Lipopolysaccharide expression within the genus *Bordetella*: influence of temperature and phase variation

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LPSs play an important role in bacterial pathogenesis. In this study, the LPS expression of the seven known *Bordetella* species and its dependency on growth temperature was analysed by oxidative silver staining of proteinase-K-treated whole bacteria separated by Tricine-SDS-PAGE. The *bordetellae* were found to have extensively variable LPS in a species-specific way. In addition, the human and ovine *Bordetella parapertussis* strains exhibited host-specific LPS expression. LPSs from human *B. parapertussis* strains grown at 37 and 25 °C were distinct. Growth temperature also affected LPS production by several *Bordetella bronchiseptica* strains. In some of these cases, BvgAS, the global regulator of virulence factors, was involved in this regulation of LPS biosynthesis. In contrast, no evidence was found for the involvement of the *Bordetella pertussis* BvgAS system in regulation of LPS synthesis. The obligate human pathogens *B. pertussis* and *Bordetella holmesii* are closely related but were shown to produce immunologically distinct LPSs. These species are isolated from the upper respiratory tract and blood, respectively. This raises several interesting questions concerning the potential role of LPS as a virulence factor in the infection processes.

Keywords: *Bordetella* species, BvgAS, lipopolysaccharide expression, phenotypic modulation, phase variation

INTRODUCTION

The genus *Bordetella* is composed of a group of Gram-negative bacteria which cause upper respiratory tract infections. The host specificities of *bordetellae* differ: *Bordetella pertussis* was isolated from humans, *Bordetella parapertussis* from humans and sheep, *Bordetella bronchiseptica* from mammals, *Bordetella avium* from birds and *Bordetella hinzii* from birds and humans (Pittman, 1984; Vandamme *et al.*, 1995). *Bordetella holmesii* was isolated from human blood (Weyant *et al.*, 1995), whereas *Bordetella trematum* was isolated from wounds and infected ears in humans (Vandamme *et al.*, 1996). The varying host specificities of *Bordetella* species raises questions about which virulence factors determine host specificity and tissue tropism.

LPSs, as major and essential components of the outer-membrane of Gram-negative bacteria, may as such be involved in determining host specificity. The lipid A

(endotoxin) part of LPS anchors the glycolipid into the membrane. It is highly conserved and a strong immunogen (Preston *et al.*, 1996). The species-specific functions of LPS are located in the extruding sugar moiety which may contain a long homopolymer of sugar structures (O-antigen) giving rise to smooth LPS (Holst *et al.*, 1996). Such polymeric O-antigens are found in some *B. parapertussis* and *B. bronchiseptica* strains (Di Fabio *et al.*, 1992). Electrophoretic analysis indicated the presence of two main LPS forms in *B. pertussis* (the principal causative agent of whooping cough), termed LPSA and the faster migrating LPSB (Peppler, 1984). Structural studies showed that the difference between these LPS forms is the presence of an additional distal trisaccharide in band A (Caroff *et al.*, 1990). mAbs directed against LPSA were able to block bacterial invasion into HeLa 229 cells (Ewanowich *et al.*, 1989). Recently, a gene cluster involved in *B. pertussis* LPS biosynthesis was identified (Allen & Maskell, 1996). One of these identified genes (*wlbG*, previously designated *bplG*) shows sequence homologies with genes required for polysaccharide biosynthesis in other bacteria, and its

Abbreviation: FHA, filamentous haemagglutinin.

Table 1. *Bordetella* strains used in this study

Strain	Host/description	Reference/source*
<i>B. pertussis</i>		
BP338	Human, Tohama I, Nal ^r	Weiss <i>et al.</i> (1983)
BP347	Tohama I, <i>bvgS::Tn5</i>	Weiss <i>et al.</i> (1983)
<i>wlbG::kan</i>	Tohama I, <i>wlbG::kan</i>	Allen & Maskell (1996)
BP338ery	Spontaneous Bvg ⁻ phase variant of BP338	This study
B112	Human, Wellcome 28, Sm ^r	Willems <i>et al.</i> (1992)
B112ery	Spontaneous Bvg ⁻ phase variant of B112	This study
18-323	Human, strain used for testing vaccines	Pittman (1984)
<i>B. holmesii</i>		
104394	Human, clinical isolate	CIP
104395	Human, clinical isolate	CIP
<i>B. parapertussis</i>		
B24	Human, clinical isolate, The Netherlands	Mooi <i>et al.</i> (1987)
MS30	Human, clinical isolate, Finland	Mertsola (1985)
SC7	Sheep, clinical isolate, Scotland	J. F. Porter, Edinburgh, UK
SC10	Sheep, clinical isolate, Scotland	J. F. Porter, Edinburgh, UK
<i>B. bronchiseptica</i>		
B14	Pig, clinical isolate	van der Zee <i>et al.</i> (1993)
B14Bvg ⁻	Spontaneous Bvg ⁻ phase variant of B14	van den Akker (1997)
B15	Pig, clinical isolate	van der Zee <i>et al.</i> (1993)
B16	Pig, clinical isolate	van der Zee <i>et al.</i> (1993)
B19	Dog, clinical isolate	van der Zee <i>et al.</i> (1993)
B19Bvg ⁻	Spontaneous Bvg ⁻ phase variant of B19	van den Akker (1997)
B20	Dog, clinical isolate	van der Zee <i>et al.</i> (1993)
B20Bvg ⁻	Spontaneous Bvg ⁻ phase variant of B20	van den Akker (1997)
B81	Dog, clinical isolate	van der Zee <i>et al.</i> (1993)
B615	Human, clinical isolate	RIVM
B618	Human, clinical isolate	RIVM
<i>B. avium</i>		
B21	Bird, clinical isolate	W. Gaastra, Utrecht, The Netherlands
B22	Bird, clinical isolate	Mooi <i>et al.</i> (1987)
<i>B. trematum</i>		
LMG 13506 ^T	Human, chronic otitis media, Germany (original designation 1779)	Vandamme <i>et al.</i> (1995)
<i>B. hinzii</i>		
LMG 10979	Chicken with slight tracheitis, Belgium	Vandamme <i>et al.</i> (1995)
LMG 13501 ^T	Chicken, trachea, Australia	Vandamme <i>et al.</i> (1995)
LMG 1872	Human, sputum, France	Vandamme <i>et al.</i> (1995)

