JOURNAL OF BACTERIOLOGY, Aug. 1991, p. 4765–4772
0021-9193/91/154765-08$02.00/0

Vol. 173, No. 15

Type 1 Fimbriae of *Salmonella enteritidis*

KARL-HEINZ MÜLLER,[1] S. KAREN COLLINSON,[2] TREVOR J. TRUST,[2] AND WILLIAM W. KAY[2]*

Department of Biochemistry and Microbiology and the Canadian Bacterial Diseases Network, University of Victoria, Victoria, British Columbia V8W 3P6,[2] and Department of Microbiology, University of British Columbia, Vancouver, British Columbia V6T 1W5,[1] Canada

Received 19 December 1990/Accepted 20 May 1991

Salmonella enteritidis **was previously shown to produce fimbriae composed of 14,000-molecular-weight (M_r) fimbrin monomers (J. Feutrier, W. W. Kay, and T. J. Trust, J. Bacteriol. 168:221–227, 1986). Another distinct fimbrial structure, comprising 21,000-M_r fimbrin monomers, has now been identified. These fimbriae are simply designated as SEF 14 and SEF 21, respectively (for *S. enteritidis* fimbriae and the M_r [in thousands] of the fimbrin monomer). A simple method for the purification of both structures was developed by using the different biochemical properties of these fimbriae. SEF 21 remained intact after being boiled in sodium dodecyl sulfate but readily dissociated into subunits of 21,000 M_r at pH 2.2. The overall amino acid composition and the N-terminal amino acid sequence of the SEF 21 fimbrin were distinct from those of SEF 14 but were virtually identical to the predicted sequence for type 1 fimbrin of *Salmonella typhimurium*. Immunoelectron microscopy of *S. enteritidis* clearly revealed fimbrial structures that reacted with immune serum specific to the 21,000-M_r fimbrin. Immune sera raised against this subunit were cross-reactive with type 1 fimbrins found in whole-cell lysates of *S. typhimurium*, *Salmonella illinois*, and *Salmonella cubana*. However, there was no cross-reaction with *Escherichia coli* type 1 fimbriae or with other fimbrins produced by *S. enteritidis*. Under certain growth conditions, *S. enteritidis* produced both SEF 14 and SEF 21. However, when *S. enteritidis* was grown at 30°C or lower, only the 21,000-M_r SEF 21 fimbrin could be detected. There was a direct correlation between mannose-sensitive hemagglutination and the presence of SEF 21.**

Pathogenic bacteria have a variety of virulence-associated surface structures: lipopolysaccharide, capsules, surface layers, flagella, and fimbriae (3, 6, 19). Fimbriae (or pili) are proteinaceous, fibrillar, surface appendages, each composed of about 10^3 helically arranged protein monomers (fimbrin), common to many bacteria, including several members of the family *Enterobacteriaceae* (4, 7, 8, 13, 38). Fimbriae are considered important, as they frequently have been shown to mediate adhesion to host tissues (12, 16, 25, 29, 39, 42, 46, 47, 51) and, in a few well-studied cases, to facilitate adhesion and colonization in the early stages of an infection (1, 22, 25, 42, 51). Although in some cases a correlation between the presence of fimbriae and bacterial virulence exists (24, 32, 42), in others the precise role of fimbriae in pathogenesis is uncertain, since little difference in virulence is seen between fimbriated and nonfimbriated mutants (2, 14). Fimbriae are also immunogenic and have therefore been used as successful vaccines in animals and are important targets for diagnostic tests.

Fimbriae of the *Enterobacteriaceae* are broadly divided into two major classes, mannose sensitive (MS) and mannose resistant, on the basis of the ability of the monosaccharide mannose to inhibit the adhesion of fimbriae to erythrocytes (hemagglutination) (38, 44). Type 1 fimbriae are about 7 nm in diameter, display a channelled appearance due to the arrangement of subunits around a hollow core, and mediate MS hemagglutination (38). They have been studied extensively in *Escherichia coli* (23, 24) but are also found in the invasive enteropathogen *Salmonella typhimurium* (8, 26, 41).

The incidence of nontyphoid salmonellosis in humans has been rising steadily in recent years (9, 43), and poultry-related infections due to *Salmonella enteritidis* are steadily increasing in North America. This organism has now become the predominant clinical isolate in the United Kingdom (10). The careful characterization of fimbrial structures, their requisite genes, and their distribution among the salmonellae are of obvious importance to the understanding of pathogenesis, to the development of genus- and species-specific diagnostic reagents, and perhaps to the design of oral vaccines for poultry and humans.

One virulent human isolate, *S. enteritidis* 27655 (17), displays MS hemagglutination and was shown to produce abundant fimbriae morphologically resembling type 1 (17). Purification and characterization of fimbriae from this strain indicated that, apart from limited N-terminal amino acid sequence homology, they differed significantly from other reported type 1 fimbriae of enteric bacteria (17), and hereafter we refer to these fimbriae as SEF 14. In the initial absence of evidence of other fimbriae in this strain, it was tentatively assumed that SEF 14 was responsible for the MS hemagglutination phenotype. Upon further investigation, we detected the presence of a second fimbria with definite type 1 morphology, hereafter referred to as SEF 21. In this communication, we report the first purification and characterization of MS type 1 fimbriae produced by *S. enteritidis* 27655 and show them to be morphologically and biochemically distinct from the previously described SEF 14 (17) as well as from the newly discovered SEF 17 (9a).

MATERIALS AND METHODS

Bacterial strains. *S. enteritidis* 27655, previously referred to as *S. enteritidis* 27655-3b (17) or *S. enteritidis* 3b (17, 36), was obtained from T. Wadström, University of Lund, Lund, Sweden. *S. enteritidis* 2-15, a Tn*phoA* Rif^r derivative of *S. enteritidis* 27655 defective in SEF 21 synthesis, was isolated by mating *E. coli* SM10(pRT733-Tn*phoA*) with a spontaneous Rif^r *S. enteritidis* isolate designated 3b-1 and selected as

* Corresponding author.

a blue colony on Luria broth agar containing kanamycin (50 μg/ml), rifampin (100 μg/ml), and 5-bromo-4-chloro-3-indolyl phosphate (167 μg/ml). SEF 21-negative clones were detected by Western blotting (immunoblotting) with immune serum against the SEF 21 fimbrin. Construction of the *E. coli* cos 48 recombinant expressing SEF 14 fimbrin was previously described (18). Other reference strains were *E. coli* A122, *Salmonella cubana* S211, *Salmonella illinois* S1093, and *S. typhimurium* ENB7, which also produce type 1 fimbriae. They were obtained from the collection of T. J. Trust. The bacteria were grown statically in liquid colonization factor antigen (CFA) medium (16) supplemented with 5 mM KH_2PO_4 and 12 mM Na_2HPO_4 or in Luria broth.

