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Isolation and Properties of Pili from Spores of *Bacillus cereus*

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Structures whose morphology is identical to that of bacterial pili have been isolated from spores of *Bacillus cereus*. The structures are absent from log-phase and sporulating cells. The pili are 6.8 nm in diameter, are of variable length, and appear to emanate randomly from the exosporium. Examination of spores from 12 *Bacillus* species showed that only those from *B. cereus* and *B. thuringiensis* have pili. Although isolated spore pili were shown to be composed of protein, their subunit nature was not discernible due to the extreme insolubility of the structure. An antiserum to spore pili was labeled with ferritin and used to examine the distribution of pilus antigen on the outer spore surface.

Many genera of bacteria possess filamentous, nonflagellar appendages called pili. Based on morphological, biochmical, and immunological data, a variety of types have been described. Although the dimensions and chemical composition of these structures vary with the organism under investigation, pili in general are composed of a single-protein monomer called pilin, which when assembled gives rise to a uniform tubular structure 3 to 25 nm wide by 0.2 to 20 μm long (23).

Numerous studies have documented the occurrence of pili on the surface of gram-negative cells, most notably on members of *Enterobacteriaceae* (1, 3) and the genera *Neisseria* (25) and *Caulobacter* (18). In comparison, few gram-positive organisms have been reported to possess pili. The pili of *Corynebacterium renale* (31) have been well studied, and more recently the cells of *Streptococcus sanguis*, *S. salivarius* (10; S. Fachon, and P. Fives-Taylor, Abstr. Annu. Meet. Am. Soc. Microbiol., 1978, J2, p. 77) and *Actinomyces naeslundii* (5) have been reported as bearing structures that resemble pili.

Hodgkiss (13) and Hachisuka and Kuno (9) showed that the spores and not the vegetative cells of 16 strains of *Bacillus cereus* possessed pili. These structures differ markedly from the appendages that have been observed on the spores of a variety of clostridia (24, 26), which due to their large size and lack of a tubular morphology have generally not been classified as pili (23). Because pili are found only infrequently among gram-positive organisms, we felt that the *Bacillus* spore pili were worthy of further study and report herein the occurrence, isolation, and preliminary characterization of pili from spores of *B. cereus* strain 9373.

MATERIALS AND METHODS

Bacterial strains and media. *B. cereus* strain 9373, used as the principal organism in this study, was obtained from A. J. Andreoli, Department of Chemistry, California State University, Los Angeles. Cells were grown in G medium (11) modified in the following manner: 0.1% sucrose was substituted for 0.2% glucose; the concentration of yeast extract was reduced from 0.2 to 0.1%, and $CoSO_4 \cdot 7H_2O$ was added to 0.00002% (wt/vol). Cultures were grown at 30°C in a New Brunswick Microferm fermentor with an aeration of 5 liters of air per min and an agitation of 150 rpm. Upon release of mature spores, the culture was placed in the cold for 24 h and harvested in a Sharples continuous-flow centrifuge. Vegetative cell debris was removed by washing the spores 15 times in distilled water.

Electron microscopy. After adsorption of samples onto parlodion-covered copper grids, negative stains were made by briefly touching the dried grid to a drop of 1.0% phosphotungstic acid (pH 7.4) and drawing off the excess stain with a strip of filter paper. All specimens were examined on a JEOL 100-B electron microscope operated at 60 kV.

Protein determinations. The amount of protein in pili samples was estimated by the UV absorption method at 260 and 280 nm (20).

SDS-polyacrylamide gel electrophoresis. For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, 10% polyacrylamide cylindrical gels were run by the method of Laemmli (17). Protein bands were visualized by staining with Coomassie brilliant blue G by standard procedures (30).

