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Serological Study of Bacterial Flagellar Hooks

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Bacterial hooks were partially purified from flagella isolated from *Salmonella* SJ25, by treatment with heat to depolymerize flagellar filaments and with *n*-butanol and calcium chloride to remove membranes. Antihook serum was obtained from a rabbit inoculated with a preparation of hooks. The serum contained antibodies directed against the flagellar filament and cell membrane. These antibodies could be removed from the serum by absorption with purified flagellar filaments and cells of a nonflagellated mutant strain. It was shown by electron microscopy that anti-SJ25-hook antibody reacts with hooks from a number of strains of *Salmonella* which differed from SJ25 in H and O antigens, flagellar shape, and motility. Hooks possessed by various strains of *Salmonella* have a common antigenicity. In addition, anti-SJ25-hook cross-reacted with hooks from *Escherichia coli* W3110 but did not react at all which those from strains of *Serratia*, *Proteus*, *Aerobacter*, and *Klebsiella*. It is well known that bacteria stop moving upon addition of antiflagella serum to the medium. However, the addition of purified antihook was found to have little effect on motility. At physiological ionic strength and pH, flagellin (*Salmonella*) can polymerize into flagellar filaments only in the presence of seeds. It was shown that a crude preparation of hooks was able to initiate in vitro polymerization of flagellin.

A bacterial flagellum is comprised of at least two parts, a proximal hook and a main helical filament. In this paper the latter portion of a flagellum will be refered to as a flagellar filament or a filament. This portion is known to consist of one kind of protein, flagellin. The filament is disintegrated into flagellin molecules under various extreme conditions of environment, whereas the hook is relatively stable and maintains its characteristic appearance. By virtue of this difference, several investigators have been able to isolate hooks from flagella (1, 4-6). The two portions have entirely different antigenic specificities, indicating that they are composed of different kinds of proteins (7, 16). Surfaces of normal bacteria are associated with O and H antigens. The former antigen is distributed over the cell envelope of each organism; the latter antigen is contained in flagellin and is therefore located along the length of each flagellar filament. Dimmitt and Simon reported that their preparations of *Bacillus* flagellar hook were immunochemically specific, that is, free from O and H antigens (7).

We attempted to isolate hooks from motile *Salmonella* strain SJ25 to prepare antihook antibody and to examine whether hooks from strains other than SJ25 are immunochemically homologous. In this paper it is shown that 18 *Salmonella* strains used, including flagella-shape mutants, paralyzed (nonmotile) mutants and strains different in H- and O-antigen types from strain SJ25, and produced hooks immunochemically homologous to SJ25. In addition, hooks of an *Escherichia* strain also are homologous to strain SJ25, whereas those of *Serratia*, *Proteus*, *Aerobacter*, and *Klebsiella* strains are not.

MATERIALS AND METHODS

Bacterial strains. Table 1 shows the *Salmonella* strains used in this study. Hooks were isolated from strain SJ25, which is a motile phase-2 monophasic strain (H-antigen type *1,2*) obtained by transduction of the phase-2 structural gene for flagellin (*H2-1,2*) from *S. typhimurium* TM2 to *S. abortusequi* SL23. Eighteen strains other than SJ25 were used to examine whether their flagellar hooks react with anti-SJ25-hook antibody. In these strains, no. 2 is a derivative of strain SL23 given the phase-1 structural gene *H1-i* of TM2 by transduction; no. 3 and no. 4 are parent strains of both strains SJ25 and SJ670 (no. 2); no. 1, 5, and 6 are flagellar-shaped mutants (12, 13);

