Some Properties of the Pili of Corynebacterium renale

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Some properties of the pili of the gram-positive bacteria Corynebacterium renale were described. A relationship was found between the morphological features of pili and the types of C. renale. Strains of types I and III usually possessed a small number of pili, whereas those of type II possessed numerous pili. Thick and long bundles of pili characteristic of C. renale were frequently observable in type II strains. Piliation of C. renale was stable under various cultural conditions. No ability to agglutinate red blood cells was demonstrated by piliated strains of C. renale. Pili were isolated from the cells of C. renale and studied serologically by immunodiffusion. The pili of a type II strain were serologically identical with the pili of another type II strain but not with those of the strains belonging to types I and III. The pili were serologically distinct from the cell wall. The pili were broken into short pieces by boiling, but their antigenicity was increased after boiling.

Filamentous appendages of bacteria, different from flagella, were designated as fimbriae (11) or pili (1). Duguid et al. (11) reported that known hemagglutinating ability of Escherichia coli was due to pili. Pili were also found in other genera, Shigella (9), Salmonella, Klebsiella, Proteus, and Chromobacterium (10). Brinton and co-workers extended the study of pili electrophoretically (4), genetically (2, 3, 5, 6), biochemically (14), morphologically, and chemically (2). Thus, the properties of bacterial pili have been considerably clarified. But the species of bacteria which have been known to possess pili were all gram-negative (5, 12).

In gram-positive bacteria, existence of pili in Corynebacterium renale was recently reported (16). Since the presence of pili had not been known before in gram-positive bacteria, it is of interest to study the properties of C. renale pili and to compare them with those of gram-negative bacteria. The present report deals with some properties of C. renale pili.

**MATERIALS AND METHODS**

**Microorganisms.** A total of 63 strains of C. renale were used. Of these, 35 were isolated in Japan and classified serologically and biochemically into types I, II, and III (15). Four strains were given by J. E. Phillips of the Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh; three strains were provided by the American Type Culture Collection, Rockville, Md. One strain isolated in Uruguay and obtained from T. Hiramune of the Hokkaido Branch Laboratory, National Institute of Animal Health, Sapporo, Japan, was identified in our laboratory as type I. Four strains sent us from A. Vallee, Institut Pasteur, Paris, were identified as type III. Eight strains, strains 1, 2, 3, and 9 (type I), strains 35 and 45 (type II), and strains 42 and 43 (type III), were used as representatives of each type for the studies of piliation under various cultural conditions and for tests for agglutination. Nutrient agar and broth were used as culture media for C. renale. Usually 1- or 2-day-old cultures were tested.

**Detection of pili by electron microscopy.** Pili were examined electron microscopically. Bacteria grown in nutrient broth were fixed by adding osmium tetroxide to the broth culture to give a final concentration of 0.5%. After fixing overnight at 4 C, the bacterial cells were sedimented by centrifugation and washed twice with distilled water. Bacteria grown on agar medium were suspended in distilled water, and, after fixing in final 0.5% osmium tetroxide overnight at 4 C, the cells were sedimented and washed likewise. The washed bacteria were mounted on the collodion grids and, after reducing the excess amount of the material by absorption with filter paper, were shadowed with palladium. Later, the procedure of fixation was frequently omitted because no marked difference was observed between the pili of fixed and nonfixed bacteria. In such cases, the bacterial cells grown on agar medium were suspended in distilled water and shadowed likewise. Preparations were examined in a JEM 7 electron microscope (Japan Electrical Optics Laboratory Co.).

**Piliation in various cultural conditions.** Piliation of C. renale was studied under the following cultural conditions: incubation at 20, 37, and 40 C; incubation for 5 days in a tube in an atmosphere of low
oxygen and elevated carbon dioxide tension (13); incubation in a desiccator in which a candle was burned to extinction; cultivation for 3 days in boiled 25% calf serum with 0.5% glucose, mannose, maltose, galactose, sucrose, or arabinose; and cultivation on agar media containing various concentrations of antibiotics, chloramphenicol (1.25 to 5.0 μg/ml), penicillin (0.2 to 0.5 unit/ml), streptomycin (0.625 to 5.0 μg/ml), and mitomycin C (0.08 to 0.32 μg/ml). The concentrations of the antibiotics were the range of the maximum concentrations which permitted the eight strains to grow. Cultivation of strain 35 on agar medium with 10% homologous hyperimmune serum was also tested. The hyperimmune rabbit serum used was that prepared for immunodiffusion.