Pili isolation. Washed spores were resuspended in distilled water to an average density of 5×10^8/ml, and their pili were sheared off in a Waring blender operated at high speed. Pili were separated from intact spores by repeated centrifugations at 6,000 × *g*. Precipitation of the pili in the supernatant fluid was effected by adding ammonium sulfate to 80% of saturation in the cold. The resulting precipitate was centrifuged, and the pellet was resuspended in a small volume of distilled water and dialyzed against 0.1 M Tris buffer (pH 7.8) containing 5×10^{-4} M EDTA. Differential centrifugation was performed again to remove further any contaminating spores.

Pili were prepared for equilibrium density centrifugation by adding CsCl to a final concentration of 31.24% (wt/wt). After centrifugation in a Beckman SW 50.1 rotor at 123,000 × *g* at 20°C for 70 h, the pili formed a visible band in the center of the gradient.

The gradients were fractionated from the bottom, and fractions containing pili, as determined by electron microscopy, were pooled and dialyzed against Tris-hydrochloride buffer and sedimented twice in the same buffer to remove any soluble protein contaminants. Equilibrium density centrifugation in CsCl was repeated as before. An estimate of 25 μg of pili per liter of original spore culture was obtained with this isolation scheme.

Chemical analyses. The chemical composition of spore pili was investigated with only those pili that had been sedimented twice to equilibrium in CsCl density gradients. The carbohydrate content was determined by the phenol sulfuric acid procedure (4). A partial amino acid analysis of the pili was performed on samples that had been dried in an ampule, sealed, and hydrolyzed in 6 N HCl at 110°C for 24 h. Amino acid analysis was performed in a Durum D500 amino acid analyzer by the method of Spackman et al. (28).

Assay for pili solubilization. Solubilization of pili was attempted by resuspending approximately 10^8 washed spores in 1 ml of the solubilizing agent in question and incubating at 37°C for 60 min. Negative stains of the treated spores were then examined in the electron microscope for the loss of pili. Agents showing positive results were subsequently used to solubilize isolated pili preparations, and the resulting mixture was examined by gel electrophoresis.

Preparation and labeling of antiserum. Isolated pili (approximately 20 μg per injection) were injected subcutaneously in three to four places on the dorsal area of female New Zealand White rabbits with Freund complete adjuvant (Difco) for the first injection. Subsequent injections were made with Freund incomplete adjuvant (Difco). The first booster was given 4 weeks after the initial immunization, and further boosters were given at 2-week intervals. Rabbits were bled from the ear 10 days after each booster.

Serum titer was monitored by using agglutination of whole spores in microtiter plates (15).

The immunoglobulin G fraction of the serum was obtained by standard methods (12) and stored at −20°C.

Antipilus serum was labeled with ferritin by the method of Singer (27).

Ferritin labeling of spores. A 1-mg amount of lyophilized spores in 100 μl of normal saline was gently mixed with 200 μl of ferritin-labeled antipilus immunoglobulin G and placed at 4°C for 64 h. The mixture was subsequently loaded on top of a 5-ml preformed 60 to 100% Renografin density gradient, and the spores were separated from the unreacted ferritin conjugate by centrifugation at 20,000 rpm in a Beckman SW 50.1 rotor for 30 min at 4°C. The spore band was removed from the side of the gradient with an 18-gauge needle, and the spores were washed 10 times with normal saline to remove further unbound antibody. The labeling pattern was determined by electron microscopy.

RESULTS

Morphology and occurrence of spore pili. Figure 1 shows that all spores of *B. cereus* 9373 grown in G medium possess pili. The structures do not appear to be artifacts of the complex medium in that they are also present on spores produced in the semidefined G medium of Nakata (21) in which vitamin-free Casamino Acids are substituted for yeast extract. Additionally, the pili are firmly bound to the spore and cannot be detached by extensive washing. These structures average eight per spore, appear to emanate randomly from the baglike exosporium, and are absent from both vegetative and sporulating cells. The pili appear tubular, having a constant 6.8-nm diameter (±0.6 nm) and a length averaging 2 μm. No subunit structure is immediately discernible, but occasionally high resolution photographs reveal either a helical or disklike arrangement (Fig. 2, arrow).