* CIP, Collection of Bacterial Strains of Institut Pasteur, Paris, France; RIVM, National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

inactivation resulted in the disappearance of LPSA and its replacement by LPSB (Allen & Maskell, 1996).

Some *Bordetella* species, e.g. *B. pertussis* and *B. bronchiseptica*, show phase variation caused by mutations in the *bvgAS* locus resulting in inactivation of the BvgAS two-component sensor transducer system (Stibitz & Miller, 1994; Akerley & Miller, 1996). BvgAS enhances in a coordinated way the expression of a number of virulence factors such as fimbriae, filamentous haemagglutinin (FHA) and adenylate cyclase toxin/haemolysin. Inactivation of BvgAS results as expected in non-virulent bacteria. Also, environmental conditions such as

lowered temperature (25 °C) and sulphate anions result in inactivation of BvgAS (phenotypic modulation).

This study focuses on the LPS expression of the seven known *Bordetella* species. They appear to exhibit extensive heterogeneity in LPS expression. As some species are not restricted to one single host, isolates from different hosts were included. In all cases the effect of culture at 25 °C on the LPS expression was investigated, since this is already known to be an important modulator of the expression of virulence factors. For some *B. bronchiseptica* isolates, LPS expression was subject to phase variation in which BvgAS is involved.

METHODS

Bacterial strains and growth conditions. *Bordetella* strains used in this study are listed in Table 1. Bacteria were routinely grown on Bordet–Gengou agar (Difco) supplemented with 15% (v/v) defibrinated sheep blood (BG agar). Plates were incubated in a humidified atmosphere containing 95% (v/v) air and 5% (v/v) CO₂ at 37 °C. For the study of LPS expression at different temperatures, the bacteria were cultured in liquid Verwey medium (Verwey *et al.*, 1949). To induce phenotypic modulation, MgSO₄ was added to the medium to a final concentration of 50 mM.

Preparation of LPS samples. Cultures of 30 ml were grown in 100 ml Erlenmeyer flasks approximately to the late exponential growth phase at 37, 25 or 41 °C, shaken at 135 r.p.m. LPS of bordetellae was prepared by proteinase K treatment of lysed bacterial cells using a modification of the method described by Hitchcock & Brown (1983). Briefly, the organisms grown in broth were harvested by centrifugation and suspended in PBS (2.68 mM KCl, 1.47 mM KH₂PO₄, 0.49 mM MgCl₂·6H₂O, 137 mM NaCl, 8.03 mM Na₂HPO₄·2H₂O). Agar-grown bacteria were collected with a sterile cotton swab and suspended in PBS. The bacteria present in 80 µl of this suspension with an OD₅₅₀ of 5.0 were pelleted and then resuspended in 100 µl water. Subsequently, an equal volume of 2 × sample buffer (6%, w/v, SDS; 6%, v/v, 2-mercaptoethanol; 10 mM DTT; 46%, v/v, glycerol; 60 mM Tris, pH 8.0; and 0.1%, w/v, bromophenol blue) was added. After incubation of the samples at 100 °C for 10 min, the bacterial proteins were digested by adding proteinase K (Merck) to a final concentration of 50 µg ml⁻¹ and incubated at 55 °C for at least 6 h. The samples were stored at -20 °C until analysis by Tricine-SDS-PAGE.

Analysis of LPS by Tricine-SDS-PAGE. Electrophoretic profiles of LPS were made by Tricine-SDS-PAGE essentially as described by Lesse *et al.* (1990). The separating gel of approximately 12 cm length was prepared with final acrylamide concentrations of 16.4% T, 6% C where T represents the total percentage (w/v) of acrylamide (both acrylamide and bisacrylamide) and C represents the percentage of bisacrylamide to the total concentration of acrylamide. The stacking gel of about 1 cm length consisted of 3.9% T, 3% C. The gel was run at a constant current of 20 mA for about 17 h at 20 °C. After electrophoresis, the gel was fixed for 24 h in 40% (v/v) ethanol and 5% (v/v) acetic acid in water. Bands were visualized by LPS silver staining (Tsai & Frasch, 1982). The following volumes of LPS samples were added per lane: *B. parapertussis*, 10 µl; *B. avium* and *B. trematum*, 7.5 µl; and *B. pertussis*, *B. holmesii*, *B. bronchiseptica* and *B. hinzii*, 5 µl. Partial bromide cleavage products of sperm whale myoglobin (17201, 14632, 8235, 6383 and 2556 Da) were used as migration markers (Pharmacia Biotech).

