Analysis of the flagellin (*hag*) gene of alkalophilic *Bacillus* sp. C-125

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Motility of the alkalophilic *Bacillus* sp. C-125, a flagellate bacterium, was demonstrated to be Na⁺- and pH-dependent. Flagellin protein from this strain was purified to homogeneity and the N-terminal sequence determined. Using the *hag* gene of *Bacillus subtilis* as a probe, the *hag* gene of *Bacillus* sp. C-125 was identified and cloned into *Escherichia coli*. Sequencing of this *hag* gene revealed that it encodes a protein of 272 amino acids (*M_r* 29995). The predicted N terminal sequence of this protein was identical to that determined by N-terminal sequencing of the flagellin protein from strain C-125. The alkalophilic *Bacillus* sp. C-125 flagellin shares homology with other known flagellins in both the N- and C-terminal regions. The middle portion, however, shows considerable differences, even from that of flagellin from the related species, *B. subtilis*.

Introduction

The flagellar apparatus has been extensively studied in neutrophilic bacteria such as *Escherichia coli* and *Bacillus subtilis* and has been shown to be a complex structure composed of a basal body, a hook and a filament. The filament accounts for 98% of the mass of the organelle and is composed exclusively of flagellin, the product of the *hag* gene (Iino, 1977; Silverman & Simon, 1977).

Flagellin plays only a passive role in motility, the mechanical energy for flagellar rotation being generated by a motor in, or associated with, the basal body structure of the organelle (Ridgeway *et al.*, 1977; Dean *et al.*, 1984; Blair & Berg, 1990). Nevertheless, flagellin has proved a focus of interest due to its self-assembling properties (Asakura *et al.*, 1964; Macnab & Aizawa, 1984), its method of transport across the cell membrane (Homma *et al.*, 1987; LaVallie & Stahl, 1989) and other features such as its antigenicity.

The primary amino acid sequences of flagellins from a number of neutrophilic bacteria have been determined (Gill & Agabian, 1983; Joys, 1985; Wei & Joys, 1985; Kuwajima *et al.*, 1986; Martin & Savage, 1988; LaVallie

& Stahl, 1989; Mirel & Chamberlin, 1989). The flagellin of alkalophilic bacteria, being exposed to the alkaline environment in which these organisms live, is likely to show special adaptations to alkaline conditions. In addition, flagellin, unlike other extracellular proteins so far studied, as a polymer is a good model for the study of subunit–subunit interactions under alkaline conditions. However, hitherto, there have been no reports on the flagellin gene of alkalophiles.

We have selected the alkalophilic *Bacillus* sp. C-125 as a model alkalophile for molecular biological study for the following reasons: (i) it grows well on minimal medium, (ii) genes from this strain are expressed well in *E. coli*, and (iii) an efficient system for plasmid transformation of this strain is now available (Kudo *et al.*, 1990). In this paper, we report the cloning and sequencing of the *hag* gene from the alkalophilic *Bacillus* sp. C-125.

Methods

Bacterial strains and media. Alkalophilic *Bacillus* sp. C-125 10596 (Trp[−] Ura[−] Cm^s) was isolated in our laboratory (Honda *et al.*, 1985; Kudo *et al.*, 1990). It was grown on Horikoshi-II medium (pH 10.3) containing 1% (w/v) soluble starch, 0.5% yeast extract, 0.5% polypeptone, 0.1% K₂HPO₄, 0.02% MgSO₄·7H₂O, and 1% Na₂CO₃ (sterilized separately). The *Bacillus* DNA was cloned in *E. coli* MV1184 (*ara* Δ(*lac-proAB*) *rpsL* *thi* (φ80 *lacZ* Δ*M15*) Δ(*srl-recA*)306::Tn10 (Tet^r) F'[*traD36 proAB lacI^a lacZ* Δ*M15*]). *E. coli* was grown on 2 × yeast/tryptone medium (Sambrook *et al.*, 1989).

Plasmids, enzymes and chemicals. pUC vectors, restriction endonucleases and T₄ DNA ligase were purchased from Takara Shuzo Co. The DIG (digoxigenin) DNA labelling and detection kit was obtained from

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The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number D10063.