To ascertain whether the presence of spore pili is a widespread phenomenon, spores from 12 species of bacilli were examined (Table 1). Structures identical to the pili of *B. cereus* 9373 were seen on the spores of two other strains of *B. cereus* and on the spores of *B. thuringiensis*. Pili were absent from spores of 10 other *Bacillus* species. Furthermore, Table 1 indicates that the spores which possess pili are a subset of those which possess a prominent exosporium.

Pilus isolation. The scheme by which spore pili have been isolated is a combination of various procedures that have been used to isolate pili from other organisms (3, 18). Pili that have been banded twice in CsCl show no contamination by spores or spore components when examined under the electron microscope (Fig. 2). Moreover, the steps taken to isolate the pili do not alter their morphology. The pilus buoyant density in CsCl was calculated refractometrically to be 1.296 g/ml.

Solubilization of pili. Isolated spore pili were subjected to electrophoresis in an attempt to ascertain further their purity and to investigate their subunit nature. Approximately 25 μg of pili was resuspended in 50 μl of 0.625 M Tris buffer containing 3% SDS and 5% 2-mercaptoethanol (SDS sample buffer), boiled for 3 to 10 min, and run on SDS-polyacrylamide gels. Under this standard protein denaturation procedure, the sample failed to penetrate the stacking gel, indicating no solubilization of the pili. Failure to solubilize the pili was confirmed by electron microscopy. Subsequent attempts to solubilize the pili by varying the denaturation conditions, i.e., increasing the concentration of SDS and mercaptoethanol or replacing the mercaptoethanol with dithioerythritol, were also unsuccessful.

Our data indicate that the spore pili are also insoluble in a wide variety of common protein-denaturing agents, including detergents, organic solvents, and various oxidizing and reducing agents. The following is a list of reagents that

