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Purification and Characterization of *Serratia marcescens* US5 Pili

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The pili of *Serratia marcescens* US5 isolated from a patient with urinary tract infection were purified and characterized. During the aeration culture, the pili were detached from the bacteria and were precipitated by the addition of ammonium sulfate. The purification of the pili was carried out by ion-exchange chromatography and gel filtration on Sepharose 4B. In electron microscopy, the purified pilus showed a filament of 3 nm in diameter and 0.3 μm in average length. The molecular weight of the protein subunit of the purified pili was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Two protein bands appeared. One has a molecular size of 19,000 daltons, and the other has a molecular size of 39,000 daltons. The isoelectric point was 3.7. The content of hydrophobic amino acids in purified pili subunits was 42% of the total amino acid content. Further purification of pili by isopycnic centrifugation failed to remove the large protein band. No identical protein bands to pili proteins were detected in the electrophoresis pattern of the outer membrane proteins extracted from *S. marcescens* US5 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These two proteins might be a dimer of a small molecule. A survey of clinically isolated strains of *S. marcescens* revealed that more than 60% of the strains had this type of pili. These results suggest that these pili are widely distributed among strains of *S. marcescens*.

Serratia marcescens is one of the bacterial species causing nosocomial infections of the respiratory and urinary tracts. Many gram-negative bacteria isolated from urinary tract infections are known to possess special cell surface appendages called pili or fimbriae. The adherence of these bacteria to tissue surfaces is thought to be mediated by these structures. The presence of pili on *S. marcescens* has also been reported (1).

In previous papers, we reported on the phenomenon of agglutination of some strains of *S. marscesens* in human urine and described the role of pili in the agglutination process (2, 12). To elucidate the influence of pili on agglutination, we undertook studies of the role of *S. marcescens* US5 pili in the agglutination of these bacteria in human urine.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. marcescens* US5 isolated from a patient with a urinary tract infection at Fukuoka University Hospital, Fukuoka, Japan, was used throughout these studies. The characterization of this strain has been described in a previous paper (2). *S. marcescens* US5 has mannose-sensitive hemagglutinating activity and is agglutinated in human urine. The strain was maintained on ordinary nutrient agar or in broth at 37°C. For the purification of pili, the strain was cultured on CFA agar or in CFA broth (6).

Purification of pili. Strain US5 was cultured in 100 ml of CFA broth by aeration at 37°C overnight. Ten milliliters of this culture was then transferred to 10 Erlenmyer flasks, each containing 400 ml of CFA broth. The cultures were incubated at 37°C by continuous shaking on a rotary shaker (G10; New Brunswick Scientific Co., Inc.) overnight. Ammonium sulfate to a final concentration of 60% saturation was added to 4 liters of the culture supernatant obtained by centrifugation at 5,000 × *g* for 30 min. The sedimented fractions containing pili were collected and dissolved in 0.1 M phosphate buffer (PB, pH 7.5) and dialyzed against PB overnight at 4°C. After the removal of the remaining bacteria in the dialyzed fluid by centrifugation (5,000 × *g*, 30 min), the fluid was concentrated to 50 ml by ultrafiltration (PM10 filter membrane, 10,000-molecular-weight exclusion; Amicon Corp., Lexington, Mass.). This concentrated fluid was designated as crude pili.

Crude pili were then adsorbed on DEAE-cellulose and eluted in high-salt buffer. Fifty grams of DEAE-cellulose was suspended in 100 ml of PB, mixed with 50 ml of crude pili in a centrifuge tube (30 × 150 mm), and allowed to stand for 30 min to allow the adsorption of pili to cellulose. After the cellulose was washed three times with PB by centrifugation (1,000 × *g*, 15 min), it was suspended in 0.2 M NaCl in PB to elute the adsorbed pili. The pili were separated from cellulose by centrifugation at 1,000 × *g* for 30 min. This pili-containing fraction was subjected to gel filtration on a Sepharose 4B column (20 by 1,000 mm; Pharmacia Fine Chemicals) equilibrated with 0.01 M Tris-hydrochloride buffer. The column was washed with 0.01 M Tris-hydrochloride buffer, and 5-ml fractions were collected. Protein contents of the fractions were measured with UV light adsorption at 280 nm, and the presence of pili structures was determined by electron microscopy. The fractions containing pili were pooled and concentrated by ultrafiltration on a PM10 filter membrane. This concentrated pool was termed purified pili.

