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Chemical Properties of the Pili of *Corynebacterium renale*

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Pili separated from the cells of *Corynebacterium renale* strain 46 (type II) by agitation at high speed in a homogenizer were purified by repeated cycles of ammonium sulfate precipitation, sonic treatment, and centrifugation. The preparation of purified pili formed a single antigen-antibody line in agar gel and showed an absorption maximum at 275 nm. The pili subjected to dodecyl sulfate-polyacrylamide gel electrophoresis formed a main band and corresponded to the molecular weight of 19,000. The fact that the total nitrogen of the amino acids of the pili was nearly equal to its nitrogen content, together with the absence of detectable carbohydrate, has led to the conclusion that the pili are protein. The pilial protein was composed of 20 amino acids. Preparations of pili which had been treated with 0.5 N NaOH, but not with 1 N HCl, no longer appeared filamentous and failed to form a precipitate with the antibody in agar gels. A comparison has been made of the amino acid composition and certain properties of the pili of *C. renale* and type I pili and F pili of gram-negative bacteria.

Recently, Yanagawa et al. (9, 10) reported the presence of pili in *Corynebacterium renale*, which is the first reported well documented example of pili on gram-positive bacteria. Strains of *C. renale* type II possessed numerous pili, whereas those of types I and III usually possessed a small number. No ability to aggregate red blood cells was demonstrated by piliated strains of *C. renale*, contrary to gram-negative bacteria.

From the viewpoint of a comparative study of bacterial pili, it will be of interest to clarify the properties of the pili of *C. renale* which are not yet thoroughly studied and to compare them with the properties of type I pili and F pili of gram-negative bacteria. This report describes the chemical properties of the pili of *C. renale* type II.

MATERIALS AND METHODS

Microorganisms. *C. renale* strain 46 (type II) was used. This strain is known to possess the most numerous pili (9, 10).

Isolation and purification of the pili. Microorganisms of strain 46 cultured on nutrient agar, *p*H 7.2, supplemented with 0.5% glucose and 1% bonito extract (Kishida Chemicals, Osaka), pH 7.2, at 37 C for 48 hr, were collected, washed three times with distilled water, and suspended in distilled water. The washed bacteria were agitated in a high-speed homogenizer at full speed for 30 min. After centrifugation at 19,000 × *g* for 20 min, a cooled, saturated ammonium sulfate solution was added slowly to the supernatant solution to 25% saturation. A small amount of precipitate was discarded, and the cooled, saturated ammonium sulfate solution was again added slowly to the supernatant fluid containing the pili to 45% saturation. The precipitate was collected, dissolved in 0.1 M glycine buffer (pH 9.0), treated sonically for 1 to 5 min in a Kubota sonifier (KMS-100; Kubota Co., Tokyo) at 10 kc/sec full power, and centrifuged at 176,600 × *g* for 60 min. The supernatant fluid was collected. The procedure of precipitation with ammonium sulfate, sonic treatment, and centrifugation was repeated five to six times, and finally the suspension was dialyzed against distilled water for 72 hr at room temperature. Approximately 60 mg of the pili was obtained from 100 g (wet weight) of the bacteria.

Immunodiffusion. The serum of a rabbit immunized against the whole cells of strain 46 was used. The method of preparation of the immune rabbit serum and the immunodiffusion method of Ouchterlony have been described previously (8).

Electron microscopy. In the course of the purification procedure, preparations of the pili were either mounted on collodion grids and shadowed with palladium or mounted on carbon-coated collodion grids and stained with 2% phosphotungstic acid (*p*H 7.2) and examined under a JEM 7 electron microscope (Japan Electrical Optics Laboratory Co., Tokyo) at magnification up to ×60,000.

Electrophoresis. The pili suspension was applied to dodecyl sulfate-polyacrylamide gel electrophoresis (7) with minor modification of staining of gels (4). Cytochrome *c*, myoglobin, chymotrypsinogen A, ovalbumin, and bovine serum albumin were migrated in parallels with the pili.

