***Actinobacillus actinomycetemcomitans* harbours type IV secretion system genes on a plasmid and in the chromosome**

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Nine contiguous genes encoding a potential type IV secretion system have been identified in the chromosome of *Actinobacillus actinomycetemcomitans* strain VT747 and on a plasmid (pVT745) in strain VT745. Seven of these genes encode predicted proteins that share significant homology with type IV secretion proteins in *Bordetella pertussis* (*ptl* operon), *Brucella melitensis* biovar *suis* and *Agrobacterium tumefaciens* (*virB* operons), where they are involved in protein secretion, pathogen intracellular survival and multiplication, and DNA transport, respectively. Results of previous studies have demonstrated that pVT745 is a conjugative plasmid and that a secondary plasmid, pMMB67, can be mobilized from strain VT745. Given these results, it was hypothesized that (1) the type IV secretion genes on pVT745 are responsible for these two functions and (2) the type IV VT747 chromosomal genes also play a role in the transport of DNA. Wild-type and mutant strains of VT745 were evaluated for their conjugative abilities. Wild-type mating efficiency was 10^{-6} transconjugants per donor, while the mutant strain yielded no transconjugants. Wild-type VT745 harbouring a co-resident plasmid, pMMB67, mobilized pMMB67 at a frequency of 10^{-6} , while VT747 was unable to mobilize this plasmid. These results support the hypothesis that the plasmid-encoded type IV secretion system on pVT745 is involved in DNA transport. However, the chromosomally encoded secretion system may not play a role in DNA transport in strain VT747. While the precise function of these chromosomal genes in strain VT747 has not been determined, Northern blot analyses demonstrated that these genes are expressed in both *Act. actinomycetemcomitans* strains VT745 and VT747.

Keywords: type IV secretion, bacterial secretion systems, extrachromosomal, chromosomal

INTRODUCTION

Actinobacillus actinomycetemcomitans is a Gram-negative pathogen associated with infective endocarditis (Chen *et al.*, 1991), brain abscesses (Martin *et al.*, 1967), prosthetic heart valve infections (Hamori & Slama, 1989) and certain forms of periodontal disease (Moore *et al.*, 1982; Zambon *et al.*, 1983). A number of proposed virulence factors, many of which are either cell-surface associated or released into the extracellular environ-

ment, have been identified in *Act. actinomycetemcomitans* (Fives-Taylor *et al.*, 1996). However, mechanisms by which *Act. actinomycetemcomitans* transports these substances from the intracellular to the extracellular environment have not been elucidated.

Type IV secretion systems have been identified in a variety of bacteria (Christie & Vogel, 2000). Specifically, a type IV system is responsible for intracellular survival and multiplication of *Bru. melitensis* bv. *suis* (O'Callaghan *et al.*, 1999), secretion of pertussis toxin from *Bordetella pertussis* (Covacci & Rappuoli, 1993; Weiss *et al.*, 1993) and for conjugal transport of Ti plasmid DNA from *Agrobacterium tumefaciens* to a susceptible plant (Shirasu & Kado, 1993). The *Bor.*

The GenBank accession number for the type IV secretion genes of *Act. actinomycetemcomitans* reported in this paper is AF332996.

Table 1. Strains and plasmids used

Strain or plasmid	Description*	Source or reference
<i>Act. actinomycetemcomitans</i> strains		
HK1651	Genome sequencing strain	Dave Dyer, University of Oklahoma
HK1651Rif	Rif ^R	This study
VT745	Harbours pVT745	LeBlanc <i>et al.</i> (1993)
VT747	Harbours type IV chromosomal genes	LeBlanc <i>et al.</i> (1993)
VT705	Does not harbour type IV genes	LeBlanc <i>et al.</i> (1993)
VT748	Does not harbour type IV genes	LeBlanc <i>et al.</i> (1993)
<i>E. coli</i> strains		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB)</i>	Yannish-Perron <i>et al.</i> (1985)
Plasmids		
pVT745	Conjugative plasmid	LeBlanc <i>et al.</i> (1993), Galli <i>et al.</i> (2001)
pUC19	Ap ^R ; high-copy-number cloning vector	Yannish-Perron <i>et al.</i> (1985)
pKN4	4·150 kb VT747 genomic clone	This study
pKN12	4·676 kb VT747 genomic clone	This study
pKN13	1·577 kb cloned PCR product from VT747	This study
pKN14	Sp ^R derivative of pVT745; created by single cross-over into 'non-essential' region	This study
pKN15	Sp ^R derivative of pVT745; created by single cross-over into <i>magB03</i>	This study
pMMB67	Ap ^R , mobilizable plasmid RSF1010 derivative	Fürste <i>et al.</i> (1986)
pUS19	Sp ^R derivative of pUC19	Benson & Haldenwang (1993)

* Rif^R, rifampicin resistance; Ap^R, ampicillin resistance; Sp^R, spectinomycin resistance.

pertussis transport system is comprised of a chromosomally encoded, 9·5 kb, nine-gene [*pertussis* toxin liberation (*ptl*)] operon (Farizo *et al.*, 1996; Weiss *et al.*, 1993). A search for proteins homologous to those predicted by the *ptl* operon revealed significant homology with seven of the 11 proteins predicted by the *virB* operon of *Agr. tumefaciens* (Shirasu & Kado, 1993; Weiss *et al.*, 1993). Genes of both the *ptl* and *virB* operons also share homology with conjugal transfer genes of the IncN plasmid pKM101 (Pohlman *et al.*, 1994) and the IncP plasmid RP4 (Lessl *et al.*, 1992). Homologous genes also have been identified in *Helicobacter pylori* (Tummuru *et al.*, 1995), *Wolbachia* spp. (Masui *et al.*, 2000), *Rickettsia prowazekii* (Andersson *et al.*, 1998) and *Legionella pneumophila* (Vogel *et al.*, 1998; Segal *et al.*, 1999). It is proposed that each of these diverse systems is involved in the secretion of macromolecules from bacteria (Weiss *et al.*, 1993; Christie & Vogel, 2000) and that they may share a common ancestry (Pohlman *et al.*, 1994; Winans *et al.*, 1996).