Purification of SEF 14 and SEF 21 fimbriae. *S. enteritidis* was grown statically in 2 liters of CFA medium at 37°C for 60 h, harvested by centrifugation, and suspended in 120 ml of 0.15 M ethanolamine buffer, pH 10.5 (17). Fimbriae were separated from the cells at room temperature by shearing them in a blender (model 909; Biospec Products, Bartlesville, Okla.) for three 1-min periods, after which cells and cellular debris were removed by centrifugation (12,000 × *g*, 15 min, 4°C). The supernatant (fraction 1) was centrifuged (100,000 × *g*, 1 h, 4°C) to remove membrane vesicles. This clarified supernatant (fraction 2) was dialyzed overnight against 10 mM Tris HCl (pH 7.5) containing 0.2% sodium dodecyl sulfate (SDS) to precipitate SEF 14. SEF 14 was pelleted by centrifugation (15,000 × *g*, 15 min, 4°C) to separate it from SEF 21, which remained in the supernatant (fraction 3). This fraction (120 ml) was concentrated to approximately 40 ml by dialysis against solid polyethylene glycol 20,000 followed by precipitation of SEF 21 with 160 ml of ice-cold acetone. The precipitated SEF 21 was recovered by centrifugation (15,000 × *g*, 20 min, 4°C). The pellet (fraction 4) was suspended in 4 ml of Laemmli sample buffer (glycerol, 10% [wt/vol]; 2-mercaptoethanol, 5% [vol/vol]; and SDS, 2% [wt/vol] in 0.125 M Tris HCl [pH 6.8] [27]) and boiled for 5 min to solubilize contaminants. The insoluble fimbriae, SEF 21, were then recovered by centrifugation (250,000 × *g*, 2 h, 4°C), boiled once more with sample buffer, and centrifuged again to yield pure SEF 21.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was conducted with a mini-slab gel apparatus (Hoeffer Scientific Instruments, San Francisco, Calif.) by the method of Laemmli (27). Whole cells or protein fractions were solubilized in SDS sample buffer, stacked in 4.5% polyacrylamide (100 V), and separated in 12.5% polyacrylamide (200 V). Samples containing SEF 21 to be analyzed by SDS-PAGE required treatment at 100°C for 5 min in SDS-PAGE sample buffer containing 0.2 M glycine (pH 2.2) before electrophoresis. Proteins were stained with Coomassie brilliant blue R-250.

Preparation of immune sera. A rabbit was initially immunized with SDS-PAGE-purified fimbrin from SEF 14 but was boosted with a native fimbrial preparation of SEF 14 that also contained SEF 21. This resulted in an immune serum (S1) containing antibodies against both fimbriae. Subsequent immune sera were generated to SEF 14 or SEF 21 preparations purified by SDS-PAGE, after which the fimbrin protein was electrophoretically transferred to nitrocellulose by using an LKB Multiphore II electrophoresis unit (LKB-Pharmacia, Broma, Sweden) at 0.8 mA/cm^2 for 90 min. Membrane-bound proteins were stained with amido black, and then the 14,000- or 21,000-M_r bands were excised, shredded, and emulsified in phosphate-buffered saline (PBS) containing Freund's complete adjuvant. Female New Zealand rabbits were immunized with an aliquot containing 100 to 200 μg of protein and then given a second injection 4 weeks later. Preimmune sera were collected 1 week prior to the first immunization.

Western blot analysis. Proteins separated on polyacrylamide gels were electrophoretically transferred to nitrocellulose as described above. The nitrocellulose sheet was immersed in blocking buffer (36) for at least 30 min, incubated in immune serum diluted 1:1,000 in 20 mM Tris HCl buffer (pH 7.5) containing 250 mM NaCl (Tris-NaCl) for 1 h, and then incubated with biotinylated goat anti-rabbit immunoglobulin G (Caltag Laboratories, San Francisco, Calif.) diluted 1:10,000 in Tris-NaCl for 1 h. This was followed by a final incubation with a streptavidin-horseradish peroxidase conjugate (Caltag) diluted 1:1,000 in Tris-NaCl. Enhanced chemiluminescence detection of protein bands was accomplished by a 1- to 2-min incubation with a chemiluminescent reagent consisting of 10 μl of 10.5-mg/ml luminol (Aldrich) in 100% *N*,*N*-dimethylformamide, 10 μl of 75-mg/ml 4 (*p*)-iodophenol (Aldrich) in 100% *N*,*N*-dimethylformamide, and 2.13 μl of 30% H_2O_2 in 10 ml of 100 mM Tris-HCl at pH 8.6 (50). The nitrocellulose membrane was covered with Saran Wrap and exposed for 30 to 60 s to a sheet of Kodak X-Omat K film (Eastman Kodak, Rochester, N.Y.). Alternatively, antiserum-treated blots were incubated with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Caltag) previously diluted 1:5,000 in blocking buffer, and stained with 5-bromo-4-chloro-3-indolyl phosphate (167 μg/ml) and Nitro Blue Tetrazolium (300 μg/ml), both from Sigma Chemical Co., St. Louis, Mo.

Amino acid composition and N-terminal amino acid sequence. A 10- to 20-μg sample of purified SEF 21 fimbriae was subjected to SDS-PAGE and transferred electrophoretically to Immobilon membranes (Millipore Corp., Bedford, Mass.). The transferred protein was stained with Coomassie brilliant blue R-250, and two samples were excised for sequence and composition analyses. One sample was sequenced directly (28) by using an Applied Biosystems model 470A gas-phase sequencer equipped with on-line phenylthiohydantoin analysis. The amino acid composition of the second sample was determined with an Applied Biosystems model 428 amino acid derivatizer-analyzer after gaseous HCl hydrolysis (165°C, 1 h).

Hemagglutination assays. *S. enteritidis* cells grown in static CFA broth were harvested by centrifugation and suspended in PBS to 0.5 × 10^9 to 1 × 10^9 cells per ml. This cell suspension (10 μl) was mixed with an equal volume of a 3% guinea pig erythrocyte suspension in PBS on a glass microscope slide and examined for agglutination. For the testing of mannose sensitivity, 5 μl of a 5% α-D-mannose solution was added to the erythrocyte suspension and gently mixed before bacterial cells were added.