Western blot analysis. To determine the reactivity of mAbs raised against *Bordetella* LPS, proteinase K digests of whole bacteria were separated on Tricine-SDS-PAGE as described above and directly after electrophoresis blotted onto a nitrocellulose membrane (Protran BA 79 cellulose nitrate, 0.1 µm; Schleicher & Schuell). Blotting was carried out in a semi-dry blot apparatus (transfer buffer: 192 mM glycine, 25 mM Tris/HCl and 20%, v/v, methanol, pH 8.3) at 1.5 mA cm⁻² for 1.5 h. Subsequently, the membrane was washed for 10 min in Tris-buffered saline with Tween 20 (TBS-T: 20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5) and blocked for at least 2 h in TBS-T containing 5% (w/v) fat-free milk powder (blocking buffer). After washing

three times for 10 min in TBS-T, the membrane was incubated with a *Bordetella* LPS mAb diluted in blocking buffer for 4 h. Following another washing procedure, the membrane was incubated for 1.5 h in blocking buffer containing secondary goat anti-mouse antibodies conjugated with peroxidase (Bio-Rad) at a dilution of 1:2500. After washing, detection was carried out by enhanced chemiluminescence (ECL, Amersham) according to the manufacturer's instructions.

For the detection of FHA expression, whole-bacterial cell lysates were separated by SDS-PAGE (Laemmli, 1970) in an 8.2% T, 2.6% C separating gel using a minigel system apparatus (Bio-Rad). Blotting on nitrocellulose was done for 2 h at 2 mA cm⁻². The next procedure was carried out similarly to that described for the LPS Western blots.

mAbs. The mAbs raised against *B. pertussis* LPS [40A1 (IgG3), 36G3 (IgG1) and 145F12 (IgG3)] and *B. pertussis* FHA [31E2 (IgG1)] have been described previously by Poolman *et al.* (1990).

RESULTS

Increased expression of LPSB in *B. pertussis* at 25 °C is not mediated by BvgAS

B. pertussis is an obligate human pathogen colonizing the upper respiratory tract and thereby causing the contagious disease whooping cough (Relman, 1995). The three *B. pertussis* strains studied were haemolytic on BG agar, indicating that the bacteria were in the Bvg⁺ state. At 37 °C, the *B. pertussis* strains expressed a strongly stained LPSA band and a faster-migrating, minor, LPSB band (Fig. 1, sets 1–3). LPSB co-migrates with the LPS of a *wlbG* mutant (Fig. 2a) which has been shown to express LPSB only (Allen & Maskell, 1996). Additionally, LPSA appeared to consist of two LPS forms which could be clearly distinguished by Tricine-SDS-PAGE using a 20% T, 6% C separation gel (data not shown). The slower-migrating LPS form of LPSA was more abundant than the faster-migrating one. Culture at 25 °C resulted in an increased level of LPSB.

Fig. 1. Electrophoretic profiles of LPS from *B. pertussis* and *B. holmesii*. Strains were cultured at 37 °C (a) or 25 °C (b). Sets: 1, BP338; 2, B112; 3, 18-323; 4, 104394; 5, 104395. Molecular mass markers are included to make comparison of the different gels possible.

Fig. 2. Effect of *bvgS* and *wlbG* inactivation on LPS expression in *B. pertussis* Tohama I. (a) Electrophoretic profiles after silver staining. Lanes: 1, BP338 (wild-type), 37 °C; 2, BP338, 25 °C; 3, BP347 (*bvgS*::Tn5), 37 °C; 4, BP347, 25 °C; 5, *wlbG*::*kan*, 37 °C. (b) Western blot of gel (a) developed with mAb 40A1 raised against *B. pertussis* LPS.

To investigate whether the BvgAS sensory system is involved in the biosynthesis of *B. pertussis* LPS and possibly responsible for the temperature-dependent LPSB expression, a Tohama I derivative (BP347) carrying a transposon insertion into *bvgS* resulting in a Bvg⁻ phenotype (Weiss *et al.*, 1983) was examined. The LPS profile of this mutant was identical to that of the parental strain; also no difference in the immunoreactivity with the *B. pertussis* LPS mAb 40A1 was observed (Fig. 2b). Furthermore, the expression of LPSB in the non-virulent *B. pertussis* mutant BP347 was increased at 25 °C compared to 37 °C, confirming that the BvgAS system is not responsible for the observed LPS temperature modulation. To further study the role of BvgAS, spontaneous Bvg⁻ phase variants of BP338 and B112 were selected by growth on BG agar containing erythromycin (0.25 µg ml⁻¹) (Weiss & Falkow, 1984). These phase variants showed no haemolytic activity and did not differ in LPS expression from their Bvg⁺ parental strains (data not shown). Taken together, the data presented show that BvgAS is not responsible for the temperature-dependent expression level of LPSB and not needed for *B. pertussis* LPS biosynthesis.

