Modulation of *Pasteurella multocida* capsular polysaccharide during growth under iron-restricted conditions and *in vivo*

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Addition of the iron chelators 2,2'-dipyridyl, deferoxamine mesylate or apo-transferrin to culture media affected the composition and the morphology of *Pasteurella multocida* cells. Cells grown under iron-restricted conditions expressed iron-regulated proteins and, in addition, iron deprivation markedly reduced the amount of capsular material covering the cells of *P. multocida*. The addition of iron neutralized the effect of these chelators on capsule production. Cells of *P. multocida* grown under iron-restricted conditions were more labelled by gold particles coated with polymyxin which is known to interact with the lipid A-core region of lipopolysaccharides, and showed increased affinity for porcine respiratory tract mucus than cells grown under iron-sufficient conditions. Bacterial cells grown *in vivo* in peritoneal chambers in rats were also only covered by a thin layer (15–20 nm) of capsular material. Although the capsule is believed to be an important virulence factor, our data indicate that under iron-restricted conditions, such as those encountered *in vivo*, *P. multocida* may not be heavily encapsulated.

Keywords: *Pasteurella multocida*, capsule, polysaccharide, iron restriction

INTRODUCTION

The environmental modulation of cell surface components of both Gram-positive and Gram-negative bacteria has been intensively investigated (Brown & Williams, 1985). Among the environmental factors that commonly influence the properties of microbial cells, the availability of essential nutrients, such as iron, assumes particular importance. Studies on various bacterial species have shown that micro-organisms frequently adapt to conditions of iron restriction, as found *in vivo*, by undergoing a number of phenotypic changes including the induction of iron-regulated proteins (IRPs). Many Gram-negative bacteria synthesize high-affinity iron-transport systems consisting of low-molecular-mass iron-chelating agents (siderophores) and receptor proteins involved in the uptake and transport of the siderophore-iron complexes across both outer and cytoplasmic membranes (Brown & Williams, 1985). In addition to the various alterations which occur in pathogenic bacteria during growth under iron restriction, the production of some classical virulence factors (e.g. certain bacterial

toxins or hydrolytic enzymes) is also affected (Griffiths & Bullen, 1987; Mekalanos, 1992).

Little is known about the effect of iron deprivation on bacterial capsule production. In general, good polysaccharide synthesis is permitted in nutrient-limited growth media where nitrogen, sulfur or phosphate provide the limiting nutrient, and a utilizable carbon source is in excess (Sutherland, 1988). Metals have been shown to influence the production of capsule exopolymers but no generalizations can be made with respect to the effects that metals and other cations have on bacterial capsule production (Geesey & Jang, 1989). Some researchers have found direct evidence that metal ions bind electrostatically with microbial polysaccharides, and various ions are known to be required as cofactors in polysaccharide synthesis (Geesey & Jang, 1989; Sutherland, 1977). The biosynthesis of capsular polysaccharide by the bacterial cell is a complex process which begins with sugar activation in the cytoplasm. Following polymerization, catalysed by cytoplasmic membrane-bound transferases, the final stage is the translocation of polysaccharide to the cell surface (Jann & Jann, 1990). Genes have been identified which are thought to encode products responsible for the translocation of the polysaccharides across the cytoplasmic and outer membranes, and the

Abbreviations: DM, deferoxamine mesylate; DP, 2,2'-dipyridyl; IRP, iron-regulated protein; OMP, outer-membrane protein.

organization of exported polysaccharides into a capsule (Boulnois & Jann, 1989).

Swine atrophic rhinitis is a multifactorial disease complex characterized by turbinate atrophy (Rutter, 1985). *Pasteurella multocida*, the etiological agent, possesses various putative virulence factors including a polysaccharide capsule, lipopolysaccharides (with endotoxic activities), and a dermonecrotic toxin (Chanter & Rutter, 1989). In this study, we have examined the effects of growth under iron limitation on *P. multocida* production of capsular material, a potentially important virulence factor.

