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Demonstration of Age-Dependent Capsular Material on *Pasteurella haemolytica* Serotype 1†

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Extracellular capsular material was demonstrated on early log-phase cells of *Pasteurella haemolytica* serotype 1 by the fluorescent-antibody and several capsular staining techniques. The presence of this material was shown to be age dependent. Wide capsules were demonstrable on cells from 2- to 12-h cultures, whereas cells from 16- to 22-h cultures had very little cell-associated capsular material. The Maneval technique most clearly demonstrated the presence of capsules on cells from young (6-h) cultures when compared with other capsule staining techniques.

Pasteurella haemolytica serotype 1 is the major cause of the severe fibrinous pneumonia that occurs in shipping fever of cattle (18). As early as 1956, the presence of soluble capsular material on *P. haemolytica* was demonstrated and its polysaccharide nature was established (5). Capsular material or diffusable surface antigens have been used subsequently to determine the serotype of this organism (1, 5, 9). Recently, material, presumably capsular, has been extracted from *P. haemolytica* by several techniques (8, 9a, 21). Vaccination of mice, hamsters (17, 21), and sheep (10, 24) with these extracts has been shown to protect them against experimentally induced pasteurellosis.

In its description of *P. haemolytica*, *Bergey's Manual* states "capsular material not usually demonstrated by microscopical methods" (20). Capsules on *P. haemolytica* from sheep have reportedly been demonstrated, and quantitative differences have been described between smooth and rough cell types (2). However, we have been unable to find any publications which include photographs of encapsulated organisms.

The purpose of the studies reported herein was to demonstrate the capsule on *P. haemolytica* serotype 1 and to examine the effect of culture age on capsular integrity. Studies with other serotypes of *P. haemolytica* were not performed.

MATERIALS AND METHODS

Culture media. Two solid types of media were used for culturing the organisms. The first was a modified

brain heart infusion agar (Difco Laboratories, Detroit, Mich.) which contained 5% bovine blood, 10% horse serum (M.A. Bioproducts, Walkersville, Md.), and 1% yeast hydrolysate (ICN Pharmaceuticals, Cleveland, Ohio). In addition, a modification of Sawata medium (14) with a brain heart infusion base was used. A corresponding Sawata broth was also used.

Microorganism. The organism used throughout the study was *P. haemolytica* serotype 1, (9), originally isolated from a feedlot calf. The organism was maintained in the lyophilized state. For each experiment, a lyophilized culture was reconstituted with distilled water and streaked for isolation onto appropriate media. After 22 h of incubation at 37°C in a candle jar, isolated colonies were suspended in 0.01 M phosphate-buffered saline (PBS) (pH 7.2), and the resulting suspension was used to inoculate fresh media. Cultures on solid media were incubated for various times at 37°C in a candle jar. Broth cultures were incubated at 37°C in a shaking water bath.

Fluorescent-antibody technique (FAT). Antiserum to *P. haemolytica* serotype 1 was made by injecting chickens intravenously with whole cells at four 1-week intervals. Pooled antisera were conjugated with fluorescein isothiocyanate (BBL Microbiology Systems, Cockeysville, Md.) (6). At 2-h intervals after inoculation (up to 22 h), thin smears of water-suspended organisms were made on glass cover slips and allowed to air dry. Subsequently, they were fixed in acetone for 10 min and stained with conjugated antiserum for 25 min at 37°C. A 1:5 dilution of antiserum was found to be optimal. The cover slips were then washed in three changes of PBS for a total of 20 min, mounted on glass microscope slides with a 90% PBS–10% glycerol mounting medium (pH 7.2), and examined microscopically with a UV light source.

