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Flagella of *Escherichia coli* Spheroplasts

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Recently, Z. Vaituzis and R. N. Doetsch (J. Bacteriol. **89**:1586, 1965) described the relationship between flagellation and motility in penicillin-induced spheroplasts of *Salmonella typhimurium*, and they concluded that the formation of flagella was dependent upon prior cell wall synthesis. This conclusion has now been confirmed with *Escherichia coli* (ATCC 13070), the results found being identical to those reported for *S. typhimurium*.

Further, spheroplasts made by deprival of diaminopimelic acid (DAP; P. Meadow et al., Biochem. J. **66**:270, 1957) were investigated by use of a DAP-requiring mutant of *E. coli* (ATCC 13071). The process of spheroplast formation differs in that no "rabbit-eared" stage was present as seen in the penicillin method. Upon DAP deprival, the cells become oval and continue to swell, becoming large spheroplasts. This mode of spheroplast formation is thought to be responsible for the general distribution of flagella around the resulting spheroplasts when observed optically by use of flagellar stains, or with an electron microscope. This is strikingly different from the flagella distribution found in penicillin-induced spheroplasts, where they are confined to areas of "rabbit-eared" fragments of the parent rod-shaped cell. Spheroplasts (formed either by DAP deprival or penicillin induction) from nonflagellated cells grown at 44 C, upon transfer to 37 C, do not regenerate flagella as do organisms possessing cell walls. We conclude that the inability of spheroplasts to form flagella is not due to one of the multiple effects of penicillin (J. T. Park, Antimicrobial Agents and Chemotherapy—1963, p. 366, 1964), since DAP deprival affects only formation of rigid mucopeptide in the cell wall.

E. coli, when exposed to ethylenediaminetetraacetic acid (EDTA)-lysozyme at *p*H 8.0 (R. G. E. Murray et al., Can. J. Microbiol. **11**:547, 1965), also became nonmotile upon loss of its rod shape. When Trypticase Soy broth (BBL), with 15% (w/v) sucrose and 0.1 M $MgSO_4$, was added after 5-min exposure of this organism to EDTA-lysozyme, reversion of approximately 40% of nonmotile spheroplasts to motile normal cells was observed. After prolonged EDTA-lysozyme treatment (15 to 20 min), reversion decreased by more than 90%, presumably due to complete removal of the mucopeptide layer, which is needed to act as a primer for new mucopeptide synthesis.

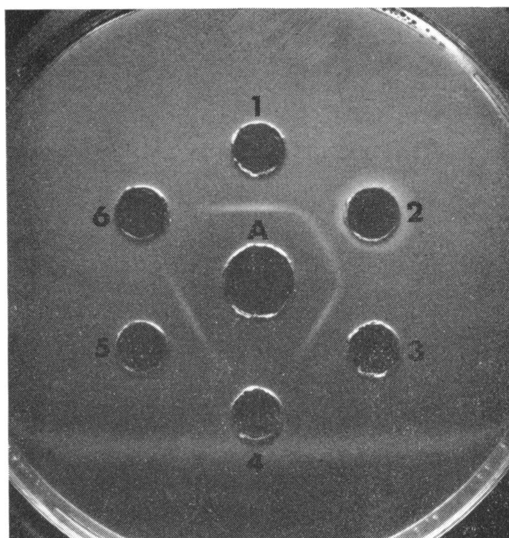

FIG. 1. *Ouchterlony analysis of soluble cell lysates of Escherichia spheroplasts. Antiserum to flagellin in center well (A); (1, 3, 5) control flagellin preparation; (2) 37 C spheroplast lysate; (4) 44 C spheroplast lysate; (6) lysate of 44 C spheroplasts incubated at 37 C for 12 hr. Only well 2, containing the 37 C spheroplast lysate, shows a line of precipitation identical to the precipitin lines of the control flagellin (1, 3).*

The detection of intracellular flagellin was accomplished by use of the Ouchterlony agar-gel diffusion technique, as applied to bacterial lysates by D. Kerridge (J. Gen. Microbiol. **33**:63, 1963). Flagellin antiserum was obtained by immunizing rabbits to flagellin prepared by maintaining purified flagella suspensions at *p*H 2 for 30 min, followed by purification on a Sephadex G-50 column with the use of a 0.01 M phosphate-saline buffer at *p*H 7.2. The antiserum was tested against penicillin-induced spheroplast lysates. Three lysate fractions containing soluble cell proteins

were examined: those of spheroplasts formed at 37 C (flagellated), at 44 C (nonflagellated), and those formed at 44 C and incubated at 37 C for up to 12 hr. Each fraction was the product of 2 liters of medium containing spheroplasts. These were lysed and centrifuged at 100,000 × *g* for 1 hr. The supernatant fluids were lyophilized and reconstituted to 2 ml with 0.85% (w/v) saline. The agar-gel diffusion technique used showed precipitin lines with a control sample containing 20 μg of flagellin per ml (determined by the method of O. Warburg and W. Christian, Biochem. Z. **310**:384, 1941). The reconstituted 37 C spheroplast lysate contained approximately 50 μg of flagellin per ml (determined by comparison of diluted lysate precipitin lines with those of control flagellin). Normal cells transferred from 44 to 37 C form flagella and become motile within 1 hr. If spheroplasts were capable of synthesizing flagellin, and only polymerization into flagella was blocked in the spheroplast state, the agar-gel diffusion method would show the presence of flagellin in the concentrated spheroplast lysates. Intracellular flagellin was not detected in the 44 C spheroplasts before or after transfer to 37 C (Fig. 1). This suggests that some critical mechanism(s) of flagellar synthesis is inoperative in the spheroplast state, and not merely the process of polymerization of flagellin into flagella. A possible explanation is that the flagellar synthesizing mechanism is closely associated with the cytoplasmic membrane, and it is inactivated when the membrane is deformed upon spheroplast formation. The conclusion that flagellin synthesis ceases is dependent upon the reliability of the agar-gel diffusion method for detection of intracellular flagellin, and this has been questioned by Kerridge.

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