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

Karen F. Novak,1 Bryan Dougherty2 and Manuel Peláez2

Author for correspondence: Karen F. Novak. Tel: +1 859 323 8705. Fax: +1 859 257 6566. e-mail: knova2@pop.uky.edu

1 University of Kentucky, College of Dentistry, Center for Oral Health Research, Lexington, KY 40536-0297, USA
2 Department of Periodontics, University of Pittsburgh School of Dental Medicine, Pittsburgh, PA 15261, USA

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 environment, 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.
pertussis transport system is comprised of a chromosomally encoded, 9.5 kb, nine-gene [pertussis toxin liberation (ptl)] operon (Farizzo 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. tumafaciens* (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

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<th>Strain or plasmid</th>
<th>Description*</th>
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<td>Genome sequencing strain</td>
<td>Dave Dyer, University of Oklahoma</td>
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<td>HK1651Rif</td>
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<td>LeBlanc et al. (1993)</td>
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<td>LeBlanc et al. (1993), Galli et al. (2001)</td>
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<td>pUC19</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;; high-copy-number cloning vector</td>
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<td>Sp&lt;sup&gt;R&lt;/sup&gt; derivative of pUC19</td>
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<sup>* Rif<sup>R</sup>, rifampicin resistance; Ap<sup>R</sup>, ampicillin resistance; Sp<sup>R</sup>, spectinomycin resistance. </sup>
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 by 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 Sau3A 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 1.2 kb segment of VT747 chromosomal DNA. 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.

**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 PstI 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 834 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 kb internal region of magB03 (as described in Methods) are indicated with converging arrows. (b) VT747 chromosomal DNA. 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.
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 PlcC and VirB4 of *Bor. pertussis* and *Agr. tumefaciens*, respectively. The 10 kb and 1056 kb PCR products described above were ligated into Smal 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 CoE1 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. 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 0.16–177 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 harbour 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 *BamHI–PstI* fragment of pVT745 or the 8.2 kb *PstI* 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/PtrH 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′ by comparing the partial ORF in pKN4, but the stop codon for VT747. Nucleotide sequence analysis of this clone encoding the VirB11

immediately 5′ by evaluating the segment of VT747 chromosomal DNA located between the PvuI and PsI 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/acr.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 ptr 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

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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 conjugal 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<sup>R</sup>) 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<sup>–6</sup> 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 conjugal plasmid and support the hypothesis that *magB* genes are necessary for conjugation (Galli et al., 2001).

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

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<td><em>Bru. melitensis</em> biovar <em>suis</em></td>
<td>VirB4</td>
<td>VirB5</td>
<td>MagB08</td>
<td>MagB07</td>
<td>MagB06</td>
<td>MagB05</td>
<td>MagB04</td>
<td>MagB03</td>
<td>ND</td>
</tr>
<tr>
<td><em>Agr. tumefaciens</em></td>
<td>VirB4</td>
<td>VirB5</td>
<td>MagB08</td>
<td>MagB07</td>
<td>MagB06</td>
<td>MagB05</td>
<td>MagB04</td>
<td>MagB03</td>
<td>ND</td>
</tr>
<tr>
<td><em>Bor. pertussis</em></td>
<td>VirB4</td>
<td>VirB5</td>
<td>MagB08</td>
<td>MagB07</td>
<td>MagB06</td>
<td>MagB05</td>
<td>MagB04</td>
<td>MagB03</td>
<td>ND</td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td>VirB4</td>
<td>VirB5</td>
<td>MagB08</td>
<td>MagB07</td>
<td>MagB06</td>
<td>MagB05</td>
<td>MagB04</td>
<td>MagB03</td>
<td>ND</td>
</tr>
</tbody>
</table>

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; N, 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.
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/PtrC 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 4676 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 VT747 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 ptx gene is transcribed from the ptx (pertussis toxin) promoter as part of a >11 kb mRNA (Baker et al., 1995). In Act. actinomycetemcomitans 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 ptx 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 ptx 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 microorganism 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 microorganism.

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pathogenicity island of Helicobacter pylori share similar hexameric ring structures. Proc Natl Acad Sci USA 97, 3067–3072.


