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white albumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.4 kDa). Repeated experiments using different preparations gave the same results.

disrupted dormant spores. No SCLE-related protein could be detected in vegetative cells collected from a stationary-phase culture, indicating that SCLE and the 36 kDa protein were spore-specific.

The coat fraction of *C. perfringens* spores was prepared as described in Methods, and the resultant coatless spores were disrupted in and extracted with 0.15 M KCl, 50 mM potassium phosphate, pH 7.0. Then, the coat fraction and the extract were electrophoresed and immunoblotted. As shown in Fig. 2, > 90% of the 36 kDa protein was detected in the coat fraction. In SDS/urea extracts of disrupted coatless spores, SCLE-related protein was not detected.

The 36 kDa protein, which was separated by SDS-PAGE, was transferred to ProBlott membrane (Applied Biosystems) and the N-terminal amino acid sequence was determined according to the method of Matsudaira (1987). The sequence was QEEVIDIQPNRLVGNFP (17 residues). We could not find any protein sharing this amino terminus (computer analysis using databases).

Nucleotide sequence of the *sleC* gene

From the gene library containing *Hind*III-digested fragments of *C. perfringens* chromosomal DNA, we obtained a clone bearing pCP22H which includes the structural gene for SCLE. Fig. 3 shows the nucleotide sequence of the 2277 bp fragment in pCP22H and its deduced amino acid sequence. The G+C content was 31.4%, which is typical for this organism (Johnson & Francis, 1975).

Two potential ORFs were identified. One (*orf1*) lacked the 5' end. Downstream of *orf1*, the 1317 bp ORF, *sleC*, was identified between nucleotides 852 and 2168. A putative ribosome-binding site, AGAAAG (843–848) was found upstream of a possible start codon (ATG) at nucleotides 852–854 of *sleC* and exhibited significant complementarity with the 3' end of 16S rRNA in *C. perfringens* (Garnier *et al.*, 1991). There was a proposed promoter region upstream of the putative ribosome-binding site, namely the –35 region, TGATTA (762–767) and the –10 region, CTTATTA (785–791), with a 17 bp space between them. Downstream of a stop codon (TAA, 2166–2168), an inverted repeat sequence (2174–2207) was found, which resembles a prokaryotic *p*-independent transcriptional terminator (ΔG –19.1 kcal mol⁻¹) followed by a poly(T) sequence. Another inverted, but imperfect, repeat (746–777) and a direct repeat (740–747 and 775–782) were found between *orf1* and *sleC*, which overlap the proposed promoter sequence of *sleC*. These may be involved in the regulation of gene expression.

Deduced amino acid sequence of *sleC*

The *sleC* gene encoded 438 amino acid residues (Fig. 3). Codon usage was consistent with that reported for *C. perfringens* (Rood *et al.*, 1991).

The N-terminal amino acid sequence of the 31 kDa mature enzyme, VLPEPVPEYIVVHN (Miyata *et al.*, 1995),

was the same as that predicted from the nucleotide sequence starting at nucleotide 1299. The deduced amino acid alignment corresponding to nucleotides 1194–1244 agreed with the N-terminal 17 residues of the 36 kDa protein, confirming that the 36 kDa protein is a pro-form of SCLE. The result shown in Fig. 3 indicates that the enzyme is produced as a precursor with a calculated molecular mass of 49 638 Da with a N-terminal prepro-region of 114 amino acid residues. It was further suggested that the molecular masses of the pro-enzyme and the mature enzyme are 37 135 Da (324 amino acids) and 33 243 Da (289 amino acids), respectively. These values were slightly higher than those determined from SDS-PAGE.

The hydropathy profile of the amino acid sequence of the *sleC* product, obtained according to Kyte & Doolittle (1982), is shown in Fig. 4. In this figure, the location of charged residues in the sequence is indicated by the bar graph. It suggests that the mature enzyme is abundant in basic amino acids, while the prepro- and pro-region have an acidic nature.