SDS-PAGE. We used the Laemmli system (14) for slab gel sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified pili were applied to the polyacrylamide slab gel (12.5%) with a 3% stacking gel and were electrophoresed 5 h at 25 mA of constant current per slab. The protein bands were stained with 0.1% Coomassie brilliant blue in acetate-methanol-water (7:43:50). For the calibration of the molecular weight, the low-molecular-weight marker kit (LKB Corp., Bromma, Sweden) was used.

Amino acid composition. The amino acid composition of purified pili was determined by the method of Spackman et al. (20) at a laboratory of chemistry in Fukuoka University

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TABLE 1. The relationship between culture conditions and percentage of piliated bacteria

Culture conditions	Piliated cell/cell counted	Piliated cells (%)	Turbidity of the culture
Nutrient agar	133/200	66.5	N.D.[a]
Nutrient broth, static	128/200	64	45
Nutrient broth, aerated	90/200	45	325
CFA agar	187/200	93.5	N.D.
CFA broth, static	192/200	96	50
CFA broth, aerated	194/200	97	300

[a] N.D., Not determined.

Medical School with a Hitachi amino acid analyzer (model no. 835).

Isoelectric focusing. Isoelectric focusing was carried out at 4°C with an Ampholine column (LKB Instruments, Inc.; model no. 8101). The column was prepared as described in the manual directions. The concentration of Ampholine was 1%, and the range of the gradient of pH was 3.5 to 10. Electrophoresis was done for 48 h at a constant electric power of 3 W.

Extraction of the outer membrane proteins. The outer membrane proteins of *S. marcescens* were extracted by the method of Gabay et al. (10). Bacterial cells were collected from 100 ml of exponentially growing culture by centrifugation, and after being washed with PB, they were suspended in 50 ml of extraction buffer (2% SDS, 10% glycerol, 2 mM $MgCl_2$, 10 mM Tris-hydrochloride [pH 7.4]). After 30 min of incubation at 60°C, the suspension was centrifuged at 100,000 × *g* for 1 h. The pellet was washed twice with 2 mM $MgCl_2$ and suspended in 50 ml of 10 mM Tris-hydrochloride buffer at pH 7.4 containing 2% SDS and 0.5 M NaCl at 37°C. After 30 min of incubation, the suspension was centrifuged at 100,000 × *g* for 1 h to remove nondisintegrated membrane. The outer membrane proteins were extracted in the supernatant.

Isopycnic centrifugation. The purified pili were further purified by isopycnic centrifugation (19). Pili (100 μg) were mixed with cesium chrolide to a density of 1.30 g/cm^3 and centrifuged at 100,000 × *g* for 68 h in a Hitachi SW 65 swing rotor. An opalescent band was formed near the top of the tube and was collected by puncturing the side of the tube with a needle. After extensively dialyzing against PB, the sample was processed for SDS-PAGE or electron microscopy.

Electron microscopy. (i) Shadowing. A drop of the bacterial suspension was placed on an electron microscope grid coated with a Formvar film, and the excess solution was blotted with a piece of filter paper. The specimen was shadowed by tungstate in a vacuum evaporator equipped with an electron beam evaporating device (Edwards type 306) at an angle of 30°. The thickness of the metal coat was monitored to 3 nm with a quartz crystal thickness monitor (Edwards FTM2D).

(ii) Negative staining. The bacterial suspension in ammonium acetate solution was mixed with 2% phosphotungstate on a Formvar-coated grid. The excess stain was removed by touching it with a piece of filter paper. The specimens were examined with a Hitachi HU-12A electron microscope at 75 kV.

Agglutination test. (i) Hemagglutination test. Guinea pig erythrocytes were suspended in a concentration of 2% in phosphate-buffered saline (pH 7.2) and mixed with pili on a slide glass. Hemagglutination occurring within a few minutes was read as a positive reaction (7, 8).

(ii) Yeast cell agglutination test. Yeast cells (*Saccharomyces cerevisiae*) were cultured on Sabouraud agar and suspended in PB. The concentration of yeast cells was adjusted to an optical density of 50 Klett units at 660 nm. The yeast cell suspension and pili were mixed on a glass slide, and agglutination was read within a few minutes (18).

(iii) Urinary mucin agglutination test. Urinary mucin was collected from the urine of human volunteers in early morning. Urine was centrifuged at 3,000 × *g* for 15 min, and pelleted mucin was suspended in PB (12). The mucin suspension and pili were mixed on a glass slide. For the determination of mannose-sensitive agglutination, the pili were suspended in PB containing 1% mannose, and agglutination tests were carried out as described above.