Capsular staining techniques. (i) Maneval method (15). One drop of PBS-suspended organisms was mixed on a glass microscope slide with an equal drop of 1% aqueous Congo red stain (Allied Chemical, New York, N.Y.). The mixture was spread into a thin film smear and allowed to air dry. Without fixation, the
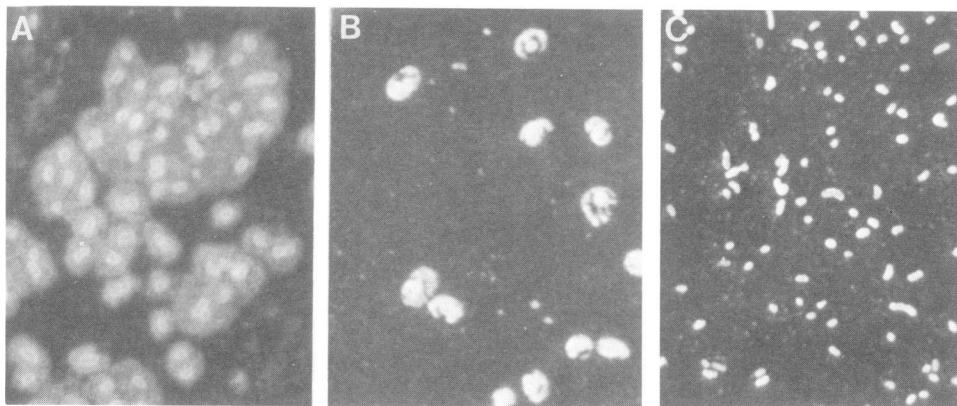
FIG. 1. *P. haemolytica* stained with fluorescein isothiocyanate-conjugated antiserum (×500). (A) Cells from a 6-h culture with wide capsules. (B) Cells from a 12-h culture. Capsules have a mottled appearance. (C) Cells from a 22-h culture. Capsular material is no longer apparent.

smears were counterstained with Maneval stain for 2 min, drained, and blotted dry.

(ii) **India ink method (4).** Bacterial growth was suspended in sterile 6% glucose on a glass microscope slide. This suspension was then mixed with a drop of waterproof ink (Pelikan, Hannover, Germany). The mixture was spread into a thin film smear and allowed to air dry. The smear was fixed for 1 min in absolute methanol, rinsed in water, counterstained with 1% aqueous crystal violet (Difco) for 1 min, and rinsed in water.

(iii) **Jasmin method (13).** Bacterial growth was suspended on a microscope slide in PBS containing 0.75% phenol and 10% horse serum. A thin film smear was made from the suspension and allowed to air dry. The smear was fixed by rapid immersion in absolute methanol followed by flaming. It was counterstained with 1% aqueous crystal violet for 1 min and rinsed in water.

(iv) **Other methods.** The eosin-serum and Hiss methods were performed as described previously (11, 12).

Smears stained by each technique were examined under a 100× oil immersion lens of a light microscope.

RESULTS

Smears stained by the FAT showed very bright fluorescent staining of the cell walls. Organisms from cultures incubated for less than 12 h were surrounded by wide capsules that stained less intensely than the cell walls (Fig. 1A). Capsules appeared to be widest around organisms from 2-h cultures. After 12 h of incubation, the capsular material had a mottled appearance and only partially encircled the cells (Fig. 1B). In smears prepared from cultures 16 h or older, capsular material usually could not be demonstrated (Fig. 1C). If present, the capsular material was often at the poles of the cells.

Capsular material also was demonstrated surrounding 6-h cells stained by the Maneval, Jasmin, and India ink methods. Capsules were not demonstrated by the eosin-serum or Hiss tech-

niques. The results of staining by the Maneval method correlated with the FAT in that capsules were clearly and consistently demonstrated on cells from 6-h cultures (Fig. 2A). The bacterial cells stained red, and the background stained blue. The capsules appeared as a wide clear zone. Capsules were larger with this method than with any of the other capsule staining techniques. The majority of the cells in a 22-h culture appeared to have very little of the extracellular material, which again was concentrated at the poles of the cells (Fig. 2B). The age-dependent character of capsular association with cells was demonstrated to occur on both solid media and Sawata broth.

In the Jasmin preparation, capsules appeared as narrow clear zones around the cells, but both cells and background stained purple, and there was not sufficient contrast to make the capsules obvious. Repeated efforts and modifications did not significantly improve the quality of the preparations. The India ink method stained the cells purple and the background black. The capsules again appeared as clear zones around the cells. However, the granularity and darkness of the background made it difficult at times to see unencapsulated organisms or to measure the size of the capsules around encapsulated ones.