METHODS

Bacterial isolates and growth conditions. Three toxigenic isolates of *P. multocida* (2305-A and 23-4, capsular type A; 30-1, capsular type D), retrieved from the nasal cavities of pigs with atrophic rhinitis, were used in the present study. Bacterial isolates were grown on Mueller-Hinton agar plates, for 18 h at 37 °C (iron-sufficient conditions). To obtain bacterial growth under conditions of iron restriction, organisms were inoculated onto plates supplemented with 2,2'-dipyridyl (DP; 60, 100 or 160 µM; Sigma), deferoxamine mesylate (DM; 50, 100, 150 or 200 µM; Sigma), or with human or bovine apo-transferrin (1 mg ml⁻¹; Sigma).

Transmission electron microscopy. (i) Polycationic ferritin labelling. Bacterial cells were prepared for transmission electron microscopy following polycationic ferritin labelling, a method which allows good preservation of capsular material (Jacques & Foiry, 1987; Jacques *et al.*, 1990). Bacterial cells were fixed in cacodylate buffer (0.1 M, pH 7.0) containing 5% (v/v) glutaraldehyde, for 2 h at 20 °C. Fixed bacteria were suspended in cacodylate buffer and allowed to react with the polycationic ferritin, final concentration 1.0 mg ml⁻¹, for 30 min at 20 °C (Jacques & Foiry, 1987). The reaction was slowed by 10-fold dilution with buffer, and the organisms were centrifuged and washed three times in cacodylate buffer. Bacterial cells were then immobilized in 4% (w/v) agar, washed five times in cacodylate buffer and post-fixed with 2% (w/v) osmium tetroxide for 2 h. Washings were repeated as above, and the samples dehydrated in a graded series of acetone washes. Samples were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin. Thin sections were post-stained with uranyl acetate and lead citrate and examined with an electron microscope (Philips 201) at an accelerating voltage of 60 kV. The determination of capsular material thickness was based on 20–25 measurements (per preparation) on micrographs taken at a magnification of 45 000×. Since capsule is not always a uniform layer, the results are expressed as a range (in nm).

(ii) Polymyxin B-gold labelling. The procedure used has been described by Morioka *et al.* (1987). Briefly, polymyxin B was conjugated with BSA using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide. Colloidal gold particles (15 nm) adjusted to pH 5.5 were then coated with polymyxin B-BSA. Gold-labelled BSA was prepared as a control. A drop of a bacterial suspension was placed on a Formvar-coated electron microscope grid for 1 min. Grids with bacteria were placed on drops of polymyxin B-BSA-gold for 30 min. After washing, the grids were negatively stained with 0.2% (w/v) phosphotungstate and examined as mentioned above.

Protein profiles. Bacterial cells were harvested and whole cell protein profiles were analysed as described by Bélanger *et al.*

(1990). Briefly, bacteria were suspended in solubilization buffer containing 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 0.0625 M Tris/HCl (pH 6.8) and 0.025% (w/v) bromophenol blue. Samples were heated for 15 min at 100 °C. Samples were separated by discontinuous SDS-PAGE using stacking gels of 4.5% (w/v) polyacrylamide and separating gels of 12.5% (w/v) polyacrylamide (Laemmli, 1970). Samples were electrophoresed at 100 V (stacking gel) and 200 V (separating gel), using a Mini-Protean II apparatus (Bio-Rad). Gels were stained with Coomassie brilliant blue R-250.

Outer-membrane protein (OMP) isolation. The method of Carlone *et al.* (1986) was followed for OMP isolation. Briefly, cells from 10 ml of a culture adjusted to an OD₆₀₀ of 0.5 were centrifuged and washed in 10 ml 10 mM HEPES (Sigma) buffer (pH 7.4). The cells were centrifuged again and the cell pellet was frozen in liquid nitrogen. After suspension in 1 ml HEPES buffer, cells were sonicated three times for 1 min each time. Total membranes were collected by centrifugation at 16 000 g for 30 min and suspended in 0.2 ml HEPES buffer. Inner membrane proteins were solubilized by the addition of an equal volume of 2% (w/v) Sarkosyl (*N*-lauroylsarcosamine; Sigma) in HEPES buffer for 30 min; OMPs were recovered by centrifugation and the pellet was suspended in 30 µl HEPES buffer. Samples were boiled for 5 min in solubilization buffer and separated by electrophoresis as described above.