Agglutination. Cow, pig, dog, cat, sheep, guinea pig, chicken, and human red blood cells were used to examine whether these red blood cells could be agglutinated by the cells of C. renale. Media and pH range used for the hemagglutination test were citrate buffer (0.15 M citric acid, 0.15 M sodium citrate; pH 5.0, 5.5, 6.0, 6.5, 7.0), 0.065 M phosphate buffer (pH 7.0, 7.2, 7.4, 7.6, 7.8, 8.0), Veronal buffer (0.15 M sodium barbital, 0.15 M HCl, pH 7.0, 7.4, 8.0), Molar borax buffer (0.15 M borax, 0.15 M borax; pH 7.5, 8.0), and (hydroxyxymethyl)aminomethane (Tris) buffer (0.15 M Tris, 0.15 M HCl, pH 7.0, 7.4, 8.0). The red blood cells were washed three times and suspended in the above buffer solutions to obtain final 0.3% (v/v) concentration. HeLa cells cultivated for 3 days, dispersed by trypsinization, washed by centrifugation, and resuspended in Hanks balanced salt solution (8 × 10⁴ cells per ml) were also used for detecting the possibility of agglutination by the cells of C. renale. In addition, bacterial cells of other species, particularly those of gram-positive bacteria such as Staphylococcus aureus, Streptococcus dysgalactiae, Listeria monocytogenes, and Bacillus subtilis, and Proteus vulgaris, which is gram-negative, were used; cells were obtained from 2-day-old cultures on nutrient agar. These bacterial cells, as well as cells of C. renale, were washed twice and resuspended in the buffer solutions to obtain a turbidity equivalent to an optical density of 0.5 at 450 nm.

Agglutination tests were carried out by mixing 0.5-ml amounts of cell suspensions of C. renale with 0.5-ml amounts of the suspensions of red blood cells, HeLa cells, or other bacterial cells in test tubes. These tubes were then incubated, respectively, for 2 hr at 37°C, overnight at room temperature, or overnight at 4°C. Then the tubes were examined for agglutination.

Isolation of pili. About 10 g (wet weight) of C. renale grown on agar medium was collected, washed three times with distilled water, resuspended in distilled water, and agitated in a high-speed mixer for 5 min, keeping the bacterial suspension cool (2). After centrifugation at 7,000 × g for 30 min, the supernatant fluid was again centrifuged. The cell-free supernatant fluid thus obtained was sedimented at 40,000 × g for 60 min. The sediment containing pili was suspended in 1 ml of distilled water, which was used for electron microscopy, and used as pili antigen.

Immunodiffusion. The serum of a rabbit which had been immunized against the whole cells of C. renale strain 35, the pilated type II strain, was used. Antigens used were the pili antigens obtained from four strains, 9, 35, 42, and 46, and the whole cell antigen extracted from strain 35. The methods of preparation of the immune rabbit serum, the agar-gel-diffusion method of Ouchterlony, and extraction of whole cell antigen by 1% sodium deoxycholate were described in a preceding report (15). Pili samples were boiled for 20 min, and pili samples treated with 0.25% trypsin (EC 3.4.4.4; Difco) in 0.065 M phosphate buffer (pH 7.8) and 0.25% Streptomyces protease (Prozyme: EC class 3.4.4; Kyowa Hakko Kogyo Co., Tokyo) in the same buffer (pH 7.2) at 37°C for 1 hr were also used.

RESULTS

Morphological features of pili in relation to C. renale types. Pili were detected in all 63 strains. No essential differences were found between the features of pili of the fixed and unfixed bacteria. Features of pili were not the same among the three types of C. renale. Pili of C. renale strains belonging to type I were small in number (Fig. 1 and 2). Not all of the bacterial population was pilated in the strains of type I, even after careful fixation of bacteria with osmium tetroxide. Usually, the pili of type I strains were short. On the contrary, all the strains of type II possessed numerous long pili (Fig. 3 and 4). Strains of type III were variable; many of them possessed few pili (Fig. 5), as did strains of type I, but some strains of type III had many pili (Fig. 6).