The C-terminal region of SCLE showed homology with the sequences of a variety of peptidoglycan hydrolases; a cell wall amidase from *B. subtilis* (CwlA; Kuroda & Sekiguchi, 1990; Foster, 1991), a cell wall amidase from *B. licheniformis* (CwlL; Oda *et al.*, 1993), a muramoylpentapeptide carboxypeptidase from *Streptomyces albus* G (DD; Joris *et al.*, 1983) and an autolytic lysozyme from *C. acetobutylicum* (Lyc; Croux & Garcia, 1991). The sequences are shown in Fig. 5. The percentages of identical amino acids between the paired 37 amino acids (residues 363–399 of the SCLE sequence) were from 38% to 44%. No other notable homologous region was found between SCLE and other proteins including peptidoglycan hydrolases.

DISCUSSION

SCLE hydrolyses intact stressed spore cortex, but not isolated unstressed cortex (Miyata *et al.*, 1995). Such substrate specificities and the sensitivities of the enzyme to temperature, pH, Hg²⁺ and Zn²⁺ (Miyata *et al.*, 1995) are similar to those of a germination-specific 29 kDa spore-cortex-lytic enzyme found in spores of *B. megaterium* KM (Foster & Johnstone, 1987). A model for the germination pathway of *B. megaterium* KM has been proposed by Foster & Johnstone (1990), in which proteolytic cleavage is a crucial event for activation of spore-cortex-lytic enzyme. We have shown here that SCLE exists as an inactive 36 kDa pro-enzyme in the dormant spore and that, during germination, the pro-form is processed to release the active enzyme. This indicates involvement of protease(s) in the germination pathway of spores of this organism. Thus, *C. perfringens* S40 possesses an activation mechanism of spore-cortex-lytic enzyme similar to that of *B. megaterium* KM.

Despite such a similarity in the activation mechanism, there is a marked difference in the mode of interaction of spore-cortex-lytic enzyme with the spore between *C. perfringens* S40 and *B. megaterium* KM. The pro-SCLE of

Fig. 2. Location of the 36 kDa protein in the dormant spore. Spore coat fraction was prepared by treatment of spores at 40 °C for 1 h with 0.1 M H_3BO_3 , pH 10.0, containing 50 mM DTT. The resultant coatless spores were washed three times with 0.1 M H_3BO_3 , pH 10.0, containing 1% SDS and further washed three times with water. Then, the coatless spores were disrupted and extracted as described in Methods. The spore coat fraction and the extract made from the coatless spore were electrophoresed and immunoblotted. Those, which were obtained from 30 mg of packed spores, were loaded on the gel. For comparison, the germination exudate (80 µg protein) was also analysed. Lanes: 1, spore coat fraction, 2, extract made from coatless spores; 3, germination exudate. Repeated experiments using different preparations gave the same results.

C. perfringens is weakly attached to some spore fraction in the dormant spore by non-covalent forces, as indicated by the observation that the pro-enzyme is liberated during mechanical disruption in 0.15 M KCl, 50 mM potassium phosphate, pH 7.0. In contrast, Foster & Johnstone (1988) indicated that an inactive pro-form of a 29 kDa spore-cortex-lytic enzyme of *B. megaterium* KM covalently binds to the cortical peptidoglycan, probably by phosphate linkage. Furthermore, they found that antiserum raised against the 29 kDa enzyme recognizes some proteins in spore fractions of other species. However, the cross-reactive proteins do not lead to the suggestion of the presence of a cortex-bound pro-enzyme in spores of organisms other than *B. megaterium* KM (Foster & Johnstone, 1988). We have observed that the anti-SCLE serum does not recognize any protein in the spores of *B.*

subtilis 168 and *B. cereus* IFO 13597 (data not shown). Although the underlying germination principle may be universal for organisms of different origins (Foster & Johnstone, 1990), it is likely that evolutionary divergence has led to a variety of spore-cortex-lytic enzymes which may differ in their mode of interaction with the spore fraction and whose activity may be regulated by different mechanisms.

The pro-enzyme was easily extracted from the dormant spore by incubation at 40 °C for 1 h in 50 mM DTT, 0.1 M H_3BO_3 solution, pH 10.0, which strips the spore coat from *C. perfringens* spores (Ando & Tsuzuki, 1984; Gombas & Labbe, 1985). Such DTT-treated spores preserved refractility as well as intact spores, suggesting that both cortex and core are not damaged, and that the pro-enzyme is not present in the compartment, cortex or core. The results suggest that the pro-enzyme in the dormant spore is localized on the inner surface of the spore coat or the outer membrane that lies underneath the coat layer (Crafts-Lighty & Ellar, 1980) and that, when activated, the enzyme initiates lysis of peptidoglycan from the exterior of the cortex layer.

