Biochemical and immunological analyses of the cell surface of *Bordetella bronchiseptica* isolates with special reference to atrophic rhinitis in swine

Ben Lugtenberg*†, Ria van Boxtel*†, Renée van den Bosch*, Marten de Jong‡ and Paul Storm§

The guinea-pig skintest was used to test the pathogenic character of nine Bordetella bronchiseptica *isolates derived from a number of different animals. One isolate was non-pathogenic, one showed a doubtful reaction whereas the others were pathogenic. Analysis of the cell envelope protein patterns by sodium dodecyl sulphate polyacrylamide gel electrophoresis revealed only minor differences. Two major protein bands and at least thirty five minor bands were observed. The major protein with an apparent molecular weight of 37 000 has properties similar to those of pore proteins of Enterobacteriaceae. Lipopolysaccharides (LPS), analysed by the same technique, could be separated into two fractions, LPS-I and LPS-II. The electrophoretic mobilities of the LPS of the strains were indistinguishable from each other with the exception of that of the non-pathogenic strain, which also differed serologically from that of the other strains. All sera of animals which had been successfully vaccinated with whole* B. bronchiseptica *cells showed a positive reaction with LPS-I whereas antibodies against the 37 000 dalton protein, LPS-II as well as against several other proteins, were also detected in several sera. These results strongly suggest that LPS-I is a protective antigen. LPS-I and the 37 000 dalton protein are accessible to antibodies in whole cells. This protein as well as a 28 000 dalton protein were found to be located at the cell surface using iodination of whole cells with Iodo-Gen. Comparison of sixteen and fourteen strains of farms on which tests for symptoms of clinical atrophic rhinitis were positive and negative, respectively, showed that all strains gave a positive guinea-pig skintest and that their cell envelope protein and LPS patterns were indistinguishable. These results show that in general the presence of pathogenic* B. bronchiseptica *alone does not cause clinically progressive atrophic rhinitis. The appearance of these clinically progressive symptoms in later stages of the investigation was related to the appearance of pathogenic* Pasteurella multocida *strains in the animals, alone or in combination with* B. bronchiseptica.

Keywords: Bacteria; *Bordetella bronchiseptica*; cell surface proteins; lipopolysaccharides; atrophic rhinitis

Introduction

Bordetella brochiseptica, a motile aerobic Gram-negative coccoid rod, has long been known as a respiratory tract pathogen of mammals[1]. The organism is taxonomically strongly related to *B. pertussis* and *B. parapertussis*[1]. *B. bronchiseptica* has been considered for a long time as a primary agent in causing swine atrophic rhinitis[2,3] while until recently *Pasteurella multocida* was thought to be the second major cause of turbinate atrophy . Combined infection with *B. bronchiseptica* and *P. multocida* was found to produce lesions similar to those caused by a single *B. bronchiseptica* infection[4]. Recent publications by Pedersen and Barfod[5] and Rutter and Rojas[6] have shown that the most severe cases of atrophic rhinitis are caused by combined infections of *B. bronchiseptica* and certain *P. multocida* strains that belong to capsular type D and produce a dermonecrotic toxin.

Department of Molecular Cell Biology* and Institute for Molecular Biology†, State University, Padualaan 8, 3584 CH Utrecht; Animal Health Service in Overijssel‡, P.O. Box 13, Zwolle; and Intervet International B.V.§, P.O. Box 31, Boxmeer, The Netherlands (Received 10 May 1984)

Correspondence to Ben Lugtenberg (present address) Department of Plant Molecular Biology, State University, Nonnensteeg 3, 1123 VJ, Leiden, The Netherlands

Whereas pathogenic *P. multocida* produces a heat-labile exotoxin, *B. bronchiseptica* contains a heat-labile endotoxin[7,8] which is inactivated by formaldehyde treatment[7] and most likely consists of a complex of protein and lipopolysaccharide with the morphological appearance of vesicular membranes[4]. As such complexes are only present in the outer membranes[9] the endotoxin must be located at the cell surface.

Knowledge of the chemical composition of the cell surface of *B. bronchiseptica* is limited. To our knowledge no information is available concerning the lipopolysaccharide (LPS) component. In a paper dealing with outer membrane proteins of *B. pertussis*, comparison with preparations of *B. bronchiseptica* showed that two proteins were not present in the latter organism[10].

In order to understand better the chemical composition of the cell surface of *B. bronchiseptica*, especially in relation to atrophic rhinitis in swine, we have analysed the cell envelope proteins and lipopolysaccharides by sodium dodecyl sulphate (SDS) gel electrophoresis. The reactions of various antisera with these cell surface constituents were studied.

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Materials and methods

Strains

All strains used are listed in *Table 1*. Strains coded 1a through 9a are isolates from various animals derived from the collection of the Central Veterinary Institute (CVI), P.O. Box 65, Lelystad. The strains were obtained through Dr J. Frik. Strain 92932 is a toxinogenic strain used in experimental CVI vaccines[11], whereas strain 53453 is a non-toxinogenic strain. Strain 31124 is also known as the weakly pathogenic or non-pathogenic strain D1[12]. These three strains were tested at the CVI on pathogenicity using the intranasal instillation of broth cultures in colostrum-deprived specific pathogen-free piglets and in the guinea-pig skintest[13]. Strains coded 1 through 30 were isolated by way of the Animal Health Service (GHD) from the noses of pigs from two different categories of pig-breeding farms, here designated as P (problem) and C (Certificate of Health) farms. P-farms harbour clinically atrophic rhinitis diseased pigs, so-called P-herds. Strains designated with the same letter code (see *Table 1*) have been isolated on the same farm. On each of the atrophic rhinitis diseased farms *B. bronchiseptica* strains were isolated from six diseased pigs, by collecting samples of nasal mucus using nose swabs (minitip, Exogen Ltd, Clydebank, UK).

