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Purification and Partial Characterization of *Bacillus subtilis* Flagellar Hooks[1]

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A method for preparing bacterial flagellar hook structures is described. The method involves isolating intact flagella from a mutant which makes thermally labile flagellar filaments and heat-treating them to disaggregate the filament preferentially. The resulting hook preparation can be separated and purified by velocity and isopycnic centrifugation. The purified hooks sediment at a relative S value of 77. On acrylamide gel electrophoresis in sodium dodecyl sulfate, they show one major and a number of minor protein bands. The purified hooks can be used to immunize rabbits, and the resulting antiserum is hook-specific. These results support the notion that hooks are composed of a protein that differs from flagellin.

Bacterial flagella terminate in hooklike structures which are in turn fixed into the cell membrane and cell wall (3-5). The hook portion of a flagellum is easily distinguished from the filament by its morphology (1) and its immunochemical reactivity (6, 11). Koffler and his coworkers showed that the hook is more stable than the filament under a variety of denaturing conditions. This property can be used as a basis for the purification of small amounts of the hook structure (2).

Although very little is known about the nature or function of the hook, this information almost certainly is implicated directly in the growth mechanism of flagella and in motility (8). Further, we have shown that the presence of the hook stabilizes the structure of the filament (7). To characterize the hooks and the other structures attached to the base of the flagella, we developed procedures for isolating relatively intact, pure flagella (7). Using these as source material, we could isolate the hook and the other structures. To enhance the difference in stability between the hook and the filament, we prepared the intact flagella from a mutant of *Bacillus subtilis* which makes heat-labile filaments. The filaments could be easily removed, leaving the hook structure intact. The hooks were purified further by differential centrifugation and isopycnic gradient techniques. A similar method was described by Koffler and his co-workers (X[th] International Congress of Microbiology).

[1] Presented in part at the Xth International Congress of Microbiology, Mexico City, August 1970.

MATERIALS AND METHODS

Bacterial strains. The strain used for the preparation of intact flagella was *B. subtilis* GD1. This strain was derived from *B. subtilis* BD71, which we obtained from D. Dubnau (*hisA1, ura, argC4*). *B. subtilis* GD1 carries a mutation in the flagellin structural gene which results in the synthesis of flagella which disaggregate when heated at 12 C lower than the temperature of disaggregation of wild-type flagellar filaments.

Media. The minimal medium of Spizizen and Anagnostapoulus was used to grow the bacteria. In experiments in which the flagella were labeled with radioactive amino acids, the Casamino Acids in the medium was replaced by a mixture of 18 amino acids at a final concentration (except for the radioactive amino acid) of 10 μg/ml.

Purification of flagellar hooks. *B. subtilis* GD1 was grown in minimal medium supplemented with ^{3}H-labeled leucine and histidine. Specific activity of the leucine was 12 mCi/μmole; that of the histidine was 3 mCi/μmole. A 30-ml amount of culture was collected, and intact flagella were prepared as previously described (7). Nonradioactive, intact *B. subtilis* GD1 flagella were added as carrier so that the final concentration of intact flagellar protein was about 100 μg/ml. The flagellar filaments then were sheared by passing them through a 24-gauge needle ten times, and the sample was heated at 65 C for 30 min. The resulting solution was layered on a gradient formed of three layers of 38, 45, and 53% sucrose in 0.01 M tris(hydroxymethyl)aminomethane (*p*H 8.0); 0.5% Brij 58 (Atlas Chemical Industries); and 0.01 M ethylenediaminetetraacetate (TBE buffer). This layered solution was centrifuged at 100,000 × g in the SW27 rotor of a Spinco model L ultracentrifuge for 5 hr at 4 C. A band formed at the 45% layer (Fig. 1). This band was removed and dialyzed against TBE buffer overnight at 4 C. The sample then was applied to a linear 30 to 60% (v/v) renografin gradient. After the sample was centri-

fuged at 144,000 × g for 11 to 12 hr at 10 C in the SW41 rotor, fractions were collected from the bottom of the tube.

Electron microscopy. Samples were placed directly on carbon-coated, Formvar-covered grids. These were negatively stained with 1% phosphotungstic acid (pH 7.3). A Phillips 200 electron microscope was used with an accelerating voltage of 60 kv.

Antigens, antibodies, and complement fixation. Antihook antibody was prepared by concentrating the front part of the renografin gradient, mixing the protein with incomplete Freunds adjuvant, and injecting it intramuscularly and into the toepads and footpads of a New Zealand White rabbit. Three weeks later the rabbit was injected again. One week later the animal was bled. The total amount of hook protein injected was derived from 90 mg of intact flagellar protein.

Complement fixation was performed by the procedure of Wasserman and Levine (12).