TABLE 1. *The Salmonella strains used in this study*

No.	Serotype	Strain	Flagellar antigen		Flagellar shape	Motility	O-antigen group
			Phase-1	Phase-2			
1	Recombinant of no. 3 and no. 4	SJ25		1,2	Normal	+	B
2	Recombinant of no. 3 and no. 4	SJ670	*i*		Normal	+	B
3	abortusequi	SL23		*e,n,x*	Normal	+	B
4	typhimurium	TM2	*i*	*1,2*	Normal	+	B
5	abortusequi	SJ30		*e,n,x*	Curly	−	B
6	typhimurium	SJ814		*1,2*	Straight	−	B
7	typhimurium	SJ597	*i*	*1,2*	Normal	−	B
8	typhimurium	SJ417	*i*	*1,2*	Normal	−	B
9	typhimurium	SJ608	*i*	*1,2*	Normal	−	B
10	paratyphi A	SW701	*a*	*1,5*	Normal	+	A
11	derby	SJ16	*fg*		Normal	+	B
12	abony	SW803	*b*	*e,n,x*	Normal	+	B
13	oranienburg	SJ1	*mt*		Normal	+	C
14	hanburg	SJ6	*gt*		Normal	+	D
15	dublin	SJ11	*gP*		Normal	+	D
16	seftenberg	SJ7	*gst*		Normal	+	E
17	wodington	SJ66	*lw*	*z*	Normal	+	G
18	wichita	SJ67	*d*	*z37*	Normal	+	G
19	poona	SJ4023	*z*	*1,6*	Normal	+	G
20	abortusequi	SJW319					B

and no. 7, 8, and 9 are paralyzed mutants of either one of the parent strains (9). Strain no. 20 produces no flagella but has the identical O antigen as SJ25 (this strain was used to purify anti-SJ25-hook serum). The remaining 10 strains (no. 10 to 19) are representative of various *Salmonella* serotypes different in O-antigen types, H-antigen types, or both, from SJ25. In addition, *E. coli* W3110, *S. marcescens* SEJ3, *P. vulgaris* IFO5988, *A. aerogenes* AJ5, and *Klebsiella* sp. KJ3 were used to examine reactivities of their hooks towards anti-SJ25-hook antibody. The latter two strains were obtained from Y. Dohi of the Institute of Infectious Diseases, Osaka University, Osaka, Japan. The motility of the cell was examined on a semisolid medium containing 0.3% agar and 8% gelatin in broth before using for serological study.

Preparation of hooks. Flagella-carrying hooks were isolated and partially purified from a large-scale culture of strain SJ25 by the method described previously (2). About 0.5 g of the partially purified material, suspended in 50 ml of a solvent containing 0.15 M NaCl and 0.01 M phosphate buffer, pH 7.0, (standard solvent), was heated at 65 C for 5 min. This treatment is known to bring about almost complete depolymerization of flagellar filaments into monomeric flagellin (2). The product of heat treatment was centrifuged at 78,000 × *g* for 1 h (high-speed centrifugation) and separated into supernatant fluid and sedimented material. The former was reserved for preparation of reconstituted flagellar filaments (see below). The latter was rich in hooks, although it was contaminated with large amounts of membrane-like material and short flagellar filament which had not completely been depolymerized by heat treatment. Therefore, it was suspended in 50 ml of solvent containing 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.2) and 3 mM $CaCl_2$ and heated again at 65 C for 3 min. A large amount of membrane-like material became insoluble during this treatment and therefore could be removed from the suspension by centrifugation at 15,000 × *g* for 15 min (low-speed centrifugation). Hooks were sedimented by high-speed centrifugation and suspended in 10 ml of 0.01 M Tris-hydrochloride buffer (pH 8.2). Next, the suspension was vigorously shaken with the addition of 4 ml of *n*-butanol for 2 min. After phase separation, a small amount of hooks was found in the aqueous phase. The butanol phase was removed and dialyzed against a large volume of 0.01 M Tris-hydrochloride buffer (pH 8.2). This dialysis gave rise to white aggregates which were removed from the solution by low-speed centrifugation. Finally, hooks were sedimented by high-speed centrifugation and resuspended in an appropriate small volume of the same buffer. The total protein amounted to 0.1 to 0.2% of the starting material (partially purified flagella). Electron micrographs of a preparation of hooks are given in Fig. 1. Many hooks are shorter than the intact ones: this may be due to breakage during purification.

