The *Moraxella bovis* RTX toxin locus *mbx* defines a pathogenicity island

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To characterize flanking regions of the *mbx* operon in *Moraxella bovis*, DNA surrounding *mbxCABDtolC* was sequenced in haemolytic and nonhaemolytic strains of *M. bovis*. In two haemolytic strains of *M. bovis*, the *mbx* operon, including the adjacent *M. bovis tolC* orthologue, was flanked by approximately 700 bp imperfect repeats. Nonhaemolytic strains of *M. bovis* had only one or no such repeats, as well as ORFs identical to those flanking the repeats from haemolytic *M. bovis*. Two nonhaemolytic strains also contained ORFs with deduced amino acid sequence similarity to bacterial *araJ* genes. The G + C content of the *mbxCABDtolC* gene region was lower than the flanking regions. The genetic organization and G + C content of *mbxCABDtolC* genes, and flanking repeats in haemolytic *M. bovis*, as well as the presence or absence of flanking repeats in nonhaemolytic *M. bovis*, suggests that this RTX operon is located on a mobile genetic element, and supports the designation of this region as a pathogenicity island, which is believed to be the first such element demonstrated in *M. bovis*.

Received 8 October 2005

Accepted 6 December 2005

INTRODUCTION

Moraxella bovis, a Gram-negative bacterium of the family *Moraxellaceae*, has been recognized as the aetiological agent of infectious bovine keratoconjunctivitis ('pinkeye'; Henson & Grumbles, 1960), the most common ocular disease of cattle. Pathogenicity of *M. bovis* is associated with expression of pilin, which is essential for adhesion of bacteria to the corneal epithelium (Annuar & Wilcox, 1985; Lehr *et al.*, 1985; Moore & Rutter, 1989), and a pore-forming cytotoxin (Clinkenbeard & Thiessen, 1991) that promotes the development of corneal ulcers by lysis of corneal epithelial cells (Beard & Moore, 1994) and host neutrophils, with subsequent leakage of neutrophil-derived degradative enzymes into the corneal stroma (Kagonyera *et al.*, 1988, 1989; Rogers *et al.*, 1987). Amino acid sequencing of a partially purified cytotoxin from *M. bovis* culture supernatants led to the identification of a gene encoding an ~100 kDa protein with amino acid sequence similarity to RTX (repeats in the structural toxin) toxins from human and animal pathogens (Angelos *et al.*, 2001). These related toxins included: *Escherichia coli* HlyA (Felmlee *et al.*, 1985), *Mannheimia haemolytica* LktA (Lo *et al.*, 1987), *Actinobacillus pleuropneumoniae* ApxIIA (Chang *et al.*, 1989) and *Actinobacillus actinomycetemcomitans* LtxA (Lally *et al.*, 1989). We subsequently identified and sequenced genes encoding *M. bovis* RTX C, B

and D proteins that together formed a classic RTX operon, designated the *mbx* operon, in the genome of haemolytic *M. bovis* (Angelos *et al.*, 2003).

The *hly* operon of uropathogenic *E. coli* is currently the most well characterized RTX operon. Typical RTX operons contain four genes arranged 5'-CABD-3'. The structural cytotoxin molecule is encoded by an *rtxA* gene. Activation of RTX A occurs by acylation of lysine residues mediated by the product of the *rtxC* gene (Stanley *et al.*, 1994). Secretion of an activated RTX A toxin is facilitated by the combined actions of the RTX B and D proteins (Koronakis *et al.*, 1992; Schlor *et al.*, 1997; Schulein *et al.*, 1994), as well as the secretion accessory protein, TolC (Wandersman & Delepelaire, 1990). Previously we demonstrated that *M. bovis* was similar to *Bordetella pertussis* (Glaser *et al.*, 1988) in having a close genetic linkage between *rtx* genes and *tolC* orthologues (Angelos *et al.*, 2003); in both organisms, *tolC* is located immediately downstream of *rtx CABD* genes.

