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Treponema denticola biofilm-induced expression of a bacteriophage, toxin–antitoxin systems and transposases

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Treponema denticola is an oral spirochaete that has been strongly associated with chronic periodontitis. The bacterium exists as part of a dense biofilm (subgingival dental plaque) accreted to the tooth. To determine *T. denticola* gene products important for persistence as a biofilm we developed a continuous-culture biofilm model and conducted a genome-wide transcriptomic analysis of biofilm and planktonic cells. A total of 126 genes were differentially expressed with a fold change of 1.5 or greater. This analysis identified the upregulation of putative prophage genes in the *T. denticola* 35405 genome. Intact bacteriophage particles were isolated from *T. denticola* and circular phage DNA was detected by PCR analysis. This represents the first, to our knowledge, functional bacteriophage isolated from *T. denticola*, which we have designated φtd1. In biofilm cells there was also an upregulation of genes encoding several virulence factors, toxin–antitoxin systems and a family of putative transposases. Together, these data indicate that there is a higher potential for genetic mobility in *T. denticola* when growing as a biofilm and that these systems are important for the biofilm persistence and therefore virulence of this bacterium.

INTRODUCTION

Treponemes are motile, asaccharolytic, anaerobic spirochaetes with characteristic helical morphology, some of which are associated with chronic diseases of humans. *Treponema denticola* is part of the microbiota of the human oral cavity and exists as part of a polymicrobial biofilm (subgingival dental plaque) accreted to the tooth root in the gingival crevice (Ellen & Galimanas, 2005). *T. denticola*, *Porphyromonas gingivalis* and *Tannerella forsythia* form a bacterial consortium that is strongly associated with the clinical progression of chronic periodontitis (Socransky *et al.*, 1998).

Treponemes are members of the phylum Spirochaetes, a clade often grouped separately from both Gram-positive and Gram-negative bacteria, and believed to have undergone extensive horizontal gene transfer with Archaea (Brown *et al.*, 2001; Ibba *et al.*, 1997; Wolf *et al.*, 2001). In addition, there is some evidence of gene transfer with eukaryotic organisms (Bond & Francklyn, 2000; Brown *et al.*, 2001). It is highly likely therefore that the treponemes as a taxon will have evolved a range of unique characteristics. Structurally, treponemes have some similarity to Gram-negative bacteria, with the cytoplasm encased in a cytoplasmic membrane and a thin peptidoglycan cell wall, which is surrounded by an outer sheath that is analogous to the Gram-negative outer membrane. However, the location of flagella in spirochaetes is unique, with periplasmic flagella wound around the protoplasmic cylinder inside the outer sheath. The outer sheath also exhibits many differences from typical Gram-negative outer membranes, including the form of glycoconjugate in the outer leaflet and the presence of lipoteichoic acids (Schultz *et al.*, 1998).

The divergence of *T. denticola* from a common treponeme ancestor was an ancient event which has resulted in the evolution of many virulence factors unique at a species level (Seshadri *et al.*, 2004). Indeed, 26 % (734) of the putative proteins encoded by the predicted 2786 protein-encoding genes in *T. denticola* 35405 have no significant similarity to any other sequenced protein, including those of *Treponema pallidum* (Seshadri *et al.*, 2004).

One of the challenges of researching treponemes is the difficulty of their cultivation *in vitro*. *T. denticola* is one of the more easily cultured species of this genus, but it is not readily grown on the surface of agar plates and has a long generation time in liquid media (Chan *et al.*, 1993, 1997). Obtaining a model system for biofilm growth of pure cultures of *T. denticola* has been reported as problematic,

Abbreviations: qRT-PCR, quantitative RT-PCR; TA, toxin–antitoxin.

The microarray data discussed in this paper are available from ArrayExpress under accession number E-MEXP-2266.

requiring the presence of a specific coating such as fibronectin to facilitate attachment of cells (Vesey & Kuramitsu, 2004), and little work has been conducted on *T. denticola* biofilm growth. Many biofilm studies focus on the initiation of biofilm formation; however, *T. denticola* is part of a polymicrobial biofilm *in vivo* and is thought to be a late colonizer (Socransky *et al.*, 1998), so the study of the later stages of well-established biofilm growth is more likely to provide relevant information.

To investigate how *T. denticola* adapts to biofilm growth we developed a continuous culture system using a chemostat, as these systems have been demonstrated to provide stable conditions for bacterial transcriptomic and proteomic bacterial analyses (Dashper *et al.*, 2009; Lo *et al.*, 2009; Shockley *et al.*, 2005). Transcriptomic analysis indicated that when in a mature biofilm compared with planktonic growth, *T. denticola* upregulates a number of systems that are potentially involved in cell viability and gene exchange, including toxin–antitoxin (TA) systems, transposases and a bacteriophage. We demonstrated that this was a functional bacteriophage and designated it φtd1. As no *T. denticola* bacteriophage has, to our knowledge, previously been isolated or genetically characterized, this work represents the discovery of the first functional bacteriophage in *T. denticola*.

METHODS

Bacterial strain and batch culture. *T. denticola* ATCC 35405 (American Type Culture Collection, Manassas, VA, USA) was grown and maintained in oral bacterial growth medium (OBGM), a derivative of modified 'new oral spirochaete' medium (mNOS) (Leschine & Canale-Parola, 1980), as described in Orth *et al.* (2010). Batch cultures were incubated anaerobically at 37 °C in a MACS MG500 anaerobic workstation (Don Whitley Scientific). For bacteriophage isolation procedures, *T. denticola* was grown in mNOS lacking haemin, as this known inhibitor of PCR co-precipitated with the bacteriophage during the extraction procedure.

Continuous culture. For transcriptomic analysis, *T. denticola* cells were grown in continuous culture using a C-30 biofermenter (New Brunswick Scientific) that was modified to incorporate four suspended glass tubes (length 6.2 cm, internal diameter 0.8 cm, glass thickness 0.15 cm) that were closed off by stainless steel housings at the top and bottom ends. The glass tubes also contained vertical slits (0.3 cm wide) to allow circulation of the planktonic cells and growth medium. The inner surface of each tube (total area ~12.5 cm^2) provided an environment with reduced shear forces to enhance biofilm attachment. Prior to insertion into the chemostat, sterile glass rods were coated with fibrinogen by incubating the rods overnight in a 10 mg fibrinogen ml^{-1} filter-sterilized solution. The culture vessel was continuously gassed with 10 % CO_2 in N_2 and the temperature was maintained at 37 °C. The working volume was 365 ml and the dilution rate was 0.027 h^{-1}, to give a mean generation time of 26 h and a μ_{rel} of 0.47. Once the cell density of the culture stabilized (OD_{650}=0.3), fibrinogen-coated glass tubes were aseptically inserted into the chemostat.