Purification of flagellar filaments. At the earlier stage in the preparation of hooks, a solution containing monomeric flagellin was obtained. To this solution was added solid ammonium sulfate with gentle stirring to give a final concentration of 0.7 M, and then it was left at room temperature for a few hours. During this period, the monomeric flagellin repolymerized into flagellar filaments which were sedimented by high-speed centrifugation and suspended in 50 ml of standard solvent. For further

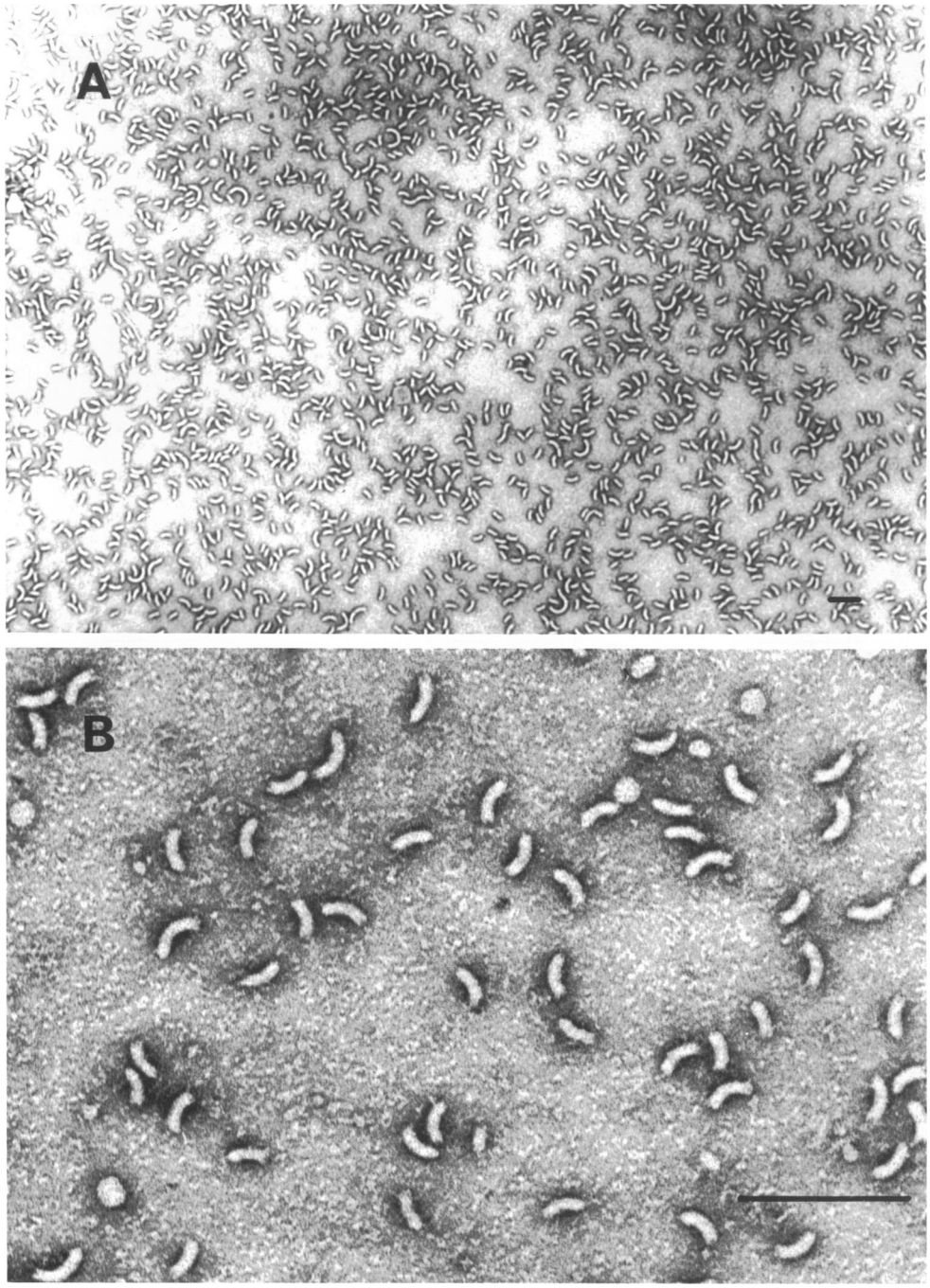

FIG. 1. *Electron micrographs of purified hooks negatively stained with phosphotungstate. A, ×21,000; B, ×120,000. Markers indicate 0.2 μm.*

purification, the same cycle of depolymerization-polymerization was repeated. This procedure made it possible to obtain flagellar filaments without hooks.