Southern blotting of multiple nonhaemolytic *M. bovis* strains revealed an absence of DNA that hybridized to individual *mbxC*, A, B or D gene probes (Angelos *et al.*, 2003). In addition, the characterization of DNA upstream of the *mbx* operon from haemolytic *M. bovis* strain Tifton I revealed an ORF with similarity to bacterial transposases. To further characterize the region flanking the *mbx* operon, and to identify possible mechanisms by which *M. bovis* could become nonhaemolytic, we sequenced DNA flanking this region in haemolytic and nonhaemolytic *M. bovis*.

Abbreviation: PAI, pathogenicity island.

The GenBank/EMBL/DDJB accession number for the complete RTX operon and flanking sequences comprising PAI_{Tifton I} is AF205359.

METHODS

Bacterial strains and culture conditions. Haemolytic *M. bovis* Tifton I and T+, as well as nonhaemolytic *M. bovis* strains T-, SFS9b and SFS100b, were generously provided by Lisle George, University of California, Davis. Nonhaemolytic strain IBH63 was a field strain originally isolated by Pugh *et al.* (1966); nonhaemolytic strain NDL68 (991R) was obtained from the National Animal Disease Laboratory, Ames, Iowa, USA. Cultures were grown in Luria-Bertani (LB) broth containing 1.5 mM calcium chloride or in tryptic soy broth. Genomic DNA was prepared from each strain (Qiagen DNeasy kit). *E. coli* DH5 α (Invitrogen) was the host for recombinant plasmids containing cloned regions of *M. bovis* genomic DNA and was grown on either LB agar or LB broth containing ampicillin (100 μ g ml⁻¹).

Determination of *mbx* operon flanking sequences. Genomic DNA flanking the *M. bovis* *mbx* operon was isolated using a PCR-based approach. Genomic DNA was restriction endonuclease digested with *Hind*III or *Hpa*II and purified (Qiagen PCR purification kit). Purified, cleaved DNA (100 ng) was ligated to pBluescript II KS (-) (Stratagene) and was digested with *Hind*III (for ligation to *Hind*III digested *M. bovis* DNA) or *Cla*I and *Eco*RI (for ligation to *Hpa*II digested *M. bovis* DNA). Ligation was performed at room temperature, overnight, using either T4 DNA ligase (Invitrogen) or LigaFast (Promega). Ligated DNA was amplified by PCR using a primer specific for the pBluescript II KS (-) vector (T3 or T7; Table 1) and a primer specific for *M. bovis* genomic sequences. The primer specific for *M. bovis* sequences was oriented to point away from the *mbx* operon. DNA sequences upstream from *mbxCABDtolC* were amplified using primers T3 and Nested. DNA sequences downstream of *mbxCABDtolC* were amplified using primers T7 and TolC dn. Successful amplification between known sequences and the pBluescript II KS (-) 'tag' resulted in the isolation of additional flanking DNA sequences. PCRs for the isolation of flanking DNA were performed using 25 cycles of the following temperatures and times: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 3 min, followed by a final 15 min step at 72 °C.

Amplification of nonhaemolytic *M. bovis* was performed using several oligonucleotide primer pairs that flanked the *mbxCABDtolC* region of haemolytic *M. bovis*. The primer pair spanning the largest region, used for sequence determinations reported here, was Mdh dn and Ap up (see Table 1). Left and right repeats were amplified using oligonucleotide primers Tsp dn and PreC up, and the following

Table 1. Primers used to amplify regions flanking *mbxCABDtolC*

Primer	Sequence
T3	5'-CAAGCGCGCAATTAACCCCTCACTAAAGGG-3'
Nested	5'-CGCTAAAAAATACGTACGGATTG-3'
T7	5'-GAGCGCGCGTAATACGACTCACTATAGGG-3'
TolC dn	5'-GAGTTTCTGGAGATATTAATTACCAG-3'
TolC dn++	5'-TATAATTTTTTGATAGCTTATGCAGCC-3'
Mdh dn	5'-ATGGAGCGTGTGACCACTCCAAG-3'
Ap up	5'-GCGAGAATGCTCCATTTTTTGTAGC-3'
Tsp dn	5'-GCTTAATAATAACAACATGACCAGC-3'
PreC up	5'-AAGACTTATCTGTGATGCTCTCA-3'
Mdh up	5'-GGCTAAACTCATCAATCTCTAAGCCTTG-3'
Ap dn	5'-ATGACCGACTTTCACAAAACCCC-3'
MidC up#2	5'-TATATTTAACCTCGTTTTCTAAACT-3'

