Conservation of Expression and N-Terminal Sequences of the *Pasteurella haemolytica* 31-Kilodalton and *Pasteurella trehalosi* 29-Kilodalton Periplasmic Iron-Regulated Proteins

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This study examined the conservation of expression of a 31-kDa iron-regulated protein by serotypes of *Pasteurella haemolytica* and *Pasteurella trehalosi* associated with pasteurellosis of cattle and sheep. A polyclonal antibody prepared against the purified 31-kDa periplasmic iron-regulated protein from *P. haemolytica* serotype A1 showed that all *P. haemolytica* serotypes expressed similar 31-kDa proteins with identical N-terminal sequences, whereas *P. trehalosi* serotypes expressed immunologically different 29-kDa proteins with a different N-terminal sequence. Antibody to the 31-kDa iron-regulated protein was a useful tool to distinguish similarities and differences of the iron-regulated proteins of *P. haemolytica* and *P. trehalosi*.

*Pasteurella haemolytica* causes respiratory disease in cattle (10) and sheep (13). *Pasteurella trehalosi*, formerly classified as *P. haemolytica* biovar T, is associated with systemic infection of sheep and also with pneumonia of sheep (13). Microbial pathogens, including *Pasteurella*, have developed an iron acquisition system for obtaining iron from host protein-bound iron which is negatively regulated by the concentration of iron in the pathogen’s environment (14). *P. haemolytica* expresses several outer membrane proteins in response to limited iron availability (5, 6, 9, 11, 20), and a transferrin receptor has been identified (23). A siderophore-mediated iron acquisition system as described by Neijlands (21) was not found in *P. haemolytica* (23). In addition to the iron-regulated outer membrane proteins, *P. haemolytica* also expresses iron-regulated periplasmic proteins (27), which are thought to play a role in iron transport across the periplasm to the cytoplasmic membrane (23). We recently identified and characterized a 31-kDa iron-regulated protein from *P. haemolytica* serotype A1 (27). *P. haemolytica* serotype A2 has been reported to express a 35-kDa iron-regulated protein (17), but no information is available on the expression of the 31-kDa protein by the 12 *P. haemolytica* serotypes and by the closely related *P. trehalosi* serotypes. The objectives of this study were (i) to examine the conservation of expression of the 31-kDa periplasmic iron-regulated protein in whole-cell extracts and osmotic shock fluids of the various serotypes of *P. haemolytica* and *P. trehalosi* by using a polyclonal antibody to the 31-kDa protein and (ii) to compare the N-terminal sequences of the conserved proteins. These experiments provided additional information on the relatedness of these two *Pasteurella* species.

*P. haemolytica* serotypes A1, A2, A5 through A9, A11 through A14, and A16 and *P. trehalosi* (formerly *P. haemolytica* biovar T) serotypes T3, T4, T10, and T15 were from the culture collection of one of us (G.H.F.). Cultures were maintained frozen at −70°C in brain heart infusion broth (BBL) containing 15% (vol/vol) glycerol. Growth on blood agar plates and subculture in RPMI 1640 medium were performed as described by Tabatabai and Frank (27), except that 2-ml broth cultures were used. For certain experiments, the RPMI 1640 medium was supplemented with 5 μg of Fe3+ (from FeCl3) per ml. The cultures were harvested after 20 h and the absorbance at 600 nm was determined.

Cell pellets were prepared by centrifuging (14,000 × g for 3 min) 1 ml of culture adjusted to an absorbance of 600 nm of 1. Periplasmic proteins were prepared by the osmotic shock method of Neu and Heppel (22) as modified by Berish et al. (3). Osmotic shock fluids were obtained after 1 h of incubation on ice followed by centrifugation at 14,000 × g. The supernatant containing the periplasmic proteins was removed, and 20-μl aliquots were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and protein analysis. The protein content of the osmotic shock fluids was determined by the method of Lowry et al. (19), with bovine serum albumin (Pentex; Miles) as a standard.

Periplasmic proteins were examined for contamination by membrane proteins by the NADH oxidase reaction (24) and for contamination by cytoplasmic proteins by the β-galactosidase reaction as follows. The reaction buffer contained 0.1 M phosphate-buffered saline (PBS) (pH 7.0), 1 mM MgSO4, and 50 mM 2-mercaptoethanol. Just before use, 4 mg of o-nitrophenyl-β-n-galactosidanoside (ONPG; Sigma Chemical Co.) was added to 10 ml of buffer, yielding a final concentration of 1.2 mM ONPG in the assay. The reaction was started by the addition of 100 μl of osmotic shock fluid to 1.0 ml of reaction buffer. The rate of change of absorbance at 405 nm was recorded. The extinction coefficient for o-nitrophenol is 1.65 × 104 M−1·cm−1. One unit of enzyme activity is defined as the production of 1 μmol of o-nitrophenol per min per mg of protein.