Fig. 3. Electrophoretic profiles of LPS from human and ovine *B. parapertussis*. Strains were cultured at 37 °C (a) or 25 °C (b). Sets: 1, B24 (human); 2, MS30 (human); 3, SC7 (sheep); 4, SC10 (sheep).

The human pathogen *B. holmesii* is closely related to *B. pertussis* but expresses a distinct LPS

Recently, a novel *Bordetella* species (*B. holmesii*) was isolated from the blood of immunocompromised patients (Weyant *et al.*, 1995; Lindquist *et al.*, 1995). The two *B. holmesii* strains investigated here were non-haemolytic on BG agar. The LPS profiles of both *B. holmesii* strains were similar and were not modulated by temperature (Fig. 1, sets 4 and 5). Three LPS bands could be distinguished: one major band and two minor ones migrating closely together. None of these bands comigrated with the *B. pertussis* LPS bands. Western blot analysis demonstrated that the LPS of *B. holmesii* strain 104394 was not recognized by mAbs 40A1, 36G3 and 145F12, indicating that *B. holmesii* LPS is distinct from the LPS found in *B. pertussis* (data not shown). The two *B. holmesii* strains exhibited a homogeneous colony morphology and screening of five randomly chosen colonies of strain 104394 did not provide evidence for intra-strain LPS variation.

Host-specific LPS expression profiles of human and ovine *B. parapertussis* strains: modulation by temperature

B. parapertussis has been isolated from both humans and sheep (Linnemann & Perry, 1977; Cullinane *et al.*, 1987; Porter *et al.*, 1994). The strains studied here differed in their haemolytic activity: the human *B. parapertussis* strains (B24 and MS30) were strongly haemolytic on BG agar whereas the ovine strains (SC7 and SC10) were weakly haemolytic. There are profound differences in the LPS profiles of bacteria isolated from the two hosts (Fig. 3). At 37 °C, the LPS of human *B. parapertussis* strains form a ladder-like pattern in the gel which is characteristic of LPS containing polymeric O-antigens. Interestingly, this pattern is absent at 25 °C and is instead replaced by a moderately faster-migrating O-antigen containing a single diffuse band. Three faster-migrating major LPS bands can be distinguished in the

Fig. 4. Electrophoretic profiles of LPS from *B. bronchiseptica* strains from pigs, dogs and humans. Strains were cultured at 37 °C (a) or 25 °C (b). Sets: 1, B14 (pig); 2, B15 (pig); 3, B16 (pig); 4, B19 (dog); 5, B20 (dog); 6, B81 (dog); 7, B615 (human); 8, B618 (human).

human strains, and culture at 25 °C has only minimal effects on their expression. No intra-strain LPS variation was detected by screening of at least 10 colonies of strain B24.

The LPS of the two ovine *B. parapertussis* strains was distinct from that of the human strains, and the LPS biosynthesis is clearly influenced by growth temperature (Fig. 3, sets 3 and 4). At 37 °C, two LPS bands are most prominent whereas culture at 25 °C induces a shift to three more slowly migrating LPS bands. The same LPS profile and temperature effects were also observed in three *B. parapertussis* strains isolated from New Zealand sheep (unpublished observations). No high-molecular-mass LPS was observed in any of the ovine *B. parapertussis* strains.

BvgAS controls LPS expression in some *B. bronchiseptica* strains

B. bronchiseptica causes respiratory infections in a broad range of mammals (Goodnow, 1980). Here, strains from three hosts were studied: pig ($n = 3$), dog ($n = 3$) and human ($n = 2$). The porcine strains were haemolytic but the canine and human isolates were not. Similarly to the human *B. parapertussis* strains (Fig. 3), most *B. bronchiseptica* strains express a ladder-forming smooth LPS at 37 °C and a faster-migrating diffuse O-antigen containing LPS at 25 °C (Fig. 4). Interestingly, whereas smooth LPS was absent in the human strain B618 at 37 °C, similar diffuse smooth LPS to that seen in other *B. bronchiseptica* strains was induced upon culture at 25 °C. The canine strains were more heterogeneous in LPS than were the strains from pigs. O-antigen containing high-molecular-mass LPS was absent in the canine *B. bronchiseptica* strain B81 at both temperatures. In all *B. bronchiseptica* strains temperature is a modulator of LPS expression. FHA was detected in *B. bronchiseptica* strain B14 grown at 37 °C, but not at 25 °C, with an anti-FHA mAb (data not shown). This indicates that culture at 25 °C results in phenotypic modulation. Growth of strains B14, B19 and B20 in the presence of sulphate anions, another modulator of

