Purification and Characterization of Flagella from the Alkalophile *Bacillus firmus* RAB

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(Received 1 February 1983; revised 22 April 1983)

Flagella from *Bacillus firmus* RAB, an alkalophilic bacterium, were purified to homogeneity. The flagella were shown to consist of a single protein subunit (flagellin) with an apparent molecular weight of 40000. The amino acid composition of *B. firmus* RAB flagellin was similar to that of other bacilli except that the former had far fewer basic amino acids. The paucity of basic amino acids may render the flagella more stable at external pH values as high as 11.0.

INTRODUCTION

The mechanism of cytoplasmic pH homeostasis (Guffanti *et al.*, 1980; Krulwich *et al.*, 1979; Mandel *et al.*, 1980) and the characterization of intracellular enzymes (Ando *et al.*, 1981; Horikoshi & Akiba, 1982) in bacteria that grow at pH values as high as 11.0 have been extensively explored. Moreover, specific transmembrane carrier-mediated transport systems in these alkalophiles have been well characterized (Guffanti *et al.*, 1978; Kitada & Horikoshi, 1980). Extracellular enzymes of acidophilic thermophiles show both activity and stability at low pH and high temperature (Buonocore *et al.*, 1976), while numerous extracellular enzymes from alkalophiles, such as protease (Kitada & Horikoshi, 1976), polygalacturonate lyase (Kelly & Fogarty, 1978), and amylase (Horikoshi & Akiba, 1982), are very stable and optimally active at high pH values.

Little, if anything, is known about the structure or function of alkalophilic cell components directly exposed to the extreme external environment. Flagella represent an excellent model system in which to explore the effect of the external milieu upon structure and function. Thus, the molecular characterization of flagella isolated from an extreme alkalophile, *Bacillus firmus* RAB, was undertaken. Evidence is presented that the amino acid composition of flagellin purified from *B. firmus* RAB is quite similar to that of other bacilli, with one striking dissimilarity – the alkalophilic flagellin has a much lower content of basic amino acids.

METHODS

Growth. *Bacillus firmus* RAB was grown with shaking on L-malate medium at pH 10.5 (Guffanti *et al.*, 1978), on a New Brunswick G25 rotary shaker.

Purification of flagella. Flagella were purified by following the techniques outlined by Smith & Koffler (1971*a*). Highly motile cultures in the late-exponential phase of growth were harvested by centrifugation at 12000 *g* for 10 min. The pellets were suspended in distilled H₂O (30 g wet wt l⁻¹) and shaken for 10 min with a New Brunswick W-8 twist-action shaker at about 500 strokes min⁻¹. Intact cells were spun down at 6000 *g* for 30 min and the resulting supernatant was centrifuged at 16000 *g* for 20 min. Flagella were then pelleted by centrifugation at 40000 *g* for 3 h. The flagella were further separated from other cell fragments by centrifugation in a Beckman SW-50.1 rotor at 12500 *g* for 30 min and then at 78000 *g* for 90 min. The clear, gelatinous flagella in the upper part of the pellet were carefully removed and suspended in distilled water. The process of separation of the flagella from other cell fractions by differential centrifugation was repeated until pellets appeared completely clear. The flagellar protein (flagellin) was further purified by acidification with HCl to a final concentration of 1 mM. After 1 h at room temperature, the insoluble residue was removed by centrifugation at 104000 *g* for 1 h. The supernatant

Fig. 1. Flagella isolated from *Bacillus firmus* RAB by mechanical agitation. The flagella were stained negatively with 1% (w/v) phosphotungstic acid. The bar marker represents 0.5 μ m.

was precipitated with 10% saturated $(\text{NH}_4)_2\text{SO}_4$ and then with 50% saturated $(\text{NH}_4)_2\text{SO}_4$ as described by Koffler & Kobayashi (1957). The 50% saturation precipitate was centrifuged at 30000 g for 30 min then resuspended in and dialysed against distilled water.