Several contiguous genes encoding a potential type IV secretion system have also been identified in *Act. actinomycetemcomitans*. However this pathogen appears unique in that the proposed system is found on a plasmid (pVT745) in one strain (VT745) (Galli *et al.*, 2001), while being present on the chromosome of a different strain (VT747). To our knowledge, this is the first report of a single pathogen harbouring this proposed system in both of these locations. While the plasmid-encoded system appears to be involved in

conjugal transfer of pVT745 (Galli *et al.*, 2001), the precise function of the chromosomal genes has not yet been identified. However, it is hypothesized that *Act. actinomycetemcomitans* may have uniquely adapted this macromolecular transport system to serve dual functions: 1) for conjugation of pVT745 in strain VT745 and 2) for macromolecular transport in strains harbouring the chromosomal genes, exemplified by VT747.

METHODS

Bacterial strains, plasmids and growth conditions. Strains and plasmids used in this study are listed in Table 1. *Act. actinomycetemcomitans* strains were grown in media composed of Tryptic Soy Broth supplemented with 0·6% yeast extract (TSBYE broth), and containing bacitracin and vancomycin at 75 and 5 µg ml⁻¹, respectively (TSBYEBV; Slots, 1982). Incubation was at 37 °C in the presence of 10% CO₂. Solid media contained 1·5% Bacto agar. The *Act. actinomycetemcomitans* recipient strain, HK1651Rif, was isolated as a spontaneous rifampicin-resistant mutant of strain HK1651. Transconjugants were selected on solid media containing appropriate antibiotics (spectinomycin, 100 µg ml⁻¹; ampicillin, 25 µg ml⁻¹; rifampicin, 50 µg ml⁻¹).

Frozen competent cultures of *Act. actinomycetemcomitans* were prepared as previously described by Sreenivasan *et al.* (1991). *Act. actinomycetemcomitans* transformants were grown on solid TSBYE plates containing spectinomycin or ampicillin. Frozen competent cultures of *Escherichia coli* strain JM109 were prepared as described in Sambrook *et al.* (1989). *E. coli* JM109 transformants screened for the presence of *Act. actinomycetemcomitans* cloned DNA were grown at

Fig. 1. Schematic representation of regions of homology shared between pVT745 and the VT747 chromosome. (a) Linearized plasmid pVT745. The schematic representation shows plasmid pVT745 linearized at the *Pst*I site located at 9.883 kb on the circular map. Other restriction sites and genes are drawn in the anticlockwise direction, consistent with transcription of the *magB* genes. 'B2' indicates one subcloned segment of pVT745 used in sequencing the plasmid that did not encode genes involved in replication or conjugation ('non-essential' region of the plasmid; Galli *et al.*, 2001). The hatched box delineates the 1.207 kb area of this region that is also present in the VT747 chromosome. The location of PCR primers used to amplify the 1.056 kb internal segment of this region (as described in Methods) are indicated with converging arrows. The 8.34 kb region of pVT745 that may have been deleted from the VT747 chromosome is noted above the line drawing. The *magB03*–11 genes are indicated by the dot-filled boxes. The location of PCR primers used to amplify the 1.0 kb internal region of *magB03* (as described in Methods) are indicated with converging arrows. (b) VT747 chromosome. White boxes represent the VT747 chromosome 5' to areas of homology shared between pVT745 and VT747. The location of PCR primers used to amplify a region of VT747 chromosomal DNA located 5' to pKN4 insert DNA is indicated with converging arrows. The hatched box represents the 1.207 kb area of homology shared between pVT745 and VT747. The seven base pairs at the end of this sequence that overlap with *tag03*, including the start codon of *tag03*, are indicated by the numbers 2307 and 2313. Dot-filled boxes represent the arrangement of the *tag* genes in the genomic clones, pKN4 and pKN12, and PCR product, pKN13, obtained from *Act. actinomycetemcomitans* strain VT747.

37 °C on solid YT medium (Miller, 1972) containing ampicillin or spectinomycin (100 µg ml⁻¹).

DNA techniques. Genomic and plasmid DNA were isolated from *Act. actinomycetemcomitans* cultures by the method of Anderson & McKay (1983) with modifications as described by LeBlanc *et al.* (1993). Caesium chloride/ethidium bromide density-gradient centrifugation (Sambrook *et al.*, 1989; Beckman VTi65 rotor, 65 000 r.p.m., 4 h, 20 °C) was used to further purify plasmids from strain VT745. *E. coli* transformants were screened for the presence of plasmid DNA by the alkaline lysis method of Birnboim & Doly (1979) with modifications as described in Sambrook *et al.* (1989). Plasmid DNA was purified from transformants using the QIAprep Midi kit (Qiagen).

DNA was digested with restriction endonucleases as recommended by the manufacturer (Life Technologies). DNA fragments were purified from agarose gels using the Elu-Quik gel extraction kit (Schleicher and Schuell). ³²P labelling of DNA fragments was accomplished using a random priming kit (Life Technologies). DNA–DNA hybridization conditions have been previously described (Novak & LeBlanc, 1994).

A genomic DNA library was constructed from *Act. actinomycetemcomitans* strain VT747 by ligating 3–5 kb fragments

obtained from a *Sau*3A partial digestion into the vector pUC19 (Life Technologies) (Sambrook *et al.*, 1989). Transformation by electroporation was used to introduce this construct into *E. coli* JM109 (Life Technologies). Colony lifts (Sambrook *et al.*, 1989), probed with ³²P-labelled fragments of pVT745, were performed to identify clones harbouring DNA homologous to selected regions of pVT745. The nucleotide sequences of identified clones were determined by automated DNA sequencing using the method of Sanger *et al.* (1977) at the University of Pittsburgh School of Medicine Biomedical Research Support Facilities. Computer analyses of the sequences were performed using MacDNASIS (Hitachi Software Engineering America). DNA and predicted protein sequences were searched against the current NCBI and GenBank databases (National Library of Medicine).