Affinity for porcine respiratory tract mucus. To screen for mucus binding by *P. multocida* isolates, dot blots were performed (Bélanger *et al.*, 1992). The trachea was removed from healthy adult pigs at the slaughter house, and crude mucus (containing approximately 1 mg protein ml⁻¹) was obtained following the procedure previously described by Letellier *et al.* (1991). A rabbit antiserum against the porcine crude respiratory tract mucus was prepared as described by Rozee *et al.* (1982). Ten microlitre samples of the bacterial suspensions were placed on a nitrocellulose membrane. All incubation steps were performed at room temperature, followed by four 3 min washes with a Tris-saline buffer (TSB; 10 mM Tris, 150 mM NaCl, pH 7.4). The membranes were first incubated for 1 h with 2% (w/v) casein, a blocking solution, and for 1 h with the filtered mucus preparation diluted 1/5. The membranes were then incubated for 2 h with a 1/50 dilution of an antiserum against the mucus preparation followed by a 1 h incubation with a goat anti-rabbit IgG conjugated to peroxidase diluted 1/10 000 (Bio-Rad). The reaction was revealed by addition of 4-chloro-1-naphthol and H₂O₂. Reference strain *P. multocida* 4-4056 (capsular type D) was used as a control with affinity for porcine respiratory tract mucus whereas *Escherichia coli* O111 was used as a negative control.

Diffusion chambers and *in vivo* growth conditions. We have examined cells of isolates 23-4 and 2305-A grown in diffusion chambers implanted in the peritoneal cavity of rat for 18 h as described by Malouin *et al.* (1990). Diffusion chambers (15 × 10 mm) were constructed from polypropylene tubing (Nalgene). Millipore MF 0.22 µm filters were cut to the diameter of the barrels and attached by melting the chambers to affix the filter. The chambers were autoclaved. Overnight cultures of bacteria diluted to 10⁸ c.f.u. ml⁻¹ in saline were injected through a needle hole melted through the side of the barrels. The hole was subsequently closed using a hot glass rod. Chamber volume was approximately 500 µl. Sprague Dawley female rats, approximately 250 g, were anaesthetized i.p. with Ketamine HCl (87 mg kg⁻¹) and xylazine (13 mg kg⁻¹). Four chambers were placed in the peritoneal cavity through a small longitudinal incision in the abdomen. Animals were sacrificed using carbon dioxide before chambers were removed.

Fig. 1. Transmission electron micrographs of thin sections of cells of *P. multocida* isolate 2305-A labelled with polycationic ferritin. Cells were grown *in vitro* under iron-sufficient conditions (a) or iron-restricted conditions obtained after the addition of 160 μM DP (b), 150 μM DM (c), or 1 mg bovine apo-transferrin ml^{-1} (d). Bars, 200 nm.

RESULTS and DISCUSSION

Cells of *P. multocida* grown under iron-sufficient conditions were covered with a dense layer of ferritin granules of approximately 55–65 nm for the isolates 30-1 and 23-4, and of 105–125 nm for the isolate 2305-A, whereas cells grown under iron-restricted conditions were covered

with a much thinner layer of 15–30 nm (Fig. 1). Growth of these isolates in the presence of increasing concentrations of an iron chelator, either DP or DM, resulted in a marked reduction in capsular material thickness (Fig. 2a).

To support the idea that the presence of a thinner capsular

Fig. 2. (a) Effects of various concentrations of DP on capsule thickness of *P. multocida* isolate 2305-A. (b) Effects of the addition of FeCl₃ on capsule thickness of *P. multocida* isolate 2305-A grown on media containing 160 μM DP.

material was due to growth under iron-restricted conditions, we first added different concentrations of FeCl₃ to media containing 160 μM DP (Fig. 2b) or 200 μM DM. Repletion of iron restored completely the capsular material production of all isolates to the level seen with cells grown under iron-sufficient conditions. It is noteworthy that growth of *P. multocida* isolates in the presence of an excess of iron (Mueller-Hinton agar plates supplemented with 10 or 25 μM FeCl₃) had no effect on their capsular material production.