conditions repeated for 25 cycles: 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Sequencing of the amplified DNA was performed by the University of California, Davis, DNA sequencing laboratory or by Davis Sequencing. Final complete sequences were assembled from multiple overlapping fragments with DNA analysis software (MacDNASIS and Lasergene Seqman; Hitachi Software Engineering and DNASTar, respectively).

RESULTS AND DISCUSSION

Isolation and characterization of regions flanking *mbxCABDtolC* in haemolytic *M. bovis*

Approximately 1.5 kb of sequence was isolated flanking the 5' end of *mbxC* and approximately 2.5 kb of sequence was isolated flanking the 3' end of *tolC*. A map detailing regions 5' and 3' to *mbxCABDtolC* is shown in Fig. 1. The complete DNA sequence of the flanking regions and previously determined *mbxCABDtolC* genes has been deposited with GenBank, accession no. AF205359. The DNA sequence of flanking regions revealed several ORFs along with a pair of approximately 700 bp repeats flanking *mbxC* and *tolC* (left repeat and right repeat, respectively; Fig. 1).

At the extreme 5' end of the DNA sequence isolated, an ORF with an ATG at position 6 through to a TAA stop codon at position 698 is present. Comparison of the peptide sequence predicted by this ORF to the GenBank database revealed similarity to malate dehydrogenases from numerous bacteria. The best match was *Polaromonas* sp. JS666 with 78 % identity (accession ZP_00364926). BLAST results further revealed that the *M. bovis* ORF is not complete, but most likely represents only the carboxy terminal ~20 % of the coding sequence.

Downstream from the partial coding sequence of the putative malate dehydrogenase, the sequence of the left repeat begins at position 946 and extends to position 1687. The DNA sequence of the left repeat contains an insertion of 80 bases (1395–1474) relative to the right repeat. Contained within the sequence of the left repeat is a single ORF extending from the beginning of the repeat to a TAG termination codon at positions 1672–1674. BLAST alignment of the peptide sequence predicted by this ORF with the GenBank database revealed similarity with numerous sequences all identified as putative transposases, including IS1548, a transposase of *Streptococcus agalactiae* (accession no. NP_688610).

At the 3' end of *mbxCABDtolC* is the second direct repeat (right repeat), which extends from position 11 617–12 278, and is 80 nucleotides shorter than the left repeat. An ORF extends from position 11 617 to 12 063; as with the left repeat, the predicted peptide sequence also exhibits similarity to transposases. This is followed by an ORF (position 12 601 to the end of the cloned sequence) tentatively identified as encoding an aminopeptidase, based on deduced amino acid sequence similarity to aminopeptidases of *Psychrobacter* sp. (GenBank accession no. ZP_00262417)

Fig. 1. Schematic diagram depicting the arrangement of DNA elements flanking *mbxCABDtolC* within *M. bovis* Tifton I. The top section shows approximately 13 kb of DNA, with the *mbxCABDtolC* genes flanked by ORFs tentatively identified as malate dehydrogenase (*mdh*) and aminopeptidase (*ap*). The bottom section of the figure represents an enlargement of the ORFs, and flanking left and right repeats, with scale bars (bp) indicated below the respective left and right hand regions; the hatched and checkerboard bars above the scale bars indicate sequences also found in common with nonhaemolytic strains, as depicted in Fig. 2. The white triangle on the left side of the left repeat represents the sequence 5'-AAATCCT-3', the inverse complement of which, 5'-AGGATTT-3', is found on the right side of the right repeat, also indicated by a white triangle. Within the left and right repeats of *M. bovis* Tifton I, at position 27 is a sequence difference, C in the left repeat and T in the right repeat, indicated above them. The black triangle over the left repeat indicates an 80 bp insertion that is not present in the right repeat. With regard to this schematic, *M. bovis* T+ has an identical structure, with the exception of a matching T at position 27 of the left and right repeats.

and *B. pertussis* (GenBank accession NP_881024). Aminopeptidases have been reported to be synthesized by *M. bovis* (Frank & Gerber, 1981).