Sodium dodecyl sulfate-acrylamide gel electrophoresis. The procedure for sodium dodecyl sulfate-acrylamide gel electrophoresis as reported by Gelfand and Hayashi (10), used 7.5% cross-linked gels. The hook sample was concentrated by centrifugation. The pellet was suspended in 1% sodium dodecyl sulfate, 1% β-mercaptanethanol, and 0.01 M tris(hydroxymethyl) aminomethane (pH 9.1) in 12% sucrose, and this suspension was heated at 100 C for 3 min. The sample was cooled and applied immediately to the gel. ^{3}H-labeled hooks and ^{3}H-labeled flagellin samples were corun with ^{14}C-labeled φX-174 proteins (a gift from M. Hayashi) as marker. The marker preparation contained four proteins (molecular weights 54,000, 45,000, 21,000, and 8,000 daltons). These proteins were sufficient to obtain clearly the positions of the unknown bands.

RESULTS

Shearing and heating intact flagella (7) resulted in a mixture of hooks, flagellin, and some membrane fragments. This mixture was layered onto a sucrose gradient and centrifuged to separate the hooks from the flagellin. Figure 1 shows the distribution of labeled flagella on the gradient. Most of the particulate material formed a band in the middle of the gradient. This material was purified further by isopycnic gradient centrifugation in renografin. Figure 2 shows the distribution of the radioactive material on renografin. When samples were removed from different regions of the gradient and examined by electron microscopy, it was found that the material at the highest density was mostly bare hooks without basal structures (Fig. 3A, B, and C). At intermediate densities, hooks bearing basal structures were most abundant. In the least dense fractions, most of the hooks were attached to relatively large pieces of membrane, and most of the particulate material appeared to be membrane-like. Subsequent work was done with the leading fractions from the renografin gradient. This material could be further purified by sedimentation. More than 50% of the radioactivity derived from the renografin gradient sediments in a relatively homogeneous band with an approximate S value of 77 (Fig. 2).

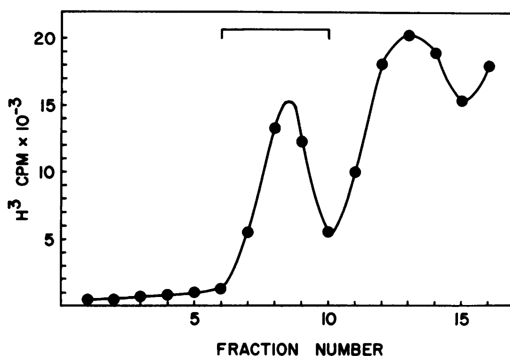

FIG. 1. *Sucrose layer centrifugation of sheared and heated flagella. Brackets indicate the region of the gradient collected for subsequent purification procedure.*

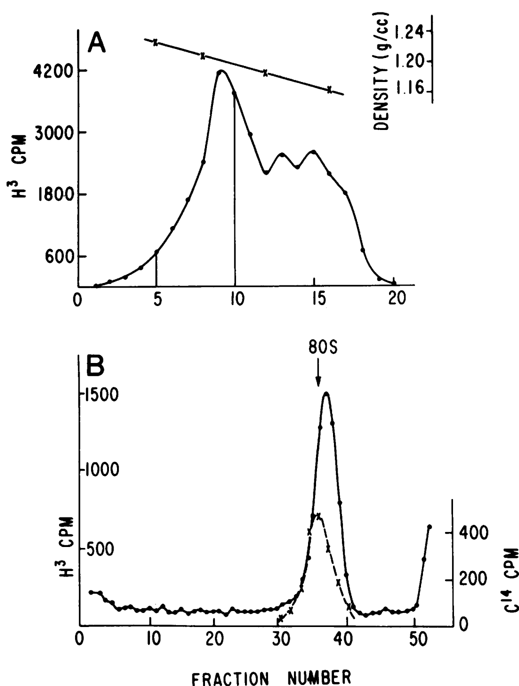

FIG. 2. *(A) Distribution of radioactive label after renografin gradient centrifugation. The band removed from the sucrose layer was dialyzed, applied to the renografin gradient, and centrifuged for 12 hr at 144,000 × g. (B) Distribution of purified hooks after sucrose gradient centrifugation. Fraction nine, taken from the renografin gradient, was dialyzed, mixed with ^{14}C-labeled MS-2 bacteriophage, layered onto a 15 to 30% sucrose gradient, and centrifuged at 180,000 × g for 90 min. The dotted line represents the marker.*

