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# A role for lipopolysaccharide in turkey tracheal colonization by *Bordetella avium* as demonstrated *in vivo* and *in vitro*

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## Summary

We isolated two insertion mutants of *Bordetella avium* that exhibited a peculiar clumped-growth phenotype and found them to be attenuated in turkey tracheal colonization. The mutants contained transposon insertions in homologues of the *wlbA* and *wlbL* genes of *Bordetella pertussis*. The *wlb* genetic locus of *B. pertussis* has been previously described as containing 12 genes involved in lipopolysaccharide (LPS) biosynthesis. Polyacrylamide gel analysis of LPS from *B. avium wlbA* and *wlbL* insertion mutants confirmed an alteration in the LPS profile. Subsequent cloning and complementation of the *wlbA* and *wlbL* mutants *in trans* with a recombinant plasmid containing the homologous *wlb* locus from *B. avium* eliminated the clumped-growth phenotype and restored the LPS profile to that of wild-type *B. avium*. Also, a parental level of tracheal colonization was restored to both mutants by the recombinant plasmid. Interestingly, complementation of the *wlbA* and *wlbL* mutants with a recombinant plasmid containing the heterologous *wlb* locus from *B. pertussis, B. bronchiseptica*, or *Bordetella parapertussis* eliminated the clumped-growth phenotype and resulted in a change in the LPS profile, although not to that of wild-type *B. avium*. The mutants also acquired resistance to a newly identified *B. avium*-specific bacteriophage, Ba1. Complementation of both *wlbA* and *wlbL* mutants with the homologous *wlb* locus of *B. avium*, but not the heterologous *B. pertussis* locus, restored sensitivity to Ba1. Complementation of the *wlbL* mutant, but not the *wlbA* mutant, with the heterologous *wlb* locus of *Bordetella bronchiseptica* or *B. parapertussis* restored partial sensitivity to Ba1. Comparisons of the LPS profile and phage sensitivity of the mutants upon complementation by *wlb* loci from the heterologous species and by *B. avium* suggested that phage sensitivity required the presence of O-antigen. At the mechanistic level, both mutants showed a dramatic decrease in serum resistance and a decrease in binding to turkey tracheal rings *in vitro*. In the case of serum resistance, complementation of both mutants with the homologous *wlb* locus of *B. avium* restored serum resistance to wild-type levels. However, in the case of epithelial cell binding, only complementation of the *wlbA* mutant completely restored binding to wild-type levels (binding was only partially restored in the *wlbL* mutant). This is the first characterization of LPS mutants of *B. avium* at the genetic level and the first report of virulence changes by both *in vivo* and *in vitro* measurements

## Introduction

*Bordetella avium* is the causative agent of bordetellosis, a disease of the upper respiratory tract of turkeys (Skeeles and Arp, 1997). As with several other members of the *Bordetella* genus, *B. avium* binds preferentially to ciliated tracheal epithelial cells (Arp *et al*., 1993). Death of the ciliated cells is thought to lead to many of the clinical signs associated with



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avian bordetellosis, which include excessive oculonasal discharge, sneezing, mouth breathing, conjunctivitis and decreased weight gain. In addition, infected turkeys are more susceptible to secondary infections by other pathogens (Barnes and Hofstad, 1978; Saif *et al*., 1980). The secondary infection, often by *Escherichia coli*, is frequently fatal and contributes significantly to losses sustained by the poultry industry from infectious agents (Saif *et al*., 1980; Skeeles and Arp, 1997).

The pathogenesis of bordetellosis and factors affecting tracheal colonization by *B. avium* are not fully understood (Gentry-Weeks *et al*., 1988; Temple *et al*., 1998). In other more well-studied members of the *Bordetellae* (*Bordetella pertussis and Bordetella bronchiseptica*), a number of traits have been associated with virulence (Goodwin and Weiss, 1990; Mooi *et al*., 1992; Geuijen *et al*., 1997; Ishikawa and Sato, 1997; Cotter *et al*., 1998; van den Berg *et al*., 1999). However, in the case of *B. pertussis*, the species that causes whooping cough in children, suspected virulence traits have been examined only in experiments utilizing heterologous hosts (Parton *et al*., 1994; Shahin and Cowell, 1994), leading to difficulties in determining the applicability of the results in guiding prevention strategies. In both *B. avium* and *B. bronchiseptica*, traits associated with virulence can be tested directly in experimental infections using the natural host (Monack and Falkow, 1993; Cotter and Miller, 1994; Ackermann *et al*., 1997; Temple *et al*., 1998). In instances where suspected virulence factors are shared amongst all *Bordetella* species, the use of the natural host-pathogen pairings has provided results that, in some cases, contradict those obtained from *B. pertussis* in heterologous hosts (Weiss *et al*., 1984; Weiss and Goodwin, 1989; Cotter *et al*., 1998; Temple *et al*., 1998).

Apart from providing possible insights into human disease, understanding avian bordetellosis is becoming increasingly important as farming practices world-wide adopt poultry production methods based upon high efficiency feed conversion where the commercial turkey particularly excels (Ensminger, 1980). Unfortunately, commercial turkeys were derived from limited genetic stock (only one breed is recognized) and their susceptibility to infectious agents is, in theory, and as tested experimentally (Temple *et al*., 1998), very uniform. This lack of natural variability, coupled with our growing reliance on them as a food source, provides incentive for a better understanding of their infectious agents.

We have been interested in discovering factors that affect *B. avium* virulence. In this report, we describe the isolation of two *B. avium* mutants defective in the normal biosynthesis of lipopolysaccharide (LPS). Our analysis revealed that these mutants were significantly reduced in colonization and had a number of interesting properties that may allow further detailed analysis of the role of LPS in producing the characteristic signs of bordetellosis.