In addition to the presence of the repeats flanking *mbxCABDtolC*, a perfect 7 bp inverted repeat flanks the left and right repeats. On the 5' side of the left repeat is 5'-AAATCCT-3' and on the 3' side of the right repeat is the sequence 5'-AGGATTT-3'; these are indicated by the white triangles in Figs 1 and 2.

Genomic DNA from *M. bovis* T+, a haemolytic strain isolated from a naturally infected cow (Angelos *et al.*, 2001) was also characterized by PCR. Individual amplification reactions with oligonucleotide primer pairs that were specific for each *mbx* operon gene were used to demonstrate the presence of each *mbx* operon gene and *tolC*. As expected from the haemolytic phenotype of *M. bovis* T+, PCR revealed the presence of *mbxCABD* genes as well as *tolC*. Further characterization by PCR revealed that the RTX operon was placed between the *mdh* and *ap* ORFs, identical to the genomic organization of *M. bovis* Tifton I. At the 5' end of the characterized region, the primers Mdh dn and MidC up#2 were used to produce an approximately 2 kb fragment. DNA sequencing of this fragment revealed near 100% identity with the sequence determined for *M. bovis* Tifton I. The only changes were transitions from cytosine to thymidine at positions 455 and 972 of the Tifton I sequence.

At the 3' end of the *M. bovis* Tifton I characterized region, T+ DNA was amplified using oligonucleotides Ap up and TolC dn++, producing a nearly 3 kb fragment. This

fragment was subject to DNA sequencing with multiple primers and the resulting sequence aligned with nearly 100% identity to the corresponding region of *M. bovis* Tifton I. The only differences were a transition from cytosine to thymidine at position 11 627 and the reverse transition, from thymidine to cytosine at position 11 270. Both of these positions are located within the right repeat; the significance of the changes is unknown.

Characterization of nonhaemolytic *M. bovis*

Previously we reported the absence of hybridization between *mbxCABD* gene-specific probes and genomic DNA from nonhaemolytic *M. bovis*, and concluded that *mbxCABD* genes were deleted from nonhaemolytic *M. bovis* (Angelos *et al.*, 2003). The existence of the repeats flanking *mbxCABDtolC* suggested that these genes might be absent because of mobilization of this region from the genome. To explore this possibility, genomic DNA from nonhaemolytic *M. bovis* was analysed by PCR using oligonucleotide primers within the putative malate dehydrogenase (*mdh*) and aminopeptidase (*ap*) sequences, as described above for *M. bovis* Tifton I. All nonhaemolytic *M. bovis* strains tested were positive for amplification by Mdh dn/Mdh up and Ap dn/Ap up primer pairs. Amplification of nonhaemolytic *M. bovis* T-, IBH63 and NDL68 genomic DNA with the Mdh dn/Ap up primer pair each produced a 2–3 kb amplicon; the amplified product from IBH63 was slightly larger than either T- or NDL68. Amplification of genomic DNA from SFS100b and SFS9b with Mdh dn and Ap up each produced a larger product of ~4 kb.