For SDS-PAGE of the samples, 50 μl of sample buffer was added to the cell pellet and the samples were boiled for 5 min (16) and loaded onto 12.5% acrylamide gels with a 4% stacking gel. Electrophoretic transfer (28) was done at 0.11 A and 30 V for 16 h with a Bio-Rad Transblot unit. The nitrocellulose blot was incubated for 15 min with 0.25% fish gelatin (Norland Products) in 0.1 M PBS (pH 7.2) containing 0.05% Tween 20. The blot was incubated with a 1:1,000 dilution of rabbit antibody to the 31-kDa protein (prepared from *P. haemolytica* serotype A1) in 0.25% fish gelatin—PBS—Tween, washed, and incubated with a 1:1,000 dilution of horseradish peroxidase-
conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Company) in fish gelatin-PBS-Tween. The blot was washed and developed with a substrate solution containing 20 ml of 0.1 M sodium phosphate buffer (pH 7.2), 4 ml of 0.3% (wt/vol) 4-chloronaphthol in methanol, and 10 μl of 30% hydrogen peroxide.

Osmotic shock fluids containing the periplasmic proteins (5 μg) were electroblotted onto a polyvinylidene difluoride (PVDF) membrane as previously described (27). N-terminal sequencing of the protein bands cut from the blots was done with an Applied Biosystems model 477 protein sequencer at the Protein Facility, Iowa State University, Ames. Yield of the N-terminal amino acids of the first cycle ranged from 5 to 8 pmol.

The 31-kDa protein was obtained from *P. haemolytica* A1 grown with iron restriction as previously described (27). Briefly, the protein was extracted by a high-salt extraction procedure followed by anion-exchange chromatography. The protein appeared as a single band in SDS-PAGE. Rabbit antiserum to the purified 31-kDa protein was prepared by mixing the protein in PBS with Freund’s incomplete adjuvant (Difco) at a 1:9 ratio of protein to adjuvant to a final concentration of 200 μg of protein/ml. Rabbits were inoculated subcutaneously in the scapular region with two 0.5-ml injections 2 weeks apart over a period of 6 weeks. Preimmune serum and bleeds after the second and third inoculations were tested for specific antibody by conventional enzyme-linked immunoassay (26) with 100 ng of periplasmic protein preparation (27) containing the 31-kDa protein per well.

The results of immunoblotting of whole-cell pellets and osmotic shock fluids prepared from *P. haemolytica* and *P. trehalosi* are shown in Fig. 1 and 2, respectively. The results showed that whole-cell pellets of *P. haemolytica* serotypes A1, A2, A5 through A9, A11 through A14, and A16 reacted strongly with the anti-31-kDa-protein antibody at the position of a 31-kDa band (Fig. 1 and 2, A lanes). In contrast, *P. trehalosi* serotype T3 and T4 proteins did not react with a band at 31 kDa, but they did react weakly with a protein at the lower molecular mass of approximately 28.6 kDa (Fig. 1, T lanes). Blots of osmotic shock fluids showed no bands with the antibody (Fig. 2, T lanes). *P. trehalosi* serotype T10 and T15 proteins showed weak reactions with the antibody at 31 and 28.6 kDa (Fig. 1, T lanes) but no bands or barely visible bands in the osmotic shock fluids (Fig. 2, T lanes). The lack of bands on blots of the osmotic shock fluids is thought to be due to a lower concentration of the proteins than in the whole-cell pellet but not due to the absence of the proteins. Concentrations determined by protein assay on the osmotic shock fluids from freshly prepared cell pellets after 1 h of incubation on ice ranged from 22 to 78 μg/140 μl for *P. haemolytica* serotypes and from 43 to 60 μg/140 μl for *P. trehalosi* serotypes.

**FIG. 1.** Immunoblot of whole-cell pellets prepared from *P. haemolytica* and *P. trehalosi* grown under iron-limited conditions. The primary antibody was prepared against the 31-kDa protein of *P. haemolytica* A1. Lane MW, amido black-stained blot of the molecular weight standards; the remaining lanes show immunoblots of the serotypes listed above the lanes. Molecular weights are given (in thousands) on both sides of the gel.

**FIG. 2.** Immunoblot of the osmotic shock fluids prepared from *P. haemolytica* and *P. trehalosi* for the identification of the 31-kDa periplasmic iron-regulated protein. The primary antibody was prepared against the 31-kDa protein from *P. haemolytica* A1. Lane MW, amido black-stained blot of the molecular weight markers; the remaining lanes show immunoblots of osmotic shock fluids from the serotypes listed above the lanes. Molecular weights are given (in thousands) on both sides of the gel.
140 μl for P. trehalosi serotypes. Thus, protein release by P. trehalosi serotypes fell within the range of that by P. haemolytica serotypes and the lack of cross-reaction with the antibody was not due to a lack of protein release in general. In addition, a Coomassie blue-stained PVDF blot (Fig. 3) of the osmotic shock fluid showed visible bands for the T serotypes at 28.6 kDa and weaker bands at 31 kDa for serotypes T10 and T15. Also, bands from the PVDF blot used for N-terminal sequencing gave initial yields ranging from 5 to 8 pmol of N-terminal amino acids. The antibody to the 31-kDa protein reacted also with two bands of more than 60 kDa and with a band at approximately 14 kDa (Fig. 1). These bands were also present in preimmune serum and could be reduced by absorption of sera with whole P. haemolytica cells (data not shown). These bands are absent from the blot with the osmotic shock fluids (Fig. 2).

