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Characterization of Type 1 Pili of *Salmonella typhimurium* LT2

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Type 1 pili from *Salmonella typhimurium* LT2 were purified and characterized. The pilus filaments were 6 nm in diameter and over 1 μm long. Estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the molecular weight of the pilin was 21,000. The isoelectric point of the filament was 4.1. Hydrophobic amino acids comprised 40.3% of the total amino acids of the pilin, which contained more proline, serine, and lysine than reported for the type 1 pilin of *Escherichia coli*. Purified pili agglutinated both horse and chicken erythrocytes and yeast cells but not bovine, sheep, or human erythrocytes. Horse erythrocyte agglutination was inhibited at lower concentrations by α-methyl-D-mannoside than by yeast mannane and D-fructose. Agglutination was not affected by D-galactose or sucrose. Results of the present study confirm the role of type 1 pili as *Salmonella* hemagglutinins and show chemical differences between the type 1 pili of *S. typhimurium* and *E. coli*.

The presence on enteric bacteria of mannose-sensitive type 1 pili is associated with agglutinating and adhesive properties (3, 4, 7). It has been demonstrated that type 1 pili purified from *Escherichia coli* K-12 agglutinate guinea pig erythrocytes (20) and that purified *Salmonella typhimurium* type 1 pili agglutinate yeast cells and erythrocytes in a manner identical to that of intact cells (8). In each case, the agglutination by type 1 pili is inhibited by D-mannose.

Most *Salmonella* species are able to form type 1 pili (3), and their synthesis has been shown to be chromosomally coded in *S. typhimurium* (21). The physiological functions of the type 1 pili in *Salmonella* are still unknown. Salmonellae bearing type 1 pili form pellicles on the surface of static, aerobic cultures and can thus reach greater culture densities than do nonpiliated variants (17, 18). It has been proposed that type 1 pili mediate the mannose-sensitive adherence of *S. typhimurium* to human intestine (3) and to both human buccal and rat urinary tract epithelial cells (T. K. Korhonen and C. Svanborg Edén, submitted for publication). In orally infected mice, the mannose-sensitive pili increase the virulence of *S. typhimurium*; this was attributed to their role in mediating bacterial adherence to the alimentary tract mucosa (6).

The chemical structure and agglutination properties of type 1 pili have been well characterized in *E. coli* (2, 8, 9, 20), but not yet in *Salmonella* species. As a first step in the study of the possible role of pili in *Salmonella* infections, we purified and characterized type 1 pili from *S. typhimurium* LT2. This communication shows that these pili are chemically different from those of *E. coli*.

MATERIALS AND METHODS

Bacteria. *S. typhimurium* SH6749 (18a), which is a *galE* mutant of the LT2 line, was grown in static Luria broth (13) for 48 h in 3- to 10-liter Erlenmeyer flasks. The medium volume was 1/10 of the volume of the flasks, and the inocula were passed three times under similar conditions before cultivation. In this medium the lipopolysaccharide (LPS) of SH6749 is of the rough Rc type; if exogenous galactose is provided, the strain incorporates it into LPS, which becomes smooth with the O antigens 4 and 12.

Purification of pili. Pili were purified by using deoxycholate and concentrated urea according to Korhonen et al. (9).

Chemicals and buffers. Unless otherwise specified, all chemicals were of analytical grade. Specially pure sodium dodecyl sulfate (SDS) from BDH (Poole, England) was used without further purification. Ampholines were obtained from LKB, Bromma, Sweden. α-Methyl-D-mannoside and yeast mannane were from Sigma Chemical Co. (St. Louis, Mo.), D-mannose, D-fructose, and D-galactose were obtained from Merck (Darmstadt, West Germany), and D-glucose and sucrose came from BDH. Phosphate-buffered saline was pH 7.1, 300 mosmol/liter.

Electron microscopy. Piliated whole bacteria and purified pilus preparations were negatively stained with 2% (wt/vol) phosphotungstic acid in 0.1 M sodium phosphate buffer (pH 6.5). All micrographs were taken with a JEM-100B electron microscope operating at 80 kV.

Polyacrylamide gel electrophoresis. Polyacryl-

amide gel electrophoresis in SDS was performed in 1-mm-thick slab gels (gel concentration, 15%), using the method of Laemmli (11). Gels were stained with Coomassie brilliant blue G-250 (1). Standard proteins were type 1 pili from *E. coli* 6013 (8, 9) and the low-molecular-weight electrophoresis calibration kit from Pharmacia (Uppsala, Sweden). Pilus samples were denaturated at low pH before the SDS treatment (15).