## Results

### Discovery, isolation and genetic characterization of LPS mutants

Isolation of an avirulent insertion mutant (strain PAS78) during a screen of signature-tagged transposon mutants (Hensel *et al*., 1995; P.A.S. *et al*., unpublished results) first alerted us to the possible importance of a peculiar clumped-growth phenotype (e.g. clumping when grown in broth culture). Subsequent screening of random insertion mutants for this clumped-growth phenotype led to the isolation and characterization of an additional mutant (strain PAS85). Both mutants showed significantly reduced colonization in turkey poults (documented in a later section). In addition to clumping when grown in broth culture, both mutants displayed a ‘rough’ phenotype that consisted of alteration in colony morphology characteristic of some LPS mutants of *Salmonella typhimurium* (Rick, 1987). These results prompted work to define the genetic defect and to further characterize these mutants.

DNA flanking each insertion was cloned and sequenced. Sequence adjacent to the insertion in strain PAS85 was homologous to the 5′ region of the *wlbA* gene and the predicted amino acid sequence was 75% identical to WlbA of *B. pertussis* (EMBL no. X90711). Sequence adjacent to the insertion in strain PAS78 was similar to the middle of the *wlbL* gene of *B. pertussis*. The predicted amino acid sequence of this region was 58% identical to WlbL of *B. pertussis* (EMBL no. X90711). Both the *wlbA* and *wlbL* genes are part of the *wlb* genetic locus consisting of 12 genes (A–L) in *B. pertussis* that are involved in LPS biosynthesis, specifically the addition of a trisaccharide to the distal portion of the core and O-antigen biosynthesis (Allen and Maskell, 1996; Allen *et al*., 1998).

In order to complement our *wlbA* and *wlbL* insertion mutants, we cloned a 15 kb region containing the *wlbL* and *wlbA* genes of *B. avium*. The resultant *B. avium wlb* locus is shown (Fig. 1), where it is compared with similar genetic loci from *B. pertussis, B. parapertussis* and *B. bronchiseptica* (Allen *et al*., 1998). Although we have not sequenced the entire region of the *B. avium* locus, restriction analysis, Southern blot analysis (data not shown), partial sequence analysis (Fig. 1) and functional analysis (below) indicated the *wlb* locus of *B. avium* is similar to the loci of the other *Bordetella* species. Partial sequence analysis included approximately 600 bp on either end of the cloned segment and the resultant sequence showed no homology to current GenBank sequences. In addition, centre sequence data (*wlbF* and *wlbG*) served to confirm the general similarity of the locus with the other species at the protein sequence level.

### *Trans-complementation of* wlbA *and* wlbL mutant phenotypes with plasmids containing homologous and heterologous wlb *genes*

In addition to clumping when grown in broth culture and reduced colonization in turkey poults, *wlbA* and *wlbL* insertion mutants displayed an alteration in their LPS profile upon PAGE and a loss of sensitivity to a *B. avium* bacteriophage, Ba1 (Shelton *et al*., submitted). The clumped-growth phenotype was readily complemented by plasmids containing the homologous and heterologous *wlb* loci (Fig. 2). In this analysis, each complementing plasmid contained the *wlb* locus of *B. avium* (pLAF-av), *B. pertussis* (pLAF-pe), *B. bronchiseptica* (pLAF-br) or *B. parapertussis* (pLAF-pa) cloned into the broad host range vector, pLAFR5 (refer to Fig. 1 and Table 1 for diagrams and sources of the plasmids respectively). The vector (pLAFR5) was the only plasmid that did not noticeably complement the clumped-growth phenotype. Similar results were seen with the restoration of a smooth colony morphology upon homologous and heterologous *wlb* loci complementation (data not shown).

Silver-stained polyacrylamide gel analysis of LPS structure showed complete restoration to the parental LPS profile when either the *wlbL* or the *wlbA* mutant was complemented with the homologous *wlb* locus (pLAF-av), evidenced by restored O-antigen production (compare lane 1 with lane 3, Fig. 3). However, only partial restoration of the profile was seen when either mutant was complemented with the *wlb* locus from other *Bordetella* species (lanes 4–6, Fig. 3). Specifically, none of the heterologous regions restored the O-antigen to parental levels, but there was a distinct change in migration of the single, fast-migrating LPS band associated with the mutants. This faster migrating band seen in the mutants [lanes 2 and 2′, Fig. 3; indicated by an asterisk (*)] was raised to a higher migrating (parental) band in the complemented mutants [lanes 4–6 compared with lane 1, Fig. 3; indicated by a double asterisk (**)].

We also found that the loss of sensitivity to a newly identified *B. avium*-specific bacteriophage, Ba1, in the *wlbA* and *wlbL* mutants could be completely restored in both mutants by complementation with the homologous wlb locus (compare rows 1–3, Fig. 4). Phage sensitivity could be partially restored by the heterologous loci only in the *wlbL*

mutant (compare rows 4–6, Fig. 4). Of the heterologous loci complementing the *wlbL* mutant, the locus from *B. pertussis* (row 4, Fig. 4) was clearly the least effective.