Thick and long bundles of pili, frequently having a thickness of more than 0.4 μm and a length of more than 10 μm, were common in the strains of type II (Fig. 7) and in three strains of type III which possessed many pili. These thick pili sometimes encircled the bacterial cell (Fig. 8). Such bundles of pili were rare in all the strains of type I and the remaining strains of type III which possessed a small number of pili. General features of pili in relation to C. renale types are summarized in Table I. Among the eight strains frequently used in the following biological studies, strains 1, 2, 3, and 9, which belonged to type I, possessed a small number of pili; strains 35 and 45 (type II) and strains 42 and 43 (type III) possessed numerous pili.

Piliation of C. renale under various cultural conditions. The original features of the pili of the eight strains were unchanged when these strains were serially subcultivated in nutrient broth or on agar plates. In nutrient broth, strains of type I possessing a small number of pili and those of type II which possessed numerous pili formed surface pellicle.
Fig. 1. *C. renale* strain 9 (type I). × 34,000.
Fig. 2. *C. renale* ATCC 10849 (type I). × 48,000.
Fig. 3. *C. renale* strain 35 (type II). × 40,000.
Fig. 4. *C. renale* strain 46 (type II). × 17,000.
FIG. 5. C. renale strain 48 (type III). \( \times 34,000 \).
FIG. 6. C. renale strain 42 (type III). \( \times 28,000 \).
FIG. 7. C. renale strain 35 (type II). \( \times 26,000 \).
FIG. 8. C. renale strain 35 (type II). \( \times 25,600 \).
In addition, no pellicle was formed in the cultures of type III strains possessing numerous pili. These findings indicated that piliation did not correlate with pellicle formation.

Piliation was not changed when the eight strains were cultivated in an atmosphere of decreased oxygen and elevated carbon dioxide concentration. Growth at 20, 37, and 40 C did not cause change in piliation. Growth in the media with one of six sugars, mannose, glucose, maltose, galactose, sucrose, or arabinose, did not induce alteration in piliation. Chloramphenicol, penicillin, streptomycin, and mitomycin C did not change piliation. Strain 35 inoculated on agar medium containing 10% homologous hyperimmune rabbit serum grew rather profusely and showed no change in the features of pili.

**Tests for hemagglutination and agglutination of other cells by C. renale.** It is well known in Enterobacteriaceae that piliated strains agglutinate various kinds of cells. The possibility of agglutination was studied with the eight strains of the three types of *C. renale*. The cells used were of human, cow, pig, chicken, cat, dog, rabbit, sheep, and guinea pig red blood cells, HeLa cells, and bacterial cells described above. No agglutination of red blood cells was observed, even in the pH range from 5.0 to 8.0 with the various buffer solutions. HeLa cells and bacterial cells were not agglutinated by the cells of *C. renale*, but were agglutinated by *P. vulgaris*. Microscopic examination showed that cells of *C. renale* did not adhere to these various cells.

**Sero logical properties of C. renale pili.** Serological properties of *C. renale* pili were studied by immunodiffusion. Prior to the study, it was necessary to isolate pili as described above.

The mixer treatment, conducted for 5 min, was effective in removing pili from the cells of *C. renale*. Longer treatments, such as 30 min, resulted in breaking the pili into pieces ranging from about 20 nm to several times that length. Such long blender treatment also resulted in contamination by the cell wall antigen.

The isolated pili of strain 35 obtained by 5 min of mixer treatment are shown in Fig. 9. Long pili are attached side by side and form thick bundles. Boiling for 10 min broke the long pili into short pieces (Fig. 10).