Boehringer Mannheim. Hybond N⁺ and Hybond N membranes were purchased from Amersham. Sequencing oligonucleotide primers were synthesized on an Applied Biosystems 380B DNA synthesizer. HindIII-digested λ HaeIII-digested ϕ X174 DNA standards (23130, 9416, 6557, 4361, 2322, 2027, 1353, 1078, 872 and 603 bp) were purchased from Toyobo. Protein standards, purchased from Pharmacia, consisted of α -lactalbumin (14400), soybean trypsin inhibitor (20100), carbonic anhydrase (30000), ovalbumin (43000), bovine serum albumin (67000) and phosphorylase *b* (94400).

Electron microscopy. Flagellation of alkalophilic *Bacillus* sp. C-125 was observed by transmission electron microscopy with a JEOL model 200CX microscope at 100 kV after negative staining with 1% (w/v) phosphotungstic acid.

Physical characterization of motility. Swimming speeds were measured using a television monitor attached to a microscope. Alkalophilic *Bacillus* sp. C-125 was grown aerobically to late exponential phase at 37 °C in Horikoshi-II medium (pH 10.3), collected and washed twice. Cells were resuspended in a 25 mM-Tris/HCl (pH 9.0), 5 mM-glucose solution containing 100 mM-NaCl unless indicated otherwise.

Purification of flagellin protein. Flagellin protein of alkalophilic *Bacillus* sp. C-125 was purified to homogeneity by the method of Martinez (1963). SDS-PAGE was carried out as described by Laemmli (1970).

N-terminal sequence of the purified flagellin. The method of Edman & Henschen (1975) was used, with an Applied Biosystems 477A protein sequencer coupled to a PTH120A analyser.

Amino acid analysis. This was done on an Applied Biosystems PTH120A analyser.

Preparation of genomic DNA. The method of Saito & Miura (1963) was used.

Preparation of probe DNA. The *B. subtilis* *hag* gene used as probe was constructed by PCR using the oligonucleotides 5' ACGTGCCTTAA-CAACATATT 3' and 5' ATGAGGAATGATTAGGAGAT 3' as primers to regions upstream and downstream of the *B. subtilis* *hag* gene, respectively (LaVallie & Stahl, 1989; Mirel & Chamberlin, 1989).

Southern blot analysis. Genomic DNA (10 μ g) of alkalophilic *Bacillus* sp. C-125 was digested with restriction endonucleases, separated on a 1% (w/v) agarose gel and then blotted using a vacuum blotter (Pharmacia LKB) on to Hybond N⁺. Blots were probed with digoxigenin-labelled *B. subtilis* *hag* gene in a 20% (v/v) formamide hybridization solution at 42 °C. Subsequent washing and detection steps were carried out according to instructions provided with the Boehringer Mannheim DIG-detection kit.

Constructing and screening a sub-genomic library. Genomic DNA (100 μ g) of alkalophilic *Bacillus* sp. C-125 was digested with the appropriate restriction endonuclease and separated by electrophoresis on a 1% (w/v) agarose gel. DNA of the desired fragment size was recovered from the agarose by electroelution, treated with phenol/chloroform and then precipitated with ethanol. The size-selected DNA was cloned into pUC vector in *E. coli* MV1184. Colony blots were prepared according to instructions provided with the Hybond N membrane and hybridized with digoxigenin-labelled *B. subtilis* *hag* gene as described above for Southern blot analysis.

DNA sequencing. This was done by the technique of Sanger *et al.* (1977) on a Dupont DNA Sequencer using single-stranded pUC as template and a Dupont sequencing kit.

Sequence alignment. Flagellin sequences were aligned using the GENETYX amino acid sequence homology program (Tokyo, Japan).