Chemical analyses. The total carbohydrate was de-

termined by the phenol sulfuric acid method (3) with glucose as the standard. The nitrogen content of the pili was determined by means of the micro-Kjeldahl analysis. The ultraviolet absorption spectrum was recorded with a Shimazu QV-50 spectrophotometer (Shimazu Co., Tokyo). The pili suspension was evaporated to dryness for amino acid analysis. The dried pili weighing 3.805 mg were hydrolyzed with 6 N HCl at 110 C for 24, 48, or 72 hr under vacuum. The amino acid composition was determined by using a Hitachi KLA-3B amino acid analyzer (Hitachi Co., Tokyo) by the method of Spackman et al. (6). The tryptophan value was calculated by the spectrophotometric method of Goodwin and Morton (5) on a sample of the pili dissolved in 0.1 N NaOH. The value was corrected for scattering. The contents of asparagine and glutamine were not determined. Half-cystine was determined after acid hydrolysis, without any preliminary performic acid oxidation.

RESULTS AND DISCUSSION

Immunodiffusion of the purified pili of *C. renale* strain 46 with anti-strain 46 serum is shown in Fig. 1. The pili formed a single precipitin line. The crude pili formed two more lines, which were thought to be derived from the cell wall. The pili were subjected to dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2). One main and nine minor bands were distinguished. The position of the main band indicated that the molecular weight of the monomer of the pili was approximately 19,000. The minor bands might correspond to polymers of the pili and to minor proteins or impurities. The ultraviolet absorption spectrum of the pili in 0.1 M phosphate buffer (*p*H 7.2) showed a strong absorption maximum at approximately 275 nm (Fig. 3). An inflection at 285 nm was observed. Purification of the pili by precipitating with 0.1 M $MgCl_2$ was unsuccessful, contrary to type I pili of gram-negative bacteria (1).

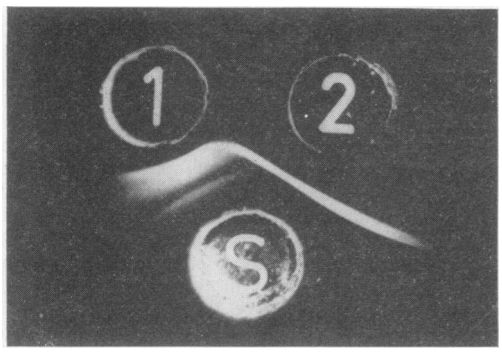

FIG. 1. *Immunodiffusion pattern of the pili. The bottom well (S) contained rabbit antiserum to whole C. renale strain 46 (type II). The upper wells contained the pili separated from the cells by homogenizer (well 1) and the pili purified (well 2).*

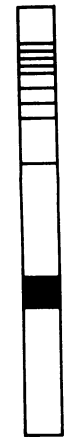

FIG. 2. *Dodecyl sulfate-polyacrylamide gel electrophoresis pattern of the pili.*

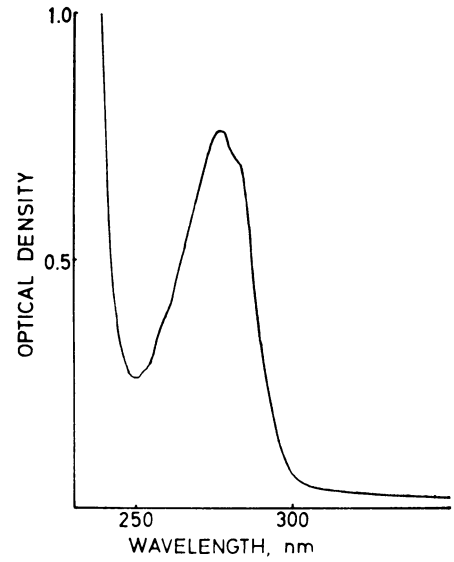

FIG. 3. *Ultraviolet absorption spectrum of the pili. The pili were dissolved in 0.1 M phosphate buffer (pH 7.2) to give a concentration of 0.91 mg/ml. The spectrum was read against a buffer blank.*

When examined by electron microscope, the purified pili appeared to be aggregated side by side and to form thick bundles. Such bundles were considered to be characteristic of the pili of *C. renale* (9, 10).

