INTRACELLULAR POLYSACCHARIDE OF ENTEROBACTERIACEAE

C. E. LANKFORD, HELEN HOYO, AND J. F. LUTTERINGER

Department of Bacteriology, University of Texas, Austin, Texas

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The periodate-Schiff polysaccharide stain of Hotchkiss (1948) and McManus (1948) has offered a new approach to the cytochemical investigation of microbial structure (Pennington, 1949). Selective staining of polysaccharide in the cell is reputed to depend upon the oxidizing action of periodate upon such chemical configurations as α, β -glycols and α -hydroxyketones.¹ Polyaldehydes generated by this selective oxidation react with sulfite-decolorized fuchsin. Provided these aldehydes are nondiffusible and are present in adequate concentration, an area of the cell is stained red corresponding to the locus of the polysaccharide. Since few components of the bacterial cell have excited more speculation concerning their exact loci than has the O antigen of the *Enterobacteriaceae* (Dubos, 1945; Miles and Pirie, 1949), a cytochemical method for its localization would be especially desirable. The results of this preliminary study are consistent with the possibility that the stainable, polar polysaccharide observed in most species of *Enterobacteriaceae* is identical with the polysaccharide haptene of their O antigens.

METHODS AND RESULTS

The staining procedure of Pennington (1949) was adopted with slight modification. The bacteria were fixed by heat, and the slide was immersed in 1 per cent aqueous potassium metaperiodate for 15 to 20 minutes. Following thorough washing with distilled water, the preparation was stained in sulfite-decolorized fuchsin for 15 minutes. Finally the slides were placed in sulfite wash water (5 ml each of 10 per cent sodium pyrosulfite and 0.1 N HCl in 100 ml water) for 10 minutes. After a final wash with distilled water the slides were air dried. The fuchsin reagent was prepared by dissolving 1 g of basic fuchsin in 100 ml of boiling water, filtering the dissolved dye, and decolorizing with 1 g sodium pyrosulfite and 5 ml N HCl per 100 ml. The straw-colored solution was placed in a colored bottle, allowed to stand 2 or 3 days, then decolorized with 1 g of norite charcoal. It should be refrigerated and renewed when it becomes colored. Unstained portions of the cell may be counterstained to advantage with 0.005 per cent aqueous methylene blue.

Stains generally were prepared from cultures grown upon 2 per cent glucose nutrient agar or tryptose agar slants for 12 to 14 hours.

¹ Since the oxidizing action of periodate requires as substrate a carbon chain with two adjacent, free hydroxyl groups, substitution at the 2 or 3 carbon blocks this reaction. Hence, pentose residues of polynucleotides, and polypyranosides with their glycosidic linkage at carbon 3, are unreactive. For discussions of specificity, refer to Hotchkiss (1948) and Jeanloz (1950).

Ninety cultures representing the genera *Shigella* (6 strains), *Salmonella* (20 strains), *Escherichia* (25 strains), *Aerobacter* (6 strains), *Paracolobactrum* (8 strains), *Klebsiella* (5 strains), *Proteus* (11 strains), and *Serratia* (9 strains) were stained. Of all the species of *Enterobacteriaceae* examined, only the 11

Figure 1. "Polysaccharide" polar bodies of representative *Enterobacteriaceae*. Twelve-hour cultures from glucose tryptose agar stained by the periodate-Schiff method. Magnification, 1,500 $\times$. Figure 1A, *Salmonella oranienburg*; 1B, *Escherichia coli*; 1C, *Aerobacter* (note variation in size of bodies, the polar "caps," a solidly-stained cell, and unipolar bodies in some cells); 1D, *Shigella paradysenteriae*, strain IV, (note predominance of unipolar cells).

Proteus strains did not exhibit stainable structures. The cell outlines of these organisms were visualized only with difficulty unless counterstained, and no internal stained structures were observed. The absence of stainable polysaccharide in *Proteus* may be due to lack of the requisite-free adjacent hydroxyls in its polysaccharide components. In contrast, cells from smooth cultures of other genera usually displayed characteristic deposits of stained material. Dense

loci of polysaccharide were situated at one or both poles of the cells as "granules," round bodies, or polar "caps" of varying size (figure 1). In many cultures these "polar bodies" were the only discernible structures to accumulate the stain, so that the surrounding cell wall and intervening cytoplasm scarcely were visible unless counterstained. In other cultures the cell boundary was rather sharply defined, and the cytoplasm was pale pink with a faintly stippled or reticulated appearance. Granules of varying size scattered at random throughout the cytoplasm between the polar bodies were observed in a portion of the cells of some cultures. An occasional cell was stained throughout. In the majority of cultures, however, the stainable polysaccharide was concentrated in the two polar bodies, located in the extreme cytoplasmic poles of the cells (figure 1A, B). These varied in size from small granules about $0.2\ \mu$ in diameter to large, round bodies filling the entire ends of the cell and occupying up to one-fifth to one-third of the apparent cell volume. Their size was more characteristic of a strain than of a particular species or genus. However, small polar bodies were observed more frequently in cultures of *Serratia* and *Paracoloobactrum*. Even within a single stained preparation considerable size variation often was apparent (figure 1C). The polar polysaccharide of some cells suggested a "cap" over each end of the cell, sometimes with a slight concavity toward the cell center. Although cells with bipolar bodies were most numerous in the majority of cultures, a few strains, particularly *Shigella paradysenteriae*, displayed a high frequency of unipolar bodies (figure 1D). Some cells with a single polar body, or with lightly stained second polar area, were observed within most of the predominantly bipolar cultures.