PCR (Mullis *et al.*, 1986), using a forward primer based on the insert DNA sequence of pKN12 (Fig. 1) and a reverse primer based on the 3' sequence of *magB11* (Galli *et al.*, 2001), was used to amplify a segment of chromosomal DNA from VT747 encoding partial ORFs for Tag10 and Tag11. PCR also was used to amplify two regions of pVT745 – a 1.0 kb internal segment of *magB03* (Galli *et al.*, 2001; Fig. 1) and a 1.056 kb internal region from a 'non-essential' area of the plasmid (Galli *et al.*, 2001; Fig. 1) – and a 1.2 kb segment of VT747

chromosomal DNA 5' to the shared areas of homology (Fig. 1). PCR was performed in a Perkin Elmer GeneAmp PCR System 2400 using the PCR Reagent System (Life Technologies) under the following profile: 25 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C. The resulting PCR products were cloned using the Original TA Cloning Kit (Invitrogen) and their nucleotide sequences determined as described above.

Construction of recombinant derivatives of pVT745. Homologous recombination was used to introduce the selectable spectinomycin-resistance marker (LeBlanc *et al.*, 1991) into two separate regions of pVT745. The first had been previously identified as a 'non-essential' area of pVT745, i.e. it did not harbour genes involved in either replication or conjugation functions of pVT745 (Fig. 1–B2; Galli *et al.*, 2001). The second was into *magB03* (Fig. 1), which encodes a protein homologous with PtlC and VirB4 of *Bor. pertussis* and *Agr. tumefaciens*, respectively. The 1.0 kb and 1.056 kb PCR products described above were ligated into *Sma*I digested, desphosphorylated vector, pUS19 (Benson & Haldenwang, 1993). These constructs were introduced by electroporation into *E. coli* JM109, with transformants selected on 100 µg spectinomycin ml⁻¹. Two resulting plasmids were used to transform *Act. actinomycetemcomitans* strain VT745 by electroporation. The ColE1 origin of replication on pUS19 does not support extrachromosomal replication in *Act. actinomycetemcomitans* (Sreenivasan *et al.*, 1991). Therefore, only transformants that had integration of either of these constructs into pVT745 by homologous recombination were able to grow in the presence of spectinomycin. Plasmid DNA from transformants was evaluated by restriction endonuclease digestion, Southern hybridization and PCR. Results from these analyses confirmed that single crossover events had occurred into both sites of pVT745. Strains harbouring these plasmid derivatives of pVT745, pKN14 and pKN15 (Table 1) were used in conjugation experiments.

Conjugation experiments. *Act. actinomycetemcomitans* strain VT745 harbouring either pKN14 or pKN15 were used as donors in mating experiments, with strain HK1651Rif serving as a recipient. Donors and recipients were grown in broth to mid-exponential phase and then mixed in a ratio of 1:10 in a total volume of 1 ml. The mixture was centrifuged and the pelleted cells resuspended in 10 µl media, spotted on a TSBYE agar plate and incubated at 37 °C for 4 h in 10% CO₂. The cells were scraped off and resuspended in TSBYE. Serial dilutions were spread on TSBYE plates containing spectinomycin and rifampicin. Serial dilutions of donor cells only were also plated. All plates were incubated at 37 °C in 10% CO₂ for 48 h. Transfer frequencies were expressed as number of transconjugants per donor cell. Selected transconjugants were evaluated by Southern blot analysis for the presence of plasmid DNA.

Mobilization experiments. Plasmid pMMB67 was introduced by electroporation into *Act. actinomycetemcomitans* strain VT747 and strain VT745. Transformants were selected on ampicillin. Transformants harbouring pMMB67 (confirmed by Southern blot analyses) were used as donors in mating experiments as described above. Transconjugants were selected on agar plates containing ampicillin and rifampicin. The presence of pMMB67 was confirmed by Southern blot analysis of selected transconjugants.

RNA isolation and Northern blot hybridization. Total RNA was isolated from *Act. actinomycetemcomitans* cells (VT747, VT745, VT705 and VT748) grown to mid-exponential phase using TRIzol reagent as described by the manufacturer

(Molecular Research Center). Each sample was treated with DNase I and a 6 µg sample subjected to electrophoresis in a denaturing 1.2% agarose/formaldehyde gel. The RNA was transferred onto nylon membranes which were hybridized (Galli & LeBlanc, 1995) with the insert DNA of clone pKN12 labelled with ³²P (Fig. 1). Either a 0.24–9.5 kb or a 0.16–1.77 kb RNA ladder (Life Technologies) was used as a size marker.

RESULTS AND DISCUSSION

pVT745 is a 25.4 kb plasmid harboured by *Act. actinomycetemcomitans* strain VT745 (LeBlanc *et al.*, 1993). Previous results from our laboratory had demonstrated that multiple regions of pVT745 shared homology with chromosomal DNA from a number of other *Act. actinomycetemcomitans* strains, none of which harboured plasmid DNA (Novak & LeBlanc, 1994). Furthermore, we previously demonstrated that these shared areas of homology did not appear to represent insertion sequence elements, transposons or bacteriophage DNA, and that the intact plasmid had not merely inserted into the chromosome of any of these strains (Novak *et al.*, 1998). Cell passaging experiments, followed by Southern blot analyses, suggested stability of the homologous DNA both within the chromosome and on the plasmid (Novak *et al.*, 1998).

