Nucleotide sequences of the *Bacillus subtilis* *flaD* locus and a *B. licheniformis* homologue affecting the autolysin level and flagellation

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A DNA fragment containing the *flaD* locus of *Bacillus subtilis*, which had been cloned into plasmid pAC3, was subcloned into an M13 phage and sequenced. The sequence contained five open reading frames (ORFs), of which ORF2 was the *flaD* gene. Unexpectedly, the sequence of the *flaD* locus was identical to that of *sin* [sporulation inhibition gene; Gaur, N. K., Dubnau, E. & Smith, I. (1986). *Journal of Bacteriology* 168, 860–869]. A *B. licheniformis* homologue (*flaL*) of the *B. subtilis* *flaD* locus was cloned into pUC19 and identified by colony hybridization. The *B. licheniformis* DNA was subcloned and sequenced. Two ORFs (ORF1, or L-ORF1; and ORF2, or *flaL*) were detected, encoding 58 and 111 amino acid residues, respectively. These are almost identical in length to ORF1 (D-ORF1; 57 amino acids) and *flaD* (111 amino acids) on the fragment of *B. subtilis* DNA. The overall interspecies differences between the nucleotide sequences of D-ORF1 and L-ORF1, and those of *flaD* and *flaL*, were 42% and 11%, respectively, and the differences in the predicted amino acid sequences were 50% and 7%, respectively. The regions 3' of the ORFs (*flaL* and *flaD*) in both species resemble rho-independent terminators of transcription. The characteristics of the amino acid sequences are also discussed.

Introduction

In *Bacillus subtilis*, autolysin(s) affect cell separation (Fein & Rogers, 1976), helical growth (Mendelson, 1982), flagellation (motility) (Fein, 1979) and competence (Ayusawa *et al.*, 1975; Akamatsu & Sekiguchi, 1987*b*), in addition to cell lysis. Since autolysins are important in cell morphology and physiology, autolysin-deficient mutants of *B. subtilis* have often been isolated, but no previous papers have indicated whether the mutations are in the structural genes of autolysins or in genes affecting their expression. One class of autolysin-deficient mutations, containing *lyt-1* and *lyt-2* (Fein & Rogers, 1976), was found to be localized in the *flaD* locus (Pooley & Karamata, 1984; Akamatsu & Sekiguchi, 1987*a, b*). Mutations at this locus led to decreases in the activities of two major autolytic enzymes (*N*-acetylmuramoyl-L-alanine amidase and endo- β -*N*-acetylglucosaminidase). Recently, we cloned a 2.8 kb *Pst*I fragment

from the chromosome of *B. subtilis* 168W which suppresses the FlaD⁻ phenotype (Sekiguchi *et al.*, 1988). An internal 1.0 kb *Xba*I–*Hind*III fragment also suppressed the FlaD⁻ phenotype, and recombination analysis indicated that the cloned fragments contained the *flaD* gene itself (Sekiguchi *et al.*, 1988).

In this communication, we report the nucleotide sequence of *flaD* and show it to be identical to *sin*, a previously reported gene responsible for inhibition of sporulation (Gaur *et al.*, 1986, 1988). We also describe the cloning and sequence analysis of a *B. licheniformis* homologue of the *flaD* locus.

Methods

Bacterial strains and plasmids. These are listed in Table 1. Plasmid pAC32R was constructed by blunt-ending an *Eco*RI–*Eco*RV fragment containing the kanamycin-resistance gene (Km^r) of pAC3 and a *B. subtilis* *ori* locus, and cloning into the blunt-ended *Eco*O109 site of pUC19. Thus, pAC32R acted as a shuttle vector between *B. subtilis* and *Escherichia coli*, expressing Km^r in both species and Lac⁺ and ampicillin resistance (Ap^r) in *E. coli*.