Fig. 2. The genomic organizations of *M. bovis* strains T+, T-, NDL68, IBH63, SFS9b and SFS100b between putative *mdh* and *ap* genes. (a) The relationship between *M. bovis* T+ and T-. The *mbxCABDtolC* region of T+ is indicated above the sequence in abbreviated form. (b) The organization of nonhaemolytic *M. bovis* strains NDL68, IBH63, SFS9b and SFS100b strains. The white triangles and black arrows delineate features as described for Fig. 1. The hatched bar below each drawing indicates DNA sequence that is collinear with *M. bovis* Tifton I from positions 1 to 945 (as shown in Fig. 1). The checkerboard bar below each drawing indicates DNA sequence that is collinear with *M. bovis* Tifton I (Fig. 1) from positions 12 278 (T- and NDL68), 12 534 (IBH63) and 11 179 (SFS9b, SFS100b) to 13 009. The *araJ*-like ORFs identified in strains IBH63, SFS9b and SFS100b are oriented to the left. The asterisk above the *araJ*-like ORF in IBH63 indicates the approximate location of a single nucleotide deletion that breaks the ORF. The white circle shown flanking the ORF in SFS9b and SFS100b indicates a 30 bp region that has no homology to *M. bovis* Tifton I sequences characterized thus far.

The identity of the amplified DNA was determined by DNA sequencing of PCR products using the original PCR primers as sequencing primers. After the initial DNA sequences were obtained, additional sequencing reactions were performed using sequencing primers internal to the amplified product. The DNA sequences of *M. bovis* T- and NDL68 had a single nucleotide difference, which corresponded to the same nucleotide difference that was found between the left and right repeats (at position 27; Fig. 2). Thus, while non-haemolytic *M. bovis* T- lacks *mbxCABDtolC*, it does contain a repeat corresponding to the right repeat of *M. bovis* Tifton I and T+. DNA sequences flanking the single repeat are a composite of DNA sequences flanking the left and right repeats of haemolytic *M. bovis*, and are identical to malate dehydrogenase (5' side) and aminopeptidase (3' side) (Fig. 2a).

M. bovis NDL68 was identical to T-, except that the single remaining repeat contains a C at position 27. Thus, *M. bovis* NDL68 represents a situation wherein *mbxCABDtolC* were precisely deleted such that one repeat remains that contains sequence characteristics of both the left and right repeats found in haemolytic *M. bovis* Tifton I (Fig. 2b).

The amplified product from Mdh dn and Ap up primers with *M. bovis* strain IBH63 was ~2380 bp. The DNA sequences at the 5' end corresponded exactly to the putative malate dehydrogenase; identity between the amplified IBH63 sequences and Tifton I ended exactly at position 945 of the Tifton I sequence (left repeat starts at position 946). At the 3' end of the amplified sequence, IBH63 exactly matches the putative aminopeptidase of *M. bovis* Tifton I (positions 12 534–12 850); however, the intervening DNA had no sequence similarity to DNA flanking *mbxCABDtolC* in *M. bovis* Tifton I (Fig. 2b). Contained within this region were two ORFs each encoding a peptide of greater than 100 aa. A GenBank BLAST search of these ORFs versus the database revealed amino acid sequence similarity to numerous proteins identified as AraJ, a sugar efflux transporter. The best match was with the AraJ protein of '*Mannheimia succiniciproducens*' (GenBank accession no. YP_088771).

M. bovis strains SFS9b and SFS100b produced the largest amplified product with the Mdh dn and Ap up primers. At the 5' end of the amplified product, DNA sequence analysis revealed the presence of malate dehydrogenase identical sequences continuing from the location of the Mdh dn

primer downstream to Tifton I position 945, the location of the left repeat. At the 3' end of the amplified product, identity was found between SFS9b and SFS100b sequences, and the aminopeptidase sequence, extending upstream to include an entire right repeat and an additional ~450 bp 5' to the repeat. That is, a 1700 bp block of identity was identified between the 3' end of the SFS9b and SFS100b PCR products, and the *M. bovis* Tifton I sequence. Tifton I sequences from position 11 179 and downstream match SFS9b/SFS100b amplified sequences from position 1966 and downstream. The repeat amplified in SFS9b/SFS100b strains was determined to be a hybrid repeat, containing the 80 nt insertion characteristic of the left repeat, and T at position 27, characteristic of the Tifton I right repeat (Fig. 2b). Intervening between the sequences homologous to the left side (*mdh* region) and the right side (*ap* region) is a region of 1.1 kb with no similarity to the *mbxCABDtolC* region of *M. bovis* Tifton I. There was 98 % nucleotide sequence identity between this sequence and the corresponding region in IBH63. Likewise, this region contained an ORF; however, the ORF in SFS9b and SFS100b extended throughout the entire 1.1 kb, representing a 369 aa peptide that also showed homology to bacterial AraJ proteins. This peptide is predicted to represent an almost full-length AraJ (~93 % of '*M. succiniciproducens*' AraJ).