Results and Discussion

Characterization of the motility of alkalophilic *Bacillus* sp. C-125

Alkalophilic *Bacillus* sp. C-125 is a flagellate bacterium (Fig. 1). Whereas in neutrophilic bacteria flagellar rotation is powered by H⁺-driven motors, in alkalophiles, apparently because the protonmotive force is too low, rotation is powered by Na⁺-driven motors (Hirota *et al.*, 1981). The effect of Na⁺ concentration on motility of alkalophilic *Bacillus* sp. C-125 cells is shown in Fig. 2. Maximum motility was observed at 50 mM-NaCl. When other cations (in the form of KCl, LiCl, NH₄Cl, RbCl, CsCl, CaCl₂ and MgCl₂) were used in place of Na⁺ no motility was observed. In addition, amiloride, a specific inhibitor of Na⁺-driven flagellar motors (Sugiyama *et al.*, 1988) inhibited movement of alkalophilic *Bacillus* sp. C-125 cells at concentrations greater than 1 mM. We conclude, therefore, that as in other alkalophiles, the flagellar motor of alkalophilic *Bacillus* sp. C-125 is driven by Na⁺.

pH also affected the motility of alkalophilic *Bacillus* sp. C-125 cells, with no motility being observed at pH values less than 7.5. Microscopic observation of cells transferred from alkaline to neutral pH revealed that cells at the new lower pH were no longer flagellate (data not shown). This loss of flagella presumably accounts for the loss of motility at pH values below 7.5. Kamiya & Asakura (1976) showed that *Salmonella* flagella displayed a remarkable reversible polymorphism when transferred from neutral pH to pH 4.7 and lower. It is possible, therefore, that the loss of flagella by alkalophi-

Fig. 1. Electron micrograph of an alkalophilic *Bacillus* sp. C-125 cell. Bar, 1 μ m.

Fig. 2. Effect of Na^+ concentration on swimming speeds of alkalophilic *Bacillus* sp. C-125 cells. Data shown are mean values of the swimming speeds of more than 28 cells for each concentration.

lic *Bacillus* sp. C-125 at pH values below 7.5 is caused by a polymorphic transition of the flagellin filament which results in the flagella becoming unstable. As yet we have no data to support this speculation; however, it may be possible to test it by observing whether alkalophilic *Bacillus* sp. C-125 filaments reassociate around seeds of polymerized flagella when the pH is raised from neutral pH back to alkaline pH.

The flagellin protein of alkalophilic *Bacillus* sp. C-125

Flagellin purified from alkalophilic *Bacillus* sp. C-125 was pure as judged from the single band obtained on SDS-PAGE (Fig. 3). By comparison with standard proteins the polypeptide M_r of the alkalophilic *Bacillus* sp. C-125 flagellin is 31 000.

The N-terminal sequence of the alkalophilic *Bacillus* sp. C-125 flagellin was determined to be as follows: Met-Ile-Ile-Asn-His-Asn-Leu-Pro-Ala-Met.

The amino acid content of the alkalophilic *Bacillus* sp. C-125 flagellin, as determined by amino acid analysis of the protein, is shown in column 2 of Table 1.

The hag gene of alkalophilic *Bacillus* sp. C-125

Fig. 4 shows the result of Southern blot analysis of *Bacillus* sp. C-125 genomic DNA cut with several endonucleases and probed with the *B. subtilis* hag gene. From this result it was apparent that the alkalophilic *Bacillus* sp. C-125 hag gene is not carried within a single *Eco*RI or *Hind*III fragment. The 1.5 kb *Hind*III fragment and the 1.2 kb *Eco*RI fragment (Fig. 4) were cloned separately, and subsequently joined at the common *Eco*RI site. The plasmid containing the complete alkalophilic *Bacillus* sp. C-125 hag gene was designated pC125Hg.

Fig. 3. SDS-PAGE of alkalophilic *Bacillus* sp. C-125 flagellin. The left lane contains flagellin purified from alkalophilic *Bacillus* sp. C-125 cells by the method of Martinez (1963) and the right lane contains standard protein size markers (see Methods).