Nucleotide sequence analysis

Nucleotide sequence analysis of pVT745 revealed the presence of eleven genes, the majority of which encode predicted proteins sharing homology with VirB proteins of *Brucella* spp. (*magB01–11*; Galli *et al.*, 2001). Southern blot hybridizations, using ³²P-labelled segments of pVT745 that contained these genes as probes, were performed against *Act. actinomycetemcomitans* strain VT747 genomic DNA. Resulting autoradiographs revealed that several of these plasmid-encoded sequences also resided in the chromosome of this strain (Novak, 1994). Screening of colonies from a genomic DNA library from strain VT747 resulted in the identification of two clones that hybridized with either the 6.9 kb *Bam*HI–*Pst*I fragment of pVT745 or the 8.2 kb *Pst*I fragment (Novak & LeBlanc, 1994; Galli *et al.*, 2001). Nucleotide sequence analysis of these two overlapping clones (pKN4 and pKN12, Fig. 1) revealed the presence of nine contiguous genes encoding complete or partial ORFs similar to those found on pVT745. These chromosomal genes were named *transport associated genes* (*tag*) for the homology several of the predicted proteins encoded by these genes demonstrated with type IV secretion system proteins involved in macromolecular transport. They were numbered *tag03–11* to be consistent with numbering of the homologous *magB* genes found on pVT745 (Galli *et al.*, 2001). A partial ORF that shared homology with MagB11 was identified at the 3' end of pKN12. PCR using a forward primer designed from the nucleotide sequence of an internal region of *tag10* and a reverse primer based on the 3' sequence of *magB11* (internal to the stop codon), was used in an attempt to amplify and clone the remainder of the gene

encoding the VirB11/PtlH homologue from strain VT747. Nucleotide sequence analysis of this clone (pKN13, Fig. 1) revealed an overlap and continuation of the partial ORF in pKN4, but the stop codon for *tag11* still could not be identified. However, a comparison with the homologous gene on pVT745 (*magB11*) suggests that the nucleotide sequence would continue in the VT747 chromosome for approximately ten additional bases prior to encoding the stop codon.

An analysis of the nucleotide sequences of these regions of homology shared between pVT745 and VT747 chromosomal DNA, including intervening segments, revealed that the DNA identity ranged from 93 to 99%. This high degree of identity at the DNA level suggests that this plasmid may have once inserted into the chromosome of strain VT747. This is further supported by evaluating the segment of VT747 chromosomal DNA immediately 5' to *tag03* (Fig. 1). This chromosomal sequence shared 98.9% homology with a 1.207 kb region located between the *PvuII* and *PstI* sites found in the 10.3 kb region of pVT745 (Novak, 1994; Galli *et al.*, 2001; Fig. 1). Although a potential ORF is encoded in this sequence, it initiates internal to the probable chromosomal integration site for pVT745 and shows no homology with predicted proteins listed in GenBank. The final seven base pairs of this sequence overlap with *tag03* and includes the start codon of *tag03* (Fig. 1), suggesting that a large segment of pVT745 may have inserted into the VT747 chromosome. However, since approximately 8.3 kb between this shared area of homology and the first 1007 base pairs of *magB03* of pVT745 are not present in the chromosomal sequence (Fig. 1), this portion of pVT745 either never inserted into, or has been deleted from, the VT747 chromosome. These results confirm our previous studies (Novak & LeBlanc, 1994), which demonstrated that the entire, intact plasmid was not present in the VT747 chromosome.

Insertion of plasmid DNA into the chromosome and subsequent rearrangement of the inserted sequence is not an uncommon event. For example, there is substantial evidence that the chromosomally encoded *vap* (virulence-associated protein) 1/3 gene regions in the animal pathogen *Dichelobacter nodosus*, evolved from the site specific integration of a native *D. nodosus* plasmid, pJIR896, into the 3' end of a tRNA gene (Billington *et al.*, 1996). Subsequent duplication/rearrangement events have been attributed to an IS element, IS1253, found on pJIR896 (Billington *et al.*, 1996). Although a similar insertion and subsequent duplication/rearrangement may have occurred between pVT745 and the VT747 chromosome, two major differences exist between the integration areas for pJIR896 and pVT745. First, the DNA sequence 5' to the VT747 type IV genes shared no homology with tRNA genes, the proposed integration site for pJIR896. Second, neither pVT745 nor the chromosomal sequence 5' to the type IV secretion system genes in VT747 harboured any DNA sequences homologous to known IS elements. Therefore, it appears that tRNA genes and IS elements

may not have been involved in the insertion and rearrangement of pVT745 in the VT747 chromosome.

Four of the predicted *Act. actinomycetemcomitans* type IV secretion proteins (Tag03, Tag09, Tag10 and Tag11) shared homology over limited regions with *cag* pathogenicity island proteins of *H. pylori* (Table 2), which may encode a protein secretion system (Censini *et al.*, 1996). Bacterial pathogenicity islands often have a different G+C content from the rest of the bacterial genome. A comparison of both the VT745 and VT747 proposed type IV secretion system genes with the *Act. actinomycetemcomitans* HK1651 database (University of Oklahoma; www.genome.ou.edu/act.html) showed that the G+C content of the type IV secretion system genes was approximately 40 mol% compared with 44 mol% in the overall *Act. actinomycetemcomitans* genome. This revealed one similar characteristic shared between pathogenicity islands and the *Act. actinomycetemcomitans* type IV genes. Other characteristics of pathogenicity islands may also include the presence of flanking and direct repeats and IS elements, origins of plasmid replication, and genes encoding mobility elements such as integrases and transposases (Hou, 1999). Because these mobility elements are active, many pathogenicity islands are unstable, excising from the genome at frequencies of 10^{-4} . Pathogenicity islands also are frequently associated with tRNA genes, which serve as proposed recombination sites (Hou, 1999). As previously stated, the DNA sequence 5' to the VT747 chromosomal type IV secretion system genes shares no homology with tRNA genes and there is no evidence for IS or other mobile genetic elements in either the pVT745 or the VT747 sequence. In addition, our previous studies have demonstrated that the type IV secretion system genes are stably maintained in the VT747 chromosome and on pVT745 (Novak *et al.*, 1998). Therefore, many of the characteristic features of previously identified pathogenicity islands do not appear to exist in this region of the VT747 chromosome or on pVT745. However, the nucleotide sequence of VT747 chromosomal DNA 3' to *tag11* is currently unknown. This sequence may contain DNA homologous to mobility elements, and/or areas of direct or inverted repeats when compared with the 5' sequence. Future experiments are designed to identify and determine the nucleotide sequence of this 3' region in an attempt to characterize the chromosomal integration site.