SCLE is possibly produced as a prepro-form. A putative promoter sequence, TGATTA-(17 bp)-CTTATTA, resembles the consensus -10 and -35 regions, TGAATA-(17-18 bp)-CATACTA, recognized by the forespore-specific RNA-polymerase-containing σ^G factor in *B. subtilis* (Nicholson *et al.*, 1989). Therefore, the precursor is likely to be produced in the forespore and this suggestion is compatible with the observation that no SCLE-related protein was detected in vegetative cells by the immunoblot assay. The prepro-region comprises 114 amino acids and has an extremely acidic nature. The sequence contains no characteristics of a signal sequence for a secreted protein. Although the actual role of the sequence remains for future study, it seems that the sequence is involved in transfer of the inactive enzyme to an appropriate locus in the dormant spore.

Structural features of the enzyme are depicted schematically in Fig. 4. The hydroplot of the pro-enzyme indicates that it is hydrophilic. The pro-region is highly acidic (the predicted pI is 4.38) in contrast to the basic nature of the mature enzyme (pI 9.24). This feature agrees with the facts that the mature protein absorbs to cation exchangers, but the pro-enzyme does not (unpublished result). The enzyme is activated by cleavage of the bond between Val₁₄₉ and Val₁₅₀ (the numbering is based on the prepro-form). It is interesting to note that the bond lies within a prolyl cluster region between Pro₁₃₁ and Pro₁₅₇ that contains nine proline residues. It appears, therefore, that the cleavage site is present in a flexible region. At least one of four cysteine residues (Cys₁₈₈, Cys₁₈₉, Cys₂₇₅ or Cys₂₈₂) might be crucial to the activity of the enzyme, because sulfhydryl reagents inactivate the enzyme (Miyata *et al.*, 1995).

As shown in Fig. 5, SCLE had a consensus motif (DG-XXGPXTXXXAXXXFQXXXXLXXXGXXXXXT) found in several cell wall hydrolases (Oda *et al.*, 1993). These enzymes differ in their substrate specificities. As

Fig. 3. Nucleotide sequence of the 2277 bp fragment and its deduced amino acid sequence. Only the non-coding DNA (mRNA-like) strand is shown, from position +1 (*HindIII* site). The deduced amino acid sequence of *sleC* (nucleotides 852–2168) and the C terminus of *orf1* (1–729) are given below the nucleotide sequence. The numbers of the nucleotides and amino acids are shown on the right. The two underlined regions denote the –35 and –10 regions of the *sleC* gene promoter as presumed by homology with those recognized by the forespore-specific RNA-polymerase-containing σ^G factor in *B. subtilis* (Nicholson *et al.*, 1989). The underlined region labelled *rb*s is a putative ribosome-binding site, showing remarkable complementarity with the 3' terminus of 16S rRNA in *C. perfringens* (Garnier *et al.*, 1991). Inverted

Fig. 4. Hydropathy plot, domain map and charge plot of the deduced amino acid sequence of the precursor of SCLE. (a) Hydropathy plot of the precursor using the algorithm and hydropathy values of Kyte & Doolittle (1982). Hydrophobicities were averaged over windows of five amino acids. (b) Alignment of the prepro-region, pro-region and mature enzyme. Arrows indicate the sites processed. A hatched box indicates the region containing a consensus motif (see Fig. 5) and locations of cysteine residues are indicated. (c) Location of charged residues indicated by a bar graph. The basic residues (K and R) are assigned a value of +1, while the acidic residues (D and E) are assigned a value of -1. The bars represent the total + or - values, above and below the horizontal axis, respectively, over 10 residues. pI values are also indicated.