Preparation of antisera against hooks and flagellar filaments. A preparation of hooks, containing 1.5 mg of protein/ml, was used to immunize a rabbit. A 0.5-ml amount of the solution was injected intraperitoneally the first time, and thereafter 0.5, 1.0, and 1.5 ml were injected intravenously at intervals of 3 or 4 days. A week after the last injection the

rabbit was bled, and the antiserum was prepared by the procedure of Kauffmann (15).

In parallel, by using a purified preparation of flagellar filaments (protein concentration 2.0 mg/ml), we prepared antiserum against filaments by the same method as described above.

Antibody labeling and electron microscopy. Specific antibody labeling of hooks of flagellar filaments was carried out by the method described previously (3). Labeled hooks or flagellar filaments were negatively stained with 0.5% phosphotungstate (pH 7.0) and observed in a JEM T7 electron microscope at magnification between 7,000 and 30,000.

RESULTS

Difference in antigenic specificities of hooks and filaments. Figure 2A shows isolated flagella of strain SJ25 treated with crude antihook serum. It is seen that not only hooks but also filaments are densely coated with antibodies. This may be due to the fact that our preparation of hooks was contaminated with H antigen, and therefore the antiserum directed against it contained antifilament antibody. This antibody could be removed from the serum by specific absorption with 0.1 volume of a solution containing 10 mg of purified flagellar filaments per ml. For further purification, the serum was absorbed by Formalin-killed cells of the nonflagellated mutant SJW319, which is identical in O-antigen type to SJ25, in order to remove antibody against O antigen. When the absorbed antiserum was used, only the hook portion was densely labeled with antibodies (Fig. 2B). On the other hand, antifilament serum contained no antihook antibody, i.e., when it was applied to isolated flagella of SJ25, filaments were labeled with antibodies, whereas hooks remained unlabeled (Fig. 2C). These experimental results support the finding by Lawn (16) and Dimmitt and Simon (7) that hooks have different antigenic specificity from flagellar filaments.

Antigenic homology of hooks in Salmonella. Strains SJ670 and SL23 (no. 2 and 3 strains in Table 1) are monophasic in phase 1 and phase 2, respectively, and different in H-antigen type from strain SJ25 and from each other. Hooks from the two strains were shown to react with anti-SJ25-hook antibody; electron micrographs very similar to Fig. 2B were obtained for the two strains. TM2 (no. 4 in Table 1) is diphasic, and the organisms of this strain produce flagella of antigen types *i* and *1,2*. Figure 3 shows flagella from TM2 treated with a crude preparation of anti-SJ25-hook serum. It can be seen that hooks are coated with antibodies, irrespective of whether they are joined to antibody-labeled *1,2*-type filaments or unlabeled *i*-type filaments. These experimental results indicate that antigenic specificity of hook is determined independently of flagellar phase and H-antigen type.

Strains SJ30 and SJ814 (no. 5 and 6 in Table 1) produce curly and straight flagella, respectively. The occurrence of such a mutant is due to a slight change in amino acid sequence of the flagellin. Therefore, it is expected that a mutational change of flagellar shape will not be associated with any change in antigenic specificity of the hook. Actually, hooks from strains SJ30 and SJ814 reacted with anti-SJ25-hook antibody (Fig. 4).

Strains SJ597, SJ417, and SJ608 (no. 7, 8, and 9 in Table 1) are paralyzed mutants, i.e., they are normally flagellated but nonmotile. The paralyzed mutants in *Salmonella* have been classified into three groups of cistrons referred to as motA, motB and motC, respectively. Strain SJ597 belongs to the first group, SJ417 to the second, and SJ608 to the third. Hooks from the three mutants reacted with anti-SJ25-hook antibody and were indistinguishable in this respect from hooks of SJ25. *Salmonella* has been classified based on O-antigen type into a large number of groups, and each group has been further subdivided into serotypes according to H-antigen specificity. Ten strains from no. 10 to 19 in Table 1 include divergent serotypes representing various groups. Hooks from these strains were reactive toward anti-SJ25-hook antibody, indicating that the antigenic specificity of hooks is common through a wide range of serotypes of *Salmonella*.