Glass tubes were incubated for 14 days within the chemostat to allow sufficient biofilm to form. Planktonic cells were harvested immediately prior to the removal of the glass tubes. A 40 ml aliquot of planktonic cells was collected and 8 ml RNA stabilization reagent (RSR; 5 % phenol in absolute ethanol) (Bhagwat *et al.*, 2003) was added. Cells were then pelleted by centrifugation (9000 ***g***, 5 min, 25 °C) and immediately frozen in liquid nitrogen after removal of the supernatant. Cell pellets were stored at −80 °C for later processing.

Additional 40 ml aliquots of cells were pelleted by centrifugation (9000 ***g***, 5 min, 25 °C) and the supernatant was used as a 'spent medium' buffer to harvest the biofilm so as to minimize changes to the expression profile. RSR (8 ml) was added to the spent medium, which was then used to sluice the cells off the glass tubes. The resuspended biofilm cells were then collected by centrifugation and frozen in the same manner as the planktonic cells. The biofilm cell pellets from four glass tubes were combined to yield enough RNA for transcriptomic analysis.

Enumeration of bacteria. Planktonic *T. denticola* cells (200 µl) were sampled from the chemostat and centrifuged (8000 ***g***, 10 min, 4 °C) to pellet cells, then suspended in an equal volume of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at −20 °C. Biofilm *T. denticola* was sampled by removing a curved glass rod from the chemostat and detaching biofilm cells from the support directly into PBS. The sample was then vortexed and 200 µl samples centrifuged (8000 ***g***, 10 min, 4 °C) before suspension in TE buffer and storage at −20 °C. Quantification of genomic DNA was performed using the Quant-iT Narrow Range DNA Assay kits (Invitrogen) according to the manufacturer's instructions.

Transcriptomic analysis

RNA extraction. Extraction of total RNA was performed in a similar manner for both biofilm and planktonic cell pellets, using a combination of mechanical lysis in the presence of TRIzol reagent (Invitrogen) followed by silica-membrane spin-column purification using the Illustra RNAspin Mini RNA Isolation kit including on-column DNase treatment (GE Healthcare), as previously described (Dashper *et al.*, 2009). RNA integrity was determined using the Experion automated electrophoresis station (Bio-Rad) and only high-quality RNA with a 23S : 16S ratio of >1.7 was used for transcriptomic analyses.

Microarray design, hybridization and analysis. Microarray slides were printed by the Australian Genome Research Facility, and consisted of custom-designed 60-mer oligonucleotide probes for 2518 predicted ORFs likely to encode proteins from the *T. denticola* 35405 genome. The full complement of probes was printed four times per microarray slide onto UltraGAPS slides (Corning).

cDNA was synthesized from 10 µg total RNA using the SuperScript Plus Indirect cDNA Labeling System (Invitrogen), with 5 µg random hexamers (Invitrogen) for priming of the cDNA synthesis reaction. cDNAs were labelled with the Amersham CyDye Post-Labeling Reactive Dye Pack (GE Lifesciences) and purified using the purification module of the Invitrogen labelling system. Microarray slides were pre-blocked, hybridized at 42 °C for 16 h and then washed (Dashper *et al.*, 2009). Microarray slides were scanned using a G2565BA microarray scanner (Agilent Technologies).

Four sets of paired biofilm and planktonic samples were harvested from three independent continuous cultures. Paired samples were compared on the same microarray using a two-colour system. A total of 10 paired microarray hybridizations were performed, incorporating technical replicates. A balanced dye design was used, in which dye swaps were performed on at least three of the paired sample sets and the overall analysis included five microarrays where planktonic samples were labelled with Cy3 and the paired biofilm samples were labelled with Cy5, and five other microarrays where samples were labelled with the opposite combination of fluorophores. Image analysis was performed using the GenePix Pro 6.0 software (Molecular Devices), and 'morph' values were used as the background

estimates in further analysis. The LIMMA software package (Smyth, 2005) was used to identify differentially expressed genes, and a detailed protocol is presented with the results on ArrayExpress (accession no. E-MEXP-2266). The Benjamini–Hochberg method was used to control the false discovery rate to correct for multiple testing.

Annotation and putative protein functions were based on the National Center for Biotechnology Information (NCBI) Refseq database (accession no. NC_002967.9), and the Clusters of Orthologous Groups of proteins (COG) database (Tatusov *et al.*, 2003). Operon predictions, COG assignments and COG functional categories were obtained from the Microbes Online database (Alm *et al.*, 2005).