BvgAS, influenced LPS expression as well (Fig. 5a, lanes 3). Since temperature and MgSO_4 also influence the bacteria in ways other than via BvgAS, it is not possible to ascribe all observed effects to this sensor system. Therefore, spontaneous phase variants of the *B. bronchiseptica* strains B14, B19 and B20 (van den Akker, 1997) were investigated. These phase variants swarmed on BG agar, indicating the presence of flagella, a characteristic of the *B. bronchiseptica* Bvg⁻ phenotype. Also, the B14 phase variant did not express FHA at 37 °C, which is also indicative of the Bvg⁻ phenotype. The LPS profiles of the three phase variants were clearly different from those of their parental strains and had several characteristics in common with the sulphate-modulated parental strains (Fig. 5a). The experiments depicted in Fig. 5(a) were repeated with progeny of independently isolated colonies with the Bvg⁺ and Bvg⁻ phenotype and displayed the same LPS profiles as shown in this figure. Some LPS molecules of strains B14 and B19 were recognized by the mAbs 145F12 and 36G3, indicating the presence of a common epitope with *B. pertussis* LPS (Fig. 5b), but no reaction with the LPS of strain B20 was detected (not shown). These immunoblots elucidate the relatedness between LPS forms seen in the LPS profiles and confirm the effect of sulphate modulation and phase variation on the LPS expression. Interestingly, the epitope recognized by mAb 36G3 is also present in the smooth LPS of both strains. In conclusion, the data on the *B. bronchiseptica* strains B14, B19 and B20 demonstrate a role for BvgAS in the LPS biosynthesis in these strains.

LPS expression in *B. avium*, *B. trematum* and *B. hinzii*

The *Bordetella* species discussed so far are genetically closely related and can be viewed as a single species (Musser *et al.*, 1986; Müller & Hildebrandt, 1993). *B. avium* and related organisms are more distinct from these bordetellae. Both *B. avium* (Kerstens *et al.*, 1984) and *B. hinzii* (Vandamme *et al.*, 1995) are pathogens of the respiratory tract of birds, where they can cause bordetellosis and tracheitis, respectively. The recently recognized *B. trematum* was isolated from ear infections

Fig. 5. (a) Effects of temperature, MgSO₄ modulation and phase variation on the LPS expression in the *B. bronchiseptica* strains B14, B19 and B20 as analysed by Tricine-SDS-PAGE. Strains were cultured in Verwey broth at 37 °C (lanes 1), 25 °C (lanes 2) or supplemented with 50 mM MgSO₄ (lanes 3). Lanes 4 contain LPS from the *B. bronchiseptica* phase variants cultured at 37 °C. (b) Western blot of gel (a) developed with mAb 145F12 (upper panel) and mAb 36G3 (lower panel) raised against *B. pertussis* LPS.

and wounds in humans but its pathogenic significance is unknown (Vandamme *et al.*, 1996). All isolates from the three species are non-haemolytic on BG agar. The two *B. avium* strains have a similar LPS expression at the body temperature of their host (41 °C) but differ in their response to culture at 37 or 25 °C (Fig. 6, sets 1 and 2). The LPS profiles of *B. avium* strain B22 are characterized

by the constitutive presence of smooth LPS and two fast-migrating LPS bands. This O-antigen containing LPS is observed in strain B21 only at 41 °C. Furthermore, some differences in the fast-migrating LPS forms are observed depending on the culture temperature in this strain.

The LPS profile of the *B. trematum* type strain (LMG 13506^T) is characterized by three strongly stained bands (Fig. 6, set 3). These bands do not possess the typical characteristics of polymeric O-antigens although they migrate slower in comparison with the LPS bands deficient of polymeric O-antigens as found in several other *Bordetella* species. No profound influence of culture temperature on LPS expression was detected.

Most remarkable in *B. hinzii* strains is the presence of O-antigen structures forming a pattern with pairs of bands ('doublets') in the gel (Fig. 7). Although the number of doublets differs from one isolate to another, their migration behaviour is comparable. The expression of this smooth LPS did not depend on the culture temperature, except for a human strain in which the structures were absent at 41 °C. Two fast-migrating LPS forms are observed in all strains independent of the culture temperature. The human *B. hinzii* isolate expresses an additional LPS at 41 °C.

To look for possible (LPS) phase variants, at least 2000 individual colonies were screened with a binocular microscope for variation in colony morphology, but no indications of phase variation were found by this method. In addition, the LPS profiles of at least five randomly selected colonies of the *B. avium* and *B. trematum* strains as well as a human and a chicken (LMG 13501^T) *B. hinzii* strain did not show LPS variation within a single strain (data not shown). Furthermore, no influence of the presence of 50 mM MgSO₄ in BG agar was observed on the LPS profiles of these three species (data not shown).

DISCUSSION

In this study, *Bordetella* LPS polymorphism was analysed by Tricine-SDS-PAGE. The data presented suggest species-specific LPS expression; however, the generalization of this notion awaits confirmation by examination of a larger number of strains. Such a study can also answer the question of whether LPS profiles are useful as an additional approach for differentiating *bordetellae*.

B. pertussis strains exhibit limited LPS variation (rarely a strain lacking LPSA) as was also observed in a study on recent clinical isolates (W. M. R. van den Akker & F. R. Mooi, unpublished) and is in agreement with other reports (e.g. Peppler, 1984; Chaby & Caroff, 1988). Based on its co-migration with the LPS of the *wlbG* mutant in *B. pertussis* Tohama I (Fig. 2), LPSB seems to be the trisaccharide-deficient LPS form. Interestingly, by use of a higher acrylamide concentration and longer electrophoresis time, it was found that LPSA consists of two LPS forms. This could possibly result from the presence or absence of a phosphate group on a 3-deoxy-D-manno-2-octulosonic acid unit in the *B. pertussis* LPS

Fig. 6. Electrophoretic profiles of LPS from *B. avium* and *B. trematum*. Strains were cultured at 37 °C (a), 25 °C (b) or 41 °C (c). Sets: 1, B21; 2, B22; 3, LMG 13506^T.