Electron microscopy. *S. enteritidis* cells or fimbriae were deposited on a Formvar-coated grid and negatively stained with 1% ammonium molybdate containing 0.1% glycerol. For immunogold labeling of whole cells of *S. enteritidis* or fimbrial preparations, the grids were incubated on a drop of PBS containing 1% skim milk and then transferred to a drop of preimmune or immune serum (diluted 1/1,000 in PBS), washed in PBS, and finally floated on a drop of protein A–15-nm gold (Auroprobe; Pharmacia, Uppsala, Sweden) diluted 1/50 in PBS. The grids were rinsed, negative stained as described above, air dried, and observed with a Philips EM300 electron microscope operated at 60 or 80 kV.

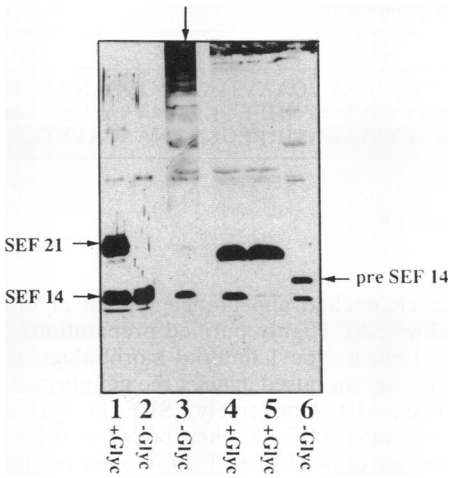

FIG. 1. Western blots of *S. enteritidis* cells and fimbriae. *S. enteritidis* 27655 cells were treated with Laemmli SDS-PAGE sample buffer (−Glyc) or sample buffer supplemented with glycine at pH 2.2 (+Glyc) prior to SDS-PAGE separation and analysis by Western blotting with a mixed immune serum raised against SEF 14 and SEF 21. Lanes: 1 and 2, semipurified fimbrial preparations; 3 and 4, lysates of cells grown at 37°C in static CFA medium; 5, cell lysates grown at 30°C in CFA medium; 6, cell lysates of the *E. coli* recombinant cos 48 containing SEF 14 (17). The vertical arrow indicates SEF 21, which does not migrate very far into the gel in the absence of acid treatment.

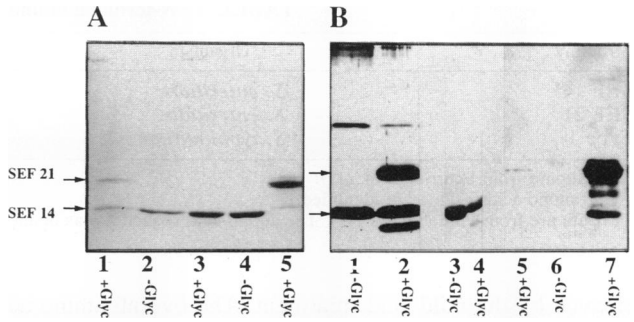

FIG. 2. Analysis of purified SEF 14 and SEF 21 from *S. enteritidis*. Both fimbriae were purified as described in Materials and Methods and were electrophoresed with (+Glyc) or without (−Glyc) prior glycine treatment, as noted in the legend to Fig. 1. (A) Coomassie-stained SDS-PAGE. Lanes: 1 and 2, fraction 1 (see Materials and Methods); 3 and 4, purified SEF 14; 5, purified SEF 21. (B) Western blot analysis. Lanes: 1 and 2, fraction 1; 3 and 4, purified SEF 14; 5, purified SEF 14; 6 and 7, purified SEF 21. Samples electrophoresed in lanes 1 to 4 were incubated with immune serum raised against SEF 14 and SEF 21 fimbriae; lanes 5 to 7 were incubated with an immune serum containing antibodies to SDS-PAGE-purified SEF 21 fimbrin.

RESULTS

Discovery of SEF 21. A nonspecific fimbrial antiserum, S1 (see Materials and Methods), was used in Western blot analysis of *S. enteritidis* cells grown in static CFA broth at 37°C and treated with Laemmli SDS-PAGE sample buffer. This antiserum revealed the previously described SEF 14 fimbrin subunit (17, 18) as well as a substantial amount of immunoreactive material which barely migrated into the gel (Fig. 1, lane 3). However, when similar samples briefly treated with SDS-PAGE sample buffer supplemented with glycine (pH 2.2) were analyzed on Western blots, the high-M_r immunoreactive material disappeared and a protein of approximately 21,000 M_r appeared (Fig. 1, lane 4). The electrophoretic migration of this 21,000-M_r protein was distinctly slower than that of pre-SEF 14 (Fig. 1, lane 6). The possibility that this 21,000-M_r protein represented the structural subunit of a second fimbrial type that required acid depolymerization to enter gels during electrophoresis was supported by the observation that it could be released from *S. enteritidis* cells by blending and was recovered in the supernatant after the cells were removed by centrifugation (Fig. 1, lanes 1 and 2). Furthermore, SDS-PAGE protein profiles of cells grown at 30°C indicated that these cells still produced the 21,000-M_r protein but that the SEF 14 fimbrin was no longer detected by Coomassie staining and was barely detected by Western blotting (Fig. 1, lane 5). However, these 30°C-grown cells still autoaggregated and formed pellicles in static broth culture to the same extent as cells grown at 37°C, suggesting the presence of some other aggregation-promoting surface structure.