Media. L broth, containing, per litre, 10 g polypeptone (Wako Pure Chemicals), 5 g yeast extract (Difco), 1 g glucose and 5 g NaCl (pH

Abbreviation: ORF, open reading frame.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M31123.

Table 1. Bacterial strains, phages and plasmids

Genotype		Source* or reference
<i>Bacillus subtilis</i>		
AG5	<i>purB6 arg-15 rgn-1 (flaD) leuB8</i>	Akamatsu & Sekiguchi (1987a)
AC333	<i>purB lyt-2 (flaD2)</i>	Akamatsu & Sekiguchi (1987a)
<i>B. licheniformis</i>		
FD0120	<i>met-1 pepA</i>	Akamatsu & Sekiguchi (1984)
<i>Escherichia coli</i>		
JM109	<i>recA1 Δ(lac-proAB) endA1 gyrA96 thi-1 hsdR17 relA1 supE44 [F': traD36 proAB lacI^qZΔM15]</i>	Takara
MV1184	<i>ara Δ(lac-pro) strA thi (φ80 lacZΔM15) Δ(srl-recA) 306::Tn10(tet^r) [F': traD36 proAB lacI^qZΔM15]</i>	Takara
Phages		
M13mp18 and M13mp19		Yanisch-Perron <i>et al.</i> (1985)
M13KO7		Takara
Plasmids		
pAC3	Ap ^r Tc ^r Km ^r	Moriyama <i>et al.</i> (1988)
pAC32R	Ap ^r Km ^r Lac ⁺ in <i>E. coli</i> Km ^r in <i>B. subtilis</i>	This study
pBRG1	Tc ^r Km ^r	Sekiguchi <i>et al.</i> (1988)
pUC19	Ap ^r Lac ⁺	Yanisch-Perron <i>et al.</i> (1985)
pUC118 and pUC119	Ap ^r Lac ⁺	Takara

* Takara is a commercial supplier: Takara Shuzo Co., Kyoto, Japan.

7.2), was used for the growth of *E. coli*. *Bacillus* spp. were grown on nutrient broth, containing, per litre, 10 g beef extract (Difco), 10 g polypeptone and 2 g NaCl (pH 7.0). Both media were solidified by the addition of 15 g agar. If necessary, kanamycin and ampicillin were added to final concentrations of 5 µg ml⁻¹ and 50 µg ml⁻¹, respectively.

For protoplast regeneration, HCP-1.5 medium (Akamatsu & Sekiguchi, 1984) was used.

DNA manipulations. Standard cloning procedures (Maniatis *et al.*, 1982) were used. Colony hybridization was also performed as described by Maniatis *et al.* (1982); plasmid pBRG1, used as a probe, was radiolabelled with a nick-translation kit (Nippon Gene).

DNA sequencing. Nucleotide sequencing was done by the dideoxy chain-termination method with a modified T7 polymerase (Sequenase; Toyobo). Phages M13mp18 and M13mp19 (Takara), or plasmids pUC118 and pUC119 (Takara) were used to generate templates for sequencing, and phage M13KO7 (Takara) was used as a helper phage. Electrophoresis was performed on 8% (w/v) polyacrylamide/8 M-urea gels.

A 1.05 kb *Hind*III fragment containing the *flaD* gene of pUC12EX (Sekiguchi *et al.*, 1988) was subcloned into the unique *Hind*III site of pUC19 DNA. The resultant plasmid, pFLAD, was then digested with restriction enzymes, exonuclease III and mungbean nuclease to construct several deletion derivatives. The *B. subtilis* DNA fragments in these derivatives were subcloned into M13mp19 and sequenced using the strategy outlined in Fig. 1.

Protoplast transformation of *B. subtilis*. This was done as described previously (Akamatsu & Sekiguchi, 1984).

Measurement of autolytic activity. The autolytic enzymes, and cell walls for their assay, were prepared as described previously (Akamatsu & Sekiguchi, 1983).

