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Detection of Motility and Putative Synthesis of Flagellar Proteins in *Salmonella pullorum* Cultures

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***Salmonella pullorum* is a host-adapted pathogen of poultry previously thought to be nonmotile and nonflagellated. We discovered that motility can be induced in this organism under special medium conditions, and this motility was observed in 39 of 44 *S. pullorum* isolates tested. The migration appeared to occur only on the medium surface and not within the medium itself, indicating that swimming may not be responsible for this event. Agar concentration, carbohydrate concentration and type, and temperature of incubation all affected the motility. Flagellar stains and transmission electron micrographs of the motile *S. pullorum* culture showed long fibrous appendages resembling flagella extending from the cells, but these appendages were thinner and less numerous than the flagella observed on *Salmonella enteritidis*. Antisera to G flagellar antigens reacted strongly with the induced-motility *S. pullorum* culture, indicating that G epitopes were expressed on these cells. These results indicate that, contrary to the paradigm which held that *S. pullorum* is nonmotile and nonflagellated, motility can be induced in *S. pullorum* and that the organism appears to have the capacity to produce flagella.**

Salmonella pullorum causes a systemic disseminated infection in poultry known as pullorum disease. The organism is host adapted for poultry and exacts its most lethal effect in young birds, aged 0 to 3 weeks (19). *S. pullorum* is vertically transmitted from the parent to the offspring via the infected egg, and the infection expands horizontally in the hatchery (19). Losses from this disease organism can be staggering, with mortalities greater than 85% in certain cases (6). Pullorum disease devastated the U.S. poultry industry in the early 1990s, but with the institution in 1935 of the National Poultry Improvement Plan, a program involving federal and state agencies in cooperation with industry, the incidence of the disease in the United States decreased dramatically in succeeding years (6, 19). The disease ceased to be a problem for U.S. commercial poultry in the 1950s, and its incidence in other developed countries similarly remained low. Such cannot be said for developing countries, where pullorum disease continues to cause substantial losses for poultry industries (4, 5, 18).

S. pullorum is a gram-negative bacillus in the family *Enterobacteriaceae*. Kauffmann-White classification places *S. pullorum* in the serogroup D_1 salmonellae along with *Salmonella gallinarum* and *Salmonella typhi*, causative agents of fowl and human typhoid, respectively (7, 16). The bulk of *Salmonella* species possess flagella and exhibit motility (7, 16). However, *S. pullorum* and *S. gallinarium* are two notable exceptions, having been shown early on to lack motility and flagella (20). However, recent evidence indicated that *S. pullorum* may possess flagellar epitopes. Ibrahim et al. (11) showed that antisera generated to *Salmonella* flagella reacted with *S. pullorum*, and Berchieri et al. showed that, on occasion, serological screening of poultry flocks by a flagellum-based enzyme-linked immunosorbent assay indicated reactions with sera from *S. pullorum*-infected birds (3). Kilger and Grimont found the genes for flagella in *S. pullorum* (12). Recently, we found that antiflagellar antibody reagents reacted to our laboratory strains of *S. pullorum*. In an effort to increase expression of the reactive epitopes, we cultured *S. pullorum* under different medium conditions shown previously to increase flagellum production in *Salmonella* species (7). These efforts were largely unsuccessful. However, using a special medium originally described by Harshey and Matsuyama (10) to induce a swarming-like motility in *Escherichia coli* and *Salmonella typhimurium*, we were able to induce motility in *S. pullorum*. This paper describes the motility produced by *S. pullorum* and the conditions which affect this motility and provides evidence for the presence of flagella on the cell.

The primary *S. pullorum* strain used in the motility studies was isolate SI0071 (our laboratory designation, SP0071), originally obtained from George Stein at the Maryland Department of Agriculture, Annapolis, Md. *Salmonella enteritidis* phage type 13a strain number 19299-52-1 (our laboratory designation, SE6) was obtained from Charles Benson, University of Pennsylvania. All cultures were stored at −20°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) containing 20% glycerol. Samples were thawed when needed, transferred to nutrient agar plates (Difco), and incubated at 37°C.