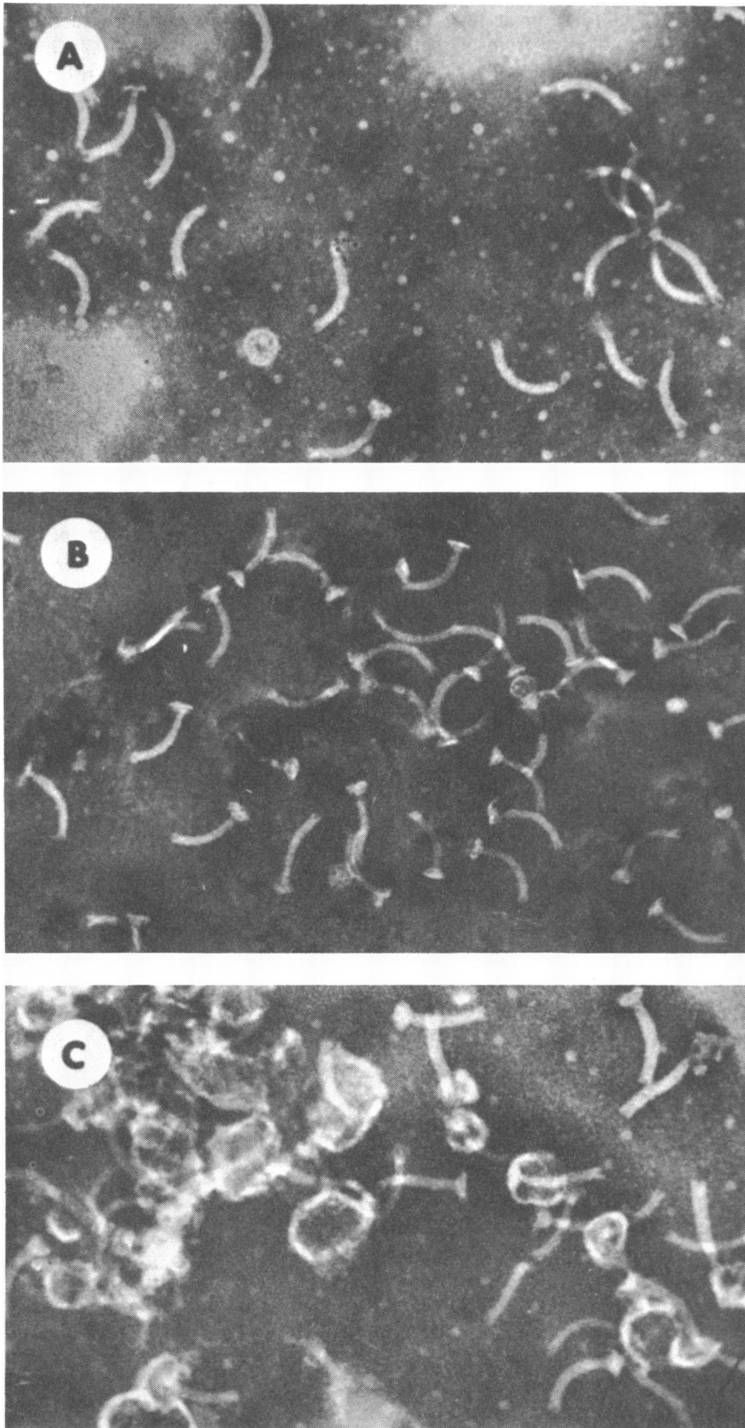

FIG. 3. *Electron micrographs of material from renografin gradient. Samples were removed from fractions 6 (A), 9 (B), and 13 (C) taken from the renografin fractionation (Fig. 2 (A)) and stained with 2% phosphotungstic acid (pH 7.0). The diameter of the hooks is about 17 nm.*

Antihook antibodies. The purified hook material could be used to prepare antihook antisera. It is known that antihook antibodies can bind to hooks (6); in electron micrographs, the hooks were covered with antibody protein, but the filaments were not affected. Results of complement fixation using antibodies prepared against renografin-purified hooks are shown in Fig. 4. The antisera reacted strongly with intact flagella. There was much less reaction with flagellar filaments obtained by shearing. No reaction was detected with flagellin or with flagellin repolymerized to form flagellar filaments.

The antibody also was used to measure the thermal denaturation of the hook structure. The hook protein maintained complete antigenic activity until it was heated above 72 C (Fig. 5). Heating at 72 C lead to a loss of about 20% of the antigenic activity in 15 min. The loss of the distinct hook structure was observable by electron microscopy. After heating at 72 C for 15 min, the preparation retained many structures with the characteristic hook morphology; after heating at 80 C, no such structures were found.