Real-time quantitative RT-PCR (qRT-PCR). Differential expression of selected *T. denticola* genes was validated by real-time qRT-PCR using SYBR Green-based detection on a Rotor Gene 3000 system (Corbett Research). cDNA synthesis and RT-PCR were carried out using the Express SYBR GreenER Two-Step qRT-PCR kit (Invitrogen) in accordance with the manufacturer's instructions. For each sample, 750 ng RNA was used in a 40 µl cDNA synthesis reaction and in a negative control reaction lacking the reverse-transcription enzyme. Following termination of the reaction cDNA reactions and negative controls were diluted 300-fold with nuclease-free water, and 2 µl was used as the template for qRT-PCR in a 20 µl reaction volume. To normalize the amount of mRNA in each reaction, a pool of 10 potential housekeeping genes from a variety of functional classes were assessed for stability using the geNorm program (Vandesompele *et al.*, 2002). Analysis indicated the geometric mean of the three most stable genes (TDE0002, TDE0601 and TDE0872; with average pair-wise variations of 0.288, 0.234 and 0.238, respectively) was sufficient to calculate a normalization factor for each sample. Primer concentrations were 200 nM, with the exception of TDE0762 and TDE1979, which were 400 nM. Primer sequences were as follows: TDE0002_F, 5′-GGACAATGGCCGAGGTATTC-3′; TDE0002_R, 5′-AACGCCTACTCCGTGCAAAC-3′; TDE0601_F, 5′-CTAAGGCCG-TTGCAGGTTTC-3′; TDE0601_R, 5′-CTGTTGCTCTGGCTGC-AATC-3′; TDE0872_F, 5′-GGATGAAGCTCTGGGAATCG-3′; TDE0872_R, 5′-ATCGATAAAGGCGGCTATGC-3′; TDE0391_F, 5′-GCAGCAATTCAATGCGACTC-3′; TDE0391_R, 5′-TTGCCTATCC-AGCTTTCAACTTC-3′; TDE0392_F, 5′-AAAGGCACAAGGGATG-AACG-3′; TDE0392_R, 5′-TTCCATGTGGGTGCGTAAAC-3′; TDE0760_F, 5′-TCCATTGTGCCTGTTTCTGC-3′; TDE0760_R, 5′-TATCCGGTTCGGCATCTACC-3′; TDE0761_F, 5′-GTTGCTGA-CCACGGAACAAG-3′; TDE0761_R, 5′-TACTACCGCCGCCTAT-ACCG-3′; TDE0762_F, 5′-GTTGAAGGCAGCGGTAGGAC-3′; TDE0762_R, 5′-TGCAAATAGCTCGGAAGTGC-3′; TDE1163_F, 5′-TAGACACCGAGGACGGAGTG-3′; TDE1163_R, 5′-AAATATTC-GATGCCGAGTACTTCC-3′; TDE1169_F, 5′-TTCCGGTATACAA-ACCCAACG-3′; TDE1169_R, 5′-TCCGCTTCTTTGTGCATCTC-3′; TDE1173_F, 5′-CCGGCACCAATATGAATCAG-3′; TDE1173_R, 5′-AGCAGGGTAGCGTGGGTATG-3′; TDE1978_F, 5′-ATTGGAC-GCGGCAATCTC-3′; TDE1978_R, 5′-ATTACCGACGCGGTAT-CTCC-3′; TDE1979_F, 5′-AAACGCCCTAAGCCGCTATC-3′; TDE1979_R, 5′-AACTCACACCGCATTTCAGC-3′; TDE2056_F, 5′-CGTGTTGGTGCAAGTCCTTC-3′; and TDE2056_R, 5′-CAAG-TGGAAATGCAGCATCG-3′.

Bacteriophage characterization

Isolation. *T. denticola* batch cultures (400 ml) were incubated for 3 weeks without agitation to allow a thick biofilm to form on the base of the vessel. A negative control containing uninoculated growth medium from the same batch was included to determine the presence of any endogenous bacteriophage. Prior to harvesting, samples were mixed vigorously to release any phage trapped in the biofilm. Bacterial cells and debris were pelleted by centrifugation (11 000 ***g***, 30 min, 4 °C) and discarded. The supernatant was collected and treated with DNase I and RNase A (each at 1 µg ml^{-1}) for 30 min at 37 °C to remove any contaminating extracellular bacterial nucleic acids. NaCl was added to each sample to a final concentration of 1 M and the culture was mixed gently until all salt had dissolved. PEG 8000 (Sigma) was added to a final concentration of 10 % and dissolved at room temperature. Samples were then chilled in ice water for 1 h to precipitate bacteriophages. Bacteriophages were pelleted by centrifugation (13 000 ***g***, 35 min, 4 °C) and the supernatant discarded. The phage pellet was then either resuspended in 2 ml SM medium (0.1 M NaCl, 0.01 M $MgSO_4$, 0.05 M Tris, pH 7.5, with a drop of chloroform) for electron microscopy, or resuspended in 360 µl Buffer ATL (from the DNeasy Blood & Tissue kit, Qiagen) for DNA extraction.

DNA extraction. DNA was obtained from both *T. denticola* lysogens and purified phage using the DNeasy Blood & Tissue kit (Qiagen) in accordance with the manufacturer's instructions for Gram-negative bacteria. Genomic DNA was extracted from both continuous and batch cultures of *T. denticola.*

PCR detection of excised bacteriophage and integrated prophage DNA. To determine whether prophages were excised from the *T. denticola* genome and circularized, PCR amplification was performed using primers directed across the *attP* integration site. The primers used should only produce an amplicon from DNA present in bacteriophages excised from the genome and ligated to form a circularized bacteriophage genome (Primers TDE1173_F and TDE1133_R). These primers should also amplify bacteriophage DNA that has been cleaved at the *cos* site and packaged in the head of functional bacteriophage particles. To detect prophage DNA still integrated within the *T. denticola* 35405 genome, each of these primers was also paired with a primer specific for adjacent DNA found within the lysogen (TDE1173_F with TDE1174_R, TDE1133_R with TDE1132_F). This should enable PCR-based detection of the *attL* and *attR* sites. In addition, PCR was carried out using a control set of primers designed to amplify a region of DNA from TDE1144 (TDE1144_F and TDE1144_R) present in both the integrated prophage and excised bacteriophage DNA.

PCRs were carried out using KAPATaq DNA polymerase (KAPA Biosystems) in accordance with the manufacturer's instructions. Each 25 µl reaction contained 0.2 mM dNTPs, 0.5 µM forward and reverse primers, 0.5 U polymerase and either genomic *T. denticola* DNA or isolated bacteriophage DNA. Primer sequences were as follows: TDE1173_F, 5′-AGGCGATAGCTCCGTTTCTC-3′; TDE1133_R, 5′-ATTGCCCTTGTACCGCTATG-3′; TDE1174_R, 5′-CGGCTCATAT-CCGTTAGGTC-3′; TDE1132_F, 5′-GCGGATGCTTTTCTGGTCTC-3′; TDE1144_F, 5′-GCTGCCTTAAAAGCACCTTG-3′; and TDE1144_R, 5′-GGGAAGCTCAAATTGCAGAG-3′.

PCR conditions were: 94 °C, 2 min; 30 times (94 °C, 30 s; 56 °C, 30 s; 72 °C, 1 min); 72 °C, 10 min. Specificity of PCR products was confirmed by DNA sequencing of the amplicons.

Electron microscopy of bacteriophage particles. Pioloform-coated 100 mesh hexagonal copper grids were incubated on droplets of phage particles for 30 min. Excess solution was drained away on filter paper and the grids incubated onto 2 % aqueous uranyl acetate for 30 s. The uranyl acetate was blotted onto filter paper and the grids dried overnight at room temperature. Coated grids with negatively stained phage were viewed with a Philips CM120 Biotwin transmission electron microscope at 120 kV and digital images acquired with a Gatan Multiscan 600 CW camera.