Table 1 *B. bronchiseptica* strains[a]

Code used in text	Strain designation	Host animal/year of isolation	Farm type[b]	Guinea pig[c] skintest
1a	CDI 92932	pig/1973	P	+
2a	CDI 94857	pig/1974	P	+
3a	ATCC 31124	dog	?	±
4a	CDI 53453	pig/1977	C	–
5a	CDI 70596	pig/1978	P	+
6a	CDI 43598	pig/1977	P	+
7a	CDI 85760	cat/1974	from a P-farm	+
8a	CDI dog	dog/1954	?	+
9a	CDI 33381	sheep/1976	?	+
1	GHD B8	pig/1982	P	+
2	GHD 3K4	pig/1982	P	+
3	GHD M5-6	pig/1982	P	+
4	GHD Ve-1	pig/1982	P	+
5	GHD JH4	pig/1982	P	+
6	GHD G-5	pig/1982	P	+
7	GHD DA7	pig/1982	P	+
8	GHD V8	pig/1982	P	+
9	GHD Hu-2	pig/1982	P	+
10	GHD E-6	pig/1982	P	+
11	GHD Lu-6	pig/1982	P	+
12	GHD GV-2	pig/1982	P	+
13	GHD S-4	pig/1982	P	+
14	GHD 90u8	pig/1982	P	+
15	GHD 2R5	pig/1982	P	+
16	GHD Ho-1	pig/1982	P	+
17	GHD Z-8-5	pig/1982	C	+
18	GHD Z-4-4	pig/1982	C	+
19	GHD Hs-7	pig/1982	C	+
20	GHD VH4	pig/1982	C	+
21	GHD He-6	pig/1982	C	+
22	GHD 7tB8	pig/1982	C	+
23	GHD OCD9	pig/1982	C	+
24	GHD 7N4	pig/1982	C	+
25	GHD 2TK	pig/1982	C	+
26	GHD Mo6	pig/1982	C	+
27	GHD Br5	pig/1982	C	+
28	GHD G10	pig/1982	C	+
29	GHD J2	pig/1982	C	+
30	GHD 8NV8	pig/1982	C	+

[a]For detailed information see Materials and methods section
[b]P, disease present on farm; C, no disease detected
[c]+, positive, ± doubtful; –, negative

On each of the C-farms samples were taken from ten pigs. These samples were examined for *B. bronchiseptica* as described previously[14].

Media and growth conditions

Strains were stored at 4°C after growth on Dorset medium for 24 h at 37°C. Cells used for the present studies were grown overnight in either Brain Heart Infusion medium, yeast broth or in L-broth at 37°C with vigorous aeration.

Plasmids

These were isolated using the method of Birnboim and Doly[15].

Guinea-pig skintest

Guinea-pigs of 200–600 g bodyweight were epilated on both sides. Broth cultures of *B. bronchiseptica* cells grown for 48 h at 37°C were injected intracutanously in volumes of 0.2 ml, three on each side. The haemorrhagic necrotizing reactions were judged daily for at least two or three days. A diameter of the black colouring area of at least ten mm was considered as positive. Reactions of five to ten mm were considered as doubtful and those of less than five mm as negative.

Isolation of cell envelope fractions

Stationary phase cells were harvested, washed and ultrasonically disrupted as described previously[16]. Cell envelopes were isolated by differential centrifugation[17]. The procedure used for treatment of cell envelopes with trypsin has been described[18]. Extraction of cell envelopes with Triton X-100 in the presence of 10 mM $MgCl_2$, which in the case of *E. coli* results in solubilization of the cytoplasmic membrane proteins whereas the outer membrane proteins remain particle-bound, was carried out as described by Schnaitman[19] with minor modifications[20]. To isolate complexes of certain proteins with peptidoglycan or with peptidoglycan-lipoprotein we followed the SDS-heat treatment of cell envelopes as described by Rosenbusch[21] with some modifications[22]. In order to optimize the isolation of such complexes temperatures below 60°C were also used.

LPS was purified from whole cells using the phenol-chloroform-petroleum ether method described by Galanos *et al.*[23]. Proteolytic degradation of cell envelopes, after solubilization by boiling for 5 min in sample buffer[16], was performed by incubation of the solution (1 mg protein/ml) for one hour at 60°C with proteinase K (50 $\mu g\ ml^{-1}$)[24].

For the analysis of proteins by SDS polyacrylamide gel electrophoresis cell envelopes were usually completely solubilized by boiling in sample buffer[16]. In a few cases boiling was replaced by incubation for 20 min at 37 or 22°C, conditions which leave more complexes of molecules, and possibly also more immunogenic determinants, intact. Three different gel systems were used for the electrophoretic analysis of the samples. System A, which is an 11% gel system developed in our laboratory[16] was routinely used. In a few cases this system was modified in that only pure SDS was used (system B) or in that 4 M urea was added to the running gel (system C) to obtain different types of resolutions (see Ref. 18). After gel electrophoresis protein bands were stained with Fast Green FCF[16]. The identity of most molecular weight standard proteins used has been described previously[16]. Soy bean trypsin inhibitor and RNA polymerase α- and β-chains with molecular weights of 21 500, 39 000 and

155 000 respectively, were purchased from Boehringer Mannheim, GmbH, West Germany. Protein bands are indicated by their apparent molecular weight multiplied by 10^{-3} and followed by the letter K. For the separation of polysaccharide-containing molecules the procedure described above was followed using gel system A, sometimes with 13% polyacrylamide. Polysaccharide was either detected with the periodic acid-Schiff (PAS) staining procedure[25] or with the modified[26] silver staining procedure of Tsai and Frasch[27].