To determine whether specific iron-regulated proteins were expressed by P. trehalosi as was shown for P. haemolytica (27), the serotypes were grown in the presence and absence of ferric chloride. SDS-PAGE of osmotic shock fluids showed that ferric chloride repressed the synthesis of a 29-kDa protein rather than a 31-kDa protein of serotypes T3, T10, and T15 (Fig. 4). Ferric chloride did not repress the expression of the 29-kDa protein of serotype T4. Also, other iron-repressible proteins were observed at approximately 37 kDa for serotypes T3, T10, and T15, but not for serotype T4.

The osmotic shock fluids were also examined for contamination with membrane protein and cytosolic proteins by using the NADH oxidase and β-galactosidase reactions, respectively. The results showed that the periplasmic preparations contained negligible contamination by membrane and cytoplasmic enzymes. NADH oxidase activities were 0.05 to 0.08 and 0.78 to 0.85 μmol/min/mg for the periplasmic and membrane fractions, respectively, and β-galactosidase activities ranged from 2.5 to 2.9 and 143 to 369 μmol/min/mg for the periplasmic and membrane fractions, respectively.

Figure 3 shows a representative Coomassie blue-stained PVDF blot of the osmotic shock fluids obtained after 1 h of incubation at 5°C. Yields of the 31- and 29-kDa proteins blotted onto PVDF were sufficient (5 to 8 pmol) for obtaining N-terminal sequences of five residues. The N termini of the 31-kDa proteins obtained from the P. haemolytica serotypes each had the sequence Glu-Pro-Val-Phe-Lys (EPVKF) (Table 1). P. trehalosi serotypes T10 and T15 also showed Coomassie blue-stained bands on PVDF at 31 kDa, and these proteins also had EPVKF at the N terminus. In contrast, the PVDF blots showed no evidence of a 31-kDa protein from P. trehalosi serotypes T3 and T4. Instead, serotypes T3, T4, T10, and T15 showed prominent protein bands at 29 kDa which were also analyzed, revealing the sequence Lys-Gln-Phe-Lys-Ala (KOFLA) (Table 1).

All P. haemolytica serotypes and three of the four serotypes of P. trehalosi expressed periplasmic iron-regulated proteins with molecular masses of 31 and 29 kDa, respectively. The function of the Pasteurella iron-regulated proteins is most likely related to iron transport because of its protein sequence similarity (27) to the Haemophilus influenzae periplasmic iron-binding protein (15), subsequently identified as the iron transport protein ferric binding protein (Fbp), encoded by the hitA gene (2). The iron transport function of the P. haemolytica 31-kDa protein will be determined when the recombinant protein becomes available. In the work reported here, we demonstrated that the antibody to the 31-kDa protein from P. haemolytica A1 is a useful tool to examine the conservation of expression of the 31-kDa protein produced by all P. haemolytica serotypes. Furthermore, the strong reactions with the antibody suggested that closely related, if not identical, 31-kDa proteins were expressed by all P. haemolytica serotypes. In contrast, the weak reactions observed with the P. trehalosi 29-kDa proteins suggested that these proteins were largely immunologically and structurally unrelated to the P. haemolytica proteins. Despite the fact that the 31-kDa proteins of P. trehalosi serotypes T10 and T15 showed some N-terminal

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**TABLE 1. N-terminal sequences of 31- and 28-kDa iron-regulated proteins from osmotic shock fluids prepared from P. haemolytica and P. trehalosi**

| Organism and serotype | Molecular mass (kDa) of protein band* | N-terminal sequence |
|-----------------------|--------------------------------------|--------------------|
| P. haemolytica         |                                      |                    |
| A1, A2, A5, A6, A7, A8, A9, A11, A12, A13, A14, A16 | 31 | EPVKF |
| P. trehalosi           |                                      |                    |
| T3, T4                | 29 | KOFLA |
| T10                   | 31 | EPVKF |
| T15                   | 31 | EPVKF |
|                       | 29 | KOFLA |

* Molecular masses of the protein bands were determined from Coomassie blue-stained PVDF blots, using molecular mass standards as described in the text.
sequence identity with the \textit{P. haemolytica} proteins, they appeared to be immunologically unrelated to the \textit{P. haemolytica} 31-kDa proteins. Others (1, 12, 18) have also noted differences in protein expression between \textit{P. haemolytica} and \textit{P. trehalosi} serotypes, supporting the findings of Sneath and Stevens (25), Davies and Quirie (7), and others (4, 8) indicating that biotype T of \textit{P. haemolytica} should be considered a different species.

In summary, we demonstrated that the antibody to the 31-kDa iron-regulated periplasmic protein revealed close relationships among the iron-regulated periplasmic proteins of the biotype A serotypes of \textit{P. haemolytica} and \textit{P. trehalosi} serotype A1. Antibody to the 31-kDa iron-regulated protein was a useful tool to distinguish similarities and differences of the iron-regulated proteins of \textit{P. haemolytica} and \textit{P. trehalosi}.

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