### *Restored turkey tracheal colonization of* wlbA *and* wlbL *mutants complemented* in trans *by a plasmid containing the* B. avium wlb *locus*

Fifty percent infectious dose ($ID_{50}$) measurements revealed a pronounced and statistically significant defect in the ability of *wlbA* and *wlbL* mutants (strains PAS78 and PAS85) to colonize turkey tracheas when compared with the parental strain (197N; Table 2). This defect in turkey tracheal colonization of the mutants was completely restored by complementation with the homologous *wlb* genetic locus from *B. avium* (Table 2). In this experiment, the *wlbA* and *wlbL* mutants, harbouring the pLAF-av recombinant plasmid, displayed an $ID_{50}$ statistically indistinguishable from that of the parental and clearly distinguishable from the mutant harbouring just the vector plasmid (pLAFR5) (Table 2). There was no statistically relevant difference in the average $ID_{50}$ between the *wlbA* and *wlbL* mutants even although the values were noticeably different.

### Resistance to naïve turkey serum and tracheal binding in vitro *are significantly altered by* wlbA *and* wlbL *lesions*

Two *in vitro* assays that measured (i) serum resistance and (ii) tracheal ring binding were performed to assess possible reasons for the decrease in colonization rate of the LPS mutants seen *in vivo*. In the case of the serum resistance assay, we took advantage of a preliminary observation that our parental *B. avium* strain was completely resistant to naïve turkey poult serum, and our prior observation that this strain also attaches to tracheal rings *in vitro* (Temple *et al*., 1998). When tested, the two LPS mutants showed a significant decrease in serum resistance and in binding to turkey tracheal rings *in vitro* (Fig. 5A and B respectively).

In the case of serum resistance, parental *B. avium* was resistant to naïve turkey serum as demonstrated by greater than 100% survival in 50% serum (Fig. 5A). The *wlbA* and *wlbL* mutants showed a decrease in resistance to serum as demonstrated by 0.004% and 0.015% survival rates respectively. Complementation of both mutants with the homologous *wlb* locus of *B. avium*, but not vector, restored serum resistance to parental levels.

In the case of tracheal ring binding, the two LPS mutants showed a significant decrease in the percentage of bacteria bound compared with the parental strain (Fig. 5B). Upon complementation with the homologous wlb locus of *B. avium*, attachment was significantly increased in both mutants when compared with the mutant containing the vector alone. However, only the *wlbA* mutant was restored to parental levels.

## Discussion

Two transposon insertion mutants of *B. avium* were identified that (i) exhibited clumping when grown in broth, (ii) had altered LPS polyacrylamide gel profiles, (iii) were significantly reduced in turkey poult colonization, (iv) were resistant to Ba1 bacteriophage, (v) showed a decrease in serum resistance, and (vi) showed a decrease in turkey tracheal epithelial cell attachment. The insertions interrupted genes associated with LPS biosynthesis. One mutant had an insertion in a gene similar to the *wlbA* gene of *B. pertussis* and the other had an insertion in a gene similar to the *wlbL* gene at the same genetic locus. Cloning of the entire *B. avium wlb* genetic locus (encoding genes *wlbA*–*wlbL*) and partial sequencing indicated similarity of the *B. avium wbl* locus to the other *Bordetella* species where the *wbl* sequence has been obtained (*B. pertussis*, *B. bronchiseptica* and *B. parapertussis*: Keen *et al*., 1988; Allen and Maskell, 1996; Allen *et al*., 1998).

Trans-complementation of *B. avium wlbA* and *wlbL* mutants with both homologous and heterologous *wlb* loci revealed that homologous genes from *B. avium* as well as heterologous genes from *B. pertussis*, *B. bronchiseptica* and *Bordetella parapertussis* could readily complement the clumped-growth phenotype in both mutants. Complementation of the clumped-growth phenotype appeared to correlate with the shift of a faster migrating LPS band in the mutants to a slower migrating band comparable to the lower band in the parent. Considering that the *wlb* genetic locus is responsible for the addition of a trisaccharide to the lipid-A-core region in the other *Bordetella* species (Peppler, 1984; Caroff *et al*., 1990; Preston *et al*., 1996; Maskell and Allen, 1997), we tentatively conclude that the *wlbA* and *wlbL* gene products of heterologous plasmids affect oligosaccharide additions to the mutant lipid-A-core region and that these additions are sufficient to complement the clumped-growth phenotype.

In contrast to the clumped-growth phenotype, restoration of *B. avium*-specific bacteriophage (Ba1) sensitivity was more dependent upon having the homologous genes, especially with respect to complementing the *wlbA* mutant. That is, in the *wlbA* mutant, phage sensitivity was, at best, poorly restored regardless of the heterologous locus employed. In the *wlbL* mutants, partial phage sensitivity was evident upon heterologous complementation by *B. bronchiseptica* and *B. parapertussis wlb* loci, but not as evident upon complementation by the *B. pertussis* genetic locus. Interestingly, the degree to which phage sensitivity was restored by the heterologous loci correlated well with the degree to which O-antigen was restored (compare appropriate regions of the gels in Fig. 2 with the cross-streaks in Fig. 3). These data suggest that phage Ba1 may bind to a portion of the O-antigen. Further examination of phage resistant phenotypes (e.g. directly selecting for phage resistant mutants) will probably provide a more sophisticated understanding of the relationship between LPS and phage sensitivity. Also, more evidence obtained from competitive binding assays are needed to show, unequivocally, that phage interact directly with O-antigen.