Purification of SEF 21 and SEF 14. Based on the hydrophobicity of SEF 14 and the insolubility of SEF 21 in boiling SDS, a simple and rapid method for the simultaneous purification of both structures was devised. Fimbriae, as well as other surface material, were sheared off the cells in the presence of 0.15 M ethanolamine, which was found to maintain SEF 14 (17) and SEF 21 in solution. Both fimbrial types were detected by SDS-PAGE or Western blotting (Fig. 2A and B, lanes 1 and 2). Only trace amounts of fimbriae sedimented under the centrifugation conditions used to eliminate cellular debris. SEF 14 selectively precipitated during dialysis against Tris buffer containing 0.2% SDS, suggesting that SEF 14 was more hydrophobic than SEF 21 (Fig. 2A and B, lanes 3 and 4). After precipitated SEF 14 was removed, the supernatant, which contained predominantly SEF 21 as well as traces of SEF 14 and other minor proteins, was concentrated and treated with acetone. The resulting precipitate was boiled in SDS, which selectively solubilized contaminating proteins except SEF 21, which was simply recovered by high-speed sedimentation. SDS-PAGE analysis and Western blotting of purified SEF 21 suggested that significant amounts of SEF 14 and other contaminating proteins were present (Fig. 2A, lane 5, and B, lane 7). However, these proteins were immunoreactive only with specific immune serum generated to SDS-PAGE-purified SEF 21 fimbrin and did not react with immune serum raised specifically to the SEF 14 fimbrin (data not shown). Moreover, SEF 14 was not immunoreactive with the immune serum raised to gel-purified SEF 21 (Fig. 2B, lane 5). Since SEF 21 and SEF 14 fimbrins were clearly not immunologically cross-reactive, the additional proteins were likely acid degradation products derived from acid-treated SEF 21. SEF 21 also appeared as a doublet (Fig. 2A, lane 5, and B, lane 7), but this was also due to the acid treatment (see below).

Amino acid composition and N-terminal sequence of the SEF 21 fimbrin. Occasionally, purified SEF 21 was resolved by SDS-PAGE into two adjacent protein bands which could be separated by using a gradient gel. The N-terminal sequence analysis of each band revealed that the first 32 amino acids of the upper band were identical to those of the subunit of type 1 fimbriae of *S. typhimurium* predicted from the DNA sequence (8) (Table 1). The lower-molecular-weight band had the same N-terminal amino acid sequence with the exception that the acid-labile Asp-Pro bond was apparently

TABLE 1. N-terminal amino acid sequences of *Salmonella* fimbriae

Structure	Organism	Residue
SEF 14[a]	*S. enteritidis*	AGFVGNKAVVQAAVTIAAQNTTSANWSQDPGFTGPA
SEF 21	*S. enteritidis*	ADPTPVSVSGGTIHFEGKLVNAAA?VS??SAD[b]
Type 1[c]	*S. typhimurium*	ADPTPVSVSGGTIHFEGKLVNAACAVSTKSAD

[a] Data are from Feutrier et al. (17).
[b] ?, amino acid residue not identified.
[c] Data are from Purcell et al. (41). The amino acid sequence was deduced from the DNA sequence.

cleaved by the mild acid treatment. The overall amino acid composition was also very similar to that of type 1 fimbriae of *S. typhimurium* (Table 2). The N-terminal sequence and composition of the 21,000-M_r SEF 21 fimbrin were clearly distinct from those of the 14,000-M_r SEF 14 fimbrin described previously (17) (Tables 1 and 2).

Hemagglutination. We examined the comparative hemagglutination properties of various *S. enteritidis* strains grown under conditions which permit the production of SEF 14 or SEF 21 or both. When grown at 37°C, *S. enteritidis* 27655, like *S. typhimurium*, which also produces type 1 fimbriae, produced SEF 14 and SEF 21 and displayed a moderate MS hemagglutination. The extent of hemagglutination of both strains was lower than that of *E. coli* producing typical type 1 fimbriae. However, *S. enteritidis* exhibited the same level of MS hemagglutination after growth at 30 or 24°C, conditions which were nonpermissive for SEF 14 fimbrin synthesis (Fig. 1, lane 5). In addition, *S. enteritidis* 2-15, a Tn*phoA* mutant conditionally defective in SEF 21 fimbrin synthesis, was nonhemagglutinating when grown under conditions nonpermissive for SEF 21 production. Also, fimbrial preparations highly enriched with SEF 21 (fraction 3; see Materials and Methods) were strongly hemagglutinating (data not shown).

Electron microscopy of *S. enteritidis* SEF 21. Fimbrial preparations of SEF 21 which had been boiled in 0.1% SDS were uniform, were approximately 7 to 8 nm in diameter, and had a channelled appearance typical of other type 1 fimbriae (Fig. 3A). Highly purified preparations of SEF 21 also had a typical type 1 fimbrial morphology and reacted with immune serum raised against the gel-purified fimbrin of SEF 21 (Fig. 3B). Conversely, SEF 14 was completely disassembled into subunits when boiled in 0.1% SDS, and the morphology of purified SEF 14 was not as characteristic of type 1 as typified by SEF 21 (Fig. 3C). SEF 14 did not cross-react with immune serum raised specifically against gel-purified SEF 21 fimbrin but reacted strongly with immune serum raised against purified SEF 14 (Fig. 3D). Purified SEF 14 was not recognized by immune serum raised against native SEF 21.

Both SEF 14 and SEF 21 were detected on cells grown in static CFA medium at 37°C by immunogold labeling with specific immune sera (Fig. 4A and B). When cells were grown at 30°C, a third fibrillar structure (SEF 17) with a morphology clearly different from that of either SEF 14 or SEF 21 was seen (Fig. 4C).

The immune sera obtained by immunization with acid-depolymerized, gel-purified SEF 21 reacted readily and specifically with denatured SEF 21 fimbrin by Western blotting (Fig. 2B, lane 7). However, the reaction with the intact structure as visualized by immunogold staining of electron micrographs was relatively weak (Fig. 4B).

Distribution of SEF 21. Several other *Salmonella* strains as well as an *E. coli* strain producing classical type 1 fimbriae

TABLE 2. Comparison of amino acid compositions of bacterial fimbrins

Amino acid	No. of residues per fimbrin				
	S. enteritidis SEF 14 (M_r 14,000)[a]	*S. enteritidis* SEF 21 (M_r 21,000)	*S. typhimurium* type 1 (M_r 22,100)[b]	*E. coli* type 1 (M_r 15,700)[a]	*Klebsiella pneumoniae* type 1 (M_r 21,500)[a]
Asn/Asp	13	25	22	19	27
Thr	17	25	25	19	25
Ser	11	16	23	10	14
Gln/Glu	14	14	19	13	17
Pro	8	12	11	2	5
Gly	22	16	23	16	18
Ala	21	33	34	31	30
Val	13	16	16	15	18
Met	0	3	tr	0	2
Ile	5	8	7	4	8
Leu	4	13	12	10	13
Tyr	2	4	4	2	6
Phe	7	8	9	7	6
His	1	1	3	2	2
Lys	4	8	9	3	8
Arg	2	4	4	3	5
Cys	0	ND[c]	0	2	4
Trp	1	ND	0	0	1

[a] Data are from Feutrier et al. (17).
[b] Data are from Korhonen et al. (26).
[c] ND, not determined.