Fig. 7. Electrophoretic profiles of LPS from *B. hinzii* strains from chickens and humans. Strains were cultured at 37 °C (a), 25 °C (b) or 41 °C (c). Sets: 1, LMG 10979; 2, LMG 13501^T; 3, LMG 1872.

(Le Dur *et al.*, 1980), or might result from variability in fatty acid substitution as described for *B. bronchiseptica* LPS (Zarrouk *et al.*, 1997). Studies of a BvgAS-deficient transposon mutant and spontaneous phase variants suggest that BvgAS is not involved in LPS biosynthesis in *B. pertussis*. *B. holmesii* is a species closely related to *B. pertussis* (Weyant *et al.*, 1995) but it had a different LPS profile and there was no cross-reactivity of *B. pertussis* LPS mAbs with the LPS of *B. holmesii*.

B. paraptentussis exhibits host-specific LPS expression. This was further confirmed by a study of more human and ovine *B. paraptentussis* strains (unpublished observations), and extends previous observations that human and ovine isolates differ in the presence of LPS epitopes (Porter *et al.*, 1995). Combined with differences found in fatty acid expression, antibiotic resistance patterns (Porter *et al.*, 1995) and the presence of insertion

sequence elements (van der Zee *et al.*, 1996), this suggests that human and ovine strains form separate lineages. Parallels in LPS structure and regulation were observed between the two human *B. paraptentussis* strains and six out of eight *B. bronchiseptica* strains. In particular, the presence and temperature modulation of two polymeric O-antigen structures was remarkable (Figs 3 and 4). One canine strain (B81), possibly a phase variant, did not produce high-molecular-mass LPS and no temperature modulation was observed (Fig. 4, set 6). In the case of a human strain (B618) smooth LPS was only expressed at 25 °C (Fig. 4, set 8). Despite these exceptions, this LPS phase variation seems to be a common phenomenon among *B. bronchiseptica* strains and was not restricted to a particular host. Cross-reactivity of LPS mAbs (Le Blay *et al.*, 1994) and the presence of a common sugar structure (a homopolymer of 1,4-linked 2,3-diacetamido-2,3-dideoxy- α -L-galactopyranosyluronic acid res-

idues) in the O-antigen (Di Fabio *et al.*, 1992) also stress the relatedness in LPS present in human *B. parapertussis* and *B. bronchiseptica* strains. Together these similarities strongly point to a mutual progenitor or acquisition of LPS genes by horizontal transfer. The effects of temperature and sulphate anions on LPS profiles, supported by the phase variants, indicate that the BvgAS system regulates LPS expression in some *B. bronchiseptica* strains. Previously, Peppler & Schrupf (1984) also reported differences in LPS profiles between Bvg⁺ and Bvg⁻ *B. bronchiseptica* phase variants. The *B. bronchiseptica* strains B14 and B19 appear to have LPS structures in common with *B. pertussis* LPS as demonstrated by cross-reactivity of mAbs. The already discussed similarities in LPS of human *B. parapertussis* isolates and *B. bronchiseptica* isolates might indicate that BvgAS plays a role in *B. parapertussis* LPS regulation. In fact, we have evidence that BvgAS is indeed implicated in *B. parapertussis* LPS expression (W. M. R. van den Akker, T. F. Meyer & F. R. Mooi, unpublished).

Gentry-Weeks *et al.* (1991) reported phase variation in a *B. avium* strain at a frequency of 2×10^{-4} . These phase variants had a different colony morphology and lacked five outer-membrane proteins. Those five proteins were also absent when the original strain was grown in the presence of MgSO₄. Neither phase variants nor an influence of MgSO₄ on LPS expression were found in the two *B. avium* strains studied here. The possibility that these strains were phase variants must be acknowledged.

The biological roles of the different LPS molecules found in the *Bordetella* species are still an enigma. It is, for example, intriguing that the closely related *B. pertussis* and *B. holmesii* species are found in different niches (respiratory tract versus blood) and differ in LPS expression. The dependency of *Bordetella* LPS expression on environmental conditions, as demonstrated here, exemplifies the dynamics of the expression of virulence determinants in these pathogenic bacteria. In this respect, it is of special interest that *B. bronchiseptica* bvgAS mutants have an advantage in survival over wild-type strains in phagocytes (Banemann & Gross, 1997), which might hint at a role for LPS in the intracellular environment. Further knowledge about heterogeneity, structure and regulation of LPS combined with information about pathogenesis is essential to be able to understand the functions of the different LPS forms in the dynamic environment that pathogens encounter during infection.

Conclusions

The main conclusions of this study can be summarized as follows. (1) There is extensive variation among the seven *Bordetella* species in LPS expression as analysed by electrophoresis. This LPS expression seems to be species-specific and may offer possibilities for determination and differentiation. (2) Temperature is a modulator of LPS expression in most *Bordetella* species. (3) The LPSs expressed by the closely related *B. pertussis*

and *B. holmesii* species are phenotypically and immunologically distinct. (4) The LPSs expressed by human and ovine *B. parapertussis* strains are clearly different from each other, but both are temperature regulated. This host-specific LPS expression concurs with the thesis that these strains constitute different lineages. (5) In some *B. bronchiseptica* strains the global regulator of the expression of virulence determinants (BvgAS) regulates LPS biosynthesis. (6) BvgAS does not seem to participate in the regulation of LPS expression in *B. pertussis*.