Immunodiffusion of rabbit antiserum to whole cells of strain 35 with the homologous whole cell antigen and the pili antigens from the four strains is shown in Fig. 11. The pili of strain 35 (well 1) gave a precipitin line which was characteristic in that the line formed close to the antigen well and extended from there in the direction opposite to the serum well. The pili of strain 35 boiled for 20 min (well 2) formed a similar precipitin line, but the line was stronger and wider. Another line was produced when deoxycholate extract of whole cells of strain 35 (well 3) was examined. This line, considered to be the reaction of cell wall antigen, appeared between the antigen and serum wells and was different from the reaction of the pili. The same figure shows that the pili of strain 46, another strain of type II (well 4), gave a precipitin line common to strain 35. However, the pili from other strains of different types of *C. renale* such as I (well 5) and III (well 6) gave no reactions.

Effects of two proteolytic enzymes, trypsin and Prozyme, on pili were also examined. Pili were considerably degraded morphologically by these enzymes. However, the enzyme-treated pili still formed a line similar to that of the original pili.

**DISCUSSION**

Cells of *C. renale*, causative agent of bovine pyelonephritis and cystitis, are unique among gram-positive bacteria in that they possess pili. Pili have been known as fine fibrillar appendages of certain bacteria, but hitherto were known only in gram-negative species (5, 12).

Morphological features of pili were not the same among three types of *C. renale*. *C. renale* was classified into three types serologically and biochemically (15). The relation of features of pili to *C. renale* types is interesting. The bundles of pili, which most frequently appeared in the strains of type II, are considered to be characteristic to *C. renale*. Such bundles of pili have not been described in gram-negative bacteria.

The diameter of an individual pili of *C. renale* was, regardless of type, about 2.5 to 3 nm (16), the diameter similar to that of type III pili of the gram-negative bacteria described by Brinton (2). Thicker pili of *C. renale* were the bundles of the fine pili.

Pili of *C. renale* were present when the bacteria were grown in an atmosphere of low O2 and elevated CO2 concentrations or at 20, 37, and 40 C. The presence of sugars including mannose, which inhibits piliation of some gram-negative bacteria (9), antibiotics, and homologous antibacterial serum did not affect piliation of *C.
piliation of *C. renale* is stable. Pili of *C. renale* differ from the pili of many gram-negative bacteria in that they do not adhere to red blood cells. In many gram-negative bacteria, piliated strains can easily be recognized by a simple hemagglutination test, for they adhere to red blood cells and cause hemagglutination (7-9, 11). No hemagglutination was caused by any strain of *C. renale*.

Although adhesive properties were not clearly shown in this report, pili of *C. renale*, particularly those of type II strains, showed a tendency to attach side by side and to form thick bundles. The adhesive property of *C. renale* pili needs further investigation.

The three types of *C. renale* possess cell wall and pili antigens, and the cell wall and pili antigens are peculiar to each type.

Serological studies of type II pili showed that antigenic specificity of pili was different from that of cell walls. Also antigenicity of pili is specific of each type of *C. renale*. *C. renale* strains were classified into three types, primarily based on the antigens extracted with deoxycholate from cell wall (15).
The peculiar position of the precipitin line formed by pili antigen might be attributed to the long filamentous shape of isolated pili which diffuse slowly in gels. After boiling, the pili fraction gave a wider and stronger precipitin line. On examination with an electron microscope, such boiled pili were not intact filaments but were broken into segments, which diffused more rapidly in agar gel. From the fact that C. renale pili, broken morphologically by boiling or by the action of proteolytic enzymes, were still serologically active, the pilus is thought to consist of small antigenic subunits assembled into a filament.

Strains of type II, which possessed the most numerous pili, were frequently isolated from apparently healthy cattle, whereas strains of types I and III, which possessed rather few pili, were frequently isolated from diseased cattle (15). Strains of type II are not very pathogenic but are parasitic in the urinary tract. Possibly these cells attach themselves to the cells of the urinary tract so that they are not easily ejected outside the animals' body. Presumably, C. renale pili are helpful for this attachment. But this is only a speculation at present. Connected with this speculation, it is interesting that saprophytic Klebsiella are piliated but pathogenic Klebsiella are not piliated (7).

At present, it is almost impossible for us to give an adequate idea of the functions of C. renale pili. Except for the function of F pili (5), the functions of pili in gram-negative bacteria are said to remain still to be discovered (8).

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LITERATURE CITED