Isoelectric focusing. Isoelectric focusing was performed in an LKB 110-ml focusing column (19). Ampholines were 3.5 to 10.

Amino acid analysis. Samples were hydrolyzed in 6 M HCl at 110°C for 24 h under nitrogen. Analysis was performed on a Jeol JLC-5AH amino acid analyzer with sodium citrate buffers. No corrections for losses of threonine and serine were made. The results represent an average of triplicate determinations from each of two separate batches of pili.

Protein estimation. Protein was estimated by the method of Markwell et al. (14) with bovine serum albumin as a standard. Prior to determination, samples were denaturated by boiling in pH 1.8 as described by McMichael and Ou (15).

Immunoelectrophoresis. Possible LPS contamination in the purified pilus preparation was checked by rocket immunoelectrophoresis using the method of Weeke (10, 22, 23; N. Kuusi and M. Nurminen, manuscript in preparation). Briefly, 3 to 30 μg of the pilus preparation in 0.2% (wt/vol) Triton X-100 was electrophoresed in a 1% agarose gel containing anti-Rc-LPS antibodies (12), in a buffer containing 0.2% Triton X-100 (10). Several different concentrations of LPS of the Rc type, also in 0.2% Triton X-100, from a *galE* mutant of *S. typhimurium* (18a) were run as standards.

Agglutination tests. Agglutination titrations, carbohydrate inhibitions, and slide tests with yeast cells (*Saccharomyces cerevisiae*; Oy ALKO Ab, Rajamäki, Finland) and bovine, sheep, human, horse, and chicken erythrocytes were done as described by Korhonen (8). Cells were washed three times with phosphate-buffered saline and suspended in it to a density of 3×10^8 cells per ml. Microtiter plates were kept at 4°C for 2 h before results were recorded.

RESULTS

Purification of pili. After growth for 48 h in static, aerobic Luria broth, the *S. typhimurium* strain formed pellicles on the culture surface and showed in slide tests a strong mannose-sensitive yeast cell agglutination. The bacteria were abundantly piliated, as is seen in the electron micrograph of a typical cell in Fig. 1. The cells were covered by pili, the longest of which were over 1.1 μm long. The diameter of a single pilus filament was about 6 nm. From 30 g (wet weight; the yield from 6 liters of medium) of such cells, the average yield of pure pilus was 1 to 3 mg.

Characterization of the SH6749 pilus protein. During purification, the pili retained their original morphology (Fig. 2). The length of the purified pilus filament was difficult to measure, but in diameter the pili were identical to those seen on the intact cells. No contaminating membrane vesicles were detected by electron microscopy.

The purified SH6749 pilus preparation gave only one band in SDS-polyacrylamide gel electrophoresis (Fig. 3), indicating for the pilin subunit an apparent molecular weight of 21,000 ± 200 (mean of 10 determinations).

Table 1 gives the amino acid composition of the SH6749 pilin and shows that 40.3% of amino acids are hydrophobic. The molecular weight calculated from the amino acid composition was 22,100.

Isolectric focusing of the purified pilus filament revealed only one fraction with an isoelectric point of 4.1.