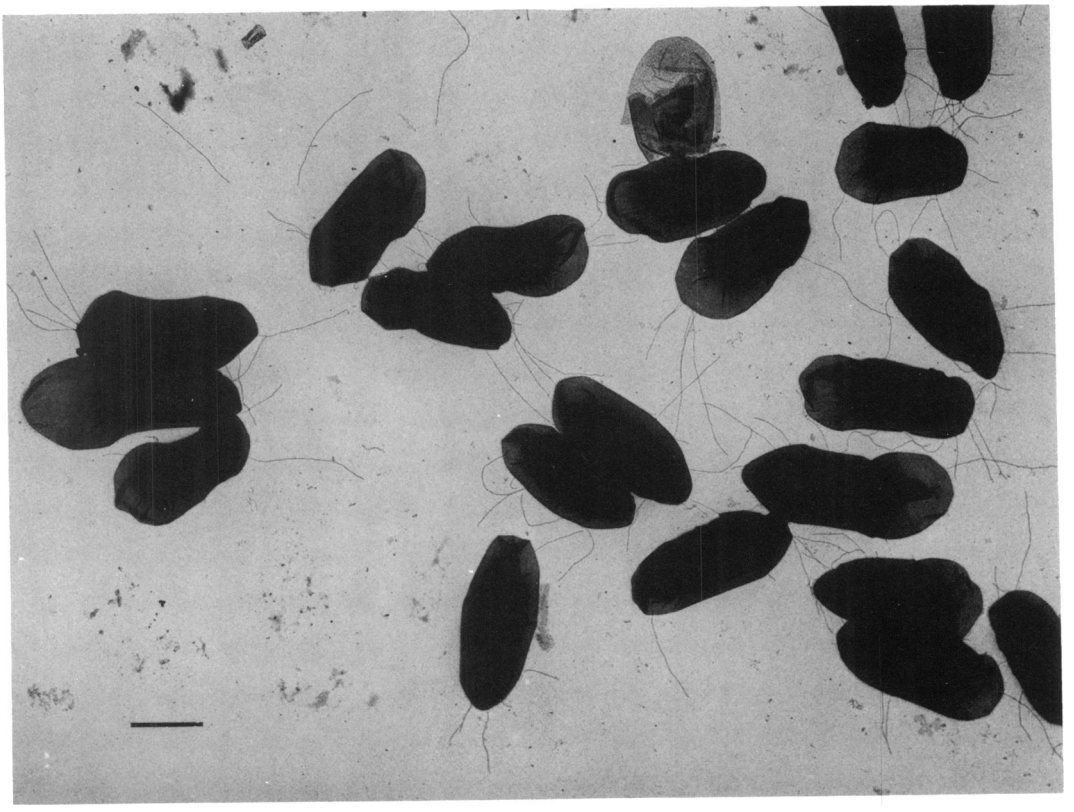

FIG. 1. *Negative stain of newly released, whole spores of B. cereus with attached pili. Bar represents 1 μm.*

were found not to solubilize *B. cereus* spore pili: SDS (3 and 30%, wt/vol), Triton X-100 (1%, vol/vol), Brij-58 (1%, wt/vol), Sarkosyl 97 (0.1%, wt/vol), phenol, chloroform, ethanol, methanol, acetone, formamide, ethylene glycol, dithiothreitol (0.1 M), dithioerythritol (0.1 M), 2-mercaptoethanol (1%, undiluted), urea (6 and 8 M), guanidine hydrochloride (6 M), guanidine thiocyanate (4 M), trypsin (10 mg/ml), alpha-chymotrypsin (10 mg/ml), subtilisin (10 mg/ml), pronase (10 mg/ml), lysozyme (300 mg/ml), sodium hypochlorite, trichloroacetic acid, (10%, wt/vol), dimethylsulfoxide, glutaraldehyde, sodium chloride (saturated), potassium chloride (saturated) magnesium chloride (0.1 M), EDTA (0.1 and 0.2 m), protosol, NaOH (0.1 and 1.0 N), formic acid (10 to 75%, vol/vol), sucrose (80%, wt/vol), ammonium persulfate (1%, wt/vol), potassium dichromate (1%, wt/vol), and osmium tetroxide (1%, wt/vol). Tests with SDS, Triton X-100, Brij 58, Sarkosyl, sodium deoxycholate, dithioerythritol, urea, and EDTA were performed at pH values ranging from 2 to 12. Many of these agents have been shown to solubilize pili from other organisms (3, 6, 18, 25).

Two treatments have resulted in the disruption of pilus integrity, but subsequent electrophoretic analysis of the solubilized material has proven inconclusive as to the subunit nature of the structure. Pili can be dissolved by treating with 0.1% $KMnO_4$ or by 1 h of refluxing in 1 M HCl. Upon electrophoresis, the first treatment yields products that never leave the stacking gel, whereas the latter shows three to four protein bands in the 90,000- to 130,000-molecular-weight range. Due to the fact that both of these treatments result in peptide bond hydolysis, it seems probable that these results do not accurately reflect the subunit composition of the spore pilus.

Carbohydrate and amino acid analysis. Isolated pili which show no contamination under the electron microscope and no bands other than that at the top of the stacking gel after SDS-mercaptoethanol denaturation and SDS gel electrophoresis have been used for further chemical analysis. The buoyant density of the pili in CsCl (1.296 g/ml) suggests that the structure is composed solely of protein. In addition, carbohydrate could not be detected by the phenol sulfuric acid method in 250-μg samples of isolated pili. In these experiments, hexoses, pen-