Fig. 4. Southern blot analysis of alkalophilic *Bacillus* sp. C-125 genomic DNA. Alkalophilic *Bacillus* sp. C-125 genomic DNA was digested with *Bam*HI (lane 3), *Eco*RI (lane 4) and *Hind*III (lane 5). Lanes 1 and 2 contain $\lambda/\phi\text{X174}$ marker DNA (see Methods) and the *B. subtilis* hag gene PCR product, respectively. The resulting Southern blot was probed with digoxigenin-labelled *B. subtilis* hag gene and digoxigenin-labelled $\lambda/\phi\text{X174}$ marker DNA. The 1.5 kb *Hind*III and 1.2 kb *Eco*RI fragments that were cloned are arrowed.

Sequencing of the inserted DNA fragment on pC125Hg resulted in the identification of an open reading frame containing the 10 amino acids determined by N-terminal sequencing of the protein (see Fig. 6). This open reading frame is immediately preceded by an ATG codon which we propose acts as the translation initiation

codon for the *hag* gene. The N-terminal sequence deduced from the gene sequence is identical to that of the N-terminal sequence determined from the purified flagellin. This indicates that, as observed for other sequenced flagellins, the export of the protein is not directed by an N-terminal signal peptide (Gill & Agabian, 1983; Kuwajima *et al.*, 1986; LaVallie & Stahl, 1989).

The open reading frame was sequenced on both DNA strands, using the strategy depicted in Fig. 5. The complete DNA sequence of the alkalophilic *Bacillus* sp. C-125 *hag* gene and its immediate 5'- and 3'-flanking regions is presented in Fig. 6. Putative promoter, ribosome-binding site and transcription-termination sequences have been identified (Fig. 6). The promoter sequence resembles that of the *B. subtilis* *hag* gene, which is thought to be used by the *B. subtilis* sigma-28 RNA polymerase (LaVallie & Stahl, 1989; Mirel & Chamberlin, 1989).

The alkalophilic *Bacillus* sp. C-125 *hag* gene encodes a protein of 272 amino acids (M_r 29995). This corresponds to the size determined for the flagellin purified from this strain (M_r 31000). This is one of the smallest amongst flagellins whose sizes have been determined: *Campylobacter* flagellins range from M_r 59500 to 63800 (Logan *et al.*, 1987); and the *E. coli* (Kuwajima *et al.*, 1986), the *Salmonella typhimurium* (SJW1103) (Joys, 1985), the *B. subtilis* (LaVallie & Stahl, 1989; Mirel & Chamberlin, 1989), the *Roseburia cecicola* (Martin & Savage, 1988) and one of the *Caulobacter crescentus* (Gill & Agabian, 1983) flagellin subunits have M_r values of 51172, 51000, 32613, 31370 and 29000, respectively. Trachtenberg & DeRosier (1988) demonstrated that the *C. crescentus* (M_r 29000) flagellin lacks the central stretch of primary amino acids of the *S. typhimurium* flagellin and that this central region corresponds to a molecular domain situated on the outer surface of the *S. typhimurium* flagellar filament. It seems, therefore, that the smallest flagellins lack all or part of an outer molecular domain present in the higher- M_r flagellins.

The amino acid composition of the alkalophilic *Bacillus* sp. C-125 flagellin, as determined from the sequence, is shown in Table 1, column 3. It compares favourably with the composition determined by direct amino acid analysis of the purified protein (column 2). For comparison, the amino acid compositions of *B. subtilis* and *Bacillus firmus* RAB flagellins are also included in Table 1. *B. firmus* RAB flagellin contains few basic amino acids, a feature that has been proposed to make the flagellum more stable at high pH (Guffanti & Eisenstein, 1983). The alkalophilic *Bacillus* sp. C-125 flagellin composition, however, does not appear to be significantly different from the *B. subtilis* composition, the lysine content being only slightly lower.

Fig. 5. Restriction map of the DNA region containing the alkalophilic *Bacillus* sp. C-125 *hag* gene. The 1.5 kb *Hind*III and the 1.2 kb *Eco*RI fragments were cloned separately and subsequently joined at the common *Eco*RI site. The direction and extent of the coding region is indicated by the upper arrow. The lower arrows indicate the direction and extent of the individual sequencing runs.