Antiserum. Anti-US5 pili antiserum was raised in rabbits

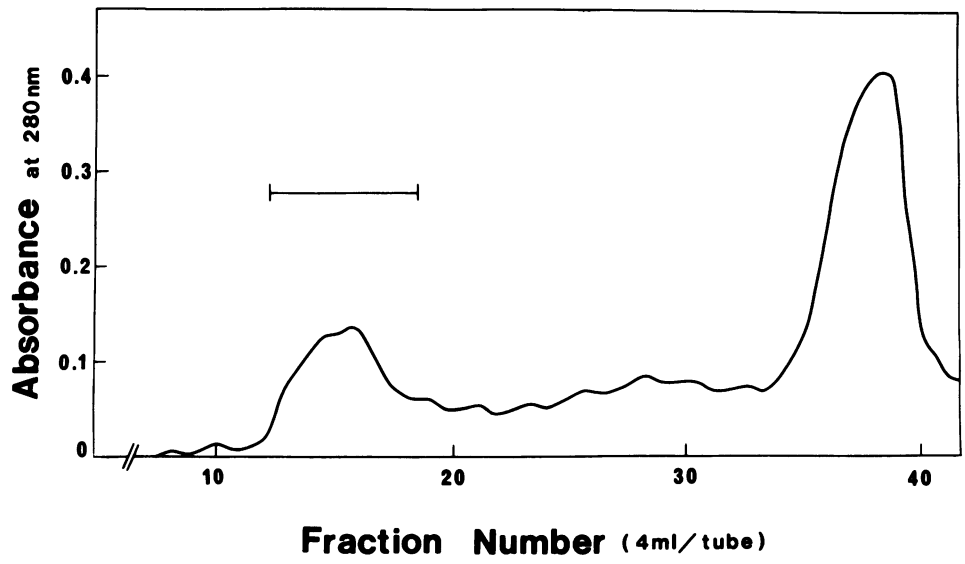

FIG. 1. Gel filtration of crude US5-type pili preparation on Sepharose 4B in Tris-hydrochloride.