Electron microscopy of *T. denticola* biofilm. Glass coverslips were carefully removed from the chemostat and excess culture was drained away on filter paper. Coverslips with adhered cells were immersed in 2.5 % glutaraldehyde in PBS for 1 h, rinsed three times in PBS for 10 min each, before being dehydrated in increasing concentrations of

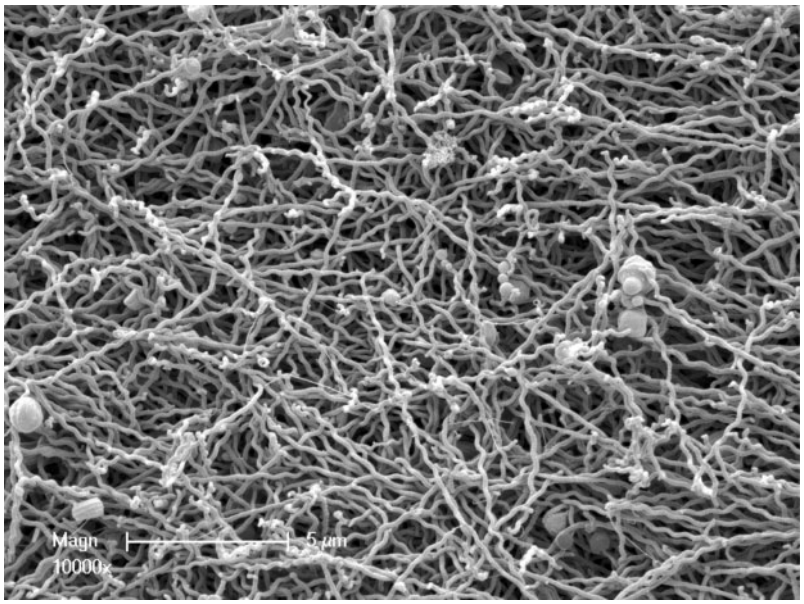

Fig. 1. Scanning electron microscope image of *T. denticola* biofilm after 14 days of incubation. Bar, 5 μm.

ethanol consisting of 10, 30, 50, 70, 90 and 100 % ethanol in water for 10 min each step. The coverslips were dried in a Balzer's critical point dryer and mounted onto 25 mm aluminium stubs with double-sided carbon tabs. The edges of the coverslips were covered with colloidal silver, dried, and then gold-coated in an Edwards S150B sputter coater. Biofilm cells on coverslips were imaged with the Philips XL30 field-emission scanning electron microscope at a voltage of 2 kV.

RESULTS

Growth of *T. denticola* biofilms within continuous culture vessels

A method for the cultivation of *T. denticola* in continuous culture in a chemostat was developed that allowed planktonic and biofilm cells to be grown simultaneously in the same vessel, thereby reducing confounding effects of gene regulation due to variations in media composition, redox potential, temperature fluctuations and other environmental variables. Approximately 7 days after inoculation the density of planktonic *T. denticola* cells in the chemostat reached steady state at 5.7×10^8 cells ml^{-1} as determined using DNA quantification. Glass tubes were suspended in the fermentation vessel of the chemostat after steady-state planktonic cell density had been reached. The thick biofilm of intertwined *T. denticola* cells that developed within the glass tubes after 14 days was visualized using scanning electron microscopy (Fig. 1). The cell density of the biofilm stabilized at 1.0×10^{10} cells cm^{-2}, as determined by DNA quantification (Table 1).

Identification and confirmation of differentially expressed genes

RNA prepared from three independent sets of biofilm and planktonic cultures was analysed for differential transcript expression using DNA microarray. A total of 126 genes were differentially expressed with a fold change of 1.5 or greater at a 5 % level of statistical significance, modified to correct for multiple testing [$\log_2$(fold change) of ± 0.58]. In biofilm cultures, 83 genes met the selection criteria for upregulation (Table 2), while 43 genes were downregulated (Table 3) relative to planktonic cells. Many of these differentially expressed genes were predicted to be polycistronic, with 13 predicted operons containing two or more upregulated genes, and seven predicted operons containing two or more downregulated genes. The direction of change of expression was found to be consistent within predicted operons, with no single operon containing both upregulated and downregulated genes. qRT-PCR analysis of 11 selected genes normalized with three housekeeping genes demonstrated the validity of the DNA microarray analysis, as the fold change data from qRT-PCR significantly correlated (Pearson $R=0.846$, $P<0.001$; Spearman $R=0.879$, $P<0.001$) with the fold change microarray data (Table 4).

Table 1. DNA quantification-based enumeration of *T. denticola* cells growing as a biofilm on the surface of glass rods inserted into a chemostat

Time after insertion (days)	Number of chromosomes* (cm^{-2})
2	0.1×10^{10}
4	0.7×10^{10}
7	0.7×10^{10}
14	1.0×10^{10}
21	1.3×10^{10}
28	1.2×10^{10}

*Represents the average of two biological replicates.

Table 2. *T. denticola* genes whose expression was significantly upregulated by more than 1.5-fold in biofilm cells relative to planktonic cells

Bold type indicates genes within predicted operons where more than one gene was significantly upregulated. TDE0207–TDE0208, TDE0394–TDE0399, TDE1163–TDE1168, TDE1188–TDE1189 and TDE1197–TDE1214 are distinct operons. *P* values were adjusted to control the false discovery rate.