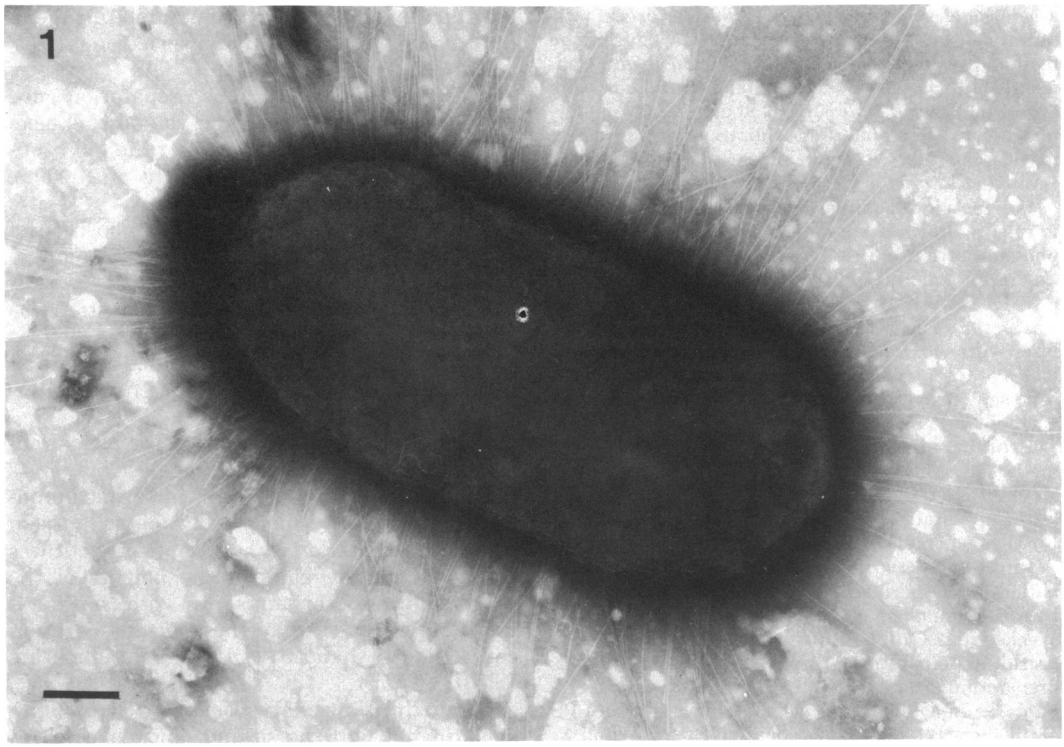
Possible LPS contaminating in the pilus preparation was checked by immunoelectrophoresis against an anti-Rc antiserum. In rocket immunoelectrophoresis, the purified pilus antigen gave no precipitation with this antiserum. It was estimated on the basis of the heights of the rockets given by this antiserum with the standard Rc type LPS that the pilus preparation contained less than 1% (wt/wt) of contaminating LPS.

Agglutination properties. The SH6749 pili agglutinated both horse and chicken erythrocytes and yeast cells but not bovine, sheep, or human erythrocytes (Table 2). The agglutination titer of yeast cells was clearly higher than that of horse or chicken erythrocytes. In each case, the agglutination by SH6749 pili was inhibited by 25 mM α-methyl-D-mannoside. Table 3 gives for various carbohydrates the minimal inhibitory concentrations required to prevent the agglutination of horse erythrocytes. α-Methyl-D-mannoside and D-mannose were very effective inhibitors, but the agglutination was also inhibited by D-fructose, by soluble yeast mannane, and, slightly, by D-glucose. D-Galactose and sucrose had no effect on the agglutination.

DISCUSSION

This communication describes the chemical and agglutination properties of type 1 pili purified from *S. typhimurium* LT2. Judged by SDS-polyacrylamide gel electrophoresis, the SH6749 pilus preparation was pure (Fig. 3), and the LPS contamination in the preparation was below 1%. The pili also retained their biological properties during the purification.

Purified SH6749 pili agglutinated horse and chicken erythrocytes and yeast cells in a mannose-sensitive manner (Table 2). The agglutination pattern of purified pili was identical to that of intact SH6749 cells (8), and Duguid et al.