In conclusion, with respect to reactivity of hooks to anti-SJ25-hook antibody, there was little difference between SJ25 and 18 *Salmonella* strains examined. It should be mentioned at this point that this experimental result does not necessarily mean that hooks from SJ25 and another strain consist of identical subunits and have identical sets of antigenic determinants. Whether antigenic determinants of hooks from two strains are identical or partially the same can not be determined by the present experimental method.

Antigenic heterogeneity of hooks among different genera of enterobacteria. Hooks from *E. coli* W3110 were found to react with anti-SJ25-hook antibody. Electron micrographs of the product were very similar to that given in Fig. 2B. On the other hand, hooks from *S. marcescens* SEJ3, *P. vulgaris* IFO5988, *A. aerogenes* AJ5, and *Klebsiella* sp. KJ3, which are phylogenically less related to *Salmonella*, never reacted with the same antibody.

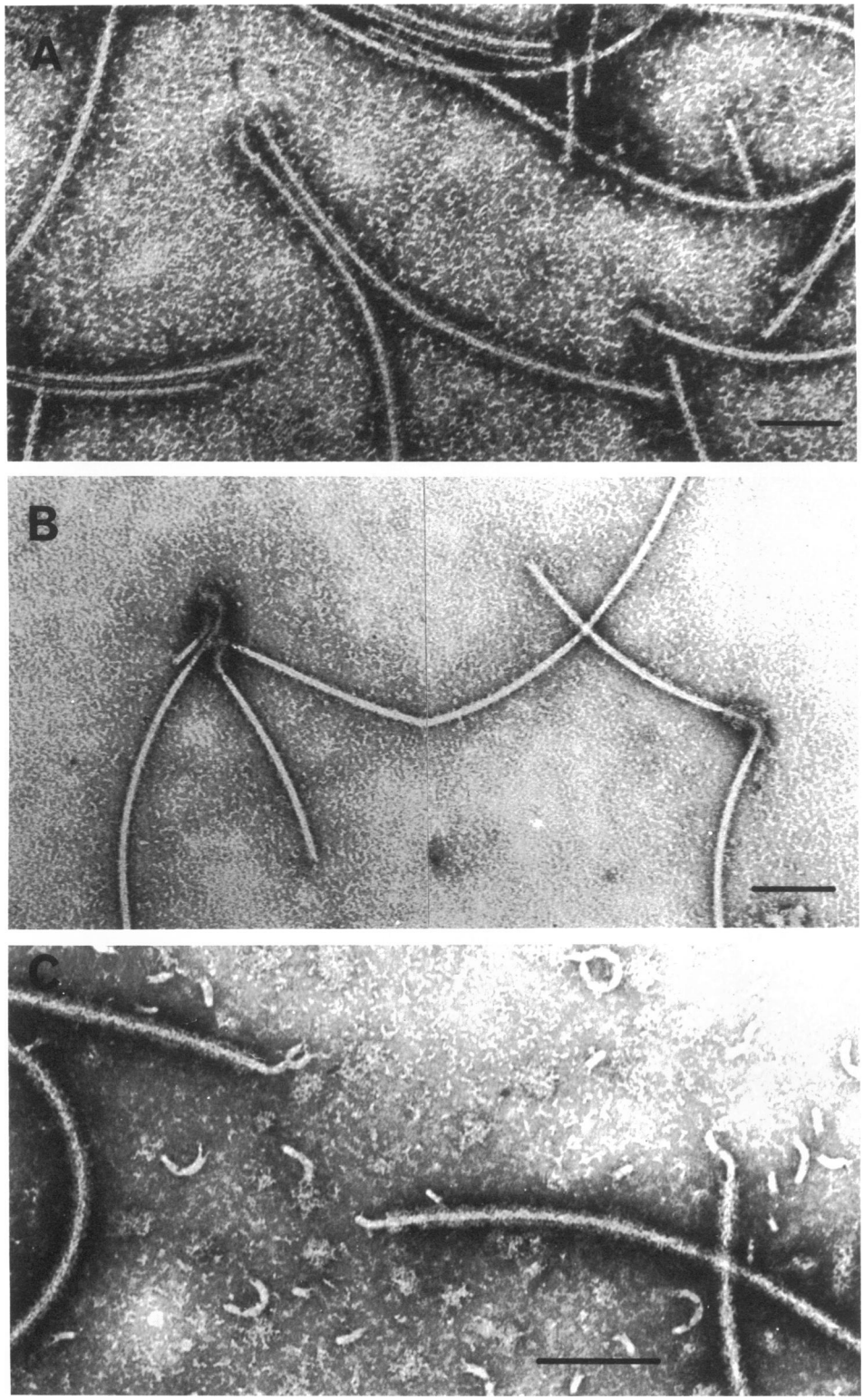

FIG. 2. *Antigen-antibody reaction of flagella from SJ25 with antisera. A, Reaction with crude anti-hook serum (×60,000); B, reaction with purified anti-hook serum (×60,000); C, reaction with anti-flagella-filament serum (×90,000); All were stained with phosphotungstate. Markers indicate 0.2 μm.*

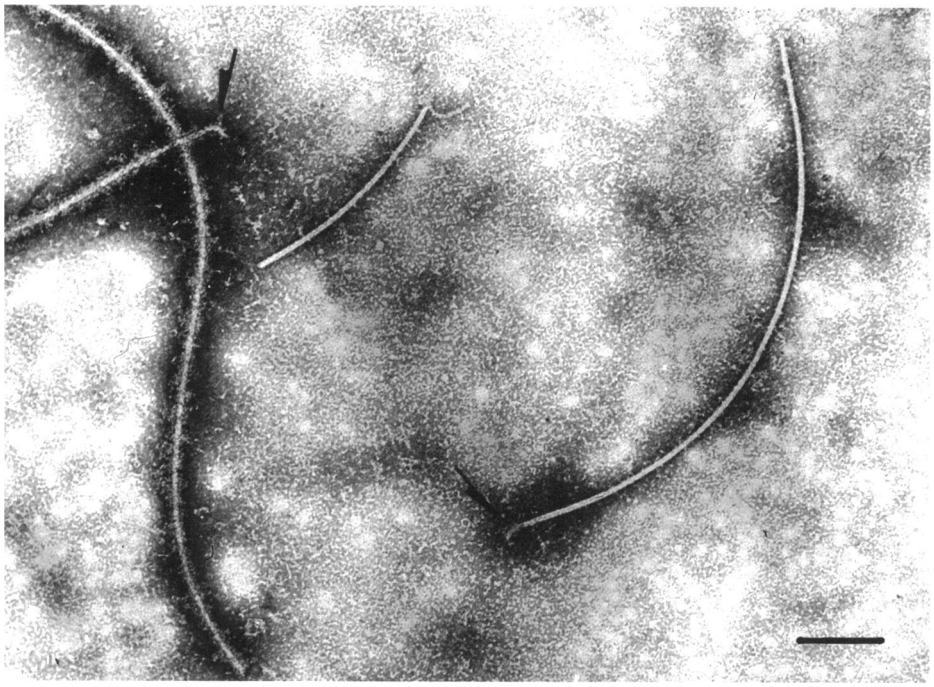

FIG. 3. *Reaction of flagella from TM2 with crude anti-SJ25-hook serum. Two hooks are shown by the arrows. Negative staining with phosphotungstate. ×60,000. Marker indicates 0.2 μm.*