The precise structural defect in LPS biosynthesis in the *wlbA* and *wlbL* mutants was not determined. However, from previous studies of *B. pertussis* LPS structure, the *wlbL* gene encodes a protein similar to enzymes typically involved in the synthesis of 6-deoxy and dideoxy sugars (Allen and Maskell, 1996). In *B. pertussis*, this corresponds to synthesis of N-acetyl-N-methylfucosamine (FucNAcMe), which is one of the trisaccharides attached to the lipid-A-core LPS. The *wlbA* gene encodes a putative dehydrogenase. Allen and Maskell (1996) propose that this gene product most probably is involved in the biosynthesis of 2,3-dideoxy-2,3-di-N-acetylmannosa-minuronic acid (2,3-diNAcManA), which is also one of the trisaccharides attached to the lipid-A-core LPS of *B. pertussis*. One might reason that the specificity of the enzymatic reaction of the *wlbL* gene product [over that of the dehydrogenase (the *wlbA* gene product)] may account for the differences in the degree of heterologous complementation found in *wlbA* and *wlbL* mutants. Another reason, other than specificity, may involve differing degrees of polarity in the *wlbA* and *wlbL* insertions. That is, the transposon insertion in *wlbA* may have polar effects on downstream *wlb* genes and be more difficult to complement due to the possibility of numerous heterologous gene products necessary for effective complementation. In contrast, the *wlbL* insertion, at the end of the locus, may not affect other relevant genes.

LPS has been shown to be important for virulence in a variety of bacterial pathogens (Sigel *et al*., 1980; Waldor *et al*., 1994; Sandlin *et al*., 1995; Bilge *et al*., 1996; Brown and Curtiss, 1996; Licht *et al*., 1996; Chiang and Mekalanos, 1999). In the Bordetellae, there have been no examinations of the virulence of LPS mutants *in vivo*; although, in *B. pertussis*, *in vitro* assays have indicated the importance of LPS in epithelial cell cytotoxicity (Flak and Goldman, 1999) and phagocytosis (Weingart and Weiss, 2000). Our results clearly show a significant defect in tracheal colonization of our *wlbA* and *wlbL* mutants, and restoration of

parental levels of colonization upon genotypic complementation. There was no statistically significant difference between the *wlbA* and *wlbL* mutants in their ability to effect tracheal colonization. This correlated with our observation that the LPS profiles of the two mutants were indistinguishable after PAGE. Nevertheless, further colonization studies and more detailed structural analyses will probably reveal differences.

In order to relate the effects of *wlbA* and *wlbL* mutations more closely to traits associated with virulence, we examined two parameters: resistance to naïve serum and tracheal attachment. These traits have been correlated with virulence *in vivo* (Fernandez and Weiss, 1994; Temple *et al*., 1998). With regard to serum sensitivity, the *wlbL* and *wlbA* mutants were both dramatically more sensitive to naïve turkey serum. This sensitivity probably decreases the ability of the mutants to initiate colonization (tracheal secretions contain serum; Suresh *et al*., 1994) and later may enhance the clearance of the microorganism (the resolution of experimental avian bordetellosis is completely correlated with the onset of humoral immunity; Suresh *et al*., 1994; Suresh and Arp, 1995). Attachment of the mutants to tracheal rings was also significantly affected. Because *B. avium*, like all *Bordetella* species, is dramatically ciliatropic (Arp and Fagerland, 1987), we infer that these mutants are impaired in binding to ciliated tracheal epithelial cells and thus would be at a disadvantage in colonizing normal turkey tracheas. Both of these effects may contribute to the decrease in colonization of turkey poults that we observed *in vivo*.

In addition to establishing the importance for the *wlb* locus in virulence, the present work suggests several avenues for further investigation. For example, the use of phage resistance as a selection for avirulent mutants may provide better understanding of the role phage receptors in virulence. Clearly, phage have been instrumental in uncovering and relating features of the bacterial cell surface to virulence (Raleigh and Signer, 1982; Nnalue *et al*., 1990). In addition, the present understanding of Bordetellae LPS structure may be exploited by using heterologous loci (Di Fabio *et al*., 1992; Preston *et al*., 1996; Maskell and Allen, 1997) in more refined complementation tests. The most important aspect of future LPS analysis will be to determine how alterations in its structure affect changes in colonization, serum resistance and tracheal cell binding.

Taken together, the above results indicate a role for LPS in *B. avium* virulence and phage sensitivity, thus providing a framework and tools for further genetic and biochemical studies of *B. avium* LPS as an important virulence factor in bordetellosis.

## Experimental procedures

### Bacterial strains and growth conditions

All bacterial strains, bacteriophage and plasmids employed in this study are listed in Table 1. Brain–heart infusion (BHI) medium (Difco) and Stainer–Scholte medium were employed under *B. avium* growth conditions that have been described previously (Temple *et al*., 1998). Antibiotics were added at the concentrations of 40 μg $ml^{-1}$, kanamycin; 20 μg $ml^{-1}$, tetracycline; 100 or 800 μg $ml^{-1}$, ampicillin; or 25 μg $ml^{-1}$, nalidixic acid when appropriate. All *E. coli* strains were grown in Luria (L) broth or agar (Miller, 1972) at 37°C overnight.

To screen for clumping in broth culture, *B. avium* was grown in 1.0 ml of BHI broth in a 48-well cell culture cluster (Costar) with rotary shaking overnight at 37°C.

### Tn5 insertion mutagenesis

Random-insertion mutants were generated by a modification of the method of Temple *et al*., 1998. Conjugation was performed between *E. coli* S17.1 (γ*pir*) containing pUTKm2 (de Lorenzo *et al*., 1990; Hensel *et al*., 1995) and *B. avium* 197N. Next, 100 μl of a 10-fold

concentrated overnight culture of *B. avium* was spread onto selective medium (BHI containing kanamycin and nalidixic acid) to create a recipient lawn. Overnight cultures of S17.1γ*pir* containing pUTKm2 plasmids were serially diluted and 20 μl was spotted onto the recipient lawn. Plates were incubated for 48 h at 37°C and resultant independent exconjugants were isolated. Single insertions and the loss of the plasmid were monitored by Southern analysis and sensitivity to 800 μg $ml^{-1}$ ampicillin. Resultant insertion mutants were grown separately in 1.0 ml of BHI broth containing kanamycin in 48-well cell culture clusters.