SCLE 360-PKIAV...KY...KRE...VKTK...KIFN...PQT...EVDYA...WYKISDYV-403
CwlA 228-KNNGI...VY...KAD...IRRS...SMYG...TQD...IYGPK...KAKLEALL-271
CwlL 316-KNNGI...VY...KAN...VKR...LMHG...SAD...IYGSD...KAKLKTL-359
DD 34-AQLAI...QF...AKA...VQR...SAYG...AAD...IAGPA...FNKIYQLQ-77
Lyc 215-GGLEV...IE...E...E...IKD...SIMG...TVD...IWGTN...SGAAQQIF-258

Fig. 5. Comparison of the deduced amino acid sequence of SCLE with that of CwlA from *B. subtilis* (Kuroda & Sekiguchi, 1990; Foster, 1991), CwlL from *B. licheniformis* (Oda et al., 1993), muramoylpentapeptide carboxypeptidase from *S. albus* (DD; Joris et al., 1983) and autolytic lysozyme from *C. acetobutylicum* (Lyc; Croux & Garcia, 1991). Identical amino acids are indicated by a black background.

noted, SCLE strictly requires *in situ* stressed spore cortex for hydrolytic activity, while CwlA hydrolyses a wide range of peptidoglycan substrates isolated from Gram-positive bacteria (Foster, 1991). The carboxypeptidase of *S. albus* cleaves a wide variety of C-terminal N^α-(D-alanyl)-D-alanine linkages which cross-link the peptidoglycan unit in certain cell walls (Ghuysen et al., 1970). The autolytic lysozyme of *C. acetobutylicum* is a muramidase (Croux & Garcia, 1991). The motif has been found in the non-catalytic regions of CwlA, CwlL, carboxypeptidase and autolytic lysozyme, and the sequence of SCLE shown in Fig. 5 lacks a cysteine residue, which is indicated to be important for activity of SCLE. It is likely, therefore, that the motif is implicated in the recognition of the peptidoglycan of cell wall and spore. Recently, De Mot & Vanderleyden (1994) found that there is a conserved C-

terminal region in a number of proteins from both Gram-positive and Gram-negative bacteria, including several peptidoglycan-associated outer-membrane proteins, and indicated that the region may be important for the interaction with peptidoglycan. However, SCLE and cell wall hydrolases shown in Fig. 5 had no homology with the conserved C-terminal sequence.

The partial amino acid sequence of a potential ORF, *orf1*, lacking the 5' end, is also shown in Fig. 3. The sequence had no homology to any known proteins.

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REFERENCES

- Ando, Y. (1979). Spore-lytic enzyme released from *Clostridium perfringens* spores during germination. *J. Bacteriol.* **140**, 59–64.
- Ando, Y. & Tsuzuki, T. (1984). Energy-dependent activation of spore-lytic enzyme precursor by germinated spores of *Clostridium perfringens*. *Biochem. Biophys. Res. Commun.* **123**, 463–467.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**, 1513–1523.

repeats and a direct repeat are indicated by solid arrows and dashed arrows, respectively. Asterisks indicate termination codons. The processing site for the prepro-peptide of the SCLE-precursor is indicated by the thick horizontal arrow. A thick vertical arrow denotes the N terminus of SCLE as determined by protein sequence analysis.