Although the chromosomal and plasmid sequences share significant homology, specific differences are noted when comparing the plasmid-encoded type IV genes with those located on the chromosome. First, the VT747 chromosome does not harbour a gene encoding a VirB1 homologue, as seen on pVT745 (*magB01*; Galli *et al.*, 2001). The *Bor. pertussis* *ptl* system also does not harbour a *virB1* homologue (Weiss *et al.*, 1993; Christie & Vogel, 2000) and VirB1 has been shown to be dispensable for plant tumourigenesis in *Agr. tumefaciens* (Berger & Christie, 1994). Second, while the predicted sizes of the homologues encoded by *Act. actinomycetemcomitans* pVT745 and the VT747 chromosome

Table 2. Comparison of selected type IV secretion system predicted proteins to *Act. actinomycetemcomitans* VT747

All columns are arranged as follows: predicted protein designation (top row); no. amino acids in predicted protein (middle row); percentage identity/similarity with *Act. actinomycetemcomitans* VT747 type IV secretion system genes (bottom row). ND, Not done; NI, none identified (no homologous protein from this bacterium identified in the GenBank database). The accession numbers for the sequences reported here are *Act. actinomycetemcomitans* VT747, AF332996; *Act. actinomycetemcomitans* VT745, AF302424; *Bru. melitensis* biovar *suis*, AF141604; *Agr. tumefaciens*, AB01180 and AF242881; *Bor. pertussis*, L10720; *H. pylori*, AF282853, AE001481.

<i>Act. actinomycetemcomitans</i> VT747	Tag03	Tag04	Tag05	Tag06	Tag07	Tag08	Tag09	Tag10	Tag11
	589	243	75	333	47	228	289	369	340
	ND	ND	ND	ND	ND	ND	ND	ND	ND
<i>Act. actinomycetemcomitans</i> VT745	MagB03	MagB04	MagB05	MagB06	MagB07	MagB08	MagB09	MagB10	MagB11
	923	258	75	333	47	228	289	369	343
	97/98	96/98	100/100	87/87	93/93	96/96	99/99	97/98	99/99
<i>Bru. melitensis</i> biovar <i>suis</i>	VirB4	VirB5	NI	VirB6	NI	VirB8	VirB9	VirB10	VirB11
	832	238	–	347	–	239	289	391	397
	42/62	20/44	–	20/39	–	37/57	36/52	37/56	55/67
<i>Agr. tumefaciens</i>	VirB4	NI	NI	NI	NI	VirB8	VirB9	VirB10	VirB11
	789	–	–	–	–	237	293	377	343
	31/50	–	–	–	–	25/46	22/35	42/64	34/52
<i>Bor. pertussis</i>	PtlC	NI	NI	NI	NI	PtlE	PtlF	PtlG	PtlH
	824	–	–	–	–	276	273	336	339
	41/60	–	–	–	–	31/55	33/48	33/48	40/60
<i>H. pylori</i>	CagE	NI	NI	NI	NI	NI	Cag	Cag	Cag
							protein	protein	protein
	981	–	–	–	–	–	522	1819	330
	30/50	–	–	–	–	–	29/51	30/53	37/55

were generally very similar, a notable exception was MagB03 and Tag03 (PtlC/VirB4 homologues). The predicted MagB03 protein encoded by pVT745 is 923 aa, compared to 589 aa for the VT747-encoded Tag03. A comparison of this predicted homologue in other bacteria (Table 2) revealed a size of 832 aa in *Bru. suis* (O'Callaghan *et al.*, 1999), 789 aa in *Agr. tumefaciens* (Shirasu *et al.*, 1990), 824 aa in *Bor. pertussis* (Weiss *et al.*, 1993) and 981 aa in *H. pylori* (Censini *et al.*, 1996). Although variability in the sizes of these proteins occurs, the pVT745-encoded protein is 334 aa larger at the amino terminus than the VT747 chromosomally encoded protein. The potential functional significance of this difference is discussed later. Finally, the VT747 chromosome did not harbour contiguous genes encoding homologues to MagB12–14 (Galli *et al.*, 2001). Since these predicted proteins do not share homology with any of those encoded by the *ptl* or *virB* operons, their absence in the chromosome of strain VT747 was not surprising.

A common feature of these secretion systems is the presence of a protein, usually cytoplasmic, that has apparent NTPase activity which provides the energy to drive the transmembrane transport (Krause *et al.*, 2000). These NTPases share conserved motifs, including Walker Box A, an Asp Box, Walker Box B and a His Box (Rivas *et al.*, 1997; Krause *et al.*, 2000). Significantly, all four of these motifs were identified in the VirB11 homologues encoded by pVT745 and VT747 (*magB11* and *tag11*; data not shown). In addition, a potential NTP-binding site, defined by the consensus

GXXGXGKT was found in the PtlC/VirB4 homologues encoded by the chromosomal and plasmid sequences (*tag03* and *magB03*; data not shown).