It has been shown that avian and bovine isolates of *P. multocida* grown under iron-restricted conditions produced a siderophore termed multocidin (Hu *et al.*, 1986) and also expressed several IRPs of molecular masses ranging from 76 to 96 kDa (Choi-Kim *et al.*, 1991; Ikeda & Hirsh, 1988; Ogunnariwo *et al.*, 1991; Snipes *et al.*, 1988). Iron-restricted conditions were also confirmed by determining the protein profiles of *P. multocida* grown

Fig. 3. Comparison of SDS-PAGE profiles of proteins, stained with Coomassie brilliant blue, from *P. multocida* 2305-A grown under iron-sufficient (lane 1), or iron-restricted conditions obtained after the addition of 160 μM DP (lane 2) or 200 μM DM (lane 3). Arrows denote the presence of iron-regulated proteins. Molecular mass markers are indicated on the left.

under iron-restricted and iron-sufficient conditions. Bacterial cells were harvested and whole cell protein profiles were analysed by SDS-PAGE. The results showed that IRPs were expressed by our porcine isolates grown in iron-restricted media. For example, IRPs of molecular masses of 80, 75, 72 and 33.5 kDa were observed for *P. multocida* 2305-A isolate (Fig. 3, arrows).

Cells grown under iron-restricted conditions were re-suspended in fresh iron-sufficient liquid medium (Mueller-Hinton broth). We observed that as soon as after 30 min of incubation in fresh iron-sufficient medium, the production of capsular material had increased; after 1–2 h of incubation the cells were well capsulated and similar in appearance to control cells grown overnight under iron-sufficient conditions.

We used polymyxin B-gold labelling to visualize the accessibility of lipopolysaccharides (LPS) at the surface of cells grown under iron-sufficient or iron-restricted conditions. Cells grown under iron-sufficient conditions were weakly labelled (Fig. 4a) whereas cells grown under iron-restricted conditions were heavily labelled by the polymyxin B-BSA-gold particles (Fig. 4b). No labelling was

Fig. 4. Transmission electron micrographs of cells of *P. multocida* isolate 23-4 grown under iron-sufficient (a) or iron-restricted conditions (b) and labelled with polyclonal BSA-gold particles (15 nm). Bars, 200 nm.

observed when BSA-gold particles were used. Results obtained using polyclonal B, a substance known to interact with the lipid A-core region of LPS molecules (Morioka *et al.*, 1987), indicate that LPS is, as anticipated, more accessible at the surface of cells grown under iron-

Fig. 5. Dot blot assay used to show binding of porcine respiratory tract mucus to isolates of *P. multocida*. Bacterial cells, immobilized on the membrane, were sequentially incubated with a mucus preparation and an antiserum against the mucus preparation. *P. multocida* 2305-A grown under iron-sufficient (a) or iron-restricted (b) conditions. *P. multocida* 23-4 grown under iron-sufficient (c) or iron-restricted (d) conditions.

restricted conditions and which are covered by a thinner layer of capsular material.

Because we have previously found that the LPS of *Actinobacillus pleuropneumoniae*, another important swine-pathogenic member of the *Pasteurellaceae*, was important for adherence to host cells and mucus (Bélangier *et al.*, 1990, 1992), we suspected that a thinner layer of capsular material could help adherence of *P. multocida*. We then used dot blotting on a nitrocellulose membrane to evaluate the binding of porcine respiratory tract mucus to *P. multocida* isolates. Bacterial cells were immobilized on the membrane and sequentially incubated with the mucus preparation and an antiserum against the mucus preparation. Bacteria grown under iron-restricted conditions on dipyrindyl-containing medium showed increased adherence to porcine respiratory tract mucus (Fig. 5). Bacteria grown under iron-restricted conditions also showed increased adherence to porcine frozen lung and trachea sections (data not shown). These results suggest that the thicker capsular material layer of *P. multocida* grown under iron-sufficient conditions masks outer-membrane components which are involved in adherence.