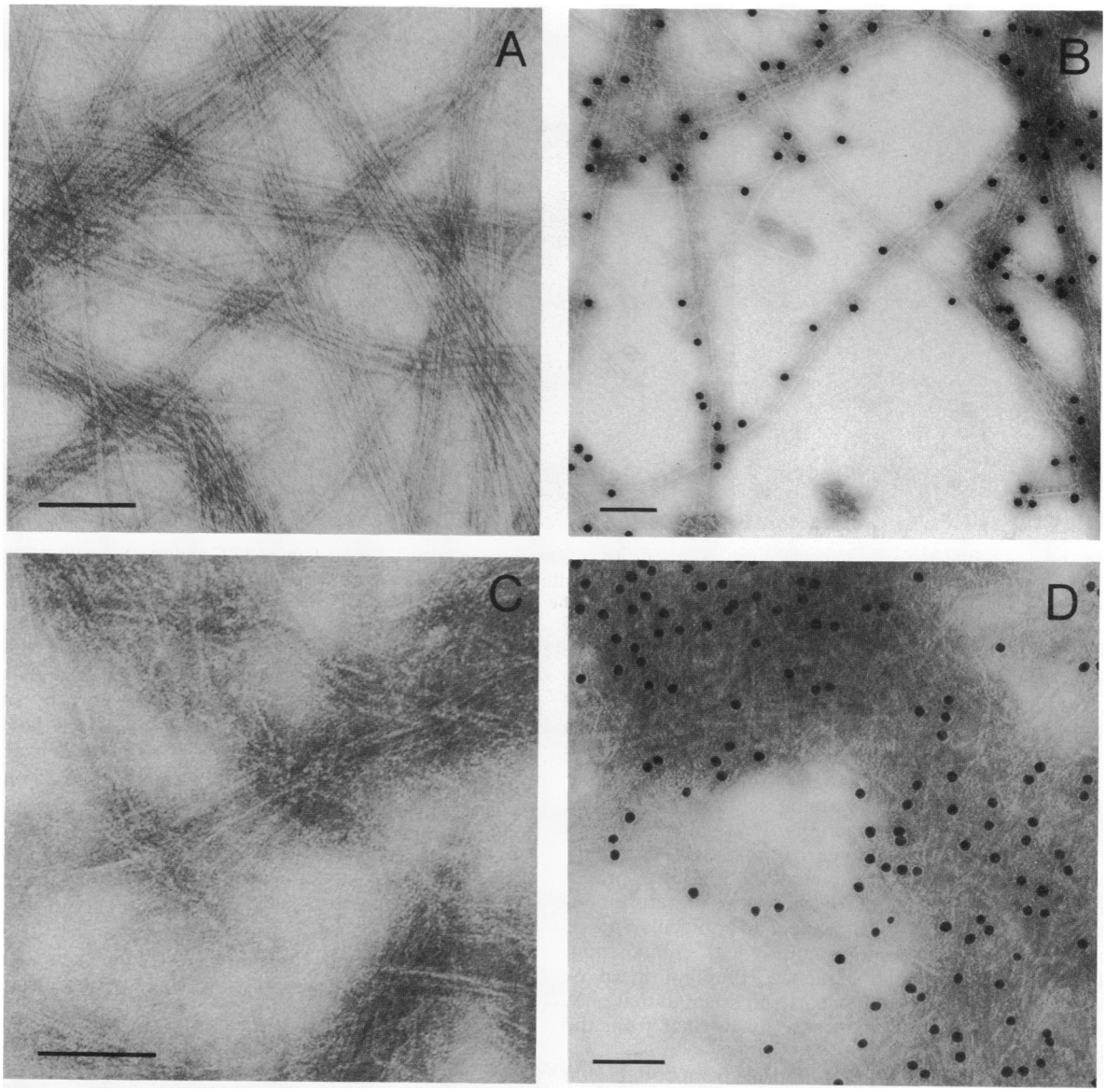

FIG. 3. Electron microscopy of negatively stained and immunogold-labeled fimbrial preparations. (A) SEF 21 obtained from a purified fimbrial preparation after solubilization of SEF 14 in boiling 0.1% SDS; (B) purified SEF 21 reacted with immune serum raised against gel-purified SEF 21 fimbrin; (C) purified SEF 14; (D) purified SEF 14 reacted with immune serum against SEF 14. Bars, 100 nm.

were also analyzed by Western blotting with the specific immune serum raised against SEF 21 fimbrin. All *Salmonella* strains tested produced fimbrin proteins which were immunologically cross-reactive. This result is consistent with the close similarity in N-terminal and overall amino acid compositions of the *S. typhimurium* type 1 fimbriae (17, 41). However, the type 1 fimbrin of *E. coli* (17,000 M_r) did not react with immune serum raised to SEF 21 fimbrin (Fig. 5, lane 6). None of the strains tested other than *S. enteritidis* were cross-reactive with specific immune serum raised against the SEF 14 fimbrin. The faint 14,000-M_r band from *S. typhimurium* ENB7 was likely an acid degradation product of type 1 fimbrin, since it was seen only in samples treated with glycine (pH 2.2) when sufficient sample was applied to the gel (Fig. 5, lane 3). Moreover, immune serum to SEF 14 did not recognize this band on Western blots (data not shown).

DISCUSSION

In addition to the previously described SEF 14 with a subunit M_r of 14,000 (17), a typical type 1 fimbria of *S. enteritidis*, designated SEF 21, has been identified, purified, and characterized. The purification procedure described in this study is straightforward, allows the simultaneous purification of both fimbriae, and results in preparations of high purity based on biochemical and immunological criteria. Initially, SEF 21 escaped our attention (17) because of the insolubility of these fimbriae in SDS-PAGE sample buffer containing SDS. Fortuitously, immune sera raised against