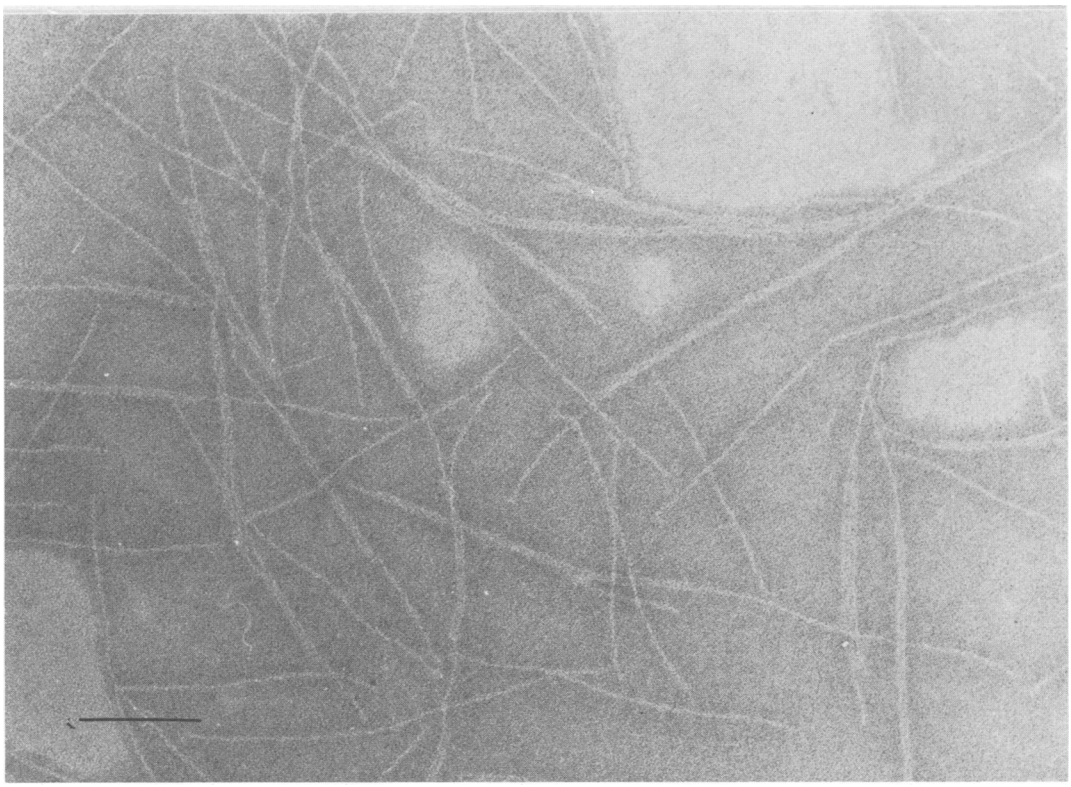

FIG. 2. Negatively stained electron micrograph of purified pili of *S. marcescens* US5. Bar, 100 nm.

by injecting intramuscularly the mixture of purified pili and Freund complete adjuvant. After two booster injections of purified pili intravenously, blood was collected from the ear vein, and the serum was separated. The antibody titer against strain US5 was 1:2,560 in the tube agglutination test.

For the determination of the distribution of US5-type pili among clinical isoltes of *S. marcescens*, a glass slide agglutination method was employed. The strains from clinical isolates were cultured on CFA agar. A loopful of the culture was mixed with serum which had been inactivated at 56°C for 30 min and diluted 100-fold in PB on a glass slide. The agglutination was read within a few minutes.

Protein estimation. Protein was estimated by the method of Lowry et al. (15) with bovine serum albumin as a standard.

RESULTS

Growth condition and piliation of bacteria. To determine the best conditions for the production of pili, strain US5 was cultured in two different media under different conditions, and the percentage of piliated cells was determined by electron microscopy (Table 1). In overnight cultures on nutrient agar or in stationary broth, more than 60% of cells were piliated, whereas in aerated nutrient broth the percentage decreased to 45% (5, 9). In contrast, in CFA broth or agar, more than 90% of cells were piliated, and the percentage of pilation was independent of the culture conditions. In the following experiment, aerated CFA broth cultures were used for the purification of pili.

Purification of pili. Pili of strain US5 were purified by ammonium sulfate precipitation, ion-exchange chromatography on DEAE-cellulose, and gel filtration. The pili were eluted from DEAE-cellulose in the buffer containing 0.2 M NaCl. The proteins were eluted in two peaks from Sepharose 4B (Fig. 1). Only the first peak contained pili (Fig. 2). The pili-containing fractions were pooled and concentrated on a PM10 filter membrane.

The purified pili migrated as two protein bands in SDS-PAGE (Fig. 3). The migration of the first band was consistent with a molecular weight of approximately 19,000,

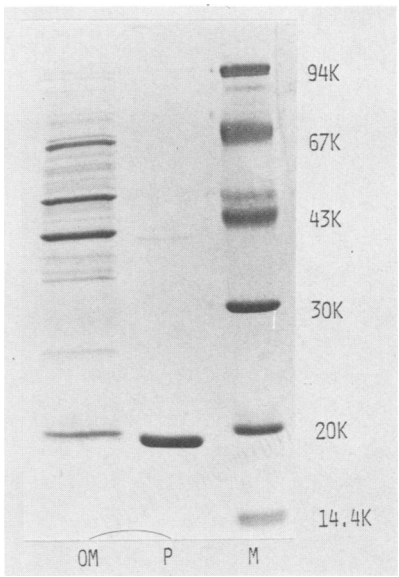

FIG. 3. SDS-PAGE of purified pili and major outer membrane protein of *S. marcescens* US5. OM, Outer membrane protein; P, pili; and M, marker.

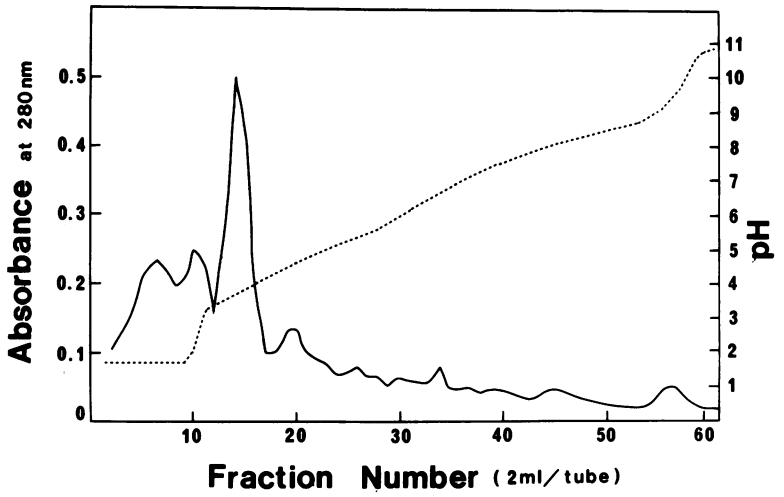

FIG. 4. Isoelectric focusing of *S. marcescens* US5 pili.

whereas the other more faintly staining band was consistent with a molecular weight of 39,000.