Finally, we have examined cells of isolates 23-4 and 2305-A grown in diffusion chambers implanted in the peritoneal cavity of rats for 18 h. The growth of *P. multocida* was monitored after implantation of diffusion chambers; the number of viable cells initially inoculated (3×10^8 c.f.u. ml⁻¹) increased a little more than three times (to 10^9 c.f.u. ml⁻¹) after 18 h incubation. Cells of *P. multocida* 23-4 grown *in vivo* were covered with a layer of capsular material much thinner (15–20 nm) than the one observed on cells grown *in vitro* under iron-sufficient conditions (Fig. 6); similar results were obtained with the

Fig. 6. Transmission electron micrographs of thin sections of cells of *P. multocida* isolate 23-4 labelled with polycationic ferritin. Cells were grown *in vitro* under iron-sufficient conditions (a) or *in vivo* in rat peritoneal diffusion chambers (b). Bars, 200 nm.

P. multocida 2305-A isolate. These reductions in capsular material thickness were not due to growth in liquid because *P. multocida* cells were well capsulated after cultivation in Mueller-Hinton broth. Rather, changes observed in chamber grown cells may be due to iron

Fig. 7. Comparison of SDS-PAGE profiles of OMPs, stained with Coomassie brilliant blue, from *P. multocida* 2305-A grown under iron-sufficient conditions (lane 1) and prior to cultivation *in vivo*, or after a 16 h growth period in diffusion chambers in rats (lane 2). Arrows denote the presence of some proteins induced by *in vivo* growth and similar in molecular mass to some of the iron-regulated proteins indicated in Fig. 3. Molecular mass markers are indicated on the left.

deprivation. Indeed, growth in diffusion chambers resulted in the expression of proteins similar to those induced by iron-restricted conditions *in vitro* (Fig. 3) and seemed to be mainly located on the outer membrane of cells (Fig. 7). However, at this point we still cannot exclude that factors other than iron limitation may be involved in the modulation of expression of capsular material *in vivo*.

To our knowledge, few studies have looked at the effects of iron limitation on bacterial ultrastructure. Large periplasmic spaces have been observed in *Haemophilus ducreyi* grown under iron limitation (Abeck *et al.*, 1990). Ferrala *et al.* (1986) observed that under iron starvation, the capsule of *Azotobacter chroococcum* appeared more diffuse and extensive, and speculated that it might serve to enhance iron sequestration. Iron limitation, an environmental condition relevant to the lungs of patients with cystic fibrosis, was shown to result in conversion of *Pseudomonas aeruginosa* to the mucoid phenotype (Terry *et al.*, 1992). Here, nutrient (iron) limitation seemed to have an opposite effect on *P. multocida* capsule production.

There is also a possibility that iron limitation leads to greater release or looser attachment of exopolysaccharides.

Bacterial pathogens have evolved highly sophisticated environmental signal transduction systems, and the expression of virulence factors is frequently coordinately controlled by a common regulatory system (Mekalanos, 1992). The capsular material regulation in *P. multocida* may be part of a response to iron-restricted growth conditions that includes derepression of genes for the synthesis of an iron-acquisition system. It is also possible that iron acts as a cofactor in the biosynthesis of *P. multocida* capsular polysaccharides.

In vivo, where free iron concentration is extremely low, the reduction in capsule formation by *P. multocida* might result in a better exposure on the bacterial cell surface of its adhesin(s) which could be advantageous for this microorganism. Our results clearly showed that adherence of *P. multocida* to porcine respiratory tract mucus, and to lung and trachea sections was enhanced for bacteria having a thinner capsular material, a morphological change induced by iron restriction *in vitro* or by growth in animals. We have previously shown that LPS molecules may be important adhesins for *A. pleuropneumoniae* (Bélanger *et al.*, 1990, 1992) and have shown here that the lipid A-core region of LPS in *P. multocida* was more accessible when cells presented less capsular material. A thinner layer of capsular material in *P. multocida* correlated with a greater adherence but more work is needed to show the importance of LPS for attachment to host cells. Thus, although capsule is believed to be an important virulence factor, *P. multocida* may not be heavily encapsulated when growing under iron-limited conditions.

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