PAGE. Non-denaturing PAGE was run on 7.5% (w/v) polyacrylamide disc gels in an LKB vertical electrophoresis unit according to the procedure of Laemmli (1970). The SDS-PAGE used 10% (w/v) polyacrylamide gradient gels. Flagellin samples were suspended in a solution containing 1% (w/v) SDS, 10% (v/v) glycerol, 5% (w/v) mercaptoethanol, 0.005% bromophenol blue, and 25 mM-Tris/HCl, pH 6.8. The samples were heated for 2 min in a boiling water bath, cooled to room temperature, and applied to the gel. The proteins were stained with 0.25% Coomassie blue in acetic acid/methanol/water (2:9:9, by vol.) for 15 min, and destained by diffusion with 10% (v/v) methanol plus 7.5% (v/v) acetic acid. Protein was determined by the Lowry method using lysozyme as the standard.

Amino acid analysis. Purified flagellin was hydrolysed in 6M-HCl and 0.2% (v/v) phenol for 24 h at 110 °C. Analysis was performed with a Beckman Model 119CL analyser.

Electron microscopy. Flagella were absorbed to Formvar-coated grids and negatively stained with 1% (w/v) phosphotungstic acid, pH 7.0, as described by Lagenaur & Agabian (1976). The grids were examined with a JEOL 100 electron microscope.

RESULTS AND DISCUSSION

Mechanical agitation of *B. firmus* RAB resulted in a suspension enriched for flagella (Fig. 1). After differential centrifugation of the suspended flagella, acidification and ammonium sulphate precipitation, only a single protein band was found in non-denaturing gels (Fig. 2). SDS-polyacrylamide gels showed one major protein band and traces of two other bands (Fig. 3). The flagellar subunit, henceforth referred to as *B. firmus* RAB flagellin, had an apparent molecular weight of 40000, similar to the flagellins of other bacteria. The molecular weights of flagellins from other bacilli range from 30000 to 50000 (DeLange *et al.*, 1976; Smith & Koffler, 1971 *b*). Most species of bacteria, like *B. firmus* RAB, appear to have flagella composed of a single subunit (Smith & Koffler, 1971 *b*), although some species may have more than one subunit (Lagenaur & Agabian, 1976; Iino, 1969).

The amino acid composition of *B. firmus* RAB flagellin (Table 1) shared many characteristics with those of other Gram-positive bacteria (Smith & Koffler, 1971 *b*). The content of glycine, alanine, leucine, isoleucine, threonine, aspartic acid and glutamic acid is high in *B. firmus* RAB flagellin and that of other bacilli (Smith & Koffler, 1971 *b*). In addition, little or no cysteine, tyrosine, tryptophan, proline and histidine residues are found in *B. firmus* RAB and related species. In contrast to neutrophilic bacilli, *B. firmus* RAB flagellin was devoid of serine. Most

Fig. 2

Fig. 3

Fig. 2. Non-denaturing polyacrylamide gel of flagella purified from *B. firmus* RAB. (a) 10% saturated ammonium sulphate precipitate; (b) 50% saturated ammonium sulphate precipitate.

Fig. 3. SDS-PAGE of purified *B. firmus* RAB flagellin. Purified flagella were acid treated as described in Methods and electrophoretically separated in 1% SDS gels. (a) Flagellin; (b) protein standards in order of decreasing molecular weight: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme.

Table 1. Amino acid composition of *B. firmus* RAB flagellin

Residue	No. of residues per molecule	Residue	No. of residues per molecule
Gly	41	Thr	43
Ala	84	Ser	0
Val	13	His	3
Leu	23	Lys	7
Ile	21	Arg	8
Met	3	Asp	66
Phe	2	Glu	34
Tyr	2	Pro	0
Trp	0	Cys	0

strikingly, the flagellin of *B. firmus* RAB had far fewer basic residues than related neutrophiles. The ϵ -amino group of lysine (pK 10.53) and the guanidine group of arginine (pK 12.48) would be, at physiological pH values for alkalophiles, very near their pK values. Such a situation, where the amino side groups would vary between the charged and uncharged forms, might conceivably lead to structural instability of the flagellum. The relative paucity of basic amino acids in *B. firmus* RAB flagellin thus leads to an overall charge that is even more negative than in its neutrophilic relatives (DeLange *et al.*, 1976). Interestingly, Horikoshi & Akiba (1982) have pointed out that the cell walls of a *Bacillus* species grown at pH 10 have a far greater content of aspartic, glutamic and uronic acids compared with the same cells grown at pH 7.0. Perhaps the highly negative charges of peripheral structures in *B. firmus* RAB and other alkalophiles represent an adaptation to the alkaline environment, repelling the negatively-charged hydroxyl ions.

This work was supported by research grant PCM 8121557 from the National Science Foundation.

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