Structure of pili. An electron micrograph of negatively stained purified pili is presented in Fig. 2. No membrane structures and flagella were found. The diameter of the pili was 3 nm, and the average length was 0.3 μm. They were seen as a twisted fine fiber. No specialized structures such as the basal body in bacterial flagella were observed on any of the pili.

Isopycnic ultracentrifugation. Purified pili preparation was further purified by isopycnic centrifugation to exclude association with proteins. After centrifugation at 100,000 × *g* for 72 h, the pili formed a single opaque band near the top of the tube which continued to migrate as two protein bands in SDS-PAGE.

Outer membrane proteins and pili. The possibility that outer membrane proteins might be associated with the purified pili preparation was investigated. The outer membrane proteins were isolated from *S. marcescens* by the method of Gabay et al. (10), and the purified pili were electrophoresed on SDS-PAGE. After being stained with Coomassie brilliant blue, more than 20 protein bands were found on the gel of the outer membrane proteins, but none of them migrated to a position identical to those of the pili proteins (Fig. 3).

TABLE 2. Amino acid composition of *S. marcescens* US5 pili

Amino acid	Approx no. of residue per mol
Asp	28
Thr	19
Ser	16
Glu	14
Ala	22
1/2Cys	N.D.[a]
Val	13
Met	N.D.
Ile	10
Leu	9
Tyr	5
Phe	10
Lys	12
His	1
Arg	4
Pro	6

[a] N.D., Not determined.

Isoelectric focusing. The isoelectric focusing profile of the pili is shown in Fig. 4. The highest peak, appearing at pI 3.7, contained pili as determined by electron microscopy. No pili were found in the other small peaks appearing at pI 3 and 4.5.

Amino acid analysis. The amino acid composition of the purified pili is shown in Table 2. The content of hydrophobic amino acids was ca. 42%. The molecular weight of the pili protein estimated from the amino acids composition was 22,000.

Agglutination of erythrocytes, yeast cells, and urinary mucin. Guinea pig erythrocytes and yeast cells were agglutinated by purified pili, and agglutination was inhibited by the addition of mannose as in the case of bacterial agglutination (Fig. 5, Table 3). The purified pili were not agglutinated in the presence of urinary mucin.

Distribution of pili antigen in clinically isolated *S. marcescens*. The distribution of pili antigen on the clinically isolated strain of *S. marcescens* was investigated with anti-pili antisera made for the purified pili of strain US5 (Table 4). Independent of the source of isolation, 63% or more of the strains tested shared pili antigens with the US5 type.

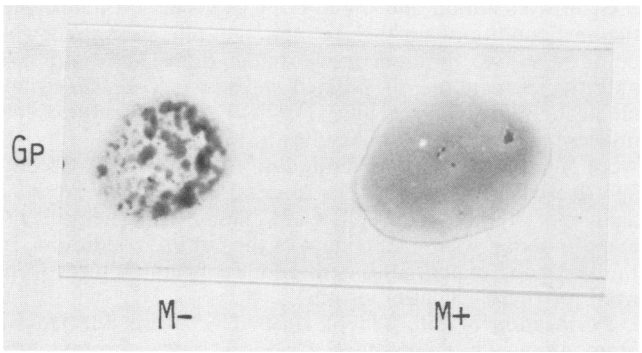

FIG. 5. Guinea pig erythrocyte agglutination by *S. marcescens* US5. M−, 1% D-mannose solution not added; M+, 1% D-mannose added.

DISCUSSION

The pili of *S. marcescens* US5, the urinary tract infection isolate, was concentrated from the culture supernatant by ammonium sulfate precipitation. Vigorous shaking of the bacteria with a blender did not increase the yield of pili. The pili were purified by the sequential steps of ion-exchange chromatography and gel filtration.