Gel immuno-radioassay (GIRA)

The GIRA technique is a radiological procedure for the detection of antigen-immunoglobin complexes in thin longitudinal sections of SDS polyacrylamide gels[28,29]. After electrophoretic separation of the antigens in SDS polyacrylamide gels, up to twenty identical thin longitudinal sections were cut. The remaining part of the gel was stained. After removal of SDS the slices were incubated with antiserum and subsequently with radioiodinated *Staphylococcus aureus* and identified by comparing the patterns of the autoradiogram and the stained gel. Modifications were introduced to reduce the background[18] and to shorten the time necessary for the procedure[30].

Surface labelling of whole cells

Overnight cultures were centrifuged and the cells washed twice with PBS (10 mM phosphate buffer, pH 7.5, 140 mM NaCl). Surface labelling was carried out using the Iodo-Gen procedure[31]. The cells were incubated with ^{125}I-iodide in a glass tube from which the surface is coated with the catalyser (1, 3, 4, 6-tetrachloro-3α, 6α-dephenyl glycoluril. Efficient labelling of a cell surface protein with radioactive iodide can only occur upon contact with the catalyser. The procedure was carried out as described[31] for five minutes at room temperature. Labelled cell envelope polypeptides were identified after SDS polyacrylamide gel electrophoresis (5 to 10×10^3 cpm/slot) and subsequent autoradiography for 24 h at $-80°$C.

Antisera

The antiserum aB was raised in a rabbit by intravenous injection with formaldehyde-treated cells of the pathogenic *B. bronchiseptica* dog isolate CDI/88624. Antisera V734, V735 and V737 were obtained from sows after three or four injections with a vaccine containing one pathogenic *B. bronchiseptica* isolate as well as two *P. multocida* isolates with different somatic antigens from which one gave a positive guinea-pig skintest. Antiserum 78 was raised in a sow using formaldehyde-treated cells of strain CDI 92932.

Antisera V734 was preadsorbed with whole cells as follows. Strains 13 (from a P-farm) and 22 (from a C-farm) were grown overnight in L-broth. Antiserum (1.5 ml) was mixed with the washed pellet of 10 ml cell suspension and the suspension was incubated for one hour at 37°C and centrifuged. The procedure was repeated twice with the supernatant fluid using fresh bacterial cells.

Results

Rationale for choice of strains

The tested isolates consisted of two groups. Strains 1a through 9a were relatively early isolates from a variety of host animals. Strains 1 through 30 were isolated in 1982 as part of a GHD atrophic rhinitis programme in which isolates from P-and C-farms were compared.

Plasmids

No plasmids were isolated from any of the strains listed in *Table 1* using the procedure of Birnboim and Doly[15].

Guinea-pig skintest

Among the early isolates the non-pathogenic strain 4a was the only strain that was isolated from a pig of the SPF-swine herd of the CVI, a C-type farm, whereas, as far as known, all skintest-positive strains were isolated on P-farms (*Table 1*). Part of these results have been described earlier[13]. Surprisingly, all isolates of the second group of strains were skintest-positive, irrespective of whether they had been isolated on a P- or on a C-farm.

Cell envelope proteins of B. bronchiseptica *strains from various host animals*

Using gel system A, the patterns of completely denatured cell envelope proteins of nine isolates grown in yeast broth medium, which differ in host animal, pathogenicity and country of origin, were found to be strikingly similar (*Figure 1*, slots 1a through 9a). Two major bands were found in electrophoretic positions corresponding with molecular weights of 37 K and 17 K (*Figure 1*). Over thirty five other bands were detected which usually correspond very well in intensity and electrophoretic position with proteins of other strains. The area of the gel corresponding with apparent molecular weights between 17 K and 24 K is relatively empty and vague, suggesting that the resolution in this area is strongly decreased by the presence of interfering non-proteinous material. A number of minor differences are worthwhile mentioning. (i) The dog isolate 3a differs from the other dog isolate 8a and from the isolates of other animals in that it has relatively heavy bands in the positions of 56 K and 45 K. (ii) The non-pathogenic swine isolate 4a (*Figure 1*) differs from all other swine isolates in that it has a very weak 28 K band and lacks the doublet in the 70 K and 67 K positions. Instead, other bands of 43 K and 68 K are present in this strain. The slightly lower electrophoretic mobilities of the 37 K and 36 K proteins of strain 4a is probably due to interference of LPS during electrophoresis (see lateron). (iii) The isolates from cat (slot 7a) and sheep (slot 9a) are strikingly similar to those of swine. (iv) Although still very similar to the other strains, the dog isolate 8a differs more strongly from the other isolates.

The growth phase of the cells or the use of tryptic soy broth or L-broth instead of yeast broth as the growth medium hardly influence the protein pattern of the tested strains 1a, 14 or 20 (not shown).

Comparison of cell envelope proteins of strains isolated from P- and C-herds

The cell envelope protein patterns of sixteen strains from P-herds and fourteen strains from C-herds were compared. The patterns were found to be extraordinarily homogeneous in that none of the proteins was significantly lacking in one of the strains or in that electrophoretic mobilities differed. In conclusion, no significant difference could be detected in the protein patterns of P- and C-strains (results not shown).