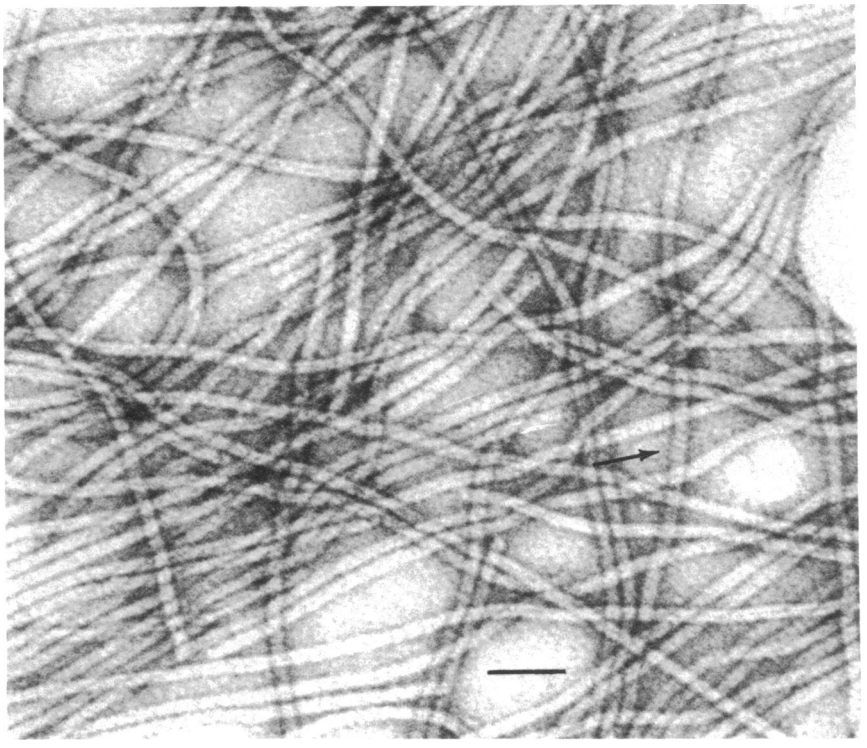

FIG. 2. *Negative stain of whole B. cereus spores that have been incubated with ferritin-labeled pili antiserum to show the distribution of pilus antigen on the spore surface. Bar represents 0.5 μm.*

TABLE 1. *Presence of spore pili in 12 species of bacilli*

Organism	Growth medium	Pili	Exosporium
B. cereus subsp. *metiens*[a] (ATCC 7039)	G	+	+
B. cereus T[b]	G	+	+
B. thuringiensis[c]	GYS (22)	+	+
B. subtilis[c]	G	−	−
B. megaterium QM B1551[b]	SNB (19)	−	−
B. licheniformis[c]	G agar	−	−
B. brevis[c]	G agar	−	−
B. natto[c]	G agar	−	−
B. globigii[c]	G agar	−	−
B. sphaericus 1593[d]	BS broth (14)	−	+
B. stearothermophilus[a]	Nutrient agar + slats (2)	−	+
B. pumilus[c]	Penassay broth (Difco)	−	+
B. amyloliquefaciens[c]	Penassy broth (Difco) + BS salts (14)	−	+

[a] University of Washington Culture Collection.
[b] J. C. Vary, University of Chicago, Chicago, Ill.
[c] H. R. Whiteley, University of Washington, Seattle, Wash.
[d] A. Walker, New Mexico State University, Las Cruces, N. Mex.

toses, methylglycosides, and uronic acids would have been detectable at the 5-μg level.

The amino acid composition of hydrolyzed pili is shown in Table 2.

Immunological analysis. As previously noted, the pili appear to arise from the exosporium. To determine whether the spore pili are a component of the exosporium, an antiserum prepared to pili has been used to explore the antigenic relationship between these two structures. The IgG fraction of antipilus serum was labeled with ferritin and reacted with whole *B. cereus*

TABLE 2. *Amino acid composition of B. cereus spore pili*

Amino acid	nmol/nmol of Tyr
Asp	9.21
Thr	8.72
Ser	6.55
Glu	7.66
Pro	5.19
Gly	8.55
Ala	4.68
Val	5.71
Ile	3.38
Leu	2.07
Tyr	1.00
Phe	2.01
Lys	1.43
Arg	1.59

spores, and the distribution of ferritin molecules on the spore surface was determined by electron microscopy.