The SE6 culture migrated 5 to 10 mm from the initial stab inoculation site in GI and S motility media (Difco), while culturing SP0071 on the same media failed to induce any kind of detectable motility. The SE6 strain administered to medium described previously to induce swarming in *S. typhimurium* (10) exhibited very rapid surface swarming, migrating 20 mm from the original streak within 6 h (Fig. 1A). No motility was initially observed with SP0071 on this medium, designated high-motility medium (HMM) in our laboratory, although small outgrowths, approximately 0.5 to 1 mm, could be seen extending from the central streak. These outcroppings were subpassaged, and a motile culture was obtained from one of these restreaks (Fig. 1B). The organism generally migrated 6 to 15 mm from the central streak following a 24-h incubation at 37°C. An isolate, SP0071-1, was saved and frozen at −20°C and used in all subsequent experiments. The culture was confirmed as being *S. pullorum* when it was submitted to the National Veterinary Services Laboratory, the U.S. Department of Agriculture reference laboratory for veterinary *Salmonella* identification. Restreaking the organism from the motile portion of the growth onto a fresh HMM plate resulted in approximately 10

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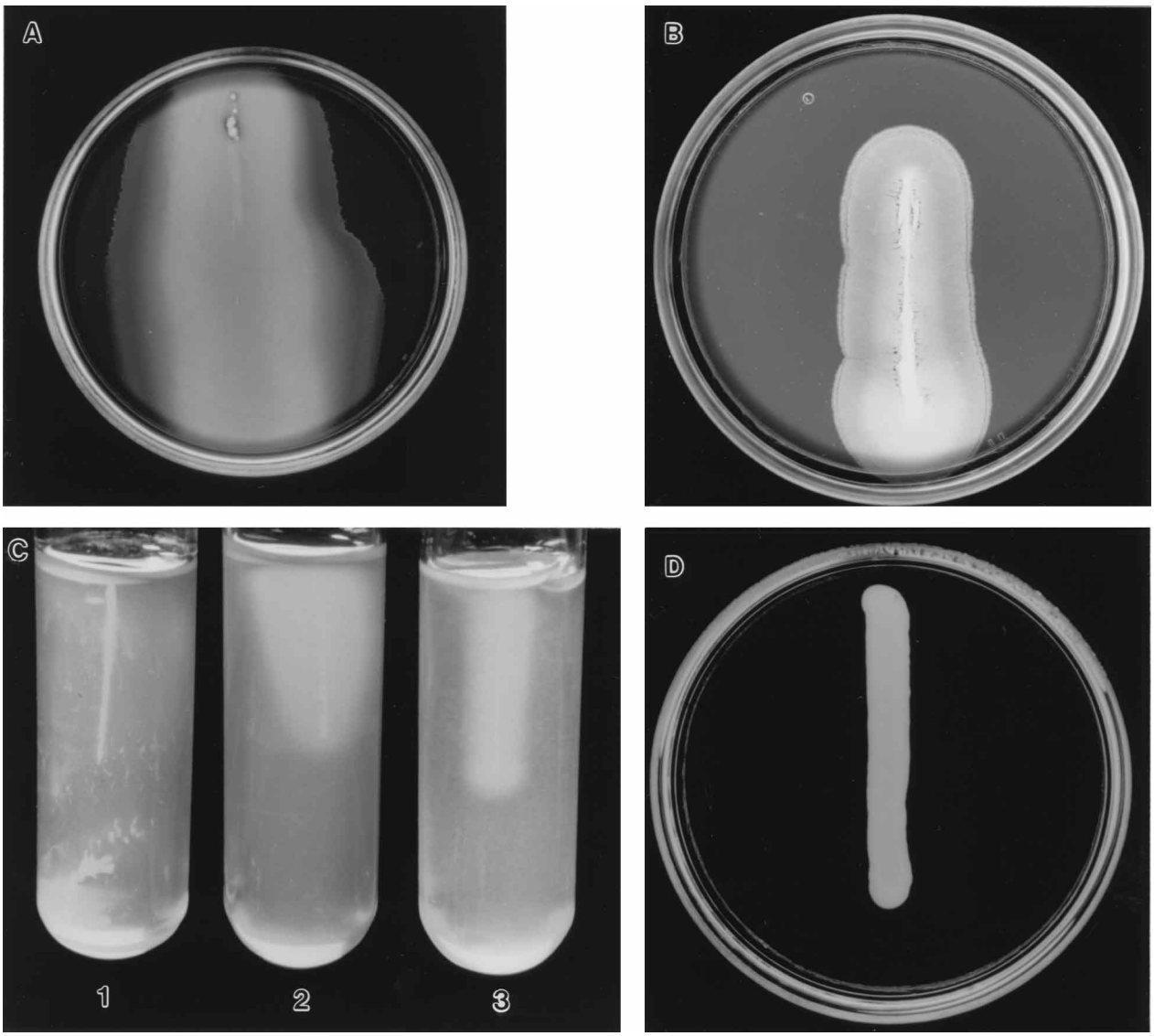