Properties of B. bronchiseptica *cell envelope proteins*

Further studies on the *B. bronchiseptica* cell envelope proteins, using procedures which have been extensively

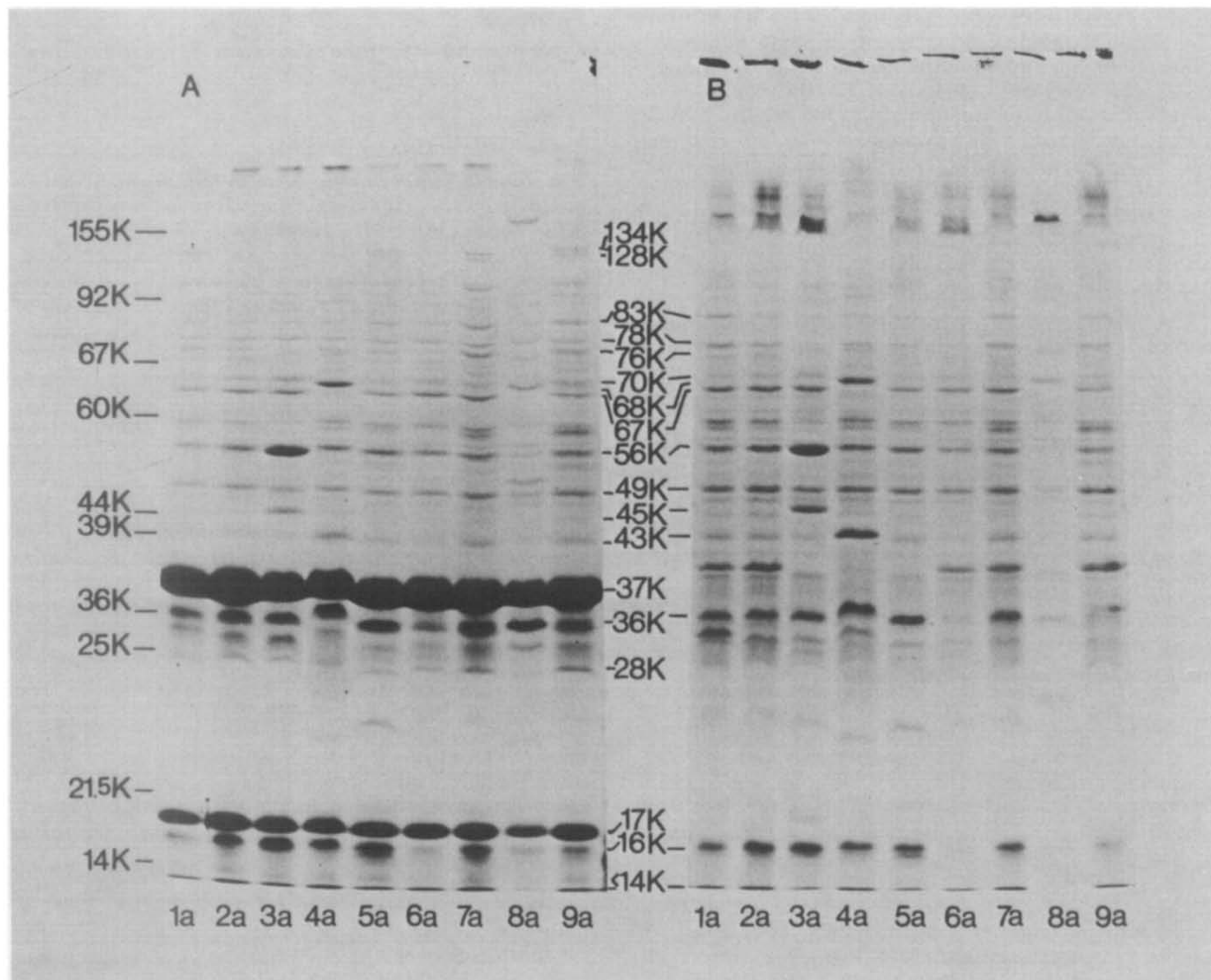

Figure 1 Patterns of cell envelope proteins after SDS polyacrylamide gel electrophoresis in gel system A after boiling in sample buffer for five minutes (A) or after incubation for twenty minutes at 37°C (B). The slots 1a through 9a represent preparations of strains coded with the same designations in *Table 1*. Stationary phase cells grown in yeast broth were used. The positions of molecular weight standard proteins are indicated at the left. The apparent molecular weights of *B. bronchiseptica* proteins were calculated from a curve obtained by plotting the logarithm of the molecular weight standard proteins against their electrophoretic mobilities. As some of these proteins showed an anomalous behaviour the calculated molecular weights sometimes are seemingly at variance with the values of the standards. *B. bronchiseptica* proteins mentioned in the text are indicated in the middle. Note the material on top of the stacking gel of the samples incubated at 37°C