Acrylamide gel electrophoresis. The hook structures were disaggregated by heating in detergent, and the constituents were studied by acrylamide gel electrophoresis. The pattern in Fig. 6 contains a major peak which includes about 35% of the radioactivity applied to the gel. The major peak is clearly different from flagellin. Its estimated molecular weight is 33,000 daltons as compared to 41,000 daltons for flagellin.

DISCUSSION

There is increasing evidence which suggests that the hook portion of the flagellum is composed of proteins that differ from the filament subunit. Koffler and his co-workers originally showed the marked heat stability of the hooks. Lawn (11) showed that antibody to the flagellar filament will coat the filament but will not coat the hook. More recently, Koffler and his co-workers showed that tryptic digests of purified hook protein give patterns similar to flagellin but also contain specific differences, further evidence of a major hook protein which differs from flagellin. Finally, we have been able to purify flagellar hooks that also contain parts of the flagellar basal structure. Antisera prepared with this material specifically coat the hook structure

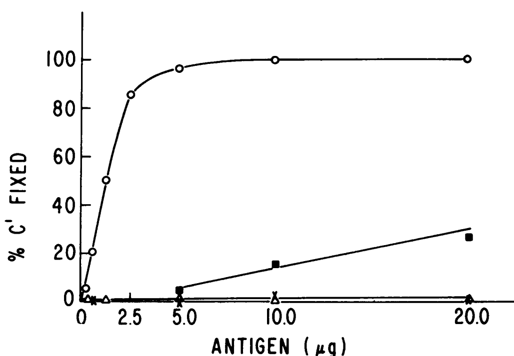

FIG. 4. *Reaction of antihook antibody measured by complement fixation. Antihook antibody was used at 1/4,000 dilution. Symbols:* O, *intact flagella;* ■, *sheared flagella;* Δ, *reaggregated flagellar filaments;* ×, *flagellin.*

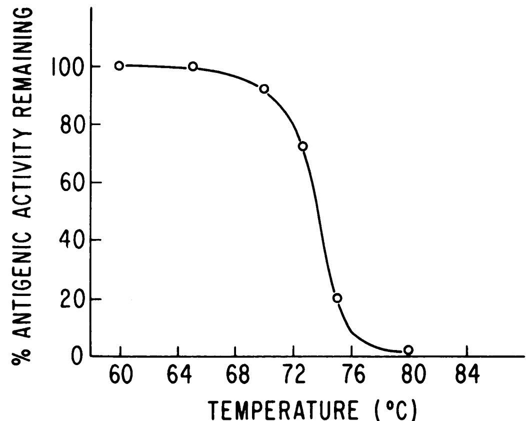

FIG. 5. *Thermal stability of purified hooks after suspension in 0.01 M tris(hydroxymethyl)aminomethane (pH 7.3) and 0.05 M NaCl buffer. A sample was heated at the temperature indicated for 15 min. After cooling, the antihook antiserum was used to measure residual antigentic activity.*

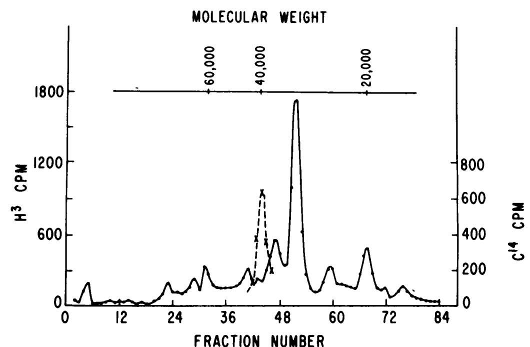

FIG. 6. *Sodium dodecyl sulfate-acrylamide gel electrophoresis of purified hooks. The material remaining in fractions 5 to 10 of the renografin gradient (Fig. 2A) was pooled and collected by high-speed centrifugation. The pellet was suspended and dissolved in sodium dodecyl sulfate buffer; half the sample was mixed with flagellin. Broken line (×) indicates the flagellin marker.*

and react with intact flagella but not with flagellin. Upon disaggregation, the hooks contained a major protein constituent with a molecular weight 20% lower than that of flagellin.

These results strongly suggest that the hooks are composed of a protein that is not flagellin. However, there are a number of possible explanations for the relationship between flagellin and the hook subunit protein. (i) The hook cistron may have been derived from a flagellin precursor cistron by gene duplication and mutation (H. Koffler, *personal communication*); (ii) the hook subunit may be a specifically modified form of flagellin; (iii) flagellin and hook subunit protein each may be derived from independent cistrons. The purification procedures described above, together with specific genetic crosses, should allow us to distinguish among these possibilities.

The minor components found on acrylamide gel electrophoresis may result from other parts of the hook structure, e.g., the basal discs and the core. Experiments designed to identify these proteins and to assign them to specific flagellar structures are in progress.

ACKNOWLEDGMENT

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