Observation of cell motility and morphology. Cell motility was investigated on nutrient agar containing 0.5% agar, by phase-contrast microscopy. Cell morphology was observed during exponential growth in nutrient broth.

Results and Discussion

Nucleotide sequence of the *flaD* gene

We have cloned a 2.8 kb *Pst*I fragment of *B. subtilis* 168W DNA carrying the *flaD* gene, and the gene was found to be located between the *Hind*III and *Xba*I sites (1.0 kb apart) (Sekiguchi *et al.*, 1988). Surprisingly, the nucleotide sequence of the 1037 bp fragment containing the *flaD* gene was identical to that containing the *sin* gene (sporulation inhibition phenotype; Gaur *et al.*, 1986). The map position of *flaD* on the *B. subtilis* chromosome was similar to that of *sin*, and the *FlaD*⁻ phenotype (filamentous, poor competence, and an increase in protease activity) was also similar to that of *Sin*⁻ cells (Akamatsu & Sekiguchi, 1983, 1987b; Gaur *et al.*, 1986). These results indicate that the *flaD* locus corresponds to the *sin* locus. Since there are five ORFs on the fragment (Fig. 1; Gaur *et al.*, 1986), we subcloned the *Xba*I–*Hind*III, *Xba*I–*Bal*I, *Sau*3A1–*Dde*I, *Dde*I–*Hind*III, *Xba*I–*Dde*I and *Sau*3A1–*Hind*III fragments into the corresponding sites of pAC32R, the cloned plasmids being designated as pFLAD0, pFLAD1, pFLAD2, pFLAD3, pFLAD12 and pFLAD23, respectively (Fig. 1). Fragments containing ORF2, but none of the other ORFs, suppressed the *FlaD*⁻ phenotype of *B. subtilis* AC333. Since the *Sin* function was located in ORF2 (Gaur *et al.*, 1986), the *flaD* gene was identical to the *sin* gene.

Gaur *et al.* (1986) suggested that the *Sin* protein is a DNA-binding protein containing a helix–turn–helix

Fig. 1. Sequencing strategy for the cloned 1.0 kb *B. subtilis* DNA insert in pFLAD, open reading frames and *flaD* suppression. Protoplasts of *B. subtilis* AC333 (*flaD2*) were transformed with various plasmids and *flaD* suppression was measured as to cell shape, motility and autolytic activity: +, rod and motile cells, and a wild-type level of autolytic activity; -, filamentous and non-motile, and a decreased level of autolytic activity. A, *Ava*I; Bl, *Bal*I; D, *Dde*I; N, *Nru*I; Sa, *Sau*3AI; H, *Hind*III; X, *Xba*I. Open arrows indicate the direction of transcription.

motif. It was therefore of interest to determine whether this motif is conserved in a homologous protein from a related organism. Gaur *et al.* (1986) reported that a strain carrying a disruption in the ORF1 (D-ORF1) of *B. subtilis* displayed an unusual aggregation of cells in the form of long chains, together with poor competence and an increase in protease activity, characteristics similar to those strains with a disruption in *sin* (*flaD*). Since long-chain cells often show decreased autolytic activity (Ayusawa *et al.*, 1975; Akamatsu & Sekiguchi, 1987b; Chatterjee *et al.*, 1976), it was thought that the D-ORF1 product might regulate expression of autolysin(s). This prompted us to clone *flaD* and D-ORF1 homologues of *B. licheniformis* and to compare their amino acid sequences with those of *flaD* and D-ORF1.

Cloning of a B. licheniformis homologue of the *flaD* gene of *B. subtilis*

We ligated *Hind*III-digested chromosomal DNA of *B. licheniformis* to the dephosphorylated *Hind*III site of pUC19, and the mixture was used to transform *E. coli* JM109, selecting ampicillin-resistant colonies. Three plates, each with about 500 colonies, were subjected to colony hybridization with pBRG1 as a probe. A hybridization-positive clone, LLYT-1, containing a 5.3 kb plasmid (pLLYT1) was selected. pLLYT1 contained two *Hind*III inserts (2.4 kb and 0.3 kb), as shown

in Fig. 2. A 2.6 kb *Pst*I fragment of pLLYT1 was subcloned into the *Pst*I site of pAC3; the resultant plasmids, pLLA and pLLB, had the *B. licheniformis* DNA fragments inserted in opposite directions (Fig. 2).