described in studies on such proteins of Enterobacteriaceae (for a review see Ref. 9), revealed the following. (i) Analysis of the cell envelope protein patterns of a number of P- and C- strains in gel systems B or C, which can result in changes in electrophoretic mobilities and therefore in the appearance of new bands[18,26], revealed very similar band patterns (not shown). (ii) Incubation of the sample for 20 min at 22°C (not shown) or 37°C (*Figure 1B*) instead of boiling for five minutes (*Figure 1A*) resulted in the disappearance of several bands among which were the two major 37 K and 17 K bands, whereas a number of new bands appeared in the upper part of the running gel and also on top of the stacking gel. Apparently, some proteins are 'heat-modifiable' (for discussion see Ref. 9) in that they are not completely unfolded by incubation at 37°C and/or remain complexed with other molecules. The new bands in the upper part of the running gel may, by analogy with *Escherichia coli* and *P. multocida*[26,32,33] consist of complexes of (pore) proteins and LPS. (iii) Mild treatment of cell envelopes with trypsin, which in the case of Enterobacteriaceae degrades most outer membrane proteins and all cytoplasmic membrane proteins[34], degrades most *B. bronchiseptica* cell envelope proteins, leaving intact most of the 37 K protein and part of several minor proteins and of the 17 K protein (*Figure 2*, slot b). (iv) Treatment of cell envelopes according to a procedure used for the isolation of peptidoglycan complexed to specific proteins, revealed that most of the 37 K protein and part of the 17 K protein copurified with the peptidoglycan, when 60°C (*Figure 2*, slot c) or 37°C (*Figure 2*, slot d) were chosen as the incubation temperatures. (v) Extraction of cell envelopes with 2% Triton X-100 in the presence of 10 mM $MgCl_2$ did not result in solubilization of any of the proteins as judged by SDS polyacrylamide gel electrophoresis (not shown). Such an aberrant behaviour of cytoplasmic membrane proteins has also been reported for *Brucella abortus*[35]

Lipopolysaccharide patterns

Staining of gels containing cell envelope constituents of various isolates of P- and C-herds with the PAS agent revealed weak but compact bands of the two major cell envelope proteins (37 K and 17 K) as well as two larger and

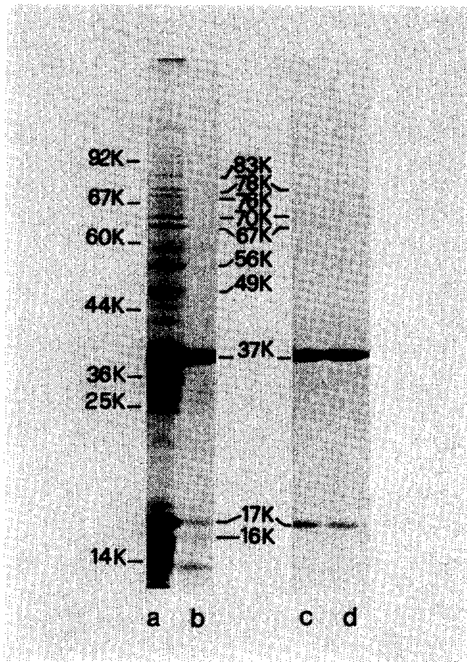

Figure 2 Influence of trypsin treatment and SDS/heat treatment on cell envelope proteins of *B. bronchiseptica*. Cell envelopes of strain 1a (slot a) were treated with trypsin to degrade sensitive proteins (slot b) or incubated in SDS at 60°C or 37°C. In the latter two cases incubation was followed by ultracentrifugation and the resulting pellets were analysed in gel system A (slots c and d, respectively). The positions of molecular weight standard proteins are indicated at the left, those of relevant *B. bronchiseptica* cell envelope proteins in the middle of the figure

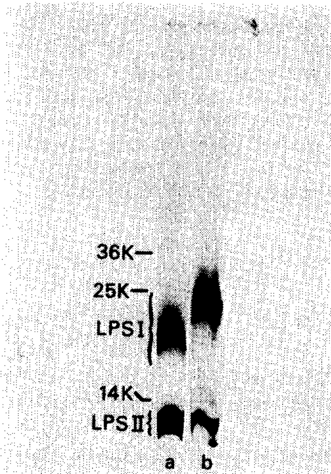

Figure 3 SDS polyacrylamide gel electrophoresis patterns of proteinase K-treated cell envelopes of the pathogenic strain 1a (slot a) and the non-pathogenic strain 4a (slot b). After electrophoresis in a 13% gel the gel was stained with silver reagent. The positions of LPS I and II and of some molecular weight standard proteins are indicated

more diffuse areas, at electrophoretic positions of proteins with apparent molecular weights of approximately 13 K and 22–24 K, respectively, (see *Figure 3a*), corresponding with regions which are strikingly devoid of protein bands (*Figure 1*). This effect was expected to be due to the presence of LPS, which is PAS-positive material. Final proof that the two PAS-positive areas indeed represent LPS came from the observation that the same two PAS-positive areas are found when LPS purified from strains 2 (P-farm) and 19 (C-farm) were used (not shown). In fact, LPS of *B. bronchiseptica* strains from P- and C-herds could not be distinguished using this method. In order to see whether LPS of the non-pathogenic isolate 4a could be distinguished from that of the pathogenic strains, proteinase K-treated cell envelopes of strains 4a and 1a were stained with the silver agent after SDS polyacrylamide gel electrophoresis. The pattern of strain 1a (*Figure 3a*) is very similar to that of other pathogenic strains, but that of the other investigated strains in that it contains relatively little of the faster moving component and in that ladder structures can be observed from which most components have a considerably lower electrophoretic mobility than that of LPS-I of the pathogenic strains. LPS purified from these strains gives the same result (not shown).

Reactions of cell surface components with antisera

Immunogenicity and antigenicity of cell surface components were tested by incubating slices of gels containing separated cell surface constituents with various antisera. All sera showed a strong reaction in the position of LPS type I from P- and C-strains. Several sera also reacted with the major 37 K protein and some sera reacted in the position of LPS type II. In addition to reactions with these three components, reactions with a number of high molecular weight components (≥ 100 K presumably proteins) were occasionally seen. None of the sera showed significant differences between strains of P- and C-herds.