## Infectious dose determinations

The 50% infectious dose ($ID_{50}$) measurement of each mutant was performed as previously described (Temple *et al*., 1998) and approved under North Carolina State University Animal Care Protocol 97-129-A. Briefly, three sets of 10 turkey poults (1 week old) were inoculated with approximately $10^6$, $10^7$ or $10^8$ colony-forming units (cfu) of a mutant *B. avium* strain. Inocula was determined by vigorous resuspension of the bacteria prior to plating dilutions. At the same time, three sets of 10 turkey poults were inoculated with approximately $10^5$, $10^6$ or $10^7$ cfu of the parental *B. avium* strain (197N). As a negative control, 10 turkey poults were inoculated with phosphate buffered saline. Prior to inoculation, four poults were removed and tested for prior exposure to *B. avium*. Fourteen days post-inoculation, one tracheal swab was collected from each turkey, expressed onto lactose MacConkey agar, and streaked for isolation. The cultures were incubated at 37°C for 48 h and the number of birds colonized (presence of any *B. avium*) was recorded. Isolates were confirmed by testing for appropriate antibiotic resistance and phenotype. $ID_{50}$s were calculated using a Reed and Muench analysis (Reed and Muench, 1938).

In the case of complementation, $ID_{50}$ determinations were carried out essentially as above. Briefly, three sets of 10 turkey poults were inoculated with approximately $10^6$, $10^7$ or $10^8$ cfu of *B. avium wlbA* mutant PAS85 or *wlbL* mutant PAS78 complemented with the plasmid vector (pLAFR5), and another three sets at the same approximate doses with PAS85 or PAS78 complemented with the recombinant plasmid containing the *wlb* locus of *B. avium* (pLAF-av). In these experiments, one set of 10 turkey poults was inoculated with approximately $10^7$ cfu of parental *B. avium* 197N containing the plasmid vector (pLAFR5) as a positive infection control. Colonizing bacteria were scored after plating directly onto lactose MacConkey agar (no antibiotic selection) and BHI agar containing tetracycline and nalidixic acid to differentiate plasmid-containing *B. avium* from those that had lost the plasmid.

## Cloning and sequence analysis of insertion mutants

*B. avium* DNA adjacent to the transposon was cloned by taking advantage of the gene encoding neomycin phosphotransferase, conferring kanamycin resistance, within the transposon. Chromosomal DNA from the *wlbL* mutant (strain PAS78) was digested with *Bgl*II and ligated into pLitmus28 (New England Biolabs) digested with *Bgl*II. The ligation mixture was introduced into competent *E. coli* DH5α by transformation and transformants were selected on L agar containing kanamycin and 100 μg $ml^{-1}$ ampicillin. One of the resulting transformants harboured a cloned DNA segment containing a portion of the transposon and downstream flanking DNA. The clone was sequenced at the UNC-CH Automated DNA Sequencing Facility on a Model 377 DNA Sequencer (Perkin Elmer, Applied Biosystems Division), using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq™ DNA polymerase, FS (Perkin Elmer, Applied Biosystems Division), using primer P7 (5′-dGCA CTT GTG TAT AAG AGT CAG; Hensel *et al*., 1995). Chromosomal DNA from the *wlbA* mutant (strain PAS85) was digested with *Not*I and ligated into pBluescript (Stratagene) digested with *Not*I. The ligation mixture was

introduced into competent *E. coli* DH5α by transformation and transformants were selected on L agar containing kanamycin and 100 μg $ml^{-1}$ ampicillin. One of the resultant transformants contained most of the transposon and the upstream flanking region. The clone was sequenced as above using primer P6 (5′-dCCT AGG CGC CCA GAT CTG AT; Hensel *et al*., 1995).

## LPS isolation and SDS–PAGE

LPS extraction from all strains were performed as previously described (Hitchcock and Brown, 1983; Apicella *et al*., 1994). Briefly, overnight broth cultures of *B. avium* were washed in PBS and diluted to 0.4 OD units $ml^{-1}$ (approximately $4 \times 10^8$ cells $ml^{-1}$). Next, 1 ml of cells was pelleted, resuspended in 100 μl lysis buffer (1 M Tris, pH 6.8, 6% SDS, 20% glycerol) and incubated at 95°C for 5–10 min. After cooling, 0.25 unit $\mu l^{-1}$ DNase I (Sigma or Boehringer Mannheim) and 1 μg $\mu l^{-1}$ RNase A (Sigma) were added and samples were incubated at room temperature for 10 min. Proteinase K (Sigma) was added (0.50 μg $\mu l^{-1}$), samples were incubated at 55°C for 2 h and the resultant lysate was stored at 4°C. In some cases, the lysate was further treated by the addition of 900 μl cold acetone, incubated on ice for 1 h followed by centrifugation. The supernatant was discarded and the pellet containing LPS was resuspended in 50–100 μl of sample loading buffer. LPS was separated by 18% SDS–PAGE (Laemmli, 1970; Ausubel *et al*., 1995) and stained by the silver staining technique of Tsai and Frasch (1982).