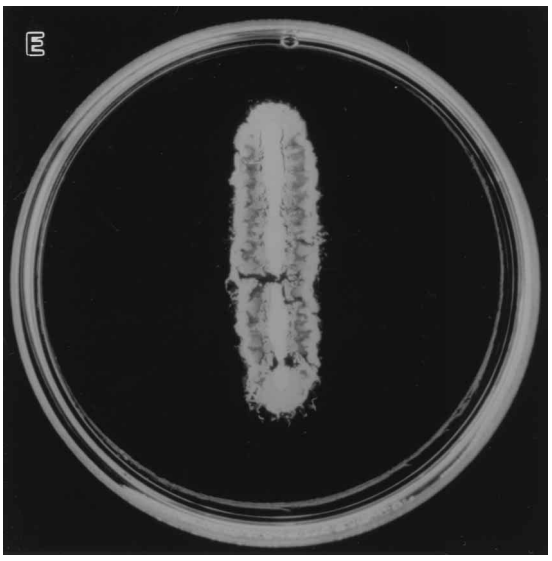

FIG. 1. Motility of *S. enteritidis* SE6 and *S. pullorum* SP0071-1 on HMM and GI motility media. (A) SE6 on HMM after a 6-h incubation at 37°C. (B) SP0071-1 on HMM after a 24-h incubation at 37°C. (C) Stab inoculations of SP0071-1 in a tube of HMM (tube 1) compared with stab inoculations of SE6 in a tube of HMM (tube 2) or GI motility medium (tube 3). Similar results were observed for the HMM tube inoculated with SP0071-1 following a 96-h incubation at 37°C. Growth of SP0071-1 at 37°C for 24 h on unsupplemented GI motility medium (D) and on GI motility medium supplemented with 0.5% dextrose (E) is shown.

mm of migration from the central streak at 24 h, while no motility was observed in a stab culture in a tube of HMM (Fig. 1C) or on a GI motility agar plate (Fig. 1D). Equal migration distances from the point of inoculation were observed when the plates were incubated aerobically or anaerobically. The presence of G flagellar epitopes was detected via slide agglutination with G complex antiserum (Difco). Reaction with serogroup D and serogroup B antisera (Difco) served as the positive and negative agglutination controls, respectively. Both SE6 and SP0071-1 reacted strongly with the G flagellum and serogroup D antisera but not with the serogroup B reagent.