Immunological analysis of samples boiled in SDS might well give an incomplete picture of the antigens present in whole cells, as the procedure might inactivate certain antigens. Therefore, and to look for differences between pathogenic and non-pathogenic strains, various antisera were incubated with samples of strains 1a and 4a solubilized at 22,37 and 100°C. The most striking results, partly illustrated in *Figure 4*, were as follows. (i) Under all tested conditions LPS-I was the predominant antigen. The intensity of the reaction increased with increasing solubilization temperature (compare slots g, h and i). (ii) Occasionally reactions were observed with LPS-II (slots i, n and p), with distinct protein bands in electrophoretic positions of approximately 40 K (slot g) and 55 K (slots g and h) and 70 K (slots m, n, o and p). (iii) Surprisingly, three of the four antisera tested showed no significant reaction with preparations of the non-pathogenic strain 4a. Only antiserum aB showed weak reactions with bands in positions of approximately 70 K (slots m and o). Interestingly, the reaction at the 70 K position was with a band of a slightly but significantly lower electrophoretic mobility than when samples of the pathogenic strain were used (compare slots m through p). (iv) In contrast to the boiled sample of strain 1a (slot i), samples solubilized at 37°C (slots h and n) and to a minor extent also those solubilized at 22°C (slots g and l), showed a positive reaction in a smear in positions corresponding with apparent molecular weights of proteins of 30 K and larger. The precise positions of the smears varied and was dependent on the incubation temperature (compare the pairs in slots g and h and in l and n) and the antiserum (compare slots h, j and k).

Molecules localized at the cell surface

Two approaches were chosen to indicate molecules that are exposed at the cell surface. First, antiserum V734 was allowed to react in a GIRA before and after preadsorption with whole cells of either strain 13 or strain 22. The results showed that both pre-incubations removed antibodies directed against LPS type I and 37 K protein (not shown). The second approach consisted of Iodo-Gen

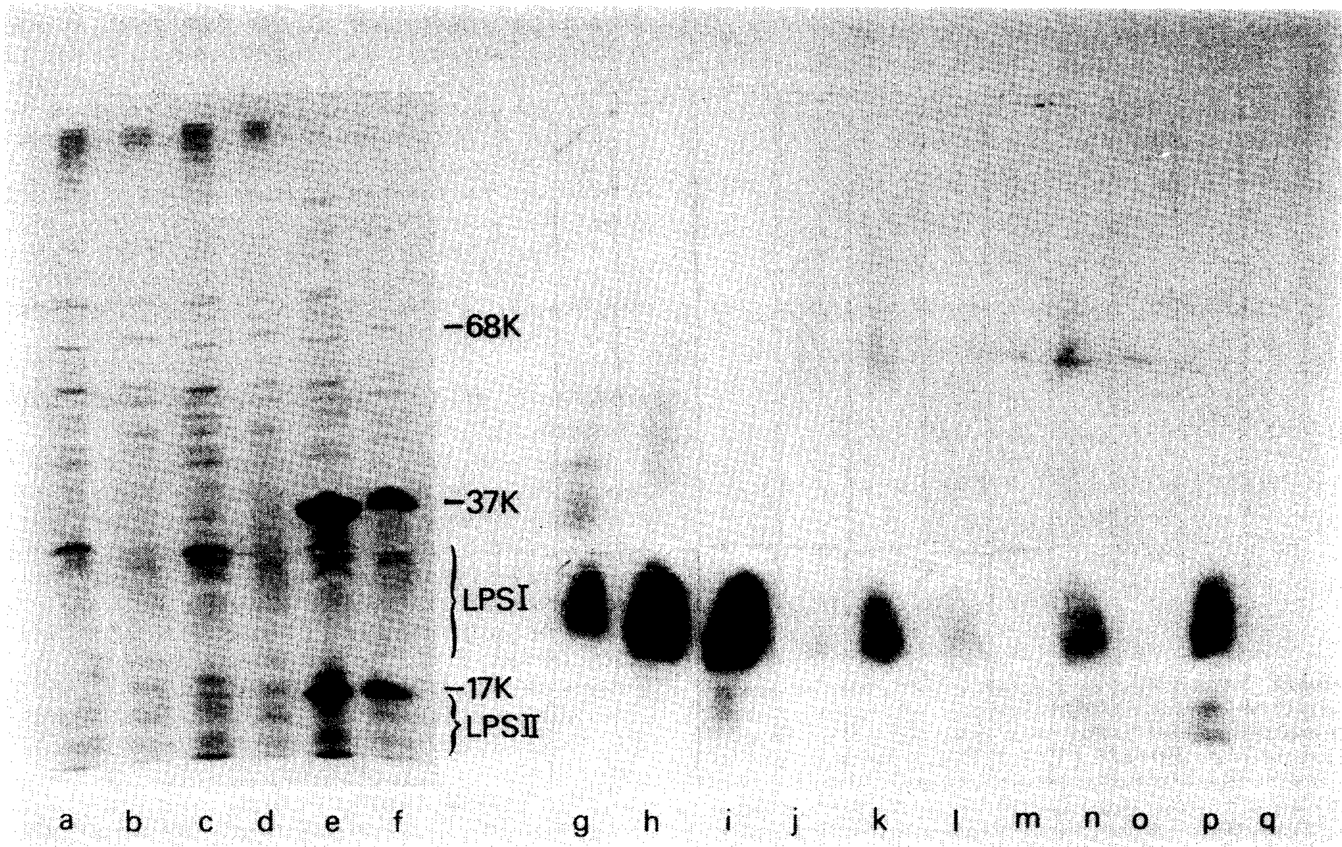

Figure 4 Antigenic activity of cell envelope constituents of the pathogenic strain 1a and the non-pathogenic strain 4a after solubilization at various temperatures. Cell envelopes of strains 1a (slots a, c and e) and 4a (slots b, d and f) were incubated in sample buffer for 20 min at 22°C (slots a and b) or 37°C (slots c and d) or boiled for 5 min (slots e and f). Its constituents were separated by SDS polyacrylamide gel electrophoresis. Before slicing, the gel was cut in four parts of about 5 x 5 cm. After removal of longitudinal slices (50 μm thick) of the gel parts, the remaining part of the gel was stained with fast green FCF (slots a-f). The slices were incubated with various antisera and the bound IgG was labelled with ^{125}I-protein A and subsequently detected by autoradiography. Lanes g, h and i, which contain the same samples as lanes a, c and e, respectively, were incubated with antiserum 78. Lanes j and k, both of which correspond with sample c, were incubated with antisera V737 and V734, respectively. Lanes l through q correspond with samples a through f and were incubated with antiserum aB