The electron micrograph of the purified pili revealed that the individual pili consisted of flexible fine filamentous structures with a diameter of 3 nm, and in high magnification, the pili fibers showed the helical array of the pilin subunits. In comparison with the type 1 or the common pili of *Escherichia coli* which also exhibit mannose-sensitive hemagglutination and a rigid rod-like shape of 7 nm in diameter (3, 17), the pili of strain US5 were more delicate and flexible.

In contrast to type 1 pili of *E. coli*, the purified pili of *S. marcescens* entered SDS-polyacrylamide gel without acid treatment (4, 17). The pili produced two protein bands on SDS-PAGE; one was a strongly staining band with a molecular weight of 19,000, and the other was a weakly staining band with a molecular weight of 39,000. The protein subunits of pili isolated from several bacterial species so far examined all have shown single peptides with molecular weights of close to 20,000 as determined by SDS-PAGE. Salit et al. (19) reported two protein bands in pili of *E. coli* in SDS-PAGE. However, one of the bands disappeared after purification by isopycnic centrifugation. They concluded that this band was a contaminating protein of the outer membrane. In our case, the second band persisted even after isopycnic centrifugation. Our pili preparation was morphologically pure; no contaminating membranous structures were observed. In isoelectric focusing, pili formed a single peak, and the material in this peak also migrated as two bands in SDS-PAGE identical to those of purified pili. No protein bands identical to those of the pili proteins were detected in the extract from the outer membrane. From these observations, we can postulate two possibilities. One is that both proteins are structural components of the pili (11), and the other is that the large protein is a dimer molecule of the small protein (4, 13).

Morphologically, the pilus of strain US5 consisted of a fine flexible filament with a diameter of 3 nm. In high magnification, the pili fibers showed a helical array, and structures such as bacterial flagellar bases were not observed on the pili filaments. Bacteriophage filamentous structures consist of two proteins, A protein and B protein (16). The A protein is a larger molecule than the B protein and is present as only one molecule per phage filament. The presence of the A protein has not yet been observed with electron microscopy. Only by SDS-PAGE can the presence of two proteins be demonstrated in these phage filaments. Therefore, the absence of a visible structure on the pili filaments by electron microscopy would not rule out the possibility that structural proteins other than pilin compose a part of the filament.

Because the molecular weight of the large protein is twice that of low-molecular-weight protein, it can be postulated that the larger protein is a dimer of the low-molecular-weight protein. If this hypothesis is true, the reasons why the dimer could not be completely separated into monomers during electrophoresis in the presence of SDS remain unclear.

The examination of the distribution of pili antigen among clinical isolates of *S. marcescens* revealed that more than 63% of strains tested possess the pili antigen. This suggests that this type of pili is widely distributed among strains of *S. marcescens* (1, 12). As previously reported (12), there was no relationship between US5-type pili and O antigen of *Serratia* species.

The strain having the US5-type pili is known to react with mucin in urine (urinary mucin) and to form large aggregates in urine (2). This phenomenon was termed urinary mucin agglutination (12). The infecting bacteria supposedly are excreted as large clumps of bacterial cells and urinary mucin by voiding. Therefore, urinary mucin agglutination was thought to be one of the host defense systems in the urinary tract. Since most strains of *S. marcescens* possess US5-type pili, urinary tract infection by these bacteria should be rare as long as normal voiding remains undisturbed.

TABLE 3. Results of agglutination by *S. marcescens* US5 and purified pili with human (A type), guinea pig, and chicken erythrocytes, yeast cells (*S. cerevisiae*), and urinary mucin

Sample	Agglutination of:				
	Erythrocytes (3%)			Yeast cells (1%)	Urinary mucin (4%)
	Human (A type)	Guinea pig	Chicken		
US5	—[a]	MS[b]	MS	MS	MS
Purified pili	—	Ms[c]	—	Ms	—

[a] —, No agglutination.
[b] MS, Mannose-sensitive agglutination. When 1% (wt/vol) D-mannose was added to bacterial suspension, the agglutination was inhibited.
[c] Ms, Agglutination by purified pili was weak and fine without D-mannose.

TABLE 4. The distribution of US5 type pili among clinically isolated strains of *S. marcescens*

Source of strains	Agglutination (%) by antiserum[a]		Total (%)
	+	–	
Urine	40[a] (69)	18 (31)	58
Sputum	49 (63)	29 (37)	78
Others	20 (77)	6 (23)	26
Total	109 (67)	53 (33)	162

[a] Antiserum = anti-US5 pili antiserum.
[b] Number of strains used for anti-US5 pili antiserum agglutination test and percentages thereof in parentheses.

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