Identification and Cloning of \textit{waaF} (\textit{rfaF}) from \textit{Bordetella pertussis} and Use To Generate Mutants of \textit{Bordetella} spp. with Deep Rough Lipopolysaccharide

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A DNA locus from \textit{Bordetella pertussis} capable of reconstituting lipopolysaccharide (LPS) O-antigen biosynthesis in \textit{Salmonella typhimurium} SL3789 (\textit{rfaF511}) has been isolated, by using selection with the antibiotic novobiocin. DNA within the locus encodes a protein with amino acid sequence similarity to heptosyltransferase II, encoded by \textit{waaF} (previously \textit{rfaF}) in other gram-negative bacteria. Mutation of this gene in \textit{B. pertussis}, \textit{Bordetella parapertussis}, and \textit{Bordetella bronchiseptica} by allelic exchange generated bacteria with deep rough LPS phenotypes consistent with the proposed function of the gene as an inner core heptosyltransferase. These are the first LPS mutants generated in \textit{B. parapertussis} and \textit{B. bronchiseptica} and the first deep rough mutants of any of the bordetellae.

\textit{Bordetella pertussis} is a gram-negative pathogen causing whooping cough in children and increasingly being implicated in respiratory infections in adults (9, 21, 24, 27). \textit{Bordetella parapertussis} is also recognized as a cause of whooping cough in children (14, 47) and also infects ovine species (10, 29–31, 46). \textit{Bordetella bronchiseptica} has only rarely been associated with human disease (13, 34, 42) and is more commonly known as a pathogen of a range of species, including rabbits, pigs, dogs, and cats, among others (1, 5, 17, 23, 35, 43, 44, 49). In the search for improved modern vaccines directed against \textit{parapertussis} is also recognized as a cause of whooping cough in children and increasingly being implicated in respiratory infections in adults (9, 21, 24, 27).

The genetics and molecular biology of LPS biosynthesis in the bordetellae have only recently been studied. The \textit{wlb} locus (previously called \textit{bpl}) (2, 33) required for the biosynthesis of band A LPS in \textit{B. pertussis} has been cloned and sequenced, and mutations have been introduced into genes within the locus with consequent loss of band A structures (2). These mutations affect only a distal structure on the LPS and leave the rest of the molecule intact. To study the role of LPS in pathogenicity and immunity, bordetellae with LPS molecules with the deepest possible rough phenotype would be desirable. The deepest rough LPS mutants of \textit{Salmonella} and \textit{Escherichia coli} result from lesions in the \textit{waaC} (\textit{rfaC}) gene (22), which encodes the glycosyltransferase responsible for the addition of the first heptose residue to Kdo (38). \textit{B. pertussis} \textit{waaC} has been identified, but attempts to mutate this gene have been unsuccessful, probably because \textit{waaC} is immediately upstream of the \textit{waaA} (previously \textit{kldA}) gene (2), which is essential for cell viability. The gene responsible for the next step in enterobacterial LPS biosynthesis is \textit{waaF} (\textit{rfaF}) (39). Consequently, we report here the identification, cloning, and sequencing of a DNA locus containing a candidate for \textit{B. pertussis} \textit{waaF} and report the construction of deep rough mutants of \textit{B. pertussis}, \textit{B. parapertussis}, and \textit{B. bronchiseptica}. These are the first mutants in these bacteria that result in a deep rough phenotype, and they are the first mutants of any kind constructed that affect LPS in \textit{B. parapertussis} and \textit{B. bronchiseptica}.

\section*{MATERIALS AND METHODS}

\textbf{Bacterial strains and plasmids.} The bordetellae used in this study were \textit{B. pertussis} BP536, \textit{B. parapertussis} CN 2591, and \textit{B. bronchiseptica} CN 7635E from our culture collection. For cloning experiments and maintenance of plasmids, \textit{E. coli} XL1-Blue (Stratagene) was used. \textit{E. coli} HU835 was used to package cosmids in vivo. SM10pir was used as the donor strain in conjugation experiments. \textit{Salmonella typhimurium} SL3789 has a mutation in the \textit{waaF} gene (\textit{rfaF511}) and was a kind gift from Brian Robertson, St. Mary's Hospital at Imperial College, London, United Kingdom. \textit{Salmonella typhimurium} AS68 was a λ− m− strain carrying the \textit{E. coli} LamB protein, enabling it to be infected by λ phage particles.
All cloning and DNA sequencing experiments used the pT7-Blue or pBlue-script II series of plasmids. The vector used in conjugation experiments for the generation of mutants in \textit{B. pertussis} was pSS2141 (41), which has an s12 allele (rplS) conferring dominant streptomycin sensitivity on streptomycin-resistant bacteria, allowing selection against maintenance of vector sequences via single-crossover events. pSS2141 is a ColEl1 replicon and thus cannot replicate in \textit{B. pertussis}. It contains an oriT mobilizable by \textit{E. coli} SM10pir.