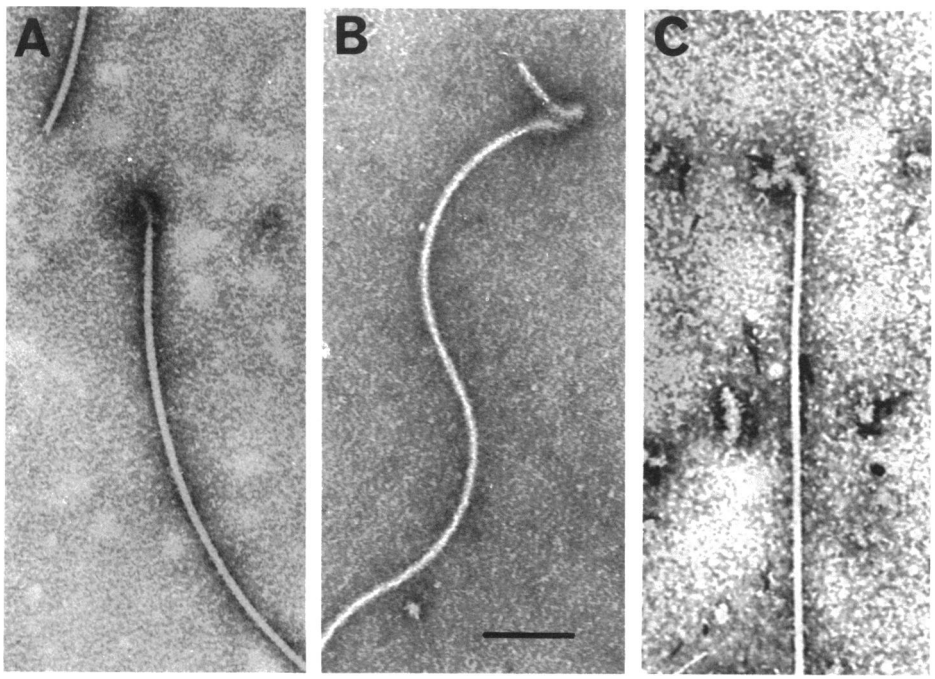

FIG. 4. *Reaction of flagella obtained from morphologically different strains with anti-SJ25-hook serum. A, SJ25; B, SJ30; C, SJ814. Negative staining with phosphotungstate. ×60,000. Marker indicates 0.2 μm.*

Other observations. It is well known that bacterial locomotion is stopped instantaniously when antiserum against flagellar filaments is added to the medium. Our crude preparation of antihook serum had the same effect on motility of the organisms of the SJ25 strain. The situation changed when the serum was absorbed with purified flagellar filaments. Gross observation by optical microscopy showed that bacterial movement was still active after the addition of the absorbed serum to the medium. Hooks exist as extracellular structures. Therefore, when antihook serum is added to a suspension of living bacteria, the hooks will be coated with antibody. Indeed, observations of negatively stained preparations of bacteria treated with antihook serum showed that the hook portions were coated with antibody. Taking into account these circumstances, we conclude that bacteria do not lose motility when their hooks are coated with specific antibody molecules.

In vitro polymerization of flagellin into flagella filaments has been reported by several authors. Asakura et al. (2) showed that in order to initiate polymerization of *Salmonella* flagellin in the presence of 0.15 M NaCl at neutral pH, it is necessary to add seeds to the solution. In these studies, fragments of flagellar filaments were used as seeds. It is of interest to determine whether isolated hooks can act as seeds. In an experiment, a preparation of hooks (strain SJ25), before heat treatment in the presence of 3 mM $CaCl_2$, was added to a solution of monomeric flagellin (strain SJ670), and the mixture was, after incubation at 26 C for a few hours, treated with anti-SJ25-filament serum. An electron micrograph of the product is shown in Fig. 5. It appears that a part of the added hooks acted as seeds, though electron microscopy can not exclude the possibility that these hooks might contain small amounts of SJ25 flagellin at their ends. We repeated similar experiments with purified preparations of hooks. In these cases it was rare to find hooks capable of initiation of polymerization.

DISCUSSION

In *Salmonella*, there exist a wide variety of serotypes differing in H-antigen specificity. In other words, flagellar filaments produced by