Gene ID	Fold change (log_2)	Adjusted *P* value	Function
TDE0090	**0.64**	**2.2×10^{-13}**	**Putative uncharacterized protein**
TDE0091	**1.04**	**5.0×10^{-17}**	**RNA polymerase sigma-24 factor, putative**
TDE0114	0.73	7.4×10^{-11}	Iron-dependent transcriptional regulator
TDE0158	0.62	2.82×10^{-9}	Putative uncharacterized protein
TDE0165	1.06	1.27×10^{-15}	Putative uncharacterized protein
TDE0178	0.80	1.2×10^{-12}	Methyl-accepting chemotaxis protein
TDE0207	**0.83**	**1.7×10^{-19}**	**Permease, GntP family**
TDE0208	**0.70**	**1.1×10^{-18}**	**Glycerate kinase (EC 2.7.1.31)**
TDE0394	**0.79**	**8.3×10^{-10}**	**Oligopeptide/dipeptide ABC transporter, permease protein**
TDE0395	**0.61**	**1.2×10^{-8}**	**Oligopeptide/dipeptide ABC transporter, permease protein**
TDE0397	**0.85**	**2.2×10^{-11}**	**Oligopeptide/dipeptide ABC transporter, ATP-binding protein**
TDE0398	**0.88**	**7.2×10^{-18}**	**Oligopeptide/dipeptide ABC transporter, periplasmic peptide-binding protein***
TDE0399	**1.27**	**7.4×10^{-18}**	**Zinc carboxypeptidase family protein**
TDE0403	0.60	3.1×10^{-13}	Conserved domain protein
TDE0409	**0.84**	**1.3×10^{-14}**	**Putative uncharacterized protein**
TDE0410	**0.60**	**1.6×10^{-13}**	**Peptidase, M48 family***
TDE0462	0.70	9.2×10^{-11}	Metallo-β-lactamase family protein
TDE0482	0.65	2.2×10^{-13}	Putative uncharacterized protein
TDE0525	0.87	1.1×10^{-18}	Putative uncharacterized protein
TDE0572	0.89	7.7×10^{-19}	Conserved domain protein*
TDE0603	0.62	4.3×10^{-14}	Putative uncharacterized protein
TDE0626	1.26	7.2×10^{-18}	Putative uncharacterized protein*
TDE0718	1.40	2.0×10^{-18}	Putative uncharacterized protein
TDE0734	1.04	2.2×10^{-23}	Conserved domain protein, degenerate
TDE0760	**0.78**	**1.4×10^{-17}**	**Conserved domain protein**
TDE0761	**0.59**	**2.6×10^{-13}**	***prcA*, Protease complex-associated polypeptide precursor (protease complex-associated polypeptide) (dentilisin-associated protein)***
TDE0816	0.73	1.2×10^{-08}	Peptidase, M20/M25/M40 family
TDE0948	1.00	8.8×10^{-22}	Conserved domain protein
TDE0975	1.32	2.2×10^{-23}	Putative uncharacterized protein
TDE0999	0.97	1.7×10^{-19}	Formate/nitrite transporter
TDE1000	0.66	1.4×10^{-17}	3-Hydroxyacid dehydrogenase family protein
TDE1072	**1.17**	**1.8×10^{-14}**	**Lipoprotein, putative***
TDE1074	**0.60**	**1.2×10^{-12}**	**Oligopeptide/dipeptide ABC transporter, permease protein**
TDE1102	1.06	3.6×10^{-18}	Putative uncharacterized protein
TDE1123	0.65	3.8×10^{-17}	Sigma factor regulatory protein, putative
TDE1163	**1.04**	**2.0×10^{-16}**	**Putative uncharacterized protein**
TDE1164	**0.79**	**2.6×10^{-11}**	**Conserved domain protein**
TDE1165	**1.25**	**7.2×10^{-19}**	**Putative uncharacterized protein**
TDE1166	**1.14**	**1.2×10^{-12}**	**Putative uncharacterized protein**
TDE1168	**1.34**	**1.8×10^{-13}**	**Putative uncharacterized protein**
TDE1188	**0.62**	**7.2×10^{-16}**	**NAD(P) transhydrogenase, beta subunit (EC 1.6.1.2)**
TDE1189	**0.89**	**1.7×10^{-16}**	**NAD(P) transhydrogenase, alpha subunit, authentic frameshift**
TDE1197	**1.24**	**2.2×10^{-23}**	**Protein *mraZ***
TDE1198	**0.80**	**1.4×10^{-17}**	***S*-Adenosyl-L-methionine-dependent methyltransferase *mraW* (EC 2.1.1.–)**
TDE1199	**0.61**	**1.5×10^{-15}**	**Cell division protein FtsL, putative**
TDE1203	**0.95**	**1.1×10^{-20}**	**Cell division protein FtsA**
TDE1214	**0.67**	**1.3×10^{-14}**	**Flagellar hook–basal body complex protein *fliE***
TDE1236	0.64	1.7×10^{-14}	Triosephosphate isomerase (EC 5.3.1.1)
TDE1246	0.68	1.1×10^{-18}	Lipoprotein, putative*

Table 2. cont.

Gene ID	Fold change (log_2)	Adjusted *P* value	Function
TDE1296	0.91	1.6×10^{-20}	Ribosomal subunit interface protein, putative
TDE1328	0.64	1.0×10^{-14}	Putative uncharacterized protein
TDE1378	0.76	4.3×10^{-09}	Conserved domain protein
TDE1383	0.90	6.0×10^{-16}	Putative uncharacterized protein
TDE1430	1.05	6.9×10^{-05}	β-1,4-Galactosyltransferase, putative
TDE1455	0.80	2.2×10^{-14}	Prevent-host-death family protein
TDE1463	0.73	3.1×10^{-12}	Putative uncharacterized protein
TDE1474	0.70	7.3×10^{-13}	Putative uncharacterized protein
TDE1480	1.01	6.9×10^{-19}	Putative uncharacterized protein
TDE1484	1.04	3.8×10^{-14}	Putative uncharacterized protein
TDE1584	0.92	4.2×10^{-16}	Lipoprotein, putative*
TDE1593	**0.62**	**2.0×10^{-14}**	**Fe-hydrogenase**
TDE1594	**1.09**	**1.1×10^{-17}**	**Pyridine nucleotide-disulphide oxidoreductase family protein**
TDE1668	**1.05**	**4.6×10^{-14}**	**Amino acid permease family protein**
TDE1669	**0.79**	**4.6×10^{-14}**	**Cystalysin (haemolysin)**
TDE1701	0.99	3.5×10^{-14}	Putative uncharacterized protein
TDE1857	1.03	2.0×10^{-17}	Putative uncharacterized protein
TDE1891	0.59	1.6×10^{-08}	Putative uncharacterized protein
TDE1936	0.72	1.3×10^{-10}	Putative uncharacterized protein
TDE1938	0.88	1.1×10^{-20}	Putative uncharacterized protein
TDE1976	1.11	1.0×10^{-15}	Putative uncharacterized protein
TDE1978	**0.82**	**6.9×10^{-09}**	**RelE toxin family protein**
TDE1979	**1.02**	**2.6×10^{-23}**	**RelB antoxin family protein**
TDE2035	0.81	4.7×10^{-18}	Glucose-1-phosphate adenylyltransferase (EC 2.7.7.27)
TDE2106	0.66	7.9×10^{-15}	Putative uncharacterized protein
TDE2122	0.63	6.5×10^{-11}	DHH superfamily protein
TDE2135	0.61	4.7×10^{-06}	Putative uncharacterized protein
TDE2199	**0.82**	**1.6×10^{-17}**	**Racemase, Asp/Glu/hydantoin family**
TDE2200	**0.70**	**3.1×10^{-15}**	**Methionine γ-lyase (EC 4.4.1.11)**
TDE2211	0.67	1.3×10^{-14}	Putative uncharacterized protein*
TDE2542	0.63	3.5×10^{-18}	Antigen, putative*
TDE2691	0.83	2.9×10^{-15}	Putative uncharacterized protein
TDE2707	0.82	5.1×10^{-14}	Putative uncharacterized protein
TDE2714	1.02	1.2×10^{-14}	Putative uncharacterized protein

*Genes predicted to encode lipoproteins.