- Brown, W. C. & Cuhel, R. L. (1975). Surface-localized cortex-lytic enzyme in spores of *Bacillus cereus* T. *J Gen Microbiol* **91**, 429–432.
- Brown, W. C., Vellom, D., Schnepf, E. & Greer, C. (1978). Purification of a surface-bound hexosaminidase from spores of *Bacillus cereus* T. *FEMS Microbiol Lett* **3**, 247–251.
- Crafts-Lighty, A. & Ellar, D. J. (1980). The structure and function of the spore outer membrane in dormant and germinating spores of *Bacillus megaterium*. *J Appl Bacteriol* **48**, 135–145.
- Croux, C. & Garcia, J. L. (1991). Sequence of the *lyc* gene encoding the autolytic lysozyme of *Clostridium acetobutylicum* ATCC824: comparison with other lytic enzymes. *Gene* **104**, 25–31.
- De Mot, R. & Vanderleyden, J. (1994). The C-terminal sequence conservation between OmpA-related outer membrane proteins and MotB suggests a common function in both Gram-positive and Gram-negative bacteria, possibly in the interaction of these domains with peptidoglycan. *Mol Microbiol* **12**, 333–334.
- Foster, S. J. (1991). Cloning, expression, sequence analysis and biochemical characterization of an autolytic amidase of *Bacillus subtilis* 168 *trpC2*. *J Gen Microbiol* **137**, 1987–1998.
- Foster, S. J. & Johnstone, K. (1987). Purification and properties of germination-specific cortex-lytic enzyme from spores of *Bacillus megaterium* KM. *Biochem J* **242**, 573–579.
- Foster, S. J. & Johnstone, K. (1988). Germination-specific cortex-lytic enzyme is activated during triggering of *Bacillus megaterium* KM spore germination. *Mol Microbiol* **2**, 729–733.
- Foster, S. J. & Johnstone, K. (1990). Pulling the triggers: the mechanism of bacterial spore germination. *Mol Microbiol* **4**, 137–141.
- Garnier, T., Canard, B. & Cole, S. T. (1991). Cloning, mapping, and molecular characterization of the rRNA operons of *Clostridium perfringens*. *J Bacteriol* **173**, 5431–5438.
- Gombas, D. E. & Labbe, R. G. (1981). Extraction of spore-lytic enzyme from *Clostridium perfringens* spores. *J Gen Microbiol* **126**, 37–44.
- Gombas, D. E. & Labbe, R. G. (1985). Purification and properties of spore-lytic enzymes from *Clostridium perfringens* type A spores. *J Gen Microbiol* **131**, 1487–1496.
- Gould, G. W., Hitchins, A. D. & King, W. L. (1966). Function and location of a germination enzyme in spores of *Bacillus cereus*. *J Gen Microbiol* **44**, 293–302.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Johnson, J. L. & Francis, B. S. (1975). Taxonomy of the clostridia: ribosomal ribonucleic acid homologies among the species. *J Gen Microbiol* **88**, 229–244.
- Joris, B., Van Beeumen, J., Casagrande, F., Gerday, C., Frere, J. M. & Ghuysen, J. M. (1983). The complete amino acid sequence of the Zn²⁺-containing D-alanyl-D-alanine-cleaving carboxypeptidase of *Streptomyces albus* G. *Eur J Biochem* **130**, 53–69.
- Kuroda, A. & Sekiguchi, J. (1990). Cloning, sequencing and genetic mapping of a *Bacillus subtilis* cell wall hydrolase gene. *J Gen Microbiol* **136**, 2209–2216.
- Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**, 105–132.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Makino, S., Ito, N., Inoue, T., Miyata, S. & Moriyama, R. (1994). A spore-lytic enzyme released from *Bacillus cereus* spores during germination. *Microbiology* **140**, 1403–1410.
- Matsudaira, P. (1987). Sequence from picomole quantities of protein electroblotted onto polyvinylidene difluoride membrane. *J Biol Chem* **262**, 10035–10038.
- Miyata, S., Moriyama, R., Sugimoto, K. & Makino, S. (1995). Purification and partial characterization of a spore-cortex-lytic enzyme of *Clostridium perfringens* S40 spores. *Biosci Biotech Biochem* **59**, 514–515.
- Moir, A. & Smith, D. A. (1990). The genetics of bacterial spore germination. *Annu Rev Microbiol* **44**, 531–553.
- Nicholson, W. L., Sun, D., Setlow, B. & Setlow, P. (1989). Promoter specificity of σ^G -containing RNA polymerase from sporulating cells of *Bacillus subtilis*: identification of a group of forespore-specific promoters. *J Bacteriol* **171**, 2708–2718.
- Oakley, B. R., Kirsch, D. R. & Morris, N. R. (1980). A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal Biochem* **105**, 361–363.
- Oda, Y., Nakayama, R., Kuroda, A. & Sekiguchi, J. (1993). Molecular cloning, sequence analysis, and characterization of a new cell wall hydrolase, CwlL, of *Bacillus licheniformis*. *Mol & Gen Genet* **241**, 380–388.
- Rood, J. I. & Cole, S. T. (1991). Molecular genetics and pathogenesis of *Clostridium perfringens*. *Microbiol Rev* **55**, 621–648.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Southern, E. M. (1975). Detection of specific sequence among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503–517.
- Tang, S. S. & Labbe, R. G. (1987). Mode of action of *Clostridium perfringens* initiation protein (spore-lytic enzyme). *Ann Inst Pasteur Microbiol* **138**, 597–608.
- Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**, 4350–4354.
- Wilson, K. (1989). Preparation of genetic DNA from bacteria. In *Current Protocols in Molecular Biology*, p. 2.4.1–2.4.2. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: John Wiley & Sons.

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