Transformation of *B. subtilis* AG5 (*flaD*) with pLLA or pLLB resulted in suppression of the *FlaD*⁻ phenotype (Fig. 3). A 1.5 kb *Eco*RI fragment of pLLYT1 was subcloned into the *Eco*RI site of pAC3 and the resultant plasmid, pEE, also suppressed the *FlaD*⁻ phenotype (Fig. 3). The 1.5 kb inserted fragment was then cut with *Xba*I into two fragments (0.9 kb and 0.6 kb), which were subcloned separately into the *Xba*I and *Eco*RI sites of pUC19 (Fig. 2). The resultant plasmids (pXE and pXHE) did not contain the *ori* locus of *B. subtilis*. Thus the inserted fragments were subcloned into the *Pst*I and *Eco*RI sites of pAC3 (Fig. 2). These fragments had lost the ability to suppress *FlaD*⁻ (Fig. 3), indicating that the *B. licheniformis* homologue (*flaL*) of *flaD* is located around the *Xba*I site.

Nucleotide sequence of the B. licheniformis homologue (*flaL*) of the *flaD* locus

The sequencing strategy is outlined in Fig. 3 and the nucleotide sequence of *flaL* region is shown in Fig. 4. The sizes of the predicted polypeptide products of ORF1 (L-ORF1), ORF2 (*flaL*) and ORF3 of *B. licheniformis* are 58, 111 and 77 amino acids, corresponding to molecular

Fig. 2. Construction of plasmids. Blocks indicate the DNA from the *B. licheniformis* chromosome. Only relevant restriction endonuclease sites are indicated. The numbers around the plasmid circles indicate the distance in kb from the basal point. mc, multiple cloning site region; Ap, ampicillin resistance; Tc, tetracycline resistance; Km, kanamycin resistance. B, BamHI; E, EcoRI; H, HindIII; P, PstI; Sm, SmaI; X, XbaI.

Fig. 3. Sequencing strategy for the cloned 0.9 kb *B. licheniformis* DNA insert in pLLYT1 and *flaD* suppression by various plasmids. Protoplasts of *B. subtilis* AG5 (*flaD*) were used as recipient and *flaD* suppression was measured as described in Fig. 1. The abbreviations are given in the legends to Figs 1 and 2, except for NT (not tested). *Sau3AI* (Sa) sites were determined only for the dotted area.

masses of 6686, 12946 and 9423 Da, respectively; those of D-ORF1, *flaD* and ORF3 of *B. subtilis* are 57, 111 and 35 amino acids, corresponding to molecular masses of 6593, 12972 and 4224 Da, respectively. The *Xba*I site was found to be in *flaL*, whose nucleotide sequence was 89% homologous to that of *flaD*. The nucleotide sequence of L-ORF1 was 58% homologous to that of D-ORF1. However, no homology was found between the ORF3s of the two species. Two presumptive ribosome-binding sites (SD1 and SD2) were found (Fig. 4), SD2 of *flaL* being completely identical to that of *flaD*. ORF3 of *B. licheniformis* contained no recognizable ribosome-binding site (Fig. 4). The relatively uncommon start codon, UUG, found in *flaD* was also observed in *flaL*.