Figure 3 shows that virtually all the ferritin labeling is found on the pili. The small amount of ferritin bound to the exosporium is seen mostly in patches. These patches of labeling are most likely due to binding of the antibody to the remaining bases of pili that had been sheared off during preparation. This ferritin labeling pattern indicates that the pilus antigen is not found on the outer exosporium surface.

DISCUSSION

This communication extends the observations on *Bacillus* spore pili by Hodgkiss (13) and Hachisuka and Kuno (9). Two strains of *B. cereus* have been added to those already known to possess pili. In addition, the method developed here for isolation of these structures from *B. cereus* strain 9373 has made possible the study of their chemical and physical properties in greater detail.

Comparison of *B. cereus* 9373 with the strains examined by Hodgkiss (13) and Hachisuka and Kuno (9) suggests that the dimensions of spore pili are variable among different organisms. Among the bacilli examined, pilus lengths of 1 to 5 μm and widths from 6.8 to 20 nm have been observed. All three reports, however, confirm the absence of pili from vegetative and sporulating cells.

Ferritin labeling data indicate that spore pili are structures whose chemistry is distinct from that of the exosporium surface. These data further suggest that although the pili appear to originate from the exosporium, they are not a structural component of that integument layer. Our unpublished observation that complete chemical solubilization of the exosporium fails to solubilize the pili supports this hypothesis.

To our knowledge, all pilus types that have been examined have been shown to be constructed from a single proteinaceous subunit. By comparison, the spore pili of *B. cereus* are also composed of protein, and occasionally a subunit arrangement was observed in negative stains (Fig. 2, arrow). However, the pilus insolubility in common protein-denaturing agents has prevented the determination of whether this structure possesses a monomeric or more complex subunit composition. The insolubility cannot be readily accounted for by the amino acid composition (Table 2) and raises the possibility that the putative subunits may be cross-linked by covalent bonds. An example of a highly insoluble, polymeric structure that is composed of covalently linked subunits is elastin. In the elastin fibers the subunits are linked through modified lysine residues and through Schiff base formation (7). Whether an analogous phenomenon exists for spore pili remains to be seen.

It is not uncommon for spore-associated structures to be highly insoluble. The *B. cereus* spore coat has been shown to be refractory to the action of many protein denaturants and proteolytic enzymes (16). Similarly, the exosporium of *B. cereus* is also highly insoluble in that the majority of the agents that failed to disrupt the pilus also failed to damage the exosporium. This insolubility is not unexpected, since the integument layers surrounding the spore are generally assumed to be protective in function (29). Whether or not the spore pili serve a protective function remains to be seen, but their presence on the outer surface of the spore seemingly would mandate that they also be highly insoluble to maintain their functional capability.

The possible significance of the pili to the spore can only be speculated upon at this time. Gerhardt and Ribi (8) suggested that the *B. cereus* spore pili may represent polymeric material that is released upon germination. However, all newly released, ungerminated spores examined in this investigation were found to possess pili (Fig. 1). Furthermore, the number of these structures does not increase upon germination, nor do structures that resemble pili reappear upon germination of spores whose pili have been mechanically removed in a Waring blender (data not shown).

Hodgkiss (13) proposed that the spore pili may act as chemosensory organelles, possibly as triggers for germination. However, no differences were found when the germination (initiated with adenosine and L-alanine) properties of spores possessing pili were compared with those whose pili had been removed in a Waring blender (data not shown). It has also been suggested that pili may serve to attach the spores