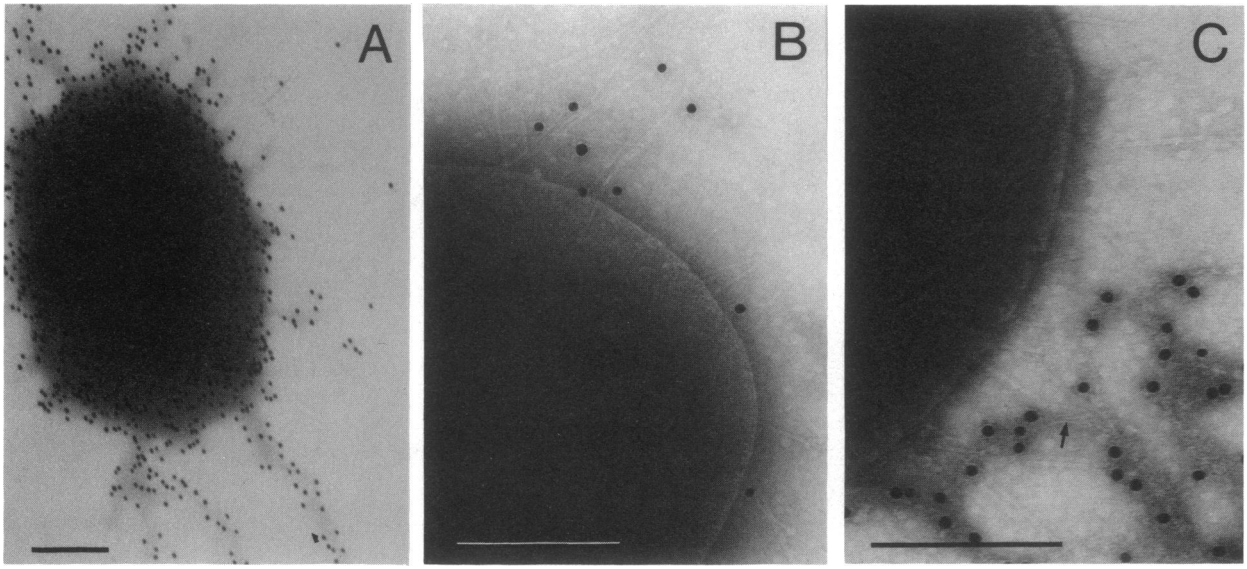

FIG. 4. Immunogold labeling of fimbriae on *S. enteritidis*. (A) Cells grown in static CFA medium at 37°C and reacted with immune serum against purified SEF 14; (B) cells grown as in panel A but reacted with immune serum raised to gel-purified SEF 21; (C) cells grown in static CFA medium at 30°C and reacted with an immune serum against the third fibrillar structure (SEF 17) (9a). Bars, 250 nm.

crude fimbrial preparations containing SEF 14 and SEF 21 recognized both fimbrin subunits in Western blots but only if acid treatment preceded electrophoresis. It is important to emphasize the necessity for acid depolymerization of SEF 21, which under these weak-acid conditions is probably little more than the titration of acidic amino acid residues critical to fimbrin subunit charge interactions. It was demonstrated some time ago that acid depolymerization of *E. coli* type 1 fimbriae was required for gel permeation (15, 33), a procedure which thereafter became routine in the literature concerning *E. coli* fimbriae. With respect to molecular mass, immunological cross-reactivity, amino acid composition, and N-terminal amino acid sequence, the fimbrin of *S. enteritidis* SEF 21 appears to be nearly identical to that of *S. typhimurium* (17, 26, 41) but is entirely distinct from the other fimbriae of *S. enteritidis*.

It is interesting that while type 1 fimbriae from both *E. coli* and *S. enteritidis* depolymerize in acid and presumably share approximately 50% DNA sequence homology by analogy to the DNA sequence of *S. typhimurium* (41), they are not immunologically cross-reactive. Perhaps conserved sequences encode structural interaction sites and nonhomologous sequences encode surface-exposed residues. Polyclonal immune serum raised against denatured SEF 14 fimbrin does not cross-react with SEF 21 fimbrin, suggesting that if sequences encoding internal residues are common, they do not give rise to immunologically common epitopes. Also, polyclonal anti-SEF 21 fimbrin antibody reacts extremely poorly with native fimbriae (Fig. 3B), suggesting that most surface epitopes on SEF 21 are conformationally dependent.

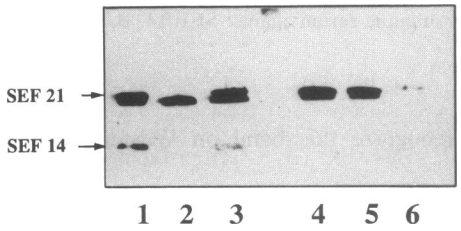

FIG. 5. Immunological comparison of type 1 fimbriae. Western blots of cell lysates from several bacterial strains after growth in static CFA or Luria broth medium at 37°C. Lanes: 1, *S. enteritidis* 27655; 2, *S. typhimurium* ENB7; 3, *S. typhimurium* ENB7 grown in static Luria broth medium at 37°C; 4, *S. cubana* S211; 5, *S. illinois*; 6, *E. coli* A122. The whole-cell lysates of these strains were subjected to SDS-PAGE and electrophoretically transferred onto nitrocellulose filters. The filters were incubated with immune serum containing antibodies against SEF 14 and SEF 21 fimbriae. Detection of cross-reactive bands was accomplished by enhanced chemiluminescence catalyzed by horseradish peroxidase as described in Materials and Methods.

The MS hemagglutination property of *S. enteritidis* 27655 appears to be due to the presence of SEF 21, since this property remains in cells grown below 30°C, temperatures not conducive to the synthesis of SEF 14. In addition, MS hemagglutination is absent in a Tn*phoA* mutant grown under conditions in which SEF 21 is missing. In a recent, more extensive hemagglutination survey with SEF 14 (49), a specific hemagglutinating role for SEF 14 could not be demonstrated, a result in agreement with our contention that SEF 21 is responsible for the MS phenotype of *S. enteritidis*. The true tissue receptor specificities of SEF 14 and SEF 21 and the role, if any, of these fimbriae in pathogenesis remain to be determined.

In addition to the SEF 14 and SEF 21 described here, *S. enteritidis* produces at least one other fibrillar structure (9a). None of these three fimbriae are immunologically related, nor are they similar in their N-terminal amino acid sequences or overall amino acid compositions. They can also be easily distinguished by the M_rs of their subunits as well as by their morphologies. SEF 21 is well defined and of uniform diameter, whereas SEF 14 is finer (approximately 3 to 5 nm in diameter) and less well defined when observed either on whole cells or in purified fimbrial preparations (Fig. 3). It is

important to clearly differentiate these fimbriae, since there is confusion regarding them (49).