Characterization of regions flanking the RTX operon of haemolytic and nonhaemolytic *M. bovis* has revealed an organization that supports the hypothesis that RTX genes within *M. bovis* are located on a mobile genetic element resembling a pathogenicity island (PAI), which therefore has been designated PAI I_{Tifton I}. Within haemolytic strains Tifton I and T+, we have identified repeated DNA sequences flanking *mbxCABDtolC* that are similar to bacterial transposases and are likely to delineate mobile elements. On each side of the repeated sequences, ORFs were identified that exhibited similarity to other bacterial gene products, and these ORFs were found in similar locations in the nonhaemolytic strains that we studied. Although isolation of these ORFs was not complete, we highly suspect that these coding regions represent bona fide *M. bovis* genes. Our data are consistent with a hypothesis that *M. bovis* Tifton I represents a typical haemolytic strain and that nonhaemolytic strains arise following deletion of *mbxCABDtolC*. Characterization of *M. bovis* T+ by PCR and limited DNA sequencing shows a gene organization identical to *M. bovis* Tifton I. It is possible that *mbxCABDtolC* will be identified at other chromosomal locations in other strains of haemolytic *M. bovis* and this would further support the mobility of this gene cluster.

The data presented support the following series of events by which RTX toxin gene acquisition could occur in *M. bovis*. First, nonhaemolytic *M. bovis* acquired the genes for RTX toxin production and secretion. This acquisition event could have been by insertion of the RTX genes (and associated *tolC*) into the locus we have identified in the Tifton I and T+ strains, or it is possible that RTX sequences were

inserted into a different locus and later moved into the current location. The close association of *mbxCABD* and *tolC* between flanking repeats suggests that these genes were associated before they integrated into the regions flanked by the putative malate dehydrogenase and aminopeptidase coding sequences. Such close association between RTX toxin genes and necessary toxin efflux proteins has been described in *B. pertussis* (Glaser *et al.*, 1988). Nonhaemolytic strains T- and NDL68 could have arisen by a single aberrant excision of the mobile element, yielding a genomic locus with only a single terminal repeat. Nonhaemolytic strains IBH63, SFS9b and SFS100b could have arisen by either a single aberrant deletion process that involved recombination with *araJ* sequences or a *mbxCABDtolC* deletion event followed by the introduction of *araJ* sequences.

The organized arrangement of genes for toxin production and secretion, bounded by direct repeats with ORFs exhibiting amino acid similarity to bacterial transposases, is similar to the genetic organization of a PAI. As described originally in *E. coli*, PAIs exhibit certain common features such as: (i) occupying large genomic DNA regions (> 20 kb), (ii) carrying at least one virulence gene, (iii) insertion within or near a tRNA gene, (iv) containing direct repeats and mobility sequences, (v) having a G+C content different from the host bacteria (Schmidt & Hensel, 2004). An additional requirement of PAIs is that they be present in the pathogenic strain, but absent from non-pathogenic strains of the same species.