DISCUSSION

Capsular material was demonstrated on young cultures of *P. haemolytica* serotype 1 by the FAT and other capsular staining techniques. The dry film India ink and Jasmin methods demonstrated capsular material, but Maneval stain was superior in that it correlated with the FAT results, and the capsules appeared larger and more distinct. The Maneval method is simple and rapid, making it excellent for following
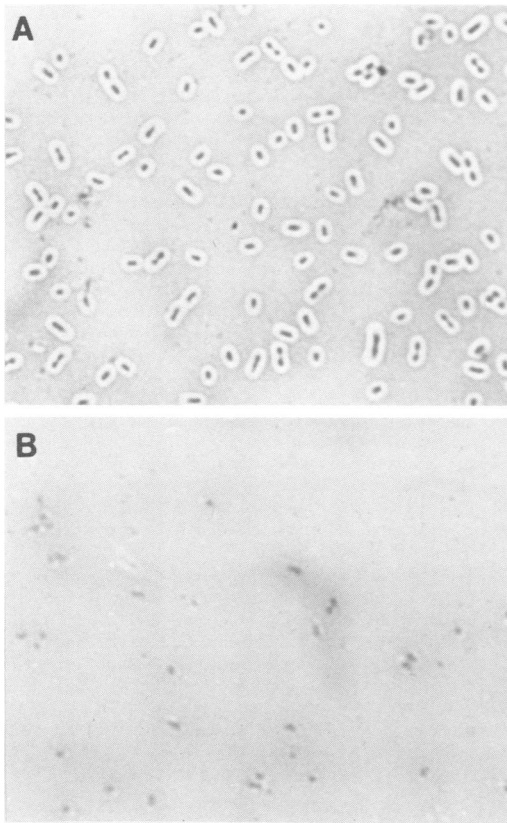
FIG. 2. *P. haemolytica* stained by the Maneval method (×1,250). (A) Cells from a 6-h culture. Capsules appear as wide clear zones. (B) Cells from a 22-h culture. Minimal capsular material is present on cells.

the dissolution of capsular material during extraction procedures, as well as for screening cultures or even individual colonies for capsule production.

Exopolysaccharide production by several organisms has been reported to be dependent on culture age, but those organisms differ in the growth stages in which production occurs. A mucoid strain of *Pseudomonas aeruginosa* was shown in batch culture to produce polysaccharide throughout the exponential growth phase, and both growth and polysaccharide production ceased simultaneously (16). For other organisms, notably *Bacillus licheniformis* (23) and *Pseudomonas* sp. strain NCIB 11264 (25), capsular polysaccharide production was not concomitant with exponential growth of the organisms, but was initiated in late log phase and occurred primarily after the cultures entered stationary phase. For type III *Pneumococcus* (3) and an organism identified as *Aerobacter aerogenes* (7), most of the total polysaccharide production occurred after cessation of logarithmic

growth when cell numbers were highest, but the rate of production per cell was greatest in the early logarithmic phase and declined progressively thereafter. It was further reported with *A. aerogenes* that the average capsule diameter increased during and after the log phase to a maximum at 48 h (7).

Our results with *P. haemolytica* serotype 1 indicate that maximum cell-associated capsular material is produced during the early logarithmic growth phase. Capsules appeared to be widest in organisms from cultures 2 to 6 h old and diminished gradually as the culture aged, until little capsular material remained on cells from cultures over 16 h old. A similar age dependency of capsular association also has been demonstrated ultrastructurally with *Klebsiella pneumoniae* (19). In that study, the majority of cells harvested in the stationary growth phase (36 h) exhibited shrunken capsules with coarse and short filaments or no capsule at all, whereas a capsule was demonstrated on almost all bacteria harvested in the logarithmic growth phase (5 to 12 h).

The cause of the age-dependent phenomenon as demonstrated in both *P. haemolytica* and *K. pneumoniae* is unknown. We have, however, observed ultrastructurally (M. J. Gentry, unpublished data) that the cell wall of *P. haemolytica* differs topographically in organisms from 6-h as compared with 22-h cultures. Alterations in cell wall integrity may allow leakage of intracellular components into the surrounding capsular material, resulting in either alterations in capsular pH or enzymatic degradation of the capsule. Such degradation has been shown to occur in *Bacillus licheniformis* (22). The polyglutamic acid produced by late log-phase cells of this organism is originally bound firmly to the cell surface as a capsule. After maximum production, however, there is a fairly rapid loss of this capsular material without cell lysis due to elaboration into the culture medium of an endo-type, poly (γ-D-glutamyl) depolymerase.

In conclusion, the amount of capsular material associated with *P. haemolytica* serotype 1 cells is maximal in young (less than 12 h) cultures. This indicates that to obtain capsular material in the quantities needed for chemical analysis or immunization studies, young cultures should be used.

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