treatment of whole cells, i.e., labelling dependent on contact between cellular constituents and a catalyser fixed at the surface of a glass tube. Using cells of strain 1a, it was observed that several proteins were labelled among which the 37 K and 28 K proteins were the most predominant ones (*Figure 5*).

Discussion

Cell surface proteins of B. bronchiseptica *strains*

To our knowledge the present paper represents the most extensive study on the chemical composition of *B. bronchiseptica* cell envelopes as studied by SDS polyacrylamide gel electrophoresis. The patterns of cell envelope proteins of isolates from different animals are surprisingly similar (*Figure 1*). Even more surprising was the observation that cell envelope protein patterns of isolates from P- and C-farms were indistinguishable.

Attempts to split up the major 37 K protein band using SDS polyacrylamide gel electrophoresis systems with a different type of resolution, which have been used successfully for this purpose with isolates of *Escherichia coli*[18] and *P. multocida*[36], did not reveal more bands. Therefore, it is likely that the major 37 K band represents one protein. Several lines of evidence show that this protein shares many properties with pore proteins of enterobacteriaceae and *P. multocida*[9,26] and therefore most likely is a pore protein. (i) Its apparent molecular weight is similar to that of pore proteins[18,22]. (ii) Relatively high temperatures are required to solubilize the protein in sample buffer (*Figure 1*). (iii) As temperatures insufficient to solubilize the 37 K protein to monomers are correlated with the presence of high molecular weight complexes in the top of the gel (*Figure 4*, slots a–d) and with a reduction in the amounts of LPS monomers (compare slots g and h of *Figure 4*), it is likely that the native form of 37 K protein is complexed with LPS, a property of most pore proteins studied so far[9]. (iv) The protein is resistant to trypsin (*Figure 2b*). (v) The protein can be isolated as a non-covalent complex with peptidoglycan[21,22] (*Figure 2c*). (vi) It is immunogenic, (vii) it is surface-exposed in whole cells (*Figure 5*). In the closely related species *B. pertussis* also major bands with apparent molecular weights similar to those of the 37 K and 17 K proteins have been observed[10,37,38] and the major 38 K protein of *B. pertussis* is one of the most surface-exposed proteins[10].

Lipopolysaccharides of B. bronchiseptica

The two PAS-positive bands observed in cell envelope preparations of strains from P- and C-farms have been identified as lipopolysaccharides as purified LPS pre-

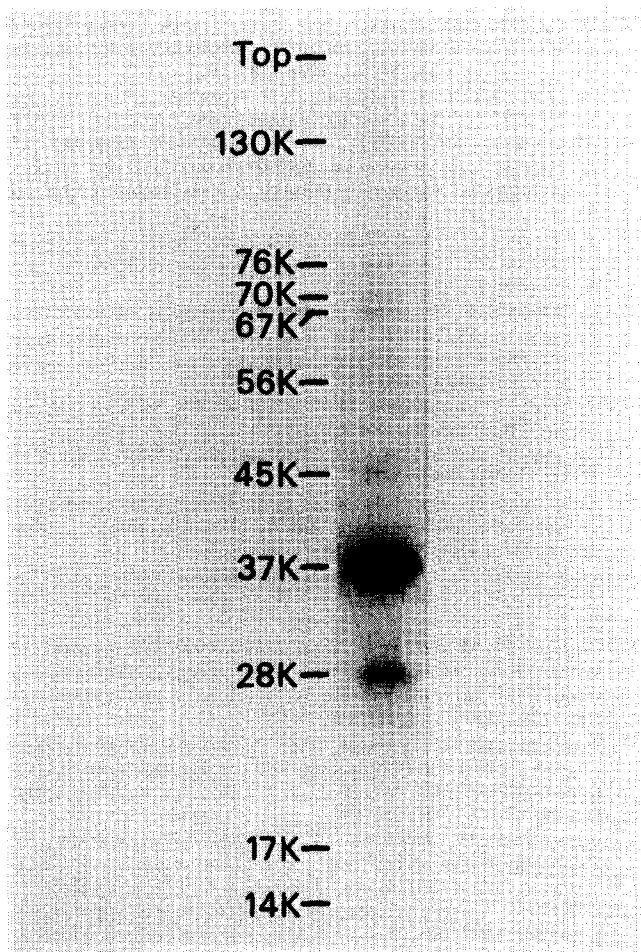

Figure 5 Surface labelling of whole cells of *B. bronchiseptica* strain 1a. Washed, stationary phase cells were incubated with ^{125}I-iodide using the Iodo-Gen method. After SDS polyacrylamide gel electrophoresis of boiled cell envelopes in gel system A, labelled polypeptides were detected by autoradiography. The estimated molecular weights of the labelled proteins are indicated