Table 1. Amino acid compositions of the flagellins of alkalophilic *Bacillus* sp. C-125 and related bacteria

Residue	No. of residues per molecule (% of total in parentheses)			
	C-125*	C-125†	<i>B. firmus</i> RAB‡	<i>B. subtilis</i> §
Gly	16 (5.8)	12 (4.4)	41 (11.7)	19 (6.3)
Thr	14 (5.1)	14 (5.2)	43 (12.3)	18 (5.9)
Ile	16 (5.8)	19 (7.0)	21 (6.0)	22 (7.2)
Met	10 (3.6)	11 (4.0)	3 (0.9)	8 (2.6)
Asp	—	20 (7.4)	—	22 (7.2)
Arg	16 (5.8)	15 (5.5)	8 (2.3)	14 (4.6)
Phe	4 (1.4)	3 (1.1)	2 (0.6)	5 (1.6)
Ala	33 (12.0)	33 (12.1)	84 (24.0)	39 (12.8)
Val	11 (4.0)	11 (4.0)	13 (3.7)	14 (4.6)
Pro	3 (1.1)	2 (0.7)	0 (0)	2 (0.7)
His	5 (1.8)	6 (2.2)	3 (0.9)	4 (1.3)
Glu	—	21 (7.7)	—	17 (5.6)
Asn	—	21 (7.7)	—	27 (8.9)
Trp	0 (0)	0 (0)	0 (0)	0 (0)
Asx	40 (14.5)	—	66 (18.9)	—
Glx	48 (17.4)	—	34 (9.7)	—
Ser	17 (6.2)	17 (6.3)	0 (0)	24 (7.9)
Leu	30 (10.9)	30 (11.0)	23 (6.5)	29 (9.5)
Cys	0 (0)	0 (0)	0 (0)	0 (0)
Tyr	2 (0.7)	1 (0.4)	2 (0.6)	1 (0.3)
Gln	—	27 (9.9)	—	24 (7.9)
Lys	11 (4.0)	9 (3.3)	7 (2.0)	15 (4.9)

* Obtained by direct amino acid analysis of the purified flagellin.

† Deduced from the *hag* gene sequence.

‡ Cited from Guffanti & Eisenstein (1983).

§ Derived from data reported by Mirel & Chamberlin (1989).

Alignment with other known flagellin sequences

The amino acid sequence of the alkalophilic *Bacillus* sp. C-125 flagellin was compared to the sequences of *E. coli* (Kuwajima *et al.*, 1986), *S. typhimurium* (Joys, 1985), *B.*

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T TAA AAA AGG ACT AAA CTC CTA TGA AAT CGT GTC GAT ATT ATT AAT GTA CCG GAA 55

AAG GAA AAG GCG GCC GAC TTT GTT CCT TTT CGC GGA TTA AGT TTA CAC CAA CCA CAA GGA 115

TGT GGG CGG AAA ACG CAT TTC ... ... . Met Ile Ile Asn His Asn Leu Pro
AAG CTT TCT TCA GGT CTT CGC ATT AAC CGT GCA GGA GAC GAT GCT GCA GGT CTT GCC ATC 175
HindIII PstI

Ala Met Asn Ala His Arg Asn Met Gly Ile Asn Leu Asn Gln Gly Gln Lys Ala Met Glu
GCA ATG AAT GCG CAT CGT AAC ATG GGT ATC AAT CTT AAC CAA GGT CAA AAA GCG ATG GAG 235

Lys Leu Ser Ser Gly Leu Arg Ile Asn Arg Ala Gly Asp Asp Ala Ala Gly Leu Ala Ile
AAG CTT TCT TCA GGT CTT CGC ATT AAC CGT GCA GGA GAC GAT GCT GCA GGT CTT GCC ATC 295
HindIII PstI

Ser Glu Lys Met Arg Ala Gln Ile Arg Gly Leu Asp Gln Ala Ser Arg Asn Ser Gln Asp
TCT GAA AAA ATG CGT GCG CAA ATC CGT GGT TTG GAT CAA GCG TCT CGT AAC TCA CAA GAC 355