## *Cloning of the* B. avium wlb *locus*

Oligonucleotides that anneal adjacent to the PAS78 and PAS85 insertion mutations were used to generate 200 bp digoxygenin-labelled PCR products within the coding regions of *wlbL* and *wlbA* respectively. DNA of the parental strain, 197N, was digested with *Not*I and resultant fragments were separated by agarose gel electrophoresis. After transfer to a nylon membrane, a 15 kb fragment showed homology to both the *wlbL* and *wlbA* dig-PCR probes (data not shown). *Not*I digested DNA fragments, from strain 197N, ranging in size from 12 to 19 kb were gel purified, ligated into pGEM11Z(f) digested with *Not*I, and introduced into competent *E. coli* DH5α by transformation. One hundred transformants were patched onto L-agar containing ampicillin and grown overnight at 37°C. Colony lifts were performed as previously described (Ausubel *et al*., 1995). Hybridization to the *wlbL* probe revealed one positive clone. Further analysis, by the PCR and Southern blot hybridization, revealed the presence of both *wlbA* and *wlbL* genes. This clone contained a 15 kb insert and was designated pGEM-av.

The *B. avium* 15 kb DNA fragment from pGEM-av was subsequently subcloned into the broad host range vector pLAFR5 as follows. The plasmid (pGEM-av) was digested with *Not*I and the 5′ overhanging ends were filled-in using Klenow polymerase to create blunt ends. *Bcl*I linkers were ligated onto the blunt ends followed by complete digestion with *Bcl*I. The resultant 15 kb fragment was gel purified after agarose gel electrophoresis and ligated into pLAFR5 digested with *Bam*HI. The ligation mixture was introduced into competent *E. coli* DH5α by transformation. This clone contained a 15 kb insert and is designated as pLAF-av.

## Complementation analysis

The plasmid pLAF-av contains the *wlb* locus of *B. avium* 197N and was constructed as described above. The plasmids pLAF-pe, pLAF-br, pLAF-pa and pLAFR5 contain the entire 12 kb *wlb* locus of *B. pertussis* (pLAF-pe), *B. bronchiseptica* (pLAF-br) or *B. parapertussis* (pLAF-pa) in the broad host range vector pLAFR5. These plasmids have been previously described (Allen *et al*., 1998; Allen and Maskell, 1996; Keen *et al*., 1988) and were kindly provided by A. Preston, Centre for Veterinary Science, University of Cambridge, UK. The

pLAF-av, pLAF-pe, pLAF-br, pLAF-pa and pLAFR5 broad host range plasmids were maintained in *E. coli* XL1-blue or DH5α and were introduced into *E. coli* S17.1(γ*pir*) by transformation prior to conjugation with *B. avium*. All *E. coli* strains containing plasmids were grown in L broth or agar containing tetracycline at 37°C. All *B. avium* strains containing the complementing plasmids were grown in BHI broth or on BHI agar containing tetracycline at 37°C.

### Ba1 sensitivity

Sensitivity to *B. avium* bacteriophage Ba1 (Shelton *et al*. submitted) was determined by cross-streaking (Silhavy *et al*., 1984). A clear plaque mutant Ba1 bacteriophage (Ba1*c*1; Shelton *et al*. submitted) was spread down the middle of a BHI-agar plate containing tetracycline and nalidixic acid. *B. avium* colonies were then picked from an agar plate with a sterile tooth pick and streaked perpendicularly across the bacteriophage streak. Plates were incubated overnight at 37°C.

### Serum resistance assay

Bacteria were grown in BHI broth with the appropriate antibiotic at 37°C overnight with shaking. The overnight culture was diluted 1:100 and grown to mid-logarithmic phase. Cell density was determined by measuring the optical density at 600 nm. Cells were washed two times in PBS and diluted to approximately $10^7$ cells $ml^{-1}$ in PBS prior to use. Each reaction contained 16 μl of diluted cells, 64 μl of PBS and 80 μl of naïve turkey poult serum. A 10 μl aliquot was removed immediately to determine the initial viable bacterial cell count. The mixture was then incubated at 37°C for 1 h. Prior to plating, the cells were collected by centrifugation and resuspended in 150 μl of PBS. Bacterial cell counts were determined by plating 10-fold serial dilutions onto BHI agar plates. Each strain was tested in triplicate bactericidal assays. At the same time, each strain was also incubated with heat inactivated serum as a control. The degree of serum resistance was quantified by determining the percent of the initial population that survived the 1 h serum treatment. In all cases, heat inactivation negated any bactericidal effect (data not shown).

### Tracheal adherence assay

Adherence assays were performed essentially as previously described (Temple *et al*., 1998) Briefly, *B. avium* to be tested for attachment were adjusted to a constant $OD_{600}$ and serial dilutions were plated to determine the initial bacterial cell count. Approximately $1 \times 10^7$ cfu were added to tissue culture wells containing three tracheal rings obtained from 26-day-old turkey embryos, and incubated at 42°C for 3 h with constant rocking. After incubation, the rings were washed and each ring was placed into a separate tube containing 1.0 ml of PBS with 1% Triton X-100, incubated at 4°C for 1–2 h, then vortexed for 1 min to distribute the bacteria. Bacterial cell counts were determined by plating 10-fold serial dilutions onto BHI agar plates. The numbers of cfu/tracheal ring were used to calculate the percent bacteria bound.

### Statistical analysis

The significance of mean differences were determined using Student's *t*-test with the aid of the Microsoft Excel statistical analysis software (version 4). In some cases, significant differences were determined by the *Z*-test, which assumes equal variances (Microsoft Excel version 4). In the cases described herein, the *Z*-test was employed when we asked the probability of a single mutant $ID_{50}$ value falling within two standard deviations of the mean $ID_{50}$ calculated using the parental strain. Standard deviation of the mean was calculated using Microsoft Excel STDEV function. Standard error was calculated as the standard deviation divided by the square root of the number of experiments.