Conjugation experiments

Results of previous experiments revealed that pVT745 was a conjugative plasmid (Galli *et al.*, 2001). The homology shared between the *magB* predicted proteins and those encoded by type IV secretion genes suggested that the *magB* genes were responsible for conjugation functions. Because the *Agr. tumefaciens* VirB4 protein is essential for Ti plasmid DNA transport (Berger & Christie, 1993) we proposed that the protein encoded by *magB03*, a VirB4 homologue, would be essential for conjugation of pVT745. A selectable marker (Sp^R) was inserted through homologous recombination into a non-essential region of pVT745 as described in Methods. Self-transfer was demonstrated in mating experiments with the recipient strain HK1651Rif, where transconjugants were obtained at a frequency of 10^{–6} per donor. Similar techniques were used to disrupt *magB03*, the *virB4* homologue. No transconjugants could be detected when matings were performed with this mutant strain. Although this disruption technique could have resulted in downstream polar effects, these results suggested that *magB03* is essential for transfer of pVT745. However, these experiments, which were performed in triplicate, confirmed that pVT745 is a conjugative plasmid and support the hypothesis that *magB* genes are necessary for conjugation (Galli *et al.*, 2001).

Mobilization experiments

Since previous experiments had demonstrated that pVT745 was capable of mobilizing a non-self-transmissible plasmid, pMMB67 (Galli *et al.*, 2001), we hypothesized that the genes found in the chromosome of strain VT747 would serve a similar function. This would be consistent with findings in *L. pneumophila* where a chromosomal homologue of *virB10*, *dotG*, has been shown to be essential for mobilization of plasmid DNA (Vogel *et al.*, 1998). Plasmid pMMB67 was introduced by electroporation into *Act. actinomycetemcomitans* strain VT745 and into strain VT747. Transformants served as donors in mating experiments with the recipient, HK1651Rif. Strain VT745 mobilized pMMB67 at a frequency of 10^{-6} transconjugants per donor, while no transconjugants were obtained using VT747/pMMB67 as a donor. These experiments were performed in triplicate. As stated previously, a potentially significant difference in the pVT745-encoded genes and the VT747 chromosomal genes lies in the size of the VirB4/PtlC homologue (MagB03 and Tag03). Specifically, the pVT745-encoded protein is 334 aa larger at the amino terminus than the VT747 chromosomally encoded protein. Although the results of the mobilization experiments suggested that the chromosomal type IV system may not be involved in DNA transport, it is also possible that the truncated version of *tag03* may be missing sequences essential for this function. To address this, the amino-terminal sequence of *magB03* will be introduced *in trans* to strain VT747 and the mobilization experiments repeated. This may result in identification of regions of the MagB03 protein that are essential for DNA transport.

Northern blot hybridization

Northern blot analyses were performed to determine if the *magB* and *tag* genes were expressed in *Act. actinomycetemcomitans* strains VT745 and VT747. The *in vivo* transcripts of the type IV genes from these two strains, as well as VT705 and VT748 (*Act. actinomycetemcomitans* strains that do not harbour these genes, either on a plasmid or in the chromosome) were analysed by Northern blotting in four separate experiments. The 32 P-labelled 4.676 kb insert of pKN12 (Fig. 1) hybridized to two major transcripts of approximately 1.8 kb and 1.2 kb in both strains (Fig. 2). Two additional transcripts visualized directly above and below the 1.8 kb and 1.2 kb bands also appeared to be present in strain VT747 (Fig. 2). These two transcripts could not be visualized in strain VT745 with a shorter exposure of the autoradiograph. Finally, strain VT745 appeared to have one large and one small transcript that may not be present in strain VT747. No hybridization was detected to total RNA isolated from strains VT705 and VT748 (Fig. 2). These results demonstrated that the type IV secretion system genes are expressed in both strains.

In *Bor. pertussis*, the *ptl* operon is transcribed from the *ptx* (pertussis toxin) promoter as part of a > 11 kb mRNA (Baker *et al.*, 1995). In *Act. actinomycetem-*

Fig. 2. Northern blot of *Act. actinomycetemcomitans* strains. Total RNA was isolated from *Act. actinomycetemcomitans* strains grown to mid-exponential phase, separated on a 1.2% agarose/formaldehyde gel and hybridized with 32 P-labelled insert DNA from pKN12. Lanes: 1, RNA ladder; 2, VT705; 3, VT745; 4, VT747; 5, VT748. Approximate sizes of two major transcripts are indicated in the right column, as well as the location of other potential transcripts as described in Results and Discussion.

comitans strains VT745 and VT747, many of the proposed ribosome-binding sites or the initiation codons for the type IV ORFs overlap with the previous ORF, suggesting that the genes may be transcriptionally linked. Future studies, including reverse-transcriptase PCR, will be conducted to determine if these genes are transcribed as part of an operon in these *Act. actinomycetemcomitans* strains. In addition, it should be noted that both the *ptl* and *virB* operons are regulated by proteins of two-component systems. *In vitro*, these systems have been shown to be regulated by substances such as nicotinamide and MgSO_4 , phenolic compounds, acidic pH and monosaccharides (Heath *et al.*, 1995). Although similar transcript sizes were found when *Act. actinomycetemcomitans* strains VT745 and VT747 were grown to either mid- or late-exponential phase, an extensive analysis of a variety of growth conditions was not performed. Therefore, the conditions under which the *Act. actinomycetemcomitans* strains were grown may not have fully simulated those required for transcriptional regulation. Those conditions may need to be identified in the future to further analyse transcription of the type IV genes in these strains.

5' nucleotide sequence analysis

The *Bor. pertussis* *ptl* genes lie directly 3' from the pertussis toxin structural genes (Weiss *et al.*, 1993). Since the exact function of the *tag* genes in VT747 has not been elucidated, we hypothesized that the DNA sequence 5' of the *tag* genes could encode a protein potentially secreted by this type IV system. To address this hypothesis, we evaluated a 1.2 kb DNA sequence immediately 5' of the areas of homology shared between

pVT745 and VT747 (Fig. 1) for the presence of ORFs. This was accomplished by comparing this 5' sequence with the database for *Act. actinomycetemcomitans* strain HK1651 (University of Oklahoma; www.genome.ou.edu/act.html). A contiguous fragment was identified in the database that matched the VT747 sequence. PCR, using primers based on pVT747 sequence (reverse primer) and HK1651 sequence (forward primer), was used to amplify a 1.2 kb fragment of VT747 chromosomal DNA. The nucleotide sequence of this fragment shared 96% homology with a PCR product amplified from HK1651 using similar techniques. Translation of this sequence revealed the presence of an ORF sharing homology with the predicted acetohydroxyacid synthase isoenzyme of *E. coli*. This protein is involved in the biosynthesis of branched-chain amino acids (Hill *et al.*, 1997). Any potential significance of this finding has yet to be determined.