Gaur *et al.* (1988) reported that the *sin* gene is transcribed from a σ^A -dependent promoter (P3) upstream of *flaD* and also from the late-transcriptional promoters (P1 and P2) upstream of D-ORF1 as polycistronic transcripts. The -10 sequence of P3 (TATAAT; consensus sequence, TATAAT) was completely conserved in the *B. licheniformis* sequence (278–283 from the *Hind*III site in Fig. 4), but the -35 sequence was rather different from the consensus sequence [CCGTAA for *B. subtilis*; CAATAA (255–260) for *B. licheniformis*; consensus sequence, TTGACA]. The P2 promoter sequence of *B. subtilis* was not conserved in the *B. licheniformis* sequence. One of the two terminators of the *flaD* and D-ORF1-*flaD* transcripts was found downstream of *flaL* in *B. licheniformis*. A short inverted repeat was observed in the upstream regions of *flaL* and *flaD*.

Strong homology of the amino acid sequences of *flaL* and *flaD* was observed (93% homologous), all the different amino acid residues (eight amino acids) being located in the C-terminal half of the sequence, with four close to the C-terminus itself (Fig. 5). The difference between the nucleotide sequences of *flaL* and *flaD* was mainly at the third position of the codons (20 of the total 36 changes), and to lesser extents at the first, second and more than two positions (7, 0 and 4, respectively). These synonymous changes suggest that the amino acids are essential for function of Sin/FlaD. It was recently reported that the Sin protein is a DNA-binding protein (Gaur *et al.*, 1989). The region (L₁₇SELAEKAGVAK-SYLSSIER₃₆), assumed to be a DNA-binding domain (Gaur *et al.*, 1986), was completely conserved in *flaL* (Fig. 5).

Comparison of the derived amino acid sequences of L-ORF1 and D-ORF1 showed the former to be one amino acid longer than the latter. The homology values (50% for identical amino acids and 64% for conservative substitutions) were not as high as those for *flaL* and *flaD* (Figs 5 and 6). The hydrophilicity profiles of their N-terminal and central regions were considerably different (Fig. 6). These results suggest that only a limited region of the amino acid sequence is required for its activity.

The *sacQ* gene, which is pleiotropic, encodes a 46 amino acid polypeptide (Yang *et al.*, 1986). The *sacQ*(Hy) mutation increases the activities of levansucrase, neutral protease, alkaline protease, α -amylase, xylanase, intracellular serine protease and β -glucanase(s) (Henner *et al.*, 1988). Henner *et al.* (1988)