In certain strains of *Salmonella* there was a tendency for the polar bodies to decrease in size and frequency with repeated transfer following their original isolation. A culture of *Salmonella enteritidis*, for example, when examined several months after its isolation from a naturally-infected mouse, possessed only small polar granules, whereas the original culture had displayed prominent polar bodies. A single mouse passage sufficed to restore the polar bodies to their former frequency and prominence in the cells.

Particular attention was devoted to the position of the polar polysaccharide with relation to the cell surface. Wet mounts of stained preparations permitted observation of cells oriented at different and changing angles in the suspending fluid. There was no apparent alteration in form or position of the polar bodies in cells which changed their presenting surface. It was concluded, therefore, that the polar polysaccharide probably is a roughly spherical or ovoid mass embedded in the cytoplasm and in contact with the polar cell membrane. However, it was recognized that the polar "caps" found in some cultures may have a more superficial location.

Even with optimum conditions for polysaccharide deposition, polar bodies usually were not observed in all cells of a culture. Both environmental and intrinsic factors appeared to control the frequency of cells bearing polar bodies. For example, variations in frequency of bipolar cells were marked between strains as well as between species. Variations in the physiological age of the

culture probably accounted for the most marked fluctuations in frequency of polar bodies within cells of a specific culture. This frequency was estimated as the percentage of cells, of 200 counted, which possessed polar deposits of polysaccharide (figure 2). The test strains, *Salmonella oranienburg* and *Escherichia coli*, attained maximum bipolarity in cultures 12 to 14 hours of age, which coincided with the early maximum stationary phase of the growth cycle. Thereafter the frequency of bipolar cells declined slowly until few remained in cultures 2 or 3 weeks old.

The addition of glucose to the culture medium was found to increase the production of polar polysaccharide by a significant factor (figure 2). The frequency of cells bearing bipolar bodies was found to be subject also to manipulation of other environmental factors, such as pH, temperature, composition of the culture medium, and the presence of partially inhibitory concentrations of

Figure 2. The effect of culture age and glucose upon frequency of polar bodies.

certain bacteriostatic agents. Although these factors will not be considered here, they may offer an approach to the study of synthesis, function, and utilization of polysaccharide.

It has been possible to demonstrate that the presence of polar polysaccharide is subject to control by genetic as well as environmental factors. This was demonstrated by degrading the original smooth cultures to rough variants. Cultures of *Salmonella typhosa*, *Salmonella enteritidis*, *Shigella alcalescens*, and *Shigella paradyserteriae*, strain IV (Boyd 103), were passed repeatedly through broth containing 1 or 2 per cent LiCl. When typical R colony types were obtained on subculture, these were subjected to biochemical tests to establish their identity, and to agglutination in homologous O antiserum in order to detect residual somatic antigen. It was necessary to subject intermediate forms of *Salmonella enteritidis*, obtained from LiCl passage, to ultraviolet irradiation in order to effect complete transition to the R type, with coincident loss of O antigen. In the O-inagglutinable, rough variants derived from the four smooth

parent cultures there was found little or no stainable, intracellular polysaccharide. Polar bodies were absent. In certain of the intermediate forms polar bodies of reduced size and frequency were observed. Some of these intermediates agglutinated strongly in the O antiserum, whereas others showed weak and delayed agglutination. These results recall the observation of White (1929) that smooth cultures of *Salmonella* give an immediate macroscopic reaction with Molish's reagent, whereas rough variants do not.

While these observations suggest the identity of polar polysaccharide and the O antigen haptene, direct evidence is lacking. However, one additional point of corroborative evidence should be mentioned. The dark-field microscopic studies of Pijper (1938, 1941) on O agglutination of *Salmonella typhosa*, supported by convincing photomicrographs, led him to state that, "... all the bacilli hang together by their ends, presenting a completely polar arrangement which produces a curiously regular type of structure." "... a slow moving bacillus may approach a small clump, then pick up speed and attach itself head on. One has the impression of fields of force at work, always in the direction of the long axis of the bacillus" (Pijper, 1938). Such an agglutinative mechanism can be explained more reasonably on the basis of polar concentration of the O antigen, rather than by the assumption of its over-all peripheral distribution.

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SUMMARY

The Hotchkiss-McManus periodate-fuchsin stain for polysaccharide has been applied to 90 strains representing 8 genera of *Enterobacteriaceae*. Although cultures of *Proteus* did not stain, nearly all smooth cultures of the other genera were characterized by deeply-stained bodies located in one or, usually, both poles of the cell cytoplasm. The size of these structures and the frequency of cells bearing them were dependent upon many factors, including age of the culture and composition of the culture medium.

Rough, O-inagglutinable variants selected from smooth parent cultures did not possess stainable polysaccharide. The possibility that the stained polar bodies represent the polysaccharide haptene of the O antigen is discussed.

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