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REFERENCES

- Akerley, B. J. & Miller, J. F. (1996). Understanding signal transduction during bacterial infection. *Trends Microbiol* **4**, 141–146.
- van den Akker, W. M. R. (1997). *Bordetella bronchiseptica* has a BvgAS-controlled cytotoxic effect upon interaction with epithelial cells. *FEMS Microbiol Lett* **156**, 239–244.
- Allen, A. & Maskell, D. (1996). The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in *Bordetella pertussis*. *Mol Microbiol* **19**, 37–52.
- Banemann, A. & Gross, R. (1997). Phase variation affects long-term survival of *Bordetella bronchiseptica* in professional phagocytes. *Infect Immun* **65**, 3469–3473.
- Caroff, M., Chaby, R., Karibian, D., Perry, J., Deprun, C. & Szabó, L. (1990). Variations in the carbohydrate regions of *Bordetella pertussis* lipopolysaccharides: electrophoretic, serological, and structural features. *J Bacteriol* **172**, 1121–1128.
- Chaby, R. & Caroff, M. (1988). Lipooligosaccharides of *Bordetella pertussis* endotoxin. In *Pathogenesis and Immunity in Pertussis*, pp. 247–271. Edited by A. C. Wardlaw & R. Parton. Chichester: Wiley.
- Cullinane, L. C., Alley, M. R., Marshall, R. B. & Manktelow, B. W. (1987). *Bordetella parapertussis* from lambs. *N Z Vet J* **35**, 175.
- Di Fabio, J. L., Caroff, M., Karibian, D., Richards, J. C. & Perry, M. B. (1992). Characterization of the common antigenic lipopolysaccharide O-chains produced by *Bordetella bronchiseptica* and *Bordetella parapertussis*. *FEMS Microbiol Lett* **97**, 275–282.
- Ewanowich, C. A., Melton, A. R., Weiss, A. A., Sherburne, R. K. & Peppler, M. S. (1989). Invasion of HeLa 229 cells by virulent *Bordetella pertussis*. *Infect Immun* **57**, 2698–2704.
- Gentry-Weeks, C. R., Provence, D. L., Keith, J. M. & Curtiss, R., III (1991). Isolation and characterization of *Bordetella avium* phase variants. *Infect Immun* **59**, 4026–4033.
- Goodnow, R. A. (1980). Biology of *Bordetella bronchiseptica*. *Microbiol Rev* **44**, 722–738.
- Hitchcock, P. J. & Brown, T. M. (1983). Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol* **154**, 269–277.
- Holst, O., Ulmer, A. J., Brade, H., Flad, H.-D. & Rietschel, E. T.