HindIII
AAGCTTGGTAAAGTGTGAGTCTCGGGTTTATCTTTGTTTATCACAAACACGGTTGTCTG 60

SD1 M N K D K N E K E
AAATACATAATGAAGTATTTTGGGGGGGAAATGTATGAATAAAGATAAAAATGAGAAAGA 120

E L D E E W T E L I K H A L E Q G I S P
AGAATTGGATGAGGAGTGGACAGAACTGATTAACACGCTCTTGAACAAGGCATTAGTCC 180

D D I R I F L N L G K K S S K P S A S I
AGACGATATACGTATTTTCTCAATTTGGGTAAGAAGTCTTCAAAACCTTCCGCATCAAT 240

E R S H S I N P F *** SD2
TGAAAGAAGTCATTCAATAAATCCTTTCTGAATGTGCTATAATATCGAAAGGAAGGTGAT 300

M I G Q R I K Q Y R K E K G Y S L S E
GACATTGATTGGCCAGCGTATTAAACAATATCGAAAAGAAAAGGCTACTCACTATCTGA 360

L A E K A G V A K S Y L S S I E R N L Q
ACTAGCTGAAAAGGCTGGGGTAGCGAAGTCTTATTTAAGTTCAATAGAAAAGAACTTGCA 420

XbaI
T N P S I Q F L E K V S A V L D V S V H
AACAAACCCCTCCATTCAATTTCTAGAAAAAGTCTCCGCTGTTCTGGACGTCTCGGTTCA 480

T L L N E K D E T E Y D G Q L D S E W E
TACCCTGCTCAATGAGAAAGATGAAACCGAATACGATGGTCAATTAGATAGTGAATGGGA 540

N L V R D A M A S G V S K K Q F R E F L
AAATCTAGTTCGTGACGCTATGGCATCAGGGGTTTCTAAAAACAATTCGAGAATTTTT 600

D Y Q K W K K R Q E K E ***
AGATTATCAAAAGTGGAAGAAAAGACAGGAAAAGGAGTAAGGGAACATATCTTACTCCC 660

M N I K K R I I
TTTGGCATTTTTACGCTGAATAAGCCGATTCTCATGAACATAAAAAACGGATCATAC 720

Sau3AI
Q I R F L N C T I I Y L H C A L F R S L
AGATCCGTTTTTTAAATTGCACCATTTATTTCTTCACTGTGCGCTCTTTCGTTCTCTTT 780

L H N L F H R H V F C R R L S S R S I E
TACATAACCTTTTTCATCGGCATGTTTTTGGCGTCGATTGTCAAGCCGTTCCATTGAGT 840

Y L R M R S Q A A Q N L L D I C S V P F
ACCTTCGAATGAGAAGTCAAGCTGCACAGAATCTCCTTGATATTTGTTCTGTACCATTCT 900

C R W R L L F C H ***
GCCGCTGGCGTCTGTTGTTTTGTCATTGACGAACCTCAATCACCATTGAACCTTGTCAAA 960

Sau3AI
GTCATATGGATC

Fig. 4. Nucleotide sequence of the 0.95 kb *HindIII*–*Sau3AI* fragment in pLLYT1. The deduced amino acid sequence is given above the nucleotide sequence. Putative Shine–Dalgarno (SD) and inverted repeat (arrows) sequences are also indicated above the nucleotide sequence. *** corresponds to a stop codon.

suggested some similarity between the *sacQ* protein and the D-ORF1 gene product. In the specified region (42 amino acid residues), there were 11 identical residues and nine conservative substitutions. However, in the case of *sacQ* and L-ORF1, only six residues were identical and there was only one conservative substitution. Therefore the sequence similarity of *sacQ* and D-ORF1 may have no meaning.

The widely used *FlaD*[−] mutants are *lyt-1*, *lyt-2* and *rgn* mutants. Complementation analysis of their mutations with a plasmid containing only the ORF2 (*flaD*) from *B. subtilis* 168W indicated that their lesions are located in the *flaD* gene itself. Recently, sequencing of the *flaD* region, containing D-ORF1 and *flaD*, of two *FlaD*[−] mutants, supported the above result (the sequence data will be published elsewhere). On the other hand, only

Fig. 6. Comparison of the deduced amino acid sequences, hydrophilicity and secondary structures of the L-ORF1 protein (a) and D-ORF1 protein (b). Underlines indicate the different amino acids in the case of the best alignment of L-ORF1 and D-ORF1 (there is assumed to be a space after the 46 amino acids from the N-terminus of D-ORF1). For the protein secondary structure and hydrophilicity, see the legend to Fig. 5.

Fig. 5. Comparison of the deduced amino acid sequences, hydrophilicity and secondary structures of the *flaL* protein (a) and *flaD* (*sin*) protein (b). Lines below the amino acid sequences indicate the amino acids that differ between the two sequences. The DNASIS program (Hitachi-SK) was used, the analysis being based on the papers of Kyte & Doolittle (1982), and Rose & Roy (1980) for the hydrophilicity profile, and of Chou & Fasman (1978) and Rose (1978) for the protein secondary structure. Hydrophilicity is presented as the running average for six amino acids.

limited research has been done on a strain carrying a disruption in D-ORF1, and the role of D-ORF1 is still obscure. Thus our research is directed toward the isolation of D-ORF1 mutants and the characterization of D-ORF1.

The identification of the *flaD* protein as the *sin* protein should help to clarify the pleiotropic effects of the *flaD* mutation. Some properties believed to be the effects of autolysin(s) may in fact not be related to the activity of autolysin(s).

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