The ~11 kb region (positions 946–12 278) we describe is smaller than has normally been associated with PAIs (10–100 kb; Hacker & Kaper, 2000). However, the most well studied PAIs are those from uropathogenic *E. coli*, and such strains typically contain genes for adherence factors, cytotoxicity, iron uptake and serum resistance (Hacker *et al.*, 1999). Because of its small size, the *mbxCABDtolC* region could be termed a pathogenicity islet, used to designate DNA regions that are too small to fit the classical definition of PAI (Schmidt & Hensel, 2004). In either case, island or islet, inasmuch as *M. bovis* MbxA (cytotoxin) is a requirement for pathogenesis, expression of *mbxCABDtolC* is expected to be essential. Indeed, previous investigators utilizing extracts of nonhaemolytic *M. bovis* have been unable to demonstrate disease or lytic effects with such nonhaemolytic strains (Beard & Moore, 1994; Gray *et al.*, 1995; Hoiem-Dalen *et al.*, 1990). Our data indicate that deletions of the *mbxCABDtolC* region do occur and result in nonhaemolytic *M. bovis*. Similar deletions have been shown to occur in pathogenic *E. coli*, resulting in non-pathogenic phenotypes (Hacker *et al.*, 1990).

BLAST searches of the DNA sequence of strain Tifton I within the *mdh/mbxCABDtolC/ap* region did not reveal any similarity to tRNA genes. In addition, searching the DNA sequence with the program TRNAscan (<http://www.genetics.wustl.edu/eddy/tRNAscan-SE/>) did not reveal any tRNA genes and it appears that *mbxCABDtolC* PAI in *M. bovis* is not located near a tRNA gene.

The fourth characteristic cited above for characteristics of PAIs indicates the possession of mobility related sequences. This relates to the transposability of mobile genetic elements, and the ability of these elements to encode enzymes capable of recognizing specific sequences and catalysing breakage and rejoining of DNA strands during the excision of an element from one location and subsequent integration into another site. Flanking the *mbxCABDtolC* region are approximately 700 bp direct repeats; as we have noted, the left repeat is 80 nt longer than the right repeat. Other than this deletion, there is only 1 nt difference between the left and right repeats in Tifton I, and none in T+. BLAST searches of the left and right repeat nucleotide sequence revealed no similarity to other DNA sequences. However, comparison of the deduced amino acid sequences of translated left and right repeats revealed similarity to various documented transposase polypeptides. In addition, a short inverted repeat flanks the left (5'-AAATCCT-3') and right (5'-AGGATTT-3') repeats, and offers additional evidence that *mbxCABDtolC* is located on a mobile genetic element.

The last piece of evidence usually considered necessary to define a PAI is the G+C content of the proposed PAI as compared to non-PAI genomic DNA. The 5' flanking region, from the partial malate dehydrogenase coding region to the beginning of the left repeat, is 49 mol% G+C. The 3' flanking region, from the end of the right repeat to the end of the isolated DNA sequence is 44 mol% G+C. The entire *mbxCABDtolC* PAI from the beginning of the left repeat through to the end of the right repeat is 36 mol% G+C. Thus, the proposed PAI has a G+C content that is different from the flanking DNA, again suggesting this region represents a PAI.

With the exception of overall size and lack of involvement of tRNA genes, the *mbxCABDtolC* region conforms to general characteristics of a PAI. Characterization of the haemolytic *M. bovis* T+ strain and the related *M. bovis* T- strain, isolated in the laboratory, provides compelling evidence that the region is a mobile gene cluster and thus, is a PAI.

The presence of an insertion sequence associated with an RTX operon was previously reported in *A. actinomycetemcomitans* (He *et al.*, 1999). In *A. actinomycetemcomitans*, a novel insertion sequence upstream of its RTX operon (*ltx* operon) is responsible for increased expression of leukotoxin in certain clinical isolates (He *et al.*, 1999). Further analysis of multiple haemolytic *M. bovis* strains will be necessary to determine whether differences in the insertion sequences reported here are associated with differential expression of cytotoxin, and whether the PAI described between malate dehydrogenase and aminopeptidase sequences is found at different loci in other strains of pathogenic *M. bovis*.

ACKNOWLEDGEMENTS

This work was supported by School of Veterinary Medicine Formula Funds, grant numbers AH-125 and 4979-H. During completion of this work, Dr Hess was supported by grant DAMD017-02-1-0664 from the US Army Medical Research and Materiel Command.

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