In conclusion, genes sharing homology with those of type IV secretion systems have been identified in *Act. actinomycetemcomitans*. This micro-organism appears unique in that the type IV genes are located on a plasmid in one strain and on the chromosome in a different strain. While there is evidence that the plasmid-encoded genes are involved in mobilization of DNA, the function of the chromosomal genes has not yet been determined. However, Northern blot analyses revealed that the genes are expressed in both strains. Further analyses will be performed to determine if these genes are cotranscribed as part of an operon. Future studies will also be performed to determine the potential origin of these sequences shared by pVT745 and *Act. actinomycetemcomitans* strain VT747. In addition, the precise role of these chromosomal genes and the nature of additional DNA sequences located 5' and 3' to the genes, will be identified. Genetic and phenotypic characterization of wild-type and mutant VT747, as well as other *Act. actinomycetemcomitans* strains harbouring these genes will help in answering these questions and in determining if these genes contribute to the virulence of this micro-organism.

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REFERENCES

- Anderson, D. G. & McKay, L. L. (1983). Simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl Environ Microbiol* **46**, 549–552.
- Andersson, S. G., Zomorodipour, A., Andersson, J. O. & 7 other authors (1998). The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* **396**, 133–140.
- Baker, S. M., Masi, A., Liu, D.-F., Novitsky, B. K. & Deich, R. A. (1995). Pertussis toxin export genes are regulated by the *ptx* promoter and may be required for efficient translation of *ptx* mRNA in *Bordetella pertussis*. *Infect Immun* **63**, 3920–3926.
- Benson, A. K. & Haldenwang, W. G. (1993). Regulation of σ^B levels and activity in *Bacillus subtilis*. *J Bacteriol* **175**, 2347–2356.
- Berger, B. R. & Christie, P. C. (1993). The *Agrobacterium tumefaciens virB4* gene product is an essential virulence protein requiring an intact nucleoside triphosphate-binding domain. *J Bacteriol* **175**, 1723–1734.
- Berger, B. R. & Christie, P. C. (1994). Genetic complementation analysis of the *Agrobacterium tumefaciens virB* operon: *virB2* through *virB11* are essential virulence genes. *J Bacteriol* **176**, 3646–3660.
- Billington, S. J., Sinistaj, M., Cheetham, B. F., Ayres, A., Moses, E. K., Katz, M. E. & Rood, J. I. (1996). Identification of native *Dichelobacter nodosus* plasmid and implications for the evolution of the *vap* regions. *Gene* **172**, 111–116.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513–1532.
- Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R. & Covacci, A. (1996). *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type 1-specific and disease-associated virulence factors. *Proc Natl Acad Sci USA* **93**, 14648–14653.
- Chen, Y. C., Chang, S. C., Luh, K. T. & Hsieh, W. C. (1991). *Actinobacillus actinomycetemcomitans* endocarditis: a report of four cases and review of the literature. *Q J Med* **81**, 871–878.
- Christie, P. J. & Vogel, J. P. (2000). Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. *Trends Microbiol* **8**, 354–360.
- Covacci, A. & Rappuoli, R. (1993). Pertussis toxin export requires accessory genes located downstream from the pertussis toxin operon. *Mol Microbiol* **8**, 429–434.
- Farizo, K. M., Cafarella, T. G. & Burns, D. L. (1996). Evidence for a ninth gene, *ptII*, in the locus encoding the pertussis toxin secretion system of *Bordetella pertussis* and formation of a PtlI-PtlF complex. *J Biol Chem* **271**, 31643–31649.
- Fives-Taylor, P. D., Meyer, D. & Mintz, K. (1996). Virulence factors of the periodontopathogen *Actinobacillus actinomycetemcomitans*. *J Periodontol* **67**, 291–297.
- Fürste, J. P., Pansegrau, W., Frank, R., Blöcker, H., Scholz, P., Bagdasarian, M. & Lanka, E. (1986). Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* **48**, 119–131.
- Galli, D. M. & LeBlanc, D. J. (1995). Transcriptional analysis of rolling circle replicating plasmid pVT736-1: evidence for replication control by antisense RNA. *J Bacteriol* **177**, 4474–4480.
- Galli, D. M., Chen, J., Novak, K. F. & LeBlanc, D. J. (2001). Nucleotide sequence analysis of conjugative plasmid pVT745. *J Bacteriol* **183**, 1585–1594.
- Hamori, P. J. & Slama, T. G. (1989). *Actinobacillus* prosthetic valve endocarditis. *Am Heart J* **118**, 853–854.
- Heath, J. D., Charles, T. C. & Nester, E. W. (1995). Ti plasmid and chromosomally encoded two-component systems important in plant cell transformation by *Agrobacterium*. In *Two-Component Signal Transduction*, pp. 367–392. Edited by J. A. Hoch and T. J. Silhavy. Washington, DC: American Society for Microbiology.
- Hill, C. M., Pang, S. S. & Duggleby, R. G. (1997). Purification of *Escherichia coli* acetohydroxyacid synthase isoenzyme II and reconstitution of active enzyme from its individual pure subunits. *Biochem J* **327**, 891–898.
- Hou, Y.-M. (1999). Transfer RNAs and pathogenicity islands. *Trends Biochem Sci* **24**, 295–298.
- Krause, S., Barcena, M., Pansegrau, W., Lurz, R., Carazo, J. M. & Lanka, E. (2000). Sequence related protein export NTPases encoded by the conjugative transfer region of RP4 and by the *cag*