- (1996). Biochemistry and cell biology of bacterial endotoxins. *FEMS Immunol Med Microbiol* 16, 83–104.
- Kerstens, K., Hinz, K.-H., Hertle, A., Segers, P., Lievens, A., Siegmann, O. & De Ley, J. (1984). *Bordetella avium* sp. nov., isolated from the respiratory tracts of turkeys and other birds. *Int J Syst Bacteriol* 34, 56–70.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Le Blay, K., Caroff, M., Richards, J. C., Perry, M. B. & Chaby, R. (1994). Specific and cross-reactive monoclonal antibodies to *Bordetella parapertussis* and *Bordetella bronchiseptica* lipopolysaccharides. *Microbiology* 140, 2459–2465.
- Le Dur, A., Chaby, R. & Szabó, L. (1980). Isolation of two protein-free and chemically different lipopolysaccharides from *Bordetella pertussis* phenol-extracted endotoxin. *J Bacteriol* 143, 78–88.
- Lesse, A. J., Campagnari, A. A., Bittner, W. E. & Apicella, M. A. (1990). Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J Immunol Methods* 126, 109–117.
- Lindquist, S. W., Weber, D. J., Mangum, M. E., Hollis, D. G. & Jordan, J. (1995). *Bordetella holmesii* sepsis in an asplenic adolescent. *Pediatr Infect Dis J* 14, 813–815.
- Linnemann, C. C. & Perry, E. B. (1977). *Bordetella parapertussis*. Recent experience and a review of the literature. *Am J Dis Child* 131, 560–563.
- Mertsola, J. (1985). Mixed outbreak of *Bordetella pertussis* and *Bordetella parapertussis* infection in Finland. *Eur J Clin Microbiol* 4, 123–128.
- Mooi, F. R., van der Heide, H. G. J., ter Avest, A. R., Welinder, K. G., Livey, I., van der Zeijst, B. A. M. & Gaastra, W. (1987). Characterization of fimbrial subunits from *Bordetella* species. *Microb Pathog* 12, 127–135.
- Müller, M. & Hildebrandt, A. (1993). Nucleotide sequences of the 23S rRNA genes from *Bordetella pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium*, and their implications for phylogenetic analysis. *Nucleic Acids Res* 21, 3320.
- Musser, J. M., Hewlett, E. L., Peppler, M. S. & Selander, R. K. (1986). Genetic diversity and relationships in populations of *Bordetella* species. *J Bacteriol* 166, 230–237.
- Peppler, M. S. (1984). Two physically and serologically distinct lipopolysaccharide profiles of *Bordetella pertussis* and their phenotypic variants. *Infect Immun* 43, 224–232.
- Peppler, M. S. & Schrumph, M. E. (1984). Phenotypic variation and modulation in *Bordetella bronchiseptica*. *Infect Immun* 44, 681–687.
- Pittman, M. (1984). Genus *Bordetella* Moreno-López 1952, 178^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 388–393. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Poolman, J. T., Kuipers, B., Vogel, M. L., Hamstra, H. J. & Nagel, J. (1990). Description of a hybridoma bank towards *Bordetella pertussis* toxin and surface antigens. *Microb Pathog* 8, 377–382.
- Porter, J. F., Connor, K. & Donachie, W. (1994). Isolation and characterization of *Bordetella parapertussis*-like bacteria from ovine lungs. *Microbiology* 140, 255–261.
- Porter, J. F., Connor, K., van der Zee, A., Reubsaet, F., Ibsen, P., Heron, I., Chaby, R., Le Blay, K. & Donachie, W. (1995). Characterisation of ovine *Bordetella parapertussis* isolates by analysis of specific endotoxin (lipopolysaccharide) epitopes, filamentous haemagglutinin production, cellular fatty acid composition and antibiotic sensitivity. *FEMS Microbiol Lett* 132, 195–201.
- Preston, A., Mandrell, R. E., Gibson, B. W. & Apicella, M. A. (1996). The lipooligosaccharides of pathogenic Gram-negative bacteria. *Crit Rev Microbiol* 22, 139–180.
- Relman, D. A. (1995). *Bordetella pertussis*: determinants of virulence. In *Bacterial Toxins and Virulence Factors in Disease. Handbook of Natural Toxins*, vol. 8, pp. 367–405. Edited by J. Moss, B. Iglewski, M. Vaughan & A. T. Tu. New York: Marcel Dekker.
- Stibitz, S. & Miller, J. F. (1994). Coordinate regulation of virulence in *Bordetella pertussis* mediated by the *vir* (*bvg*) locus. In *Molecular Genetics of Bacterial Pathogenesis*, pp. 407–422. Edited by V. L. Miller, J. B. Kaper, D. A. Portnoy & R. R. Isberg. Washington, DC: American Society for Microbiology.
- Tsai, C.-M. & Frasch, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* 119, 115–119.
- Vandamme, P., Hommez, J., Vancanneyt, M., Monsieurs, M., Hoste, B., Cookson, B., Wirsing von König, C. H., Kersters, K. & Blackall, P. J. (1995). *Bordetella hinzii* sp. nov., isolated from poultry and humans. *Int J Syst Bacteriol* 45, 37–45.
- Vandamme, P., Heyndrickx, M., Vancanneyt, M., Hoste, B., de Vos, P., Falsen, E., Kersters, K. & Hinz, K.-H. (1996). *Bordetella trematum* sp. nov., isolated from wounds and ear infections in humans, and reassessment of *Alcaligenes denitrificans* Rüger and Tan 1983. *Int J Syst Bacteriol* 46, 849–858.
- Verwey, W. F., Thiele, E. H., Sage, D. N. & Schuchardt, L. F. (1949). A simplified liquid medium for the growth of *Haemophilus pertussis*. *J Bacteriol* 58, 127–134.
- Weiss, A. A. & Falkow, S. (1984). Genetic analysis of phase change in *Bordetella pertussis*. *Infect Immun* 43, 263–269.
- Weiss, A. A., Hewlett, E. L., Myers, G. A. & Falkow, S. (1983). Tn-5 induced mutations affecting virulence factors of *Bordetella pertussis*. *Infect Immun* 42, 33–41.
- Weyant, R. S., Hollis, D. G., Weaver, R. E. & 7 other authors (1995). *Bordetella holmesii* sp. nov., a new gram-negative species associated with septicemia. *J Clin Microbiol* 33, 1–7.
- Willems, R. J. L., van der Heide, H. G. J. & Mooi, F. R. (1992). Characterization of a *Bordetella pertussis* fimbrial gene cluster which is located directly downstream of the filamentous haemagglutinin gene. *Mol Microbiol* 6, 2661–2671.
- Zarrouk, H., Karibian, D., Bodie, S., Perry, M. B., Richards, J. C. & Caroff, M. (1997). Structural characterization of the lipid A of three *Bordetella bronchiseptica* strains: variability of fatty acid substitution. *J Bacteriol* 179, 3756–3760.
- van der Zee, A., Agterberg, C., van Agterveld, M., Peeters, M. & Mooi, F. R. (1993). Characterization of IS1001, an insertion sequence element of *Bordetella parapertussis*. *J Bacteriol* 175, 141–147.
- van der Zee, A., Groenendijk, H., Peeters, M. & Mooi, F. R. (1996). The differentiation of *Bordetella parapertussis* and *Bordetella bronchiseptica* from humans and animals as determined by DNA polymorphism mediated by two different insertion sequence elements suggests their phylogenetic relationship. *Int J Syst Bacteriol* 46, 640–647.

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