Consistent with the high level (50 %) of genes in the *T. denticola* 35405 genome for which there is no known function (Seshadri *et al.*, 2004), a high proportion (41 %) of differentially expressed genes were also of unknown function, including 44 genes predicted to encode uncharacterized proteins and eight genes encoding domains conserved in other organisms but with no known function.

The major virulence factor cystalysin, encoded by TDE1669, is predicted to be co-transcribed with TDE1668 and both genes showed very similar upregulation of expression in biofilm cells (Table 2). Adherence plays a critical role in the formation of thick, multilayered biofilms, and transcripts encoding several outer sheath proteins, including lipoproteins, were upregulated in the biofilm. The *T. denticola* genome is predicted to encode 166 lipoproteins based on leader sequence analysis (Setubal *et al.*, 2006). The level of transcript expression was higher in the biofilm for 10 of these predicted lipoproteins, whereas four lipoproteins showed a higher level of expression in planktonic cells (Tables 2 and 3). The outer membrane dentilisin lipoprotein complex is encoded by the TDE0761 and TDE0762 genes, which are predicted to be co-transcribed with TDE0760. TDE0760 and TDE0761 were significantly upregulated in the biofilm compared with the planktonic cells (Table 2), and TDE0762 showed a similar trend, with a statistically significant 1.3-fold increase. qRT-PCR analysis confirmed that transcription of the TDE0760–0762 operon was statistically significantly upregulated. Interestingly, the qRT-PCR analysis also confirmed that the section of the transcript from the first gene in the operon was more abundant than those sections of the transcript from the second and third genes,

Table 3. *T. denticola* genes whose expression was significantly downregulated by more than 1.5-fold in biofilm cells relative to planktonic cells

Bold type indicates genes within predicted operons where more than one gene was significantly upregulated. TDE2421–TDE2425, and TDE2466–TDE2467 are distinct operons. *P* values were adjusted to control the false discovery rate.

Gene ID	Fold change (log_2)	Adjusted *P* value	Function
TDE0001	−0.66	7.0×10^{-05}	Chromosomal replication initiator protein *dnaA*
TDE0011	−1.15	1.9×10^{-12}	Probable peroxiredoxin (EC 1.11.1.15)
TDE0130	−0.80	4.3×10^{-13}	Sodium/dicarboxylate symporter family protein
TDE0306	−0.63	9.5×10^{-13}	Signal peptide peptidase SppA
TDE0362	−0.62	4.4×10^{-10}	Bacterial immunoglobulin-like domain protein*
TDE0386	−0.62	4.4×10^{-09}	ABC transporter, periplasmic substrate-binding protein
TDE0387	**−0.71**	**1.8×10^{-12}**	**(*R*)-Hydroxyglutaryl-CoA dehydratase activator**
TDE0389	**−0.85**	**2.9×10^{-11}**	**(*R*)-2-Hydroxyglutaryl-CoA dehydratase, β subunit, putative**
TDE0392	−1.04	3.3×10^{-17}	(*R*)-2-Hydroxyglutaryl-CoA dehydratase, β subunit, putative
TDE0484	−0.67	4.2×10^{-15}	Methyl-accepting chemotaxis protein
TDE0591	−0.65	3.1×10^{-14}	Acetyl-CoA carboxylase, biotin carboxylase (EC 6.4.1.2)
TDE0629	−0.66	5.1×10^{-15}	Chaperone protein *dnaJ*
TDE0681	**−0.66**	**3.5×10^{-08}**	**Protein-export membrane protein SecD**
TDE0682	**−0.68**	**5.1×10^{-13}**	**Preprotein translocase, YajC subunit**
TDE0748	−0.62	3.8×10^{-13}	Iron compound ABC transporter, periplasmic iron compound-binding protein, putative
TDE0937	−1.04	2.0×10^{-16}	RNA polymerase sigma-70 factor family protein
TDE1009	−0.66	1.7×10^{-15}	Methyl-accepting chemotaxis protein
TDE1012	−0.64	7.1×10^{-11}	Tex protein, putative
TDE1028	**−1.12**	**3.2×10^{-12}**	**Putative uncharacterized protein**
TDE1029	**−1.21**	**6.0×10^{-16}**	**Hsp20/α-crystallin family protein**
TDE1109	−0.60	3.6×10^{-11}	Decarboxylase, pyridoxal-dependent family
TDE1112	−0.72	2.2×10^{-14}	Adenylate kinase (EC 2.7.4.3) (ATP–AMP transphosphorylase)
TDE1585	**−0.83**	**3.9×10^{-11}**	**Putative uncharacterized protein**
TDE1586	**−0.85**	**1.1×10^{-13}**	**Putative uncharacterized protein**
TDE2056	−0.70	6.6×10^{-10}	Outer membrane haemin-binding protein A*
TDE2078	−0.64	3.4×10^{-14}	Tetratricopeptide repeat (TPR) domain protein
TDE2130	**−0.63**	**1.4×10^{-17}**	**Putative uncharacterized protein**
TDE2131	**−0.69**	**1.5×10^{-15}**	**Cobalamin biosynthesis protein CbiM, putative**
TDE2144	−0.66	1.1×10^{-17}	Putative uncharacterized protein
TDE2339	−0.60	1.2×10^{-10}	Leucyl-tRNA synthetase (EC 6.1.1.4)
TDE2347	−0.67	5.7×10^{-15}	30S ribosomal protein S2
TDE2372	−0.69	2.1×10^{-16}	Putative uncharacterized protein*
TDE2421	**−0.63**	**5.7×10^{-08}**	**DNA-directed RNA polymerase subunit β (EC 2.7.7.6) (RNAP subunit β) (transcriptase subunit beta) (RNA polymerase subunit β)**
TDE2425	**−0.62**	**9.6×10^{-11}**	**50S ribosomal protein L11**
TDE2466	**−0.63**	**2.4×10^{-10}**	**Putative uncharacterized protein**
TDE2467	**−0.81**	**1.8×10^{-14}**	**Conserved domain protein***
TDE2473	−1.02	1.2×10^{-14}	30S ribosomal protein S21
TDE2476	−0.74	6.3×10^{-13}	Carbamate kinase (EC 2.7.2.2)
TDE2526	−0.78	1.8×10^{-12}	Putative uncharacterized protein
TDE2547	−0.59	2.6×10^{-11}	Putative uncharacterized protein
TDE2549	−0.59	6.9×10^{-14}	Methyl-accepting chemotaxis protein
TDE2608	−0.61	2.1×10^{-13}	Putative uncharacterized protein
TDE2661	−0.69	4.3×10^{-13}	Putative uncharacterized protein