- pathogenicity island of *Helicobacter pylori* share similar hexameric ring structures. *Proc Natl Acad Sci USA* **97**, 3067–3072.
- LeBlanc, D. J., Lee, L. N. & Inamine, J. M. (1991). Cloning and nucleotide base sequence analysis of a spectinomycin adenylyltransferase AAD(9) determinant from *Enterococcus faecalis*. *Antimicrob Agents Chemother* **35**, 1204–1210.
- LeBlanc, D. J., Lee, L. N., Abu-Al-Jaibat, A. R., Sreenivasan, P. K. & Fives-Taylor, P. M. (1993). Identification of plasmids in *Actinobacillus actinomycetemcomitans* and construction of intergeneric shuttle plasmids. *Oral Microbiol Immunol* **8**, 94–99.
- Lessl, M., Balzer, D., Pansegrau, W. & Lanka, E. (1992). Sequence similarities between the RP4 Tra2 and the Ti VirB region strongly support the conjugation model for T-DNA transfer. *J Biol Chem* **267**, 20471–20480.
- Martin, B. F., Derby, B. M., Budzilovich, G. N. & Ransohoff, J. (1967). Brain abscess due to *Actinobacillus actinomycetemcomitans*. *Neurology* **17**, 833–837.
- Masui, S., Tetsuhiki, S. & Ishikawa, H. (2000). Genes for the type IV secretion system in an intracellular symbiont, *Wolbachia*, a causative agent of various sexual alterations in arthropods. *J Bacteriol* **182**, 6529–6531.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Moore, W. E. C., Holdeman, L. V., Smibert, R. M., Hash, D. E., Burmeister, J. A. & Ranney, R. R. (1982). Bacteriology of severe periodontitis in young adults. *Infect Immun* **38**, 1137–1148.
- Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. & Erlich, H. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symp Quant Biol* **51**, 263–273.
- Novak, K. F. (1994). Characterization of plasmid pVT745 isolated from *Actinobacillus actinomycetemcomitans*. PhD thesis, University of Texas Health Science Center, San Antonio, TX, USA.
- Novak, K. F. & LeBlanc, D. J. (1994). Characterization of plasmid pVT745 isolated from *Actinobacillus actinomycetemcomitans*. *Plasmid* **31**, 31–39.
- Novak, K. F., Lee, L. N. & LeBlanc, D. J. (1998). Functional analysis of pVT745, a plasmid from *Actinobacillus actinomycetemcomitans*. *Oral Microbiol Immunol* **13**, 124–128.
- O'Callaghan, D. O., Cazeville, C., Allardet-Servent, A., Boschioli, M. L., Bourg, G., Foulongne, V., Frutos, P., Kulakov, Y. & Ramuz, M. (1999). A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. *Mol Microbiol* **33**, 1210–1220.
- Pohlman, R. F., Genetti, H. D. & Winans, S. C. (1994). Common ancestry between IncN conjugal transfer genes and macromolecular export systems of plant and animal pathogens. *Mol Microbiol* **14**, 655–668.
- Rivas, S., Bolland, S., Cabezon, E., Goni, F. M. & de la Cruz, F. (1997). TrwD, a protein encoded by the IncW plasmid R388, displays an ATP hydrolase activity essential for bacterial conjugation. *J Biol Chem* **272**, 25583–25590.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Segal, G., Russo, J. J. & Shuman, H. A. (1999). Relationships between a new type IV secretion system and the *icm/dot* virulence system of *Legionella pneumophila*. *Mol Microbiol* **34**, 799–809.
- Shirasu, K. & Kado, C. I. (1993). The *virB* operon of the *Agrobacterium tumefaciens* virulence regulon has sequence similarities to B, C, and D open reading frames downstream of the pertussis toxin-operon and to the DNA transfer-operons of broad-host-range conjugative plasmids. *Nucleic Acids Res* **21**, 353–354.
- Shirasu, K., Morel, P. & Kado, C. I. (1990). Characterization of the *virB* operon of an *Agrobacterium tumefaciens* Ti plasmid: nucleotide sequence and protein analysis. *Mol Microbiol* **4**, 1153–1163.
- Slots, J. (1982). Selective medium for isolation of *Actinobacillus actinomycetemcomitans*. *J Clin Periodontol* **15**, 606–609.
- Sreenivasan, P. K., LeBlanc, D. J., Lee, L. N. & Fives-Taylor, P. (1991). Transformation of *Actinobacillus actinomycetemcomitans* by electroporation, utilizing constructed shuttle plasmids. *Infect Immun* **59**, 4621–4627.
- Tummuru, M. K. R., Sharma, S. A. & Blaser, M. J. (1995). *Helicobacter pylori* *picB*, a homologue of the *Bordetella pertussis* toxin secretion protein, is required for induction of IL-8 in gastric epithelial cells. *Mol Microbiol* **18**, 867–876.
- Vogel, J. P., Andrews, H. L., Wong, S. K. & Isberg, R. R. (1998). Conjugative transfer by the virulence system of *Legionella pneumophila*. *Science* **279**, 873–876.
- Weiss, A. A., Johnson, F. D. & Burns, D. L. (1993). Molecular characterization of an operon required for pertussis toxin secretion. *Proc Natl Acad Sci USA* **90**, 2970–2974.
- Winans, S. C., Burns, D. L. & Christie, P. J. (1996). Adaptation of a conjugal transfer system for the export of pathogenic macromolecules. *Trends Microbiol* **4**, 64–68.
- Yannish-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
- Zambon, J. J., Christersson, L. A. & Slots, J. (1983). *Actinobacillus actinomycetemcomitans* in human periodontal disease: prevalence in patient groups and distribution of biotypes and serotypes within families. *J Periodontol* **54**, 707–711.

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