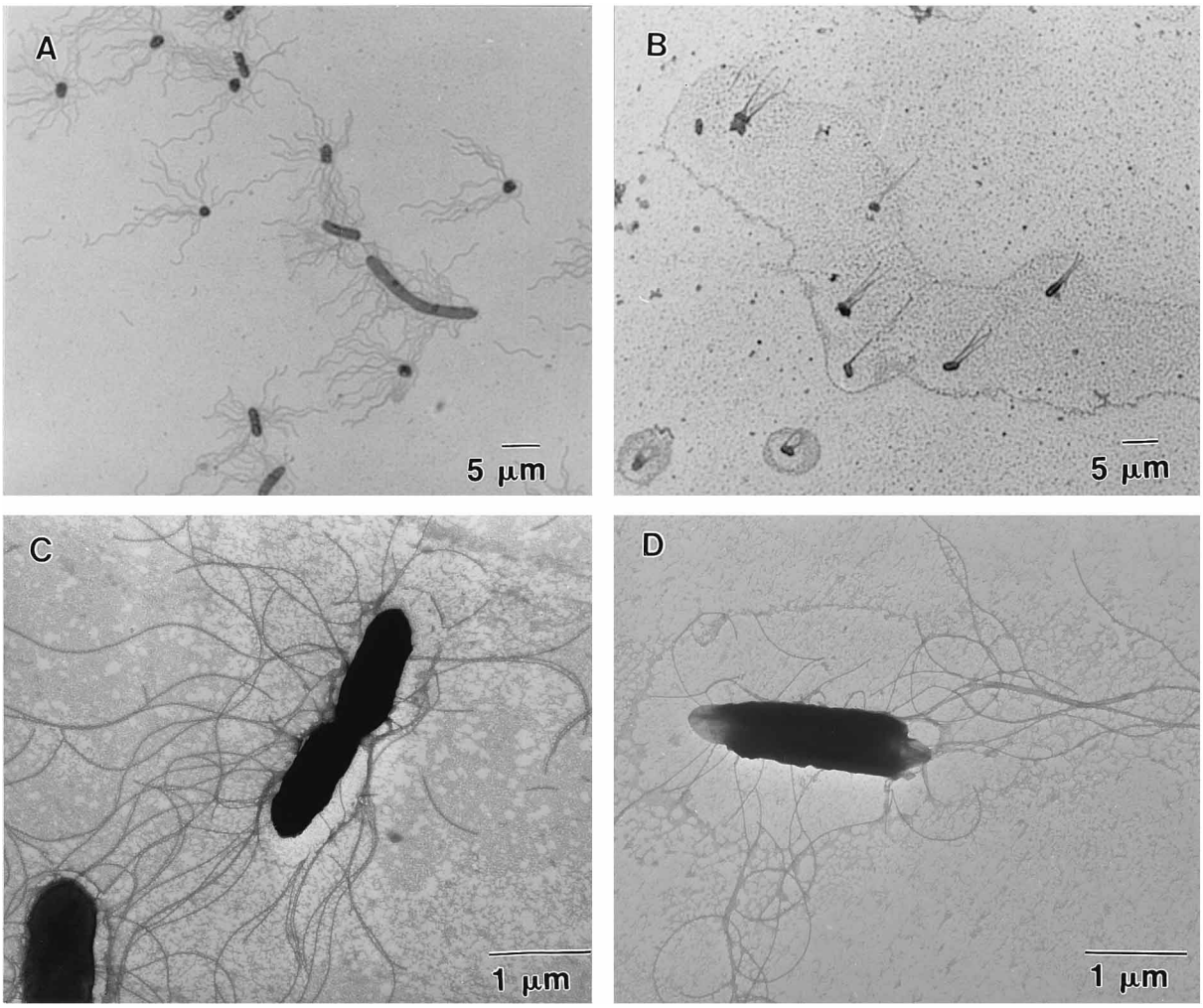

FIG. 2. Photomicrographs of *S. enteritidis* SE6 (A and C) and *S. pullorum* SP0071-1 (B and D) grown on HMM for 6 h (SE6) and 24 h (SP0071). Samples were taken from the edge of the motile colony. Flagellar stains of SE6 (A) and SP0071-1 (B) viewed at a magnification of ×1,000 with an oil immersion objective are shown. Transmission electron micrographs of SE6 (C) and SP0071 (D) are shown.

Optimal motility by SP0071-1 was observed on HMM plates supplemented with 0.5 or 0.75% dextrose and was dramatically reduced at dextrose levels above or below this range. Similar dextrose levels were necessary for expression of swarming behavior by *S. typhimurium* (10). Commercially available GI motility medium with dextrose levels adjusted to give a final concentration of 0.5% also supported motility of SP0071-1 (Fig. 1E). Subtle differences in the carbon source also had dramatic effects on motility. Migration distances equal to those produced in dextrose were observed on plates supplemented with mannose, an epimer of dextrose at carbon atom 2. Supplementing the medium instead with mannitol, a sugar alcohol of mannose, induced a 1.5-fold increase in the SP0071-1 rate of movement, while no migration of organisms cultured on HMM containing sorbitol, a sugar alcohol of dextrose, was observed. Moderate motility was observed with D-galactose– and D-fructose–HMM, and no motility was observed with maltose, sucrose, lactose, dulcitol, or arabinose.

The motility displayed by an organism can be dramatically affected by the viscosity of the medium. Swarming by *Proteus mirabilis* is inhibited on media containing 4% agar (2). Too high an agar concentration will not allow free spread of an organism (14). Agar concentration also played a significant role in observed motility by *S. pullorum* in that optimal migration by the organism was observed in medium containing 0.5% agar while in media containing 1, 1.5, and 2% agar, motility was substantially reduced, similar to previously described conditions for *Shigella* species (9) and *S. typhimurium* (10).

The temperature of incubation can also impact motility. Most *Enterobacteriaceae* optimally exhibit motility at 35°C, while cell migration by *Yersinia enterocolitica* and *Listeria monocytogenes* occurs more readily at 22 to 25°C (14). Harshey and Matsuyama observed that swarming by *S. typhimurium* occurred optimally at 37°C while the best swarming by *E. coli* occurred at 30°C (10). In our study, good motility by *S. pullorum* occurred at 37°C but the rate of movement was increased 1.5-fold at 42°C. *S. pullorum* is host adapted for poultry, and the chicken core body temperature is 40 to 42°C (8). Such a temperature optimum would be necessary if the motility trait occurred in vivo. The manifestation of such traits in birds during infection remains to be determined, although we have found that sera from birds infected with *S. pullorum* react with several different flagellum serotypes in an enzyme-linked immunosorbent assay format (data not shown), indicating that in

vivo production of determinants cross-reactive with these flagella does appear to occur.