Micro-Kjeldahl analysis of the pili gave a nitrogen content of 16.63%. No carbohydrate was detectable. The results of the amino acid analysis are given in Table 1. The total nitrogen content of the amino acids of the pili was almost equal to the nitrogen content of the pili. From these findings, the pili were considered to be protein.

TABLE 1. *Amino acid composition of the pili of Corynebacterium renale strain 46 compared with that of type I pili and F pili of gram-negative bacteria*

Amino acid	Amt of amino acid residue (moles/10^5 g of protein)		
	Pili of *C. renale* strain 46	Type I pili[a]	F pili[b]
Lysine	59	18	85
Histidine	15	12	0
NH_3	96	181	
Arginine	31	18	0
Aspartic acid	113	120	68
Threonine	85	120	68
Serine	40	60	93
Glutamic acid	117	78	34
Proline	46	12	0
Glycine	79	102	127
Alanine	90	205	127
Half-cystine	4[c]	12	0
Valine	74	78	178
Methionine	6	0	68
Isoleucine	29	24	34
Leucine	50	60	76
Tyrosine	30	12	17
Phenylalanine	20	48	59
Tryptophan	5[d]	0	17

[a] Calculated from the data of Brinton (1).
[b] Calculated from the data of Brinton (2).
[c] Half-cystine value was determined after acid hydrolysis without preliminary performic acid oxidation.
[d] Tryptophan value was calculated from ultraviolet spectra (5).

The content of hydrophilic amino acid residues was higher than the content of hydrophobic amino acid residues. The minimum molecular weight of the monomer of the pili was calculated to be 19,400, based on the amino acid analysis. This agrees well with the value of 19,000 obtained by dodecyl sulfate-polyacrylamide gel electrophoresis.

For comparison, the amino acid compositions of type I pili (1) and F pili (2) of gram-negative bacteria are shown in Table 1. Glycine, alanine, and phenylalanine are lower but tyrosine and glutamic acid are higher in the pili of *C. renale* than in type I pili and F pili of gram-negative bacteria. Methionine and tryptophan are absent in type I pili of gram-negative bacteria, and arginine, histidine, proline, and cysteine are absent in F pili; all of these amino acids are present in the pili of *C. renale*. However, there is a possibility that half-cystine, methionine, and tryptophan of the pili of *C. renale* originate from impurities. As a whole, the content of hydrophilic amino acid residues is higher and that of hydrophobic amino acid residues is lower in the pili of