**Media, chemicals, and reagents.** \textit{B. pertussis} was routinely cultured on Bordet-Gengou medium supplemented with 15% horse blood. \textit{E. coli} was cultured on Luria broth or agar (36). Media were purchased from Difco Ltd. or Oxoid Ltd. Antibiotics were used where appropriate. For the bordetellae, gentamicin at 10 \(\mu\)g/ml, ampicillin at 100 \(\mu\)g/ml, and streptomycin at 200 \(\mu\)g/ml were used. For \textit{E. coli} and \textit{S. typhimurium}, ampicillin was used at 100 \(\mu\)g/ml. SL3789 with its \textit{waaF} lesion complemented by the BP536 \textit{waaF} gene was selected on novobiocin at 2.5 \(\mu\)g/ml. All antibiotics and routine chemicals were purchased from Sigma Chemical Company. Restriction and modifying enzymes were purchased from Boehringer Mannheim. DNA ligase was purchased from Gibco-BRL. Sequenase sequencing kits were purchased from Amersham International.

**Cloning of \textit{LPS} genes.** A cosmid library was constructed in the vector pHC79 (16) from BP536 chromosomal DNA partially digested with Sau3AI. Size selection of 35- to 45-kb DNA fragments was performed with a 0.8% low-melting-point agarose gel in pulsed-field gel electrophoresis. This DNA was purified from the gel with agarase, and then ligated with pHC79, and packaged with Gigapack Gold III packaging mixes (Stratagene). These packaged cosmids were transfected into \textit{E. coli} strain SM10pir, and 1,000 resultant colonies were maintained as a representative library. The packaged library was also amplified with the in vivo packaging strain \textit{E. coli} HU3835. Before using purified cosmids to infect the \textit{S. typhimurium} SL3789 \textit{waaF} selection strain, the cosmids were used to infect \textit{S. typhimurium} AS68 and SL3770 (positive) and SL3789 alone (negative) were selected on various concentrations of novobiocin with or without ampicillin. SL3789 with its \textit{waaF} lesion complemented by the BP536 \textit{waaF} gene; lane 2, SL3789 alone; lane 3, \textit{S. typhimurium} wild type (SL3770). The \textit{waaF} mutant displays the deep rough LPS phenotype expected, while the wild-type control shows the ladder pattern expected for a full-length smooth LPS. The complemented mutant also has the O-antigen ladder, but a rough core molecule is also observed in the LPS preparation, suggesting that the complementation is not completely effective.

**RESULTS AND DISCUSSION**

**Identification and cloning of \textit{waaF}.** A cosmid library of \textit{B. pertussis} BP536 DNA, constructed in the vector pHC79, was analyzed on and mapped as described in Materials and Methods. This cosmid DNA was isolated and used to electroporate \textit{S. typhimurium} SL3789 (waf511), which has a deep rough LPS phenotype. Complementation of this genetic lesion would enable the bacteria to synthesize complete, smooth LPS. To select for complementation by recombiant cosmid, bacteria were plated on media containing novobiocin, since this antibiotic selectively kills rough bacteria at much lower concentrations than are needed to kill smooth bacteria (4). Transformants and controls consisting of wild-type \textit{S. typhimurium} SL3770 (positive) and SL3789 alone (negative) were selected on various concentrations of novobiocin with or without ampicillin. SL3789, being smooth, was capable of growth on novobiocin at 2.5 \(\mu\)g/ml, whereas SL3789 was sensitive to this concentration as a consequence of having rough LPS. Electroporation of SL3789 with the cosmid library produced four colonies resistant to both ampicillin and novobiocin at 2.5 \(\mu\)g/ml. LPS was purified from one of these and analyzed by silver-stained SDS-PAGE, confirming the restoration of the O-antigen phenotype (Fig. 1). The complemented bacteria were also agglutinable with anti-O4,5 antiserum. These data...