*Genes predicted to encode lipoproteins.

indicating further regulation of this operon (Table 4). Conversely, some predicted surface-exposed proteins such as TDE0362 and TDE1012 showed higher transcript levels in planktonic cells (Table 3).

Several putative annotated homologues of TA systems had higher transcript levels in the biofilm than in planktonic cells (TDE1455, TDE1978 and TDE1979; Table 2). The RASTA-Bacteria tool (Sevin & Barloy-Hubler, 2007) was

Table 4. Expression of selected *T. denticola* genes determined by qRT-PCR

Fold change is expressed as the $\log_2$ of the ratio of transcript abundance in biofilm cells to that in planktonic cells. Three genes (TDE0002, TDE0601 and TDE0872) were used as references. Bold type indicates genes within predicted operons. Distinct operons are delineated by sequential numbering.

Gene ID	qRT-PCR fold change ($\log_2$)	qRT-PCR *P* value	Microarray fold change ($\log_2$)	Function
TDE0002	0.18	0.16	−0.04	*gyrB* DNA gyrase subunit B
TDE0601	−0.10	0.17	−0.10	*fabD* Malonyl-CoA-acyl carrier protein transacylase
TDE0872	−0.08	0.35	−0.19	*recA* Recombinase A protein
TDE0391	**−2.03**	**0.005**	**−0.13**	**Putative uncharacterized protein**
TDE0392	**−1.72**	**0.013**	**−1.04**	**(*R*)-2-Hydroxyglutaryl-CoA dehydratase, beta subunit, putative**
TDE0760	**1.09**	**0.037**	**0.78**	**Predicted membrane-associated protein**
TDE0761	**0.61**	**0.009**	**0.59**	***prcA* Protease complex-associated polypeptide, dentilisin-associated protein (lipoprotein)**
TDE0762	**0.48**	**0.046**	**0.36**	***prtP* Dentilisin**
TDE1163	0.84	0.038	1.04	Putative uncharacterized protein (prophage)
TDE1169	2.02	0.008	Not determined*	Phage DNA-binding protein, excisionase family
TDE1173	−0.50	0.53	0.04	Site-specific recombinase, phage integrase family (prophage)
TDE1978	**0.87**	**0.004**	**0.82**	**Putative RelE toxin**
TDE1979	**1.30**	**0.016**	**1.02**	**Putative RelB antitoxin**
TDE2056	−0.73	0.012	−0.70	Outer membrane haemin-binding protein A (putative lipoprotein)

*No microarray probe present for predicted ORF.

used to globally predict potential TA systems for *T. denticola* 35405, and these genes and their respective expression ratios are shown in Table 5. A high proportion of these loci (25/33) showed statistically significant increases in the transcript levels present in the biofilm for one or more members of the predicted TA system. Only one TA locus (TDE1837) was significantly downregulated in the biofilm cells. qRT-PCR was used to confirm the upregulation of the TDE1978/9 TA genes (Table 4).

A family of putative transposases were upregulated in the biofilm cells. *T. denticola* 35405 has 35 predicted members of the Interpro protein family IPR010106 that show some similarity to protein domains found in known transposases; however, none of these proteins has been functionally characterized. Over 70 % (25/35) of the genes encoding these proteins showed a statistically significant higher level of expression in the biofilm (Table 6).

Expression of prophage genes in biofilm culture

The genomic DNA sequence of *T. denticola* 35405 (Seshadri *et al.*, 2004) revealed several regions of unusual trinucleotide composition, including a cluster of 41 genes (TDE1133–TDE1173), some of which encode putative proteins that have significant amino acid sequence similarity to known bacteriophage proteins. Bioinformatic analyses of the prophage indicated the presence of a complete lysogenic phage. Three of the putative prophage genes were amongst the most upregulated genes during biofilm growth. In total, 19 of the putative prophage genes were statistically significantly upregulated and no bacteriophage genes were significantly downregulated (Table 7). The upregulation of TDE1163 was confirmed by qRT-PCR, and the fourfold upregulation of TDE1169, the putative phage excisionase gene, for which there was no microarray probe, was demonstrated. The lack of differential expression of TDE1173, the phage integrase gene, was also confirmed (Table 4).

Detection of functional bacteriophage

To demonstrate lytic phage activity, PCR primers were designed to amplify across the *att* region of the circularized bacteriophage genome (Fig. 2, region from TDE1133 to TDE1173). During insertion into the bacterial chromosome, the bacteriophage genome is linearized at the *att* site, so this PCR amplicon should only be produced when the DNA from excised, circularized (or packaged) bacteriophage is present. No amplicon should be produced when only integrated prophage is present. PCR amplification detected an amplicon of the correct size (~900 bp) specific for excised bacteriophage, using template DNA extracted from the purified bacteriophage preparation from biofilm cells (results not shown). Excised bacteriophage was also detected when using purified bacterial DNA as the template from steady-state planktonic chemostat cultures of

Table 5. Changes in mRNA expression of predicted TA-encoding genes in *T. denticola* 35405 between biofilm and planktonic cells

Bold type indicates differentially expressed with an adjusted *P* value of <0.05.