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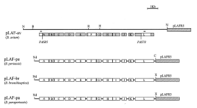

**Fig. 1.**

Diagrams of plasmids containing the *wlb* genetic loci of *B. avium* (pLAF-av), *B. pertussis* (pLAF-pe), *B. bronchiseptica* (pLAF-br) and *B. parapertussis* (pLAF-pa). Shaded boxes in the *B. avium* locus indicate proposed genetic structure based upon similarity to other members of the Bordetellae shown. White boxes in the *B. avium* locus indicate sequenced areas (*wlbA*, Accession No. AF248033; *wlbF*, Accession No. AF248035and No. AF248036; *wlbG*, Accession No. AF248036; *wlbL*, Accession No. AF248034). Gene organization of the heterologous *wlb* loci have been redrawn from published work for comparison (Allen *et al*., 1998). Insertion sites of *B. avium* mutants are indicated. Lines above *wlbA* and *wlbL* genes indicate the location of digoxigenin-labelled PCR probes. Restriction sites are B, *Bam*HI; C, *Cla*I; R, *Eco*RI; H, *Hind*III; Nd, *Nde*I; N, *Not*I; X, *Xba*I.

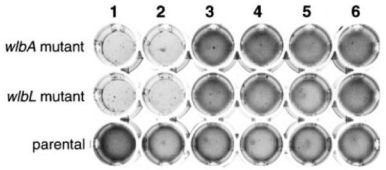


**Fig. 2.**

Clumped-growth phenotype of *B. avium wlb* mutants. Cultures were grown in a 48 well tissue culture tray at 37°C overnight with rotary shaking. Indicated at left are the genotypes. Lane 1 contains the strains without any recombinant plasmids. Lanes 2–6 contain the plasmids pLAFR5, pLAF-av, pLAF-pe, pLAF-br or pLAF-pa respectively.

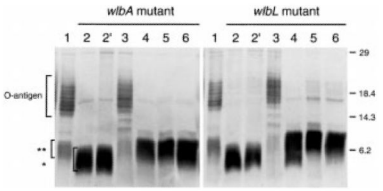


**Fig. 3.**

Silver-stained polyacrylamide gel analysis of *B. avium* LPS mutants and complemented mutants (*wlbA*, first panel; *wlbL*, second panel). First panel: lane 1, parental *B avium* strain 197N; lane 2 and 2′, *wlbA* mutant strain (PAS85) with no addition or addition of the pLAFR5 vector respectively; lanes 3–6, strain PAS85 complimented with pLAF-av, pLAF-pe, pLAF-br, or pLAF-pa respectively. Second panel: lane 1, parental *B. avium* strain 197N; lane 2 and 2′, *wlbL* mutant strain (PAS78) with no addition or addition of the pLAFR5 vector respectively; lanes 3–6, strain PAS78 complimented with pLAF-av, pLAF-pe, pLAF-br, or pLAF-pa, respectively. Molecular weight markers are indicated in kdaltons. Lower (*) and higher (**) migrating lipid-A-associated bands are indicated by brackets. O-antigen bands are indicated by the top bracketed area.

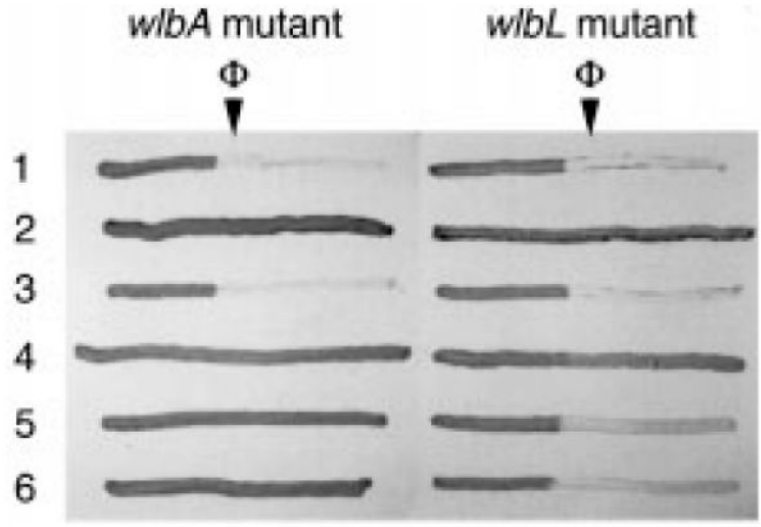


**Fig. 4.**

Cross-streak analysis of *wlbA* (first panel) and *wlbL* (second panel) mutants and complemented mutants with a clear plaque-forming derivative of bacteriophage Ba1, Ba1*c*1. Streak 1, parental *B. avium* strain 197N with the vector plasmid pLAFR5. Streaks 2–6, *wlb* mutant (PAS85 or PAS78) complemented with pLAFR5, pLAF-av, pLAF-pe, pLAF-br or pLAF-pa respectively. The arrow denotes where phage lysates were vertically streaked prior to making the cross-streaks.