![Fig 1](https://example.com/f1.png)

**Nucleotide sequence accession number.** The DNA sequence described here is deposited with the EMBL database under accession no. Y13475.
indicate the presence of a functional waaF homolog within the locus. The fact that the deep rough LPS molecule from the S. typhimurium waaF mutant is efficiently restored to the wild-type phenotype by the B. pertussis waaF homolog shows that the bordetella protein can recognize the S. typhimurium waaF mutant LPS as a substrate. This might not be immediately expected, as the inner core structures of Salmonella and Bordetella are different in a number of respects (6, 7, 18, 19, 22). For example, two Kdo residues are present between lipid A and the first heptose in the S. typhimurium core, whereas in the equivalent region of the B. pertussis LPS molecule, only one Kdo residue is observed. This difference does not seem to interfere with the correct functioning of the bordetella enzyme.

The cosmid DNA isolated from these four transformants was digested with NarI, revealing several common fragments between the cosmids. One cosmid was partially digested with NarI, then self-ligated, and electroporated into SL3789 with selection again on novobiocin and ampicillin. Plasmids from resultant colonies, when digested with NarI, revealed a mini-

FIG. 2. BOXSHADE of a PILEUP performed in the GCG DNA analysis package with MsbA protein sequences from E. coli (Ecoli) and H. influenzae (Hin) and the proposed homolog from B. pertussis (BP536). The black shading surrounds blocks of amino acids which are identical, and the grey shading surrounds blocks with conservative substitutions. The B. pertussis sequence is shown as starting with a leucine residue since it has TTG as a start codon. This sequence is truncated at the position of the SacI site where the DNA sequence published here starts. Only the parts of the E. coli and H. influenzae sequences corresponding to the truncated B. pertussis sequence are shown.
of these waaF loci is ongoing and may allow the differences between \textit{B. parapertussis} core and the other \textit{Bordetella} LPS molecules to be addressed at the molecular genetic level.

\textbf{Construction of waaF mutants in the bordetellae.} To confirm that waaF was required for inner core LPS biosynthesis in the bordetellae, allelic exchange mutants were generated. A single-crossover strategy was chosen to ensure the successful mutagenesis of \textit{waaF} in all three strains. The same 439-bp PCR product was used to mutagenize the three bordetellae (see Materials and Methods). Following mutagenesis, the LPS phe-

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig3}
\caption{BOXSHADE of a PILEUP performed in the GCG DNA analysis package with WaaF protein sequences from \textit{N. gonorrhoeae} (Ngon), \textit{N. meningitidis} (Nmen), \textit{E. coli} (Ecoli), \textit{S. typhimurium} (Salty), \textit{H. influenzae} (Hin), and \textit{P. aeruginosa} (Psaer) and the proposed \textit{B. pertussis} (BP536) WaaF protein. See Fig. 2 legend for an explanation of the shading.}
\end{figure}
notypes of resultant colonies were analyzed by silver-stained SDS-PAGE. Each of the waaF mutants had single LPS bands that migrated equally with each other and much faster than band B seen in the controls (Fig. 4). This is consistent with the LPS molecule having a deep rough phenotype. In addition, band A was absent from the B. pertussis mutant allele exchange mutants from B. pertussis mutant. In addition, that migrated equally with each other and much faster than mutant; lane 3, B. bronchiseptica. Lane 1, B. parapertussis from the waaF linked to the first heptose, with this second heptose being band A trisaccharide, and the O antigen are built. The second glucose residue upon which the rest of the core main chain, the Bordetella mutants may not have been entirely predictable. The deeply truncated LPS phenotype observed in the three B. pertussis differences in structure of the observed in the rest of the core intact and potentially allowing the addition of B. pertussis mutated this gene in sequenced the waaF currently being investigated. of the rest of the LPS core molecule. These possibilities are complex, may have polar effects on genes downstream of waaF structures constructed so far in the bordetellae. FIG. 4. Silver-stained Tris-tricine polyacrylamide gel of wild type and waaF mutants of B. pertussis, B. bronchiseptica, and B. parapertussis. Lane 1, B. pertussis (BP536) wild type; lane 2, B. pertussis waaF mutant; lane 3, B. bronchiseptica (CN 7635E) wild type; lane 4, B. bronchiseptica waaF mutant; lane 5, B. parapertussis (CN 2591) wild type; lane 6, B. parapertussis waaF mutant.

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