S. enteritidis 27655 is apparently capable of producing a multiplicity of fibrillar structures, a characteristic of some other successful pathogens (30, 31, 37, 45, 48). To avoid confusion, we devised a simple nomenclature (used here for *S. enteritidis*) based on the genus and species in which the fimbriae are found and the M_rs of the different fimbrin subunits. The assignment of the requisite genotypes responsible for the synthesis and assembly of each fimbrial type will hereafter be *sef* for SEF 14 and *fim* for SEF 21, which permits the distinction of genes for each fimbrial type and is still in keeping with the nomenclature for type 1 fimbriae of *S. typhimurium* and *E. coli*. Previously (18, 36), we referred to the fimbrin structural gene of SEF 14 as *fimA*, but it will hereafter be referred to as *sefA*. This system is flexible and allows us to assign common names and genes for newly discovered fimbriae, such as the newly described third fimbrial structure of *S. enteritidis* (Fig. 4C) (9a).

The production of at least two (SEF 14 and SEF 21) of the three fibrillar structures is now known to be regulated by a variety of environmental factors (36a). Since *S. enteritidis* is capable of colonizing different hosts and occupying different niches within a host (40), the capacity to produce several environmentally regulated yet antigenically distinct fimbriae may contribute to this pathogen's current success. Presumably, *S. enteritidis* deploys a responsive and sophisticated regulatory system that ensures survival in the different, often hostile environments encountered during the course of an infection. It has been demonstrated, in *S. typhimurium*, for example, that several regulatory networks seem to be involved in virulence (5, 11, 20, 21, 34, 35). So far, there is little information on the nature of regulatory networks involved in the production of the fimbriae of *S. enteritidis*. Important questions concerning fimbrial genetic organization, common components, and roles in adhesion and virulence remain.

ACKNOWLEDGMENTS

This research was supported by a grant to W.W.K. from the British Columbia Health Care Research Foundation and by a Programme Grant to T.J.T. and W.W.K. from the Medical Research Council. S.K.C. was supported by a Natural Science and Engineering Research Council of Canada postdoctoral fellowship.

The technical assistance of Sharon Clouthier is gratefully acknowledged, as is that of S. Kielland for N-terminal amino acid sequence and composition analyses. We also thank J. L. Doran and L. Emödy for helpful advice and editorial comments.

REFERENCES

1. **Aronson, M., O. Medalia, L. Schori, D. Mirelman, N. Sharon, and I. Ofek.** 1979. Prevention of colonization of the urinary tract of mice with *Escherichia coli* by blocking of bacterial adherence with methyl α-D-mannopyranoside. J. Infect. Dis. **139**:329–332.
2. **Bloch, C. A., and P. Orndorff.** 1990. Impaired colonization by and full invasiveness of *Escherichia coli* K1 bearing a site-directed mutation in the type 1 pilin gene. Infect. Immun. **58**:275–278.
3. **Brubaker, R. R.** 1985. Mechanisms of bacterial virulence. Annu. Rev. Microbiol. **39**:21–50.
4. **Buchanan, K., S. Falkow, R. A. Hull, and S. I. Hull.** 1985. Frequency among *Enterobacteriaceae* of the DNA sequences encoding type 1 pili. J. Bacteriol. **162**:799–803.
5. **Buchmeier, N. A., and F. Heffron.** 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science **248**:730–732.
6. **Carsiotis, M., D. L. Weinstein, H. Karch, I. A. Holder, and A. D. O'Brien.** 1984. Flagella of *Salmonella typhimurium* are a virulence factor in infected C57BL/6J mice. Infect. Immun. **46**:814–818.
7. **Clegg, S., and G. F. Gerlach.** 1987. Enterobacterial fimbriae. J. Bacteriol. **169**:934–938.
8. **Clegg, S., B. K. Purcell, and J. Pruckler.** 1987. Characterization of genes encoding type 1 fimbriae of *Klebsiella pneumoniae*, *Salmonella typhimurium*, and *Serratia marcescens*. Infect. Immun. **55**:281–287.
9. **Cohen, M. L., and R. V. Tauxe.** 1986. Drug resistant *Salmonella* in the United States: an epidemiological perspective. Science **234**:964–969.

9a. **Collinson, S. K., L. Emödy, K.-H. Müller, T. J. Trust, and W. W. Kay.** 1991. Purification and characterization of thin, aggregative fimbriae from *Salmonella enteritidis*. J. Bacteriol. **173**:4773–4781.

10. **Cooke, E. M.** 1990. Epidemiology of foodborne illness: UK. Lancet **336**:790–793.
11. **Curtiss, R., III, and S. M. Kelly.** 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. **55**:3035–3043.
12. **Doig, P., P. A. Sastry, R. S. Hodges, K. K. Lee, W. Paranchych, and R. T. Irvin.** 1990. Inhibition of pilus-mediated adhesion of *Pseudomonas aeruginosa* to human buccal epithelial cells by monoclonal antibodies directed against pili. Infect. Immun. **58**:124–130.
13. **Dorman, C. J., S. Chatfield, C. F. Higgins, C. Hayward, J. P. Duguid, and D. C. Old.** 1980. Adhesive properties of *Enterobacteriaceae*, p. 185–227. *In* E. H. Beachey (ed.), Bacterial adherence. Chapman and Hall, Ltd., London.
14. **Duguid, J. P., M. R. Darekar, and D. W. F. Wheater.** 1976. Fimbriae and infectivity in *Salmonella typhimurium*. J. Med. Microbiol. **9**:459–473.
15. **Eshdat, Y., F. J. Silverblatt, and N. Sharon.** 1981. Dissociation and reassembly of *Escherichia coli* type 1 pili. J. Bacteriol. **148**:308–314.
16. **Evans, D. G., D. J. Evans, Jr., and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18**:330–337.
17. **Feutrier, J., W. W. Kay, and T. J. Trust.** 1986. Purification and characterization of fimbriae from *Salmonella enteritidis*. J. Bacteriol. **168**:221–227.
18. **Feutrier, J., W. W. Kay, and T. J. Trust.** 1988. Cloning and expression of *Salmonella enteritidis* fimbrin genes in *Escherichia coli*. J. Bacteriol. **170**:4216–4222.
19. **Finlay, B. B., and S. Falkow.** 1989. Common themes in microbial pathogenicity. Microbiol. Rev. **53**:210–230.
20. **Finlay, B. B., F. Heffron, and S. Falkow.** 1989. Epithelial cell surfaces induce *Salmonella* proteins required for bacterial adherence and invasion. Science **243**:940–943.
21. **Groisman, E. A., and M. H. Saier, Jr.** 1990. *Salmonella* virulence: new clues to intramacrophage survival. Trends Biochem. Sci. **15**:30–33.
22. **Hohmann, A. W., G. Schmidt, and D. Rowley.** 1978. Intestinal colonization and virulence of *Salmonella* in mice. Infect. Immun. **22**:763–770.
23. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33**:933–938.
24. **Ihawi, T., Y. Abe, M. Nakao, A. Imada, and K. Tsuchiya.** 1983. Role of type 1 fimbriae in the pathogenesis of ascending urinary tract infection induced by *Escherichia coli* in mice. Infect. Immun. **39**:1307–1315.
25. **Jones, G. W., and L. A. Richardson.** 1981. The attachment to and invasion of HeLa cells by *Salmonella typhimurium*: the contribution of mannose-sensitive and mannose-resistant haemagglutinating activities. J. Gen. Microbiol. **127**:361–370.
26. **Korhonen, T. K., K. Lounatmaa, H. Ranta, and N. Kuusi.** 1980. Characterization of type 1 pili of *Salmonella typhimurium*. J. Bacteriol. **144**:800–805.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the

assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
28. **LeGendre, N., and P. Matsudaira.** 1988. Direct protein microsequencing from Immobilon™-P transfer membrane. Biotechniques **6**:154–159.
29. **Lindquist, B. L., E. Lebenthal, P.-C. Lee, M. W. Stinson, and J. M. Merrick.** 1987. Adherence of *Salmonella typhimurium* to small-intestinal enterocytes of the rat. Infect. Immun. **55**:3044–3050.
30. **Lund, B., F. P. Lindberg, M. Båga, and S. Normark.** 1985. Globoside-specific adhesins of uropathogenic *Escherichia coli* are encoded by similar *trans*-complementable gene clusters. J. Bacteriol. **162**:1293–1301.
31. **Lund, B., B.-I. Marklund, N. Strömberg, F. Lindberg, K.-A. Karlsson, and S. Normark.** 1988. Uropathogenic *Escherichia coli* can express serologically identical pili of different receptor binding specificities. Mol. Microbiol. **2**:255–263.
32. **McCormick, B. A., D. P. Franklin, D. C. Laux, and P. S. Cohen.** 1989. Type 1 pili are not necessary for colonization of the streptomycin-treated mouse large intestine by type 1-piliated *Escherichia coli* F-18 and *E. coli* K-12. Infect. Immun. **57**:3022–3029.
33. **McMichael, J. C., and J. T. Ou.** 1979. Structure of common pili from *Escherichia coli*. J. Bacteriol. **138**:969–975.
34. **Miller, J. F., J. J. Mekalanos, and S. Falkow.** 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science **243**:916–922.
35. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86**: 5054–5058.
36. **Müller, K.-H., T. J. Trust, and W. W. Kay.** 1989. Fimbriation genes of *Salmonella enteritidis*. J. Bacteriol. **171**:4648–4654.
36a. **Müller, K.-H., T. J. Trust, and W. W. Kay.** Unpublished data.
37. **Nowicki, B., M. Rhen, V. Väisänen-Rhen, A. Pere, and T. K. Korhonen.** 1984. Immunofluorescence study of fimbrial phase variation in *Escherichia coli* KS71. J. Bacteriol. **160**:691–695.
38. **Paranchych, W., and L. S. Frost.** 1988. The physiology and biochemistry of pili. Adv. Microb. Physiol. **29**:53–114.
39. **Parkkinen, J., A. Ristimäke, and B. Westerlund.** 1989. Binding of *Escherichia coli* S fimbriae to cultured human endothelial cells. Infect. Immun. **57**:2256–2259.
40. **Popiel, I., and P. C. B. Turnbull.** 1985. Passage of *Salmonella enteritidis* and *Salmonella thompson* through chick ileocecal mucosa. Infect. Immun. **47**:786–792.
41. **Purcell, B. K., J. Pruckler, and S. Clegg.** 1987. Nucleotide sequence of the genes encoding type 1 fimbrial subunits of *Klebsiella pneumoniae* and *Salmonella typhimurium*. J. Bacteriol. **169**:5831–5834.
42. **Reid, G., and J. D. Sobel.** 1987. Bacterial adherence in the pathogenesis of urinary tract infection: a review. Rev. Infect. Dis. **9**:470–487.
43. **Rodrigue, D. C., R. V. Tauxe, and B. Rowe.** 1990. International increase in *Salmonella enteritidis*: a new pandemic? Epidemiol. Infect. **105**:21–27.
44. **Salit, I. E., and E. C. Gotschlich.** 1977. Hemagglutination by purified type 1 *Escherichia coli* pili. J. Exp. Med. **146**:1169–1181.
45. **Sareneva, T., H. Holthöfer, and T. K. Korhonen.** 1990. Tissue-binding affinity of *Proteus mirabilis* fimbriae in the human urinary tract. Infect. Immun. **58**:3330–3336.
46. **Silverblatt, F. J.** 1974. Host-parasite interaction in the rat renal pelvis. A possible role for pili in the pathogenesis of pyelonephritis. J. Exp. Med. **140**:1696–1711.
47. **Silverblatt, F. J., and I. Ofek.** 1978. Influence of pili on the virulence of *Proteus mirabilis* in experimental hematogenous pyelonephritis. J. Infect. Dis. **138**:664–667.
48. **Tennent, J. M., S. Hultgren, B.-I. Marklund, K. Forsman, M. Göranson, B. E. Uhlin, and S. Normark.** 1990. Genetics of adhesin expression in *Escherichia coli*, p. 79–110. *In* B. H. Iglewski and V. L. Clark (ed.), Molecular basis of bacterial pathogenesis. Academic Press, Inc., New York.
49. **Thorns, C. J., M. G. Sojka, and D. Chasey.** 1990. Detection of a novel fimbrial structure on the surface of *Salmonella enteritidis* by using a monoclonal antibody. J. Clin. Microbiol. **28**:2409–2414.
50. **Thorpe, G. H. G., and L. J. Kricka.** 1986. Enhanced chemiluminescent reactions catalyzed by horseradish peroxidase. Methods Enzymol. **133**:331–353.
51. **Yamamoto, T., A. Ariyoshi, and K. Amako.** 1985. Fimbria-mediated adherence of *Serratia marcescens* strain US5 to human urinary bladder surface. Microbiol. Immunol. **29**:677–681.