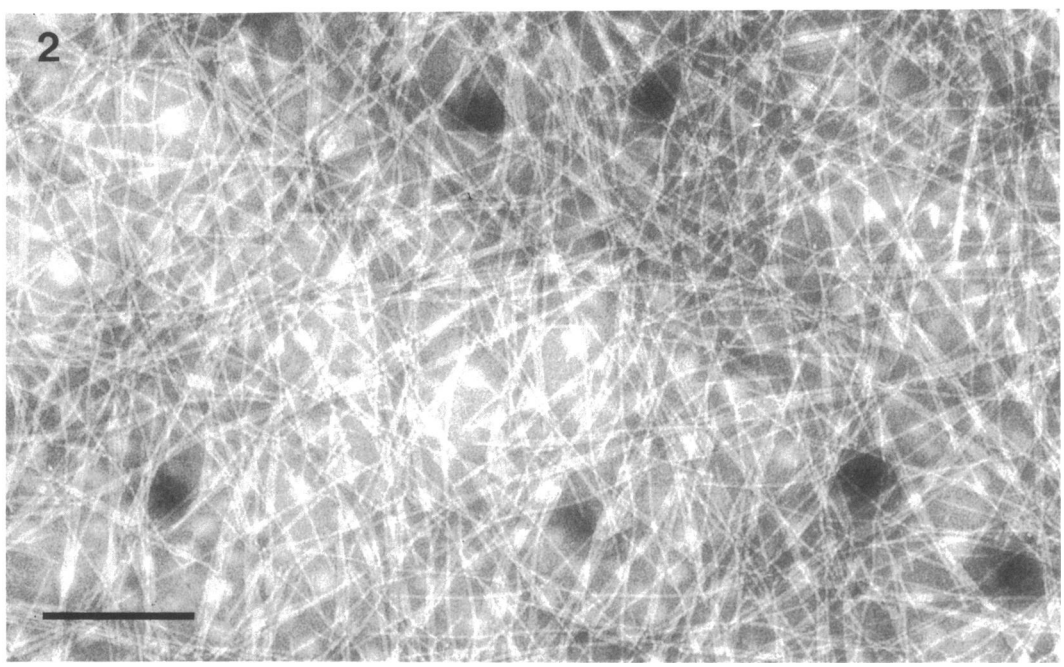


FIG. 1. *S. typhimurium SH6749 after growth for 48 h in Luria broth. The cell is surrounded by pili, which are 6 nm in width and over 1.1 μm in length. The bar represents 200 nm.*

FIG. 2. *Type 1 pili purified from S. typhimurium SH6749. The bar represents 200 nm.*

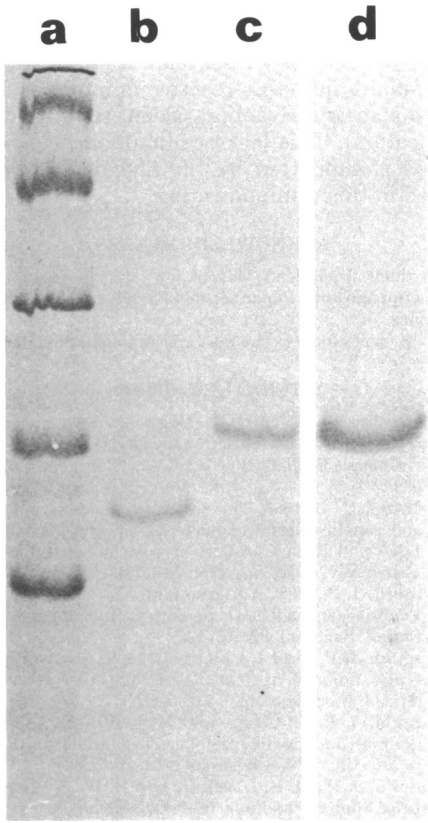


FIG. 3. *Comparison of type 1 pili from S. typhimurium SH6749 and E. coli 6013 (8, 9) in SDS-polyacrylamide gel electrophoresis. (a) Standard proteins (molecular weight): phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and α-lactalbumin (14,000). (b) Purified 6013 pili (15 μg). (c) Purified SH6749 pili, 15 μg and (d) 45 μg, run in the same gel.*

(3) have reported a similar agglutination pattern for various piliated species of *Salmonella*. The SH6749 pili agglutinated yeast cells more readily than horse or chicken erythrocytes (Table 2). α-Methyl-D-mannoside and D-mannose inhibited the agglutination of horse erythrocytes at concentrations below 4 μM, and D-fructose and yeast mannane inhibited agglutination at below 100 μM (Table 3). The agglutination was also sensitive, to some extent, to D-glucose, but not to D-galactose or sucrose. This inhibition pattern is similar to that in hemagglutination by *E. coli* K-12 type 1 pili (20) and by piliated *S. typhimurium* cells (16) and also to that in yeast cell agglutination by purified SH6749 pili (8). Old (16) studied the carbohydrate inhibition of hemagglutination by piliated *S. typhimurium* cells and showed that the α-configuration at the C-1 position in the mannopyranoside molecule and unmodified hydroxyl groups at C-2, C-3, C-4, and C-6 of the D-mannose molecule are required for maximum binding to the pilus protein. The results obtained with purified *Salmonella* pili in this study agree with these results and confirm the role of type 1 pili as *Salmonella* hemagglutinins.