parations, isolated by a method in which rough LPS is preferentially extracted, shows the same two bands. This result strongly suggests that LPS of these strains is rough, i.e. does not, or hardly contains O-antigenic repeating units. This notion is supported by the electrophoretic mobility of LPS on gels, which is considerably higher than that of complete *Escherichia coli*[39] or *Salmonella typhimurium*[40] LPS. Frasch has recently proposed that LPS of a number of Gram-negative bacteria, including *Neisseria gonorrhoeae, N. meningitidis, B. pertussis* and *P. multocida*, which have high electrophoretic mobilities in SDS polyacrylamide gels, have a structure which differs fundamentally from the classical LPS structure of Enterobacteriaceae (C.E. Frasch, personal communication). From our recent experience with strains related to atrophic rhinitis it seems that the LPS of *P. multocida* (Ref. 35) and *B. bronchiseptica* (*Figure 3a*) also belong to this class of molecule.

As LPS-I has the lowest electrophoretic mobility it probably has the longest polysaccharide chain of the two LPS species. This idea agrees with the observation that it is much more immunogenic than LPS-II (*Figure 4*). The observation that LPS-I of whole cells is effectively accessible to antibodies shows that it is surface-located, as is generally accepted for LPS[9].

The LPS of strain 4a is an exception among the studied strains as its LPS moves slower upon electrophoresis than that of the other strains and in that it shows the ladder pattern known from LPS species with various numbers of repeating units of the O-antigen[39,40]. Although this behaviour suggests that this LPS is 'smooth', it should be noted that it can be easily extracted with the method of Galanos *et al.*[23] by which rough LPS is preferentially extracted.

Cell envelope constituents in relation to vaccines against atrophic rhinitis in swine

Strain 4a, which is the only clear non-toxinogenic strain as judged from the guinea-pig skintest (*Table 1*), is also the only strain from which the LPS clearly differs from that of other strains, both chemically (*Figure 3*) as well as immunologically (*Figure 4*).

The pig isolates of *B. bronchiseptica* from P- and C-herds are indistinguishable with respect to cell envelope protein patterns, LPS patterns or immunological reactions (text and *Figure 4*). Although the *B. brochiseptica* toxin must be a surface-located protein–LPS complex (see Introduction), no component could be detected that is specifically present in P-farm strains and absent in C-farm strains.

The sow antisera used in this work are raised against a pathogenic *B. bronchiseptica* isolate. This vaccine has been used successfully in the field to practically eliminate this bacterium from the noses of vaccinated swine (M. de Jong and P. Storm, unpublished results). From the reactions of these antisera with solubilized antigens (*Figure 4*) and from their cell surface localization (Ref. 8 and *Figure 5*) it is tempting to speculate that LPS-I and perhaps also the 37 K protein could be protective antigens. However, it is premature to draw such a conclusion as (i) it is possible that the major protective antigen can not be detected because it might have been inactivated by the solubilization procedure, and, (ii) the growth conditions used may not allow the synthesis of the major protective antigen.

It is worthwhile to compare our results with those published in a recent abstract by Novotny *et al.*[40] Using an ELISA based on a K-antigen preparation which was found to consist mainly of fibrillar antigen and outer membrane proteins, the latter authors were able to use the measured level of antibodies to predict whether or not a mouse would survive an intraperitoneal *B. bronchiseptica* challenge. Upon further fractionation of the antigen preparation it was found that the level of protection correlated with the level of antibodies against outer membrane proteins whereas no such relationship was found when the fibrillar antigen and/or LPS was used. Using an immunological detection of antigens after separation by SDS polyacrylamide gel electrophoresis, they found that the most reactive proteins had apparent molecular weights of 33 K, 39 K, 44 K and 54 K[41]. It is likely that most of the latter proteins correspond with proteins observed by us (*Figure 1*). Their experiments have the same limitations as ours. The suggestion of Novotny *et al.* that the protective antigen likely is an outer membrane protein, and not LPS as is suggested by our experiments, may be due to the choice of the animal used in protection experiments.

Role of B. bronchiseptica *in atrophic rhinitis*

The *B. bronchiseptica* pig isolates from P- and C-herds were indistinguishable with respect to their cell envelope protein and LPS patients and in their serological reactions.

Although the *B. bronchiseptica* endotoxin seems to be a surface-located protein–LPS complex (see Introduction)

no component could be detected that is specifically present in P-strains and absent in C-strains. This is in agreement with the findings of Rutter *et al.*[42] that *B. bronchiseptica* isolates from herds with atrophic rhinitis did not differ in virulence from isolates from herds unaffected by atrophic rhinitis. Our observation that all isolates from both P- and C-farms are positive in the guinea-pig skintest supports their conclusion.

The following observation is important for the role of *B. bronchiseptica* in causing atrophic rhinitis. After the strains described in this paper had been isolated, clinical atrophic rhinitis has been diagnosed at a number of farms that had been certified as C-farms. In approximately 90% of the cases the appearance of clinical atrophic rhinitis was accompanied by the appearance of pathogenic *P. multocida* (M. de Jong, unpublished observations). These observations make a number of theories on the role of *B. bronchiseptica* in atrophic rhinitis unlikely and support the following theory of events. (i) The presence of pathogenic *B. bronchiseptica* does not result in clear clinically progressive atrophic rhinitis symptoms like deviated or twisted noses and shortened snouts. In this stage the disease can only be detected after dissection of the noses. (ii) The infection with toxin-producing *P. multocida* can result in clear progressive atrophic rhinitis symptoms. The pathogenic *P. multocida* in combination with *B. bronchiseptica* causes these symptoms to a larger extent than infection with *P. multocida* alone.

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