Gly Ile Ser Leu Ile Gln Thr Ala Glu Gly Ala Leu Asp Glu Val His Ser Ile Leu Gln
GGT ATT TCG TTA ATT CAA ACA GCT GAA GGT GCG CTT GAT GAA GTA CAT TCT ATT CTT CAA 415

Arg Met Arg Glu Leu Ala Val Gln Ser Ser Asn Glu Thr Asn Val Glu Gln Asp Gln Ala
CGT ATG CGT GAG CTA GCG GTT CAA TCT TCG AAC GAA ACG AAT GTT GAG CAA GAT CAA GCA 475

Ala Leu Asn Asp Glu Phe Gln Gln Leu Val Glu Glu Ile Glu Arg Ile Lys Asp Thr Thr
GCT CTT AAC GAT GAA TTC CAA CAA TTA GTT GAG GAA ATT GAA AGA ATC AAA GAT ACA ACT 535
EcoRI

Gln Phe Asn Thr Gln Lys Leu Leu Asp Asp Thr Val Asp Thr Val Gln Leu Gln Val Gly
CAA TTT AAT ACG CAA AAA TTA CTC GAT GAT ACA GTA GAT ACT GTA CAA CTT CAA GTT GGT 595

Ala Asn Ser Gly Glu Leu Ile Glu Leu Asp Leu Thr Lys Val Asp Leu Ser Ala Ile His
GCT AAT TCT GGT GAA TTA ATT GAA CTT GAT TTA ACA AAA GTT GAT TTA TCA GCT ATC CAT 655

Thr Ala Leu Ala Ala Glu Asp Ile Thr Asp His Thr Asn Ala Gln Ser Ala Ile Asp Ala
ACA GCT TTG GCG GCT GAG GAT ATT ACT GAC CAC ACT AAT GCA CAA TCA GCT ATT GAC GCT 715

Ile Asp Glu Gln Leu Lys Ala Val Ser Glu Gly Arg Ser Tyr Leu Gly Ala Met Gln Asn
ATT GAT GAG CAA TTA AAA GCT GTT TCA GAA GGT CGC TCT TAC CTA GGA GCT ATG CAA AAC 775

Arg Leu Glu His Thr Ile Lys Asn Leu Asp Asn Ala Ser Glu Asn Leu Gln Ala Ala Glu
CGC CTA GAG CAT ACA ATC AAA AAC CTT GAT AAT GCT TCT GAA AAC CTT CAA GCT GCT GAG 835

Ser Arg Ile Arg Asp Val Asp Met Ala Lys Glu Met Met Glu Phe Thr Arg Thr Asn Ile
TCT CGT ATC CGT GAC GTA GAC ATG GCG AAA GAA ATG ATG GAG TTC ACA AGA ACA AAC ATC 895
AccI

Leu Asn Gln Ala Ser Gln Ala Met Leu Ala Gln Ala Asn Gln Gln Pro Gln Ala Val Leu
TTA AAC CAA GCG TCT CAA GCG ATG CTT GCT CAA GCA AAC CAA CAG CCA CAA GCT GTA TTA 955

Gln Leu Leu Arg TER
CAA TTA CTT CGT TAA TTT GCT TCC ATT TAA AGA TCT GGA TTT ATT CCA GGT CTT TTT TAT 1015

TTT CCG CTC AAC CGT TAC TTT GCT GAT AGG TTG TTA AAG TTT AGG AAT 1062

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Fig. 6. Nucleotide sequence of the alkalophilic *Bacillus* sp. C-125 *hag* gene and corresponding amino acid sequence. The nucleotides underlined represent the putative promoter regions. The nucleotides overlaid with dots represent a potential ribosome-binding site. The nucleotides overlined may form a hair-pin loop structure which results in termination of transcription.