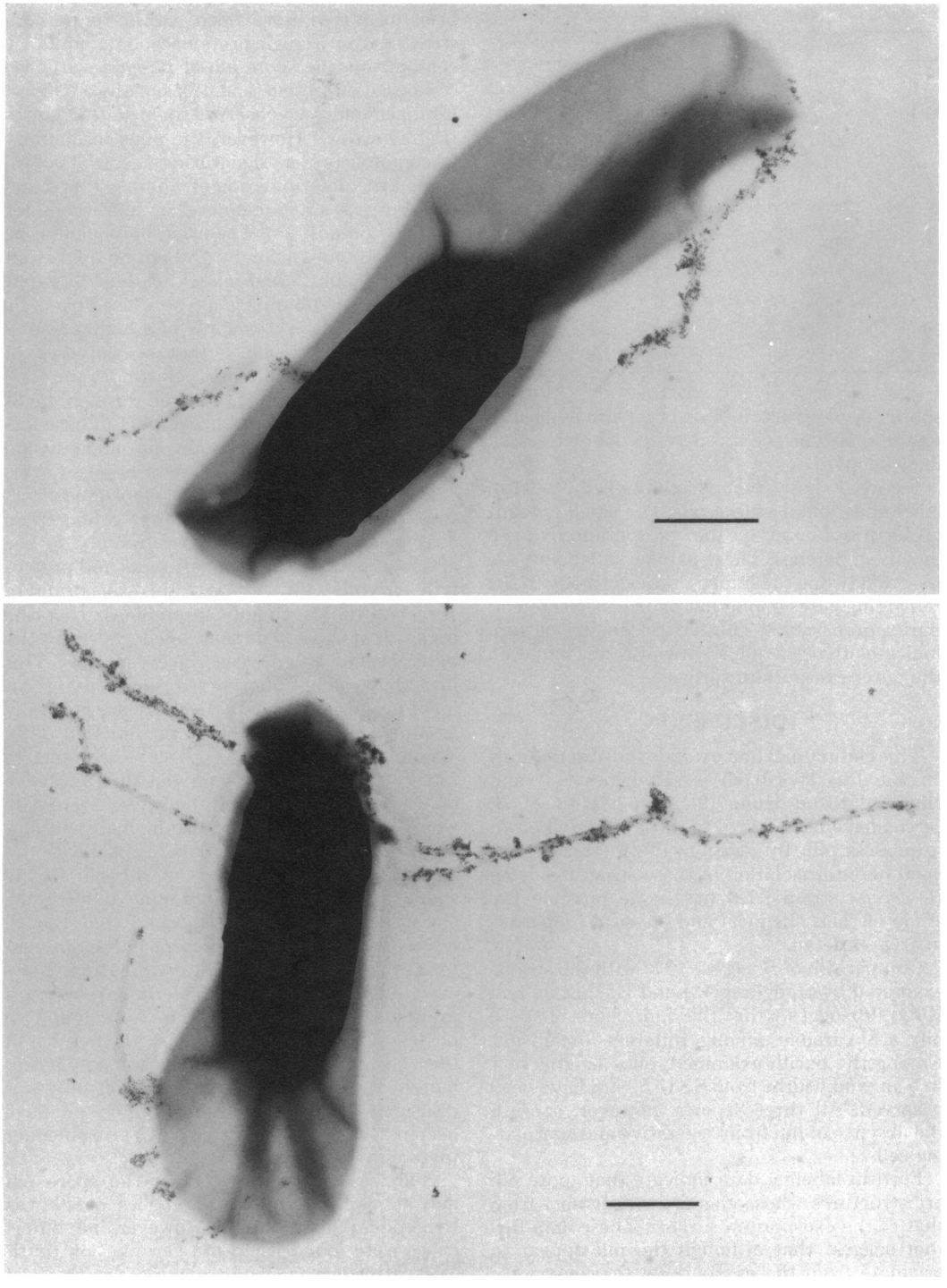

FIG. 3. *Negative stain of spore pili twice banded to equilibrium in CsCl. Arrow denotes an occasionally seen area of periodicity. Bar represents 50 nm.*

to particles or to other cells. Whatever hypothesis is ventured as to their significance to the spore, the observation that not all *Bacillus* species have pili on their spores must be taken into consideration (Table 1).

Because pili are absent from *B. cereus* vegetative cells, it is possible that these structures may be the product of a gene expressed only during sporulation. If so, it would be of interest to know when this gene is first expressed and whether its expression occurs in the mother cell or forespore compartment or both.

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