Type 1 pili of *E. coli* are protein filaments of 7 nm in width and consist of one protein subunit with an apparent molecular weight of 17,000 (2, 20). Hydrophobic amino acids comprise 44 to 45% of the total amino acid composition (2, 20), and the isoelectric point of the pili has been

TABLE 1. *Amino acid compositions of the type 1 pili from S. typhimurium SH6749 and E. coli K-12*

Amino acid	Residues per molecule	
	S. typhimurium SH6749[a]	*E. coli* K-12 (20)
Aspartic acid	22	18
Threonine	25	20
Serine	23	9
Glutamic acid	19	16
Proline	11	2
Glycine	23	21
Alanine	34	34
Cystine (half)	ND[b]	2
Valine	16	14
Methionine	Tr[b]	0
Isoleucine	7	5
Leucine	12	14
Tyrosine	4	2
Phenylalanine	9	8
Lysine	9	4
Histidine	3	2
Arginine	4	2

[a] The proportion of hydrophobic amino acids (proline, alanine, valine, methionine, isoleucine, leucine, and phenylalanine) was 40.3%. The calculated molecular weight was 22,100.

[b] ND, Not detected; Tr, traces.

TABLE 2. *Agglutination titers of S. typhimurium SH6749 pilus with yeast cells and various erythrocytes*

Cells	Titer (μg/ml)[a]	
	−αMM[b]	+αMM[b]
Yeast cells	2.3	>150.0
Horse erythrocytes	12.4	>150.0
Chicken erythrocytes	24.4	>150.0
Bovine erythrocytes	>150.0[c]	>150.0
Sheep erythrocytes	>150.0	>150.0
Human erythrocytes	>150.0	>150.0

[a] Means of duplicate determinations from two batches of purified pili.

[b] −αMM and +αMM, In the absence and presence, respectively, of α-methyl-D-mannoside (25 mM).

[c] No agglutination at 150.0 μg/ml.

TABLE 3. *Inhibition by carbohydrates of horse erythrocyte agglutination by pili purified from S. typhimurium SH6749*

Carbohydrate	Concn[a]
α-Methyl-D-mannoside	2.5
D-Mannose	3.4
D-Fructose	54.2
Yeast mannane	86.7[b]
D-Glucose	8,670.0
D-Galactose	>50,000.0[c]
Sucrose	>50,000.0

[a] Minimal inhibitory concentration required to prevent complete hemagglutination. Pilus concentration was four times the agglutination titer.

[b] Calculated as D-mannose molecules (actual result, 15.6 μg/ml).

[c] No inhibition at 50 mM.

reported to be 3.9 (2). This is close to the isoelectric point of 4.1 obtained for the SH6749 pili. Duguid et al. (5, 7) reported that *E. coli* strains carrying type 1 pili agglutinated horse and fowl erythrocytes strongly, human erythrocytes moderately strongly, sheep erthrocytes weakly, and bovine erythrocytes not at all. However, purified *E. coli* type 1 pili did not agglutinate human erythrocytes (8, 20) and showed an agglutination pattern identical to that of the SH6749 pili (8).

The SH6749 pili differed from *E. coli* type 1 pili in the molecular weight of their subunits (Fig. 3): the SH6749 pilin had an apparent molecular weight of 21,000, and *E. coli* type 1 pilin was 17,000 (2, 9, 20). The calculated molecular weights were 22,100 (Table 1) and 17, 099 (20), respectively. Although both proteins have a high content of hydrophobic amino acids and lack methionine, the amino acid compositions of these proteins are somewhat different (Table 1). *S. typhimurium* SH6749 pili contained more serine, proline, and lysine than those of *E. coli* K-12 (2, 20). These differences are large enough to be significant even when based on separate studies. Despite these differences, type 1 pili of *S. typhimurium* SH6749 and *E. coli* K-12 are similar in their morphology and isoelectric point. The fact that they could be purified by the same procedure (9) is another indication of how they are related. We also found that the low-pH treatment used for the solubilization of type 1 pili of *E. coli* K-12 (15) is needed to solubilize the SH6749 pili for SDS-polyacrylamide gel electrophoresis analysis.

Duguid and Campbell (4) demonstrated that various piliated *Salmonella* strains were not agglutinated by anti-pilus antiserum against type 1 pili of an *E. coli* strain and vice versa. The anti-pilus antisera were prepared by absorbing antiserum against piliated whole bacteria by nonpiliated variants of the same strain. Using enzyme-linked immunosorbent assay, we have recently demonstrated that type 1 pili purified from two *E. coli* strains and *S. typhimurium* SH6749 are not cross-reactive immunologically (Korhonen and Svanborg Edén, submitted for publication). This lack of immunological relatedness is supported by the chemical data reported in this communication.

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