A total of 44 *S. pullorum* isolates were inoculated in duplicate onto HMM-dextrose for possible motility induction. The isolates were derived from the *Salmonella* stocks maintained at the Southeast Poultry Research Laboratory and also obtained from George Stein at the Maryland Department of Agriculture and Lee Ann Thomas at the National Veterinary Services Laboratory in Ames, Iowa. The strains represent *S. pullorum* isolated from various poultry organ sites and from different geographical locations in the United States. The plates were incubated at 37 or 42°C for 96 h, and each nonmotile strain was subcultured onto a fresh HMM plate for a further 96-h incubation. After six rounds of subculture, motility could be induced in 57% of *S. pullorum* cultures incubated at 37°C while 89% of the strains incubated at 42°C exhibited this characteristic. More than 50% of the *S. pullorum* isolates could be induced to motility at 42°C after the first subculture compared with 20% of *S. pullorum* isolates incubated at 37°C. No correlation between the migration capacity and organ of isolation could be determined.

As motility is mediated in many cases by flagella (1, 2, 17), the presence of these organelles on SP0071-1 was examined via flagellum staining and transmission electron microscopy. For staining of flagellar proteins, the organisms were grown until motility was evident, at which time a sample was removed from the forward edge of the growth and applied to a drop of distilled water on a microscope slide. The procedures for staining SE6 and SP0071-1 were adapted from previously described methods of Kodaka et al. (13) and Leifson (15). Flagellar stains of the SE6 cells grown on HMM showed a peritrichous array of flagellation around the cell periphery (Fig. 2A). Similarly, appendages which resemble those of SE6 were observed extending from HMM-grown SP0071-1 cells (Fig. 2B), but they existed in fewer numbers, one to two per cell, appeared to be thinner, and were arrayed in a more polar fashion. For transmission electron microscopy, carbon-Formvar grids (Electron Microscopy Sciences, Fort Washington, Pa.) were floated on a drop of cells suspended in distilled water and then allowed to dry. The grids were stained in 2% uranyl acetate (Electron Microscopy Sciences) and viewed on a JEOL (Peabody, Mass.) model 100 CX transmission electron microscope at an 80-kV accelerating voltage. The SE6 cells grown on HMM showed results similar to those of the flagellar stain with a peritrichous array of flagellation around the cell periphery (Fig. 2C). The SP0071-1 culture produced thin, fibrous appendages resembling flagella, but the appendages were not as thick as the SE6 flagella nor as numerous on the cell surfaces (Fig. 2D). The appendages were extremely fragile, and extra manipulations of the cells, such as multiple washing steps, caused shearing and loss of the proteins. Similar fragility problems have been described previously for *Serratia marcescens* flagella (1). Physical characterization of the proteins and attempts at immunogold labeling of the cells have so far been unsuccessful.

The paradigm regarding *S. pullorum* states that this organism is nonmotile (16, 19) and nonflagellated (6, 7, 20). The nonflagellated nature of this organism was described over 60 years ago (20), and belief in this characteristic has remained constant over the succeeding years. This study indicates that *S. pullorum* is indeed motile and does appear to produce cellular appendages consistent with flagella. Why the evidence for flagellation in *S. pullorum* has remained elusive for such a long period may be due to a couple of reasons. First, *S. pullorum* is nonmotile on regular culture media (16, 19), which reduces the inclination to search intensively for flagella. Special conditions are necessary before motility can be detected (Fig. 1). A second reason may be the status of the disease itself. *S. pullorum* seldom infects humans and thus is of little interest to those conducting human pathogenesis research, and with the declining incidence of clinical disease caused in poultry by this organism, the need for research on pullorum disease diminished significantly (6, 19). Little substantive research has been conducted with *S. pullorum* since the 1950s, and it is therefore understandable why motility or flagella were not discovered until now.

The potential impact of the current information is twofold. First, the long-accepted fact that *S. pullorum* is nonmotile and nonflagellated is disproved. This will affect *Salmonella* classification schemes used by poultry diagnostic laboratories and will require modifying the flagellum status of *S. pullorum* within the Kauffmann-White serological scheme. Second, both *S. pullorum* and *S. gallinarum* are two of the most pathogenic *Salmonella* species for poultry and yet were thought to be nonmotile, in contrast to the other salmonellae which infect poultry. Motility is generally a highly conserved property within the *Salmonella* genus, indicating that motility plays an important role in the survival of the species (17). The nonmotile nature of *S. pullorum* called into question the role of this property in the pathogenesis of an organism. However, the identification of a motile phase in the *S. pullorum* growth cycle renews the possible link between the ability of a microorganism to migrate and its ability to cause disease. Again, as alluded to above, the expression of motility in vivo needs to elucidated before the role of this property in pullorum pathogenesis can be determined.

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