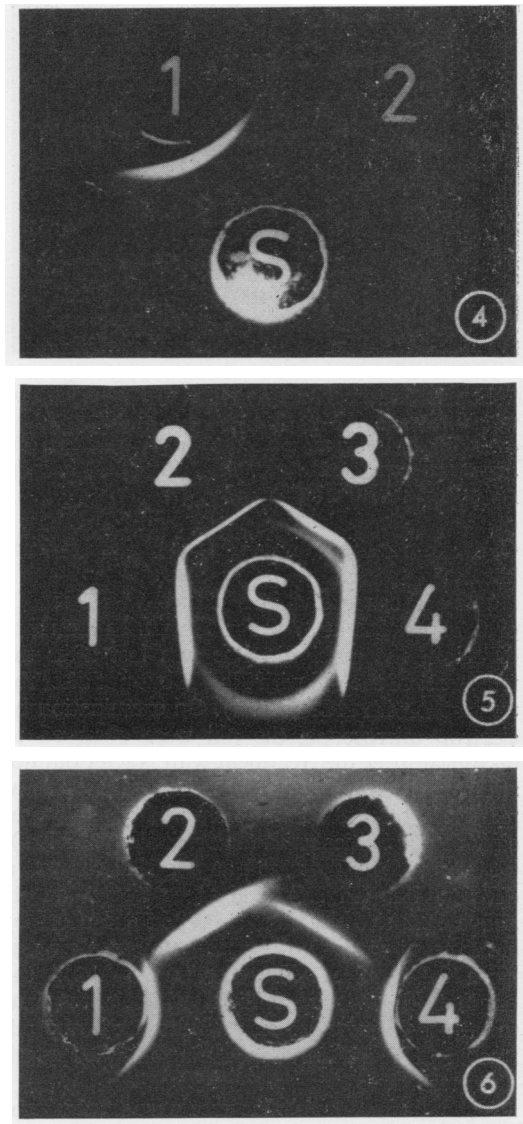

FIG. 4. *Immunodiffusion pattern of the pili. The bottom well (S) contained rabbit antiserum to whole C. renale strain 46 (type II). The upper wells contained the pili treated with 1 N HCl (well 1) and those treated with 0.5 N NaOH (well 2).*

FIG. 5. *Immunodiffusion pattern of the pili treated at pH 11.8 or boiled for 120 min. The central well (S) contained rabbit antiserum to whole C. renale strain 46 (type II). The peripheral wells contained the pili treated at pH 11.8 (well 3) and boiled for 120 min (wells 1 and 4) and the native pili (well 2).*

FIG. 6. *Immunodiffusion pattern of the pili treated at pH 12.0 or boiled for 180 min. The central well (S) contained rabbit antiserum to whole C. renale strain 46 (type II). The peripheral wells contained the pili treated at pH 12.0 (well 3) and boiled for 180 min (well 2) and the native pili (wells 1 and 4).*

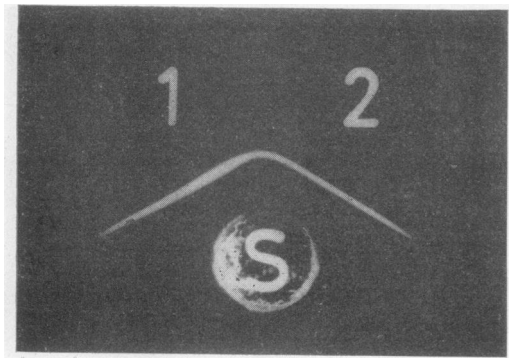

FIG. 7. *Immunodiffusion pattern of the pili treated with 8 M urea. The bottom well (S) contained rabbit antiserum to whole C. renale strain 46 (type II). The upper wells contained the native pili (well 1) and the pili treated with 8 M urea (well 2).*

C. renale than in type I pili and F pili of gram-negative bacteria.

The pili suspension was treated with 1 N HCl or 0.5 N NaOH at 37 C for 60 min. In immunodiffusion, the precipitin line was lost after the treatment of the pili with NaOH but not after the treatment with HCl (Fig. 4). On examination by electron microscope, the pili were observed to disappear after the treatment with NaOH but not after the treatment with HCl. The pili treated at *p*H range from 8.0 to 11.8 showed a single precipitin line identical with the original one (Fig. 5). However, the pili treated at *p*H 12.0 showed a different line (Fig. 6), and those treated at *p*H above 12.2 showed no line. When examined by electron microscope, the pili treated at *p*H range from 8.0 to 11.8 were observed to hold the original filamentous feature, whereas the filamentous pili were reduced in number in the pili treated at *p*H 12.0. At *p*H above 12.2, the pili disappeared.

The pili treated with 8 M urea at room temperature for 24 hr gave a precipitin line identical with that of the native pili (Fig. 7). When examined by electron microscope, the pili treated with 8 M urea were found to be dissociated into pieces of various lengths, 20 to 200 nm.

The pili boiled for 120 min showed a precipitin line identical with that of the native pili (Fig. 5), whereas the pili boiled for 180 min showed a line different not only from the native pili but also from the pili treated at *p*H 12.0 (Fig. 6). The pili of strain 35 of *C. renale* type II were also resistant to boiling (9). The thermostable property of the pili of *C. renale* type II is thus clear.

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