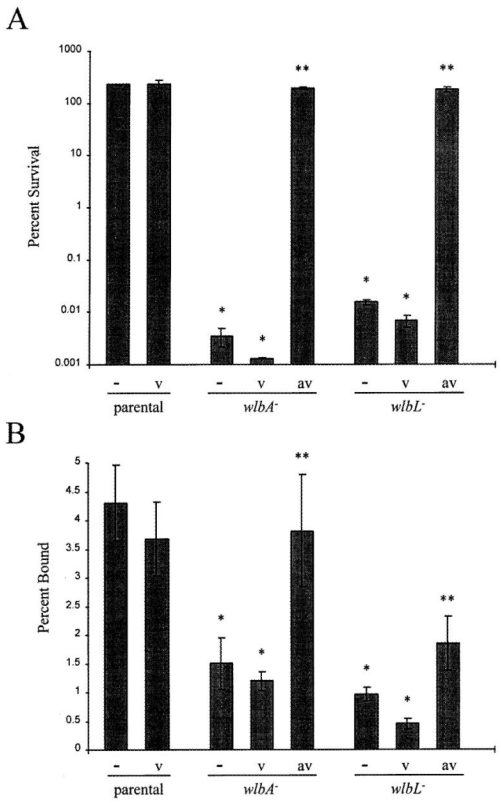


**Fig. 5.**

Measurements of serum resistance (A) and tracheal ring binding (B) by parent, mutants and complemented strains. Strains are indicated below the solid lines as follows: parental, *B. avium* 197N; *wlbA*$^{-}$, *wlbA* mutant PAS85; *wlbL*$^{-}$, *wlbL* mutant PAS78. Abbreviations along the *x*-axis refer to the following: −, indicates strain containing no plasmid; v, indicates strain containing vector plasmid pLAFR5; av, indicates strain containing recombinant plasmid pLAF-av. Single asterisk indicates significant difference ($P < 0.05$) from parent *B. avium* 197N. Double asterisk indicates significant difference ($P < 0.05$) from mutant containing pLAFR5 as determined by Student's *t*-test. Error bars indicate standard error. Refer to text for a description of the assays.

**Table 1**

Bacteria, bacteriophage and plasmids.

| Bacteria/bacteriophage/ plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| Bacteria | | |
| 197N | parental *B. avium* strain; $Nal^R$ | Temple *et al.* (1998) |
| PAS78 | *wlbL* insertion mutant of *B. avium* 197N; *wlbL*::Tn5; $Kan^R$, $Nal^R$ | This study |
| PAS85 | *wlbA* insertion mutant of *B. avium* 197N; *wlbA*::Tn5; $Kan^R$, $Nal^R$ | This study |
| *E. coli* S17.1(γ*pir*) | $Sm^R$ | Simon *et al.* (1983) |
| *E. coli* DH5α | | Life Technologies |
| Bacteriophage | | |
| Ba1*c*1 | clear plaque derivative of *B. avium* phage Ba1 | Shelton *et al.* (submitted) |
| Plasmids | | |
| pGEM-11z(f) | *E. coli* cloning vector; $Ap^R$ | Promega |
| pGEM-av | *Not*I 15 kb *wlb* locus of *B. avium* in pGEM-11zf; $Ap^R$ | This study |
| pLAFR5 | broad host range vector; $Tc^R$ | Keen *et al.* (1988) |
| pLAF-av | *Not*I 15 kb *wlb* locus of *B. avium* from pGEM-av cloned into pLAFR5; $Tc^R$ | This study |
| pLAF-pe | *wlb* locus of *B. pertussis* in pLAFR5; $Tc^R$ | Allen *et al.* (1998) |
| pLAF-br | *wlb* locus of *B. bronchiseptica* in pLAFR5; $Tc^R$ | Allen *et al.* (1998) |
| pLAF-pa | *wlb* locus of *B. parapertussis* in pLAFR5; $Tc^R$ | Allen *et al.* (1998) |

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**Table 2**

Infectious dose measurements (50%) of LPS mutants in turkey poults.

| Strain[a] | Plasmid[b] | Relevant properties[c] genotype (phenotype) | $ID_{50}$[d] (× $10^6$) | Significantly different from parental[g] strain |
|---|---|---|---|---|
| 197N | – | Parental strain ($WlbA^+$, $WlbL^+$) | 7 ± 8.7 | NA |
| PAS85 | – | *wlbA*::Tn*5* ($WlbA^-$) | 81 ± 73 | Yes |
| | pLAFR5 | *wlbA*::Tn*5* ($WlbA^-$) | 110 | Yes |
| | pLAF-av | *wlbA*::Tn*5*/*wlbA-L* ($WlbA^+$) | 9 | No |
| PAS78 | – | *wlbL*::Tn*5* ($WlbL^-$) | 1900 ± 1100[e] | Yes |
| | pLAFR5 | *wlbL*::Tn*5* ($WlbL^-$) | > 500[f] | Yes |
| | pLAF-av | *wlbL*::Tn*5*/*wlbA-L* ($WlbL^+$) | 2 | No |

[a] Strain numbers refer to specific insertion mutant isolates.

[b] Plasmid pLAFR5 refers to the vector; plasmid pLAF-av refers to the pLAFR5 vector containing the *wlb* locus of *B. avium*.

[c] The *wlb* genetic mnemonics refer to LPS biosynthesis genes interrupted by an insertion.

[d] Fifty percent infectious dose ($ID_{50}$) measurements were performed as previously described (in the text). Averages or individual measurements are indicated. When two or more experiments were performed, the standard deviation of the mean is indicated (+/−).

[e] These $ID_{50}$ values were extrapolated from colonization rates obtained from birds infected at dose of ~$10^7$ and ~$10^8$ cfu.

[f] The inequality refers to the fact that no birds were infected at any dose given. The numeric value shown is lowest possible $ID_{50}$ achievable (i.e. it represents the $ID_{50}$ value generated if all animals were infected at a dose one order of magnitude higher than the highest dose employed).

[g] $ID_{50}$ measurements of both mutant strains (PAS85 and PAS78) were found to be significantly different ($P < 0.05$) from the parental strain using the Student's *t*-test. Mutants containing either the vector or complementing plasmid were found to be significantly different ($P < 0.05$) from the parental strain using the *Z*-test analysis. NA; not applicable.