subtilis (LaVallie & Stahl, 1989; Mirel & Chamberlin, 1989) and *R. cecicola* (Martin & Savage, 1988) flagellins. The alignment with the *B. subtilis* flagellin sequence is shown in Fig. 7. Whereas the N-terminal region (amino acids 1–96) and C-terminal region (amino acids 200–272)

show 74 and 82% identity, respectively, to the comparable regions in the *B. subtilis* flagellin, the central region (amino acids 97–199) shows only 32% identity. Such a pattern of similarity in N- and C-terminal regions and dissimilarity in the central regions has been observed for

Fig. 7. Alignment of the alkalophilic *Bacillus* sp. C-125 (lower) and the *B. subtilis* (upper) flagellin amino acid sequences. Identical amino acids and homologous amino acids are highlighted by asterisks and dots, respectively. Amino acids conserved in neutrophilic flagellins but altered in the alkalophilic flagellin are indicated by the vertical arrows. Domains 1, 2 and 3 [as proposed by Namba *et al.* (1989) for the *S. typhimurium* flagellin] are shown.

It has been proposed that the conserved regions of flagellin play an important structural role in the flagellar filament. Homma *et al.* (1987) proposed a hairpin loop structure for the *S. typhimurium* flagellin in which both the N- and the C-terminus point into the interior of the filament. This model is supported by the findings of Namba *et al.* (1989), who have assigned the primary amino acid sequence of *S. typhimurium* flagellin to three domains identified from electron-density maps. Domain 1, the innermost domain, and domain 2, the middle domain, appear to be made up of amino acids from the N- and C-terminal regions, whereas domain 3, situated on the filament surface, contains the highly variable, central sequence of amino acids.

The central, variable region of the flagellin peptide has not been implicated with any structural or functional properties of the protein. In the structures proposed by Homma *et al.* (1987) and Namba *et al.* (1989) it is the central portion that forms the outer surface of the flagellin filament, and thus, this portion is exposed to the outer environment of the cell. For *Bacillus* sp. C-125 the environment is alkaline and so the central portion of the

flagellin, on the outer surface of the flagellar filament, is likely to require special adaptations to these alkaline conditions. This may explain the very low level of homology observed between the central regions of the alkalophilic *Bacillus* sp. C-125 and *B. subtilis* flagellins.

Decreased amounts of acidic and/or basic amino acids have been observed in some alkalophilic proteins (Guffanti & Eisenstein, 1983; Van der Laan *et al.*, 1991). The overall amino acid content of the alkalophilic *Bacillus* sp. C-125 does not appear to be significantly different from that of the *B. subtilis* flagellin (Table 1). However, the alkalophilic *Bacillus* sp. C-125 domain 3 does appear to have fewer charged amino acids than the equivalent region in the *B. subtilis* flagellin (Fig. 7). Thus, it seems that, if we confine the comparison to regions that are presumed to be exposed to alkaline conditions, the low content of basic or acidic amino acids observed in other alkalophilic proteins (Guffanti & Eisenstein, 1983; Van der Laan *et al.*, 1991) can also be observed in the alkalophilic *Bacillus* sp. C-125 flagellin.

Due to the high level of variation among the flagellin central regions it is difficult to pinpoint and investigate possible changes in the alkalophilic *Bacillus* sp. C-125 flagellin that may contribute to its adaptation to alkaline conditions. However, the conserved N- and C-terminal regions (domains 1 and 2) are also likely to show adaptations to alkalinity since these, although not situated on the outer surface of the flagellum, may, for example, be exposed during the assembly process. Moreover, Kanto *et al.* (1991) have shown that amino acids responsible for flagellin shape are located in the terminal regions of the *S. typhimurium* flagellin. A comparison of the N- and C-terminal regions revealed that only two amino acids, an Ala (Gln 22 of *Bacillus* sp. C-125 flagellin, domain 1) and a Val (Met 206 of *Bacillus* sp. C-125 flagellin, domain 2) (Fig. 7), conserved in all four neutrophilic bacterial flagellins are different in the alkalophilic *Bacillus* sp. C-125 flagellin. Site-directed mutagenesis work is currently in progress to investigate whether these changes contribute to the alkaline adaptation of alkalophilic *Bacillus* sp. C-125 flagellin.

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