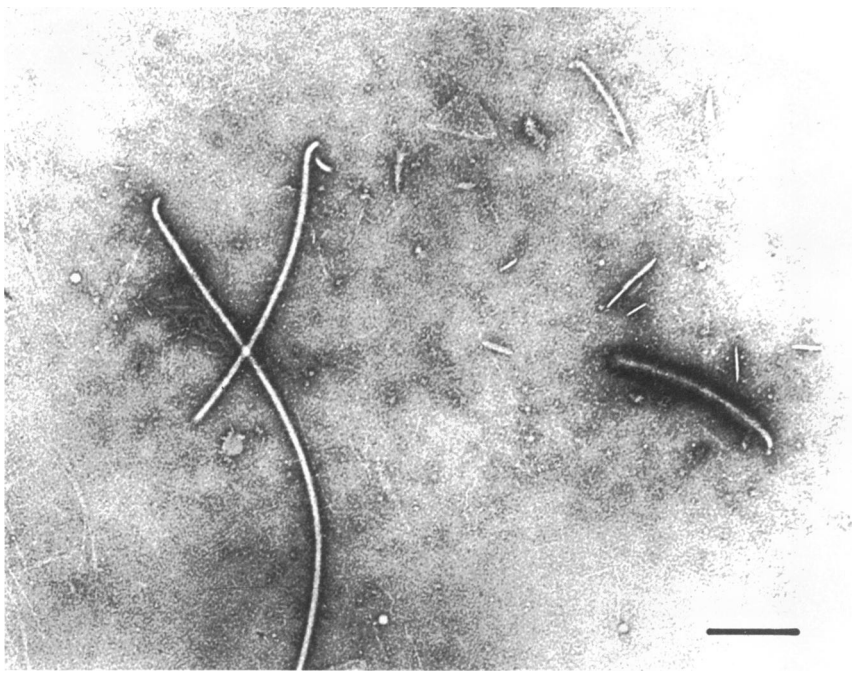

FIG. 5. *Polymerization of flagellin initiated by the addition of hooks. A partially purified preparation of hook (strain SJ25) was added to a solution of monomeric flagellin (strain SJ670), and the mixture was left at 26 C for a few hours and then treated with anti-SJ25-filament serum. Note that two filaments on the left-hand side of the micrograph are associated to hooks but have no parts coated with antibody. A filament on the right-hand side, coated with antibody, came from the preparation of hooks used. Negative staining with phosphotungstate. ×60,000. Marker indicates 0.2 μm.*

each serotype have narrow ranges of antigenic specificity which do not overlap with each other. On the contrary, hooks derived from different serotypes were shown to share common antigenicity. A similar result has been reported with two strains of *Bacillus subtilis* (7). In the present investigation, the results were more pronounced. Homology in antigenic specificity of hooks was found not only among various serotypes in a genus but also between different genera, namely *Salmonella* and *Escherichia*.

From the evolutionary point of view, it is interesting that two parts of an organelle are so diverse in antigenic specificity. Why is antigenicity of flagellar filaments remarkably polymorphic whereas that of hooks homogeneous? It may be worth noting here that flagella are locomotive organelles and the bacterial motility is inhibited by antifilament serum but not by antihook serum. Therefore bacteria carrying new mutant H-antigens perhaps can escape more efficiently from immunological protection of the host animal than those carrying the preexisting H antigen. Thus, mutations of filaments are selectively advantageous for establishing a new line, whereas mutations of hook-antigen are selectively neutral. If this is so, the rate of evolutionary change of flagellin structure might be much faster than that of hook protein structure (17).

Among the enterobacteria examined, only *E. coli* was found to have common hook antigen with *Salmonella*, indicating that the antigenic homology of hook is confined to these related genera. It may be taken as another reflection of phylogenetic intimacy between *Salmonella* and *Escherichia*, consistent with accumulated genetic information (10). The extensive comparative studies of hook antigen promise to provide a clue to the phylogenetic relations among flagellate bacteria.

It has been shown that in vivo growth of bacterial flagella takes place at their distal ends (8, 11, 14). At the earliest stage of this process, hooks must act as seeds for polymerization of flagellin (Fig. 5). However, the purified hooks were shown to be unable to act as seeds. This result might be at least partly due to the fact that hooks were exposed to extreme conditions of environment during preparation. The isolated hook might involve partial breakage in structure. Yamaguchi et al. have obtained *Salmonella* mutants which possess hooks but no flagella. These strains have a defect in the structural gene of flagellin (18). It has been found that the addition of normal flagellin to a suspension of the organisms of these strains gives rise to flagellation, though the number of flagella possessed by each bacterium was very small (Iino, Suzuki, and Yamaguchi, unpublished data).

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