Antitoxin			Toxin			Putative TA family
Gene ID	Fold change (log_2)	Adjusted *P* value	Gene ID	Fold change (log_2)	Adjusted *P* value	
TDE0036	**0.19**	**0.01**	TDE0035	0.01	0.9	*vapBC*
TDE0196	**0.33**	**1.4×10^{-03}**	**TDE0197**	**0.33**	**8.9×10^{-05}**	*vapBC*
TDE0225	0.05	0.5	TDE0224	0.13	0.06	*phd/doc*
TDE0235	0.11	0.2	**TDE0236**	**0.32**	**7.0×10^{-06}**	*vapBC*
TDE0278	**0.33**	**2.7×10^{-06}**	TDE0277	0.08	0.3	*vapBC*
TDE0281	**0.54**	**3.4×10^{-11}**	**TDE0280**	**0.23**	**1.3×10^{-04}**	*vapBC*
TDE0302	0.11	0.2	TDE0303	−0.03	0.9	*relBE*
TDE0452	Not determined*		**TDE0453**	**0.50**	**3.2×10^{-14}**	*hicAB*
TDE0465	**0.20**	**0.03**	**TDE0464**	**0.22**	**9.7×10^{-04}**	*vapBC*
TDE0481	**0.51**	**6.9×10^{-09}**	**TDE0482**	**0.65**	**2.2×10^{-13}**	*hicAB*
TDE0495	**0.21**	**0.01**	TDE0494	0.16	0.1	*hipBA*
TDE0506	**0.44**	**1.3×10^{-08}**	**TDE0505**	**0.16**	**0.05**	*relBE*
TDE0600	−0.06	0.3	TDE0599	−0.05	0.5	*vapBC*
TDE0663	**0.22**	**0.01**	TDE0662	Not determined*		*relBE*
TDE0699	**0.51**	**1.6×10^{-07}**	TDE0698	0.10	0.2	*vapBC*
TDE0736	**0.52**	**1.6×10^{-13}**	**TDE0735**	**0.52**	**4.1×10^{-10}**	*relBE*
TDE0738	0.04	0.7	TDE0737	0.01	0.9	*relBE*
TDE0888	**0.23**	**2.8×10^{-03}**	**TDE0887**	**0.20**	**2.7×10^{-03}**	*vapBC*
TDE0990	**0.27**	**7.1×10^{-06}**	**TDE0989**	**0.25**	**2.5×10^{-03}**	*hicAB*
TDE1116	**0.46**	**2.6×10^{-07}**	**TDE1115**	**0.51**	**1.2×10^{-09}**	*relBE*
TDE1304	Not determined*		**TDE1305**	**0.40**	**9.2×10^{-08}**	*hipBA*
TDE1648	0.15	0.06	TDE1647	Not determined*		*relBE*
TDE1790	0.10	0.2	**TDE1789**	**0.24**	**8.3×10^{-05}**	*higBA*
TDE1813	0.11	0.2	TDE1812	0.10	0.1	*higBA*
TDE1837	**–0.11**	**0.04**	TDE1838	0.10	0.3	*hicAB*
TDE1962	**0.43**	**2.6×10^{-08}**	**TDE1961**	**0.21**	**2.0×10^{-03}**	*vapBC*
TDE1979	**1.04**	**2.6×10^{-23}**	**TDE1978**	**0.82**	**6.9×10^{-09}**	*relBE*
TDE2126	**0.18**	**2.2×10^{-03}**	TDE2127	0.02	0.8	*vapBC*
TDE2214	**0.24**	**1.7×10^{-04}**	**TDE2213**	**0.37**	**2.5×10^{-05}**	*hicAB*
TDE2531	**0.37**	**2.5×10^{-07}**	TDE2532	−0.04	0.6	*vapBC*
TDE2563	**0.34**	**8.0×10^{-05}**	**TDE2564**	**0.32**	**6.3×10^{-04}**	*mazEF*
TDE2578	0.16	0.05	TDE2577	0.19	0.08	*vapBC*
TDE2779	**0.32**	**1.2×10^{-8}**	**TDE2778**	**0.24**	**3.7×10^{-7}**	*pspBC*

*No microarray probe present for predicted ORF.

T. denticola 35405. Sequencing confirmed that these amplicons were identical and had the sequence expected for excised, circularized bacteriophage (EMBL accession no. FN 599804). Electron microscopy of material precipitated from the *T. denticola* culture by PEG revealed the presence of many bacteriophage-like particles ~50 nm in diameter (Fig. 3). No such particles were observed in material from uninoculated growth medium (results not shown).

Bioinformatic analysis of bacteriophage φtd1

An examination of the annotation of the prophage-coding sequences (TDE1133–TD1173), intergenic regions and putative *att* sites detected several differences and additions from the published annotation of *T. denticola* 35405 (Fig. 2, Table 7). The predicted functions, order and relative gene orientations within the predicted prophage are consistent with tailed, double-stranded DNA, temperate bacteriophages from the order Caudovirales. Previously discovered spirochaete bacteriophages are of the order Caudovirales and have been observed to have contractile tails, indicating that they are members of the family *Myoviridae* (Eggers *et al.*, 2000).

The bacteriophage is 37 920 bp in length and has a GC content of 37.3 %, similar to that of the *T. denticola* host (37.9 %). Sequencing of the *attP* region of the excised bacteriophage revealed the location of the attachment site in the genome of *T. denticola* 35405. The prophage is inserted into a Met tRNA at a 17 bp predicted *attB* site, 5′-TAGCGGCAATAGGGCTT-3′, identical to the predicted

Table 6. Changes in mRNA expression of genes from a putative transposase family in *T. denticola* 35405 between biofilm and planktonic cells

Bold type indicates differentially expressed with an adjusted *P* value of <0.05.

Gene ID	Fold change (log_2)	Adjusted *P* value
TDE0049	**0.25**	**4.9×10^{-06}**
TDE0165	**1.06**	**1.3×10^{-15}**
TDE0403	**0.60**	**3.1×10^{-13}**
TDE0669	**0.55**	**7.0×10^{-10}**
TDE0734	**1.04**	**2.2×10^{-23}**
TDE0830	**0.26**	**1.7×10^{-03}**
TDE0831	**0.23**	**4.6×10^{-02}**
TDE0862	**0.34**	**6.2×10^{-05}**
TDE0948	**1.00**	**8.8×10^{-22}**
TDE0975	**1.32**	**2.2×10^{-23}**
TDE1032	**0.33**	**1.4×10^{-03}**
TDE1058	−0.04	6.1×10^{-01}
TDE1344	**0.36**	**1.5×10^{-03}**
TDE1691	0.04	5.6×10^{-01}
TDE1891	**0.59**	**1.6×10^{-08}**
TDE1904	0.04	6.9×10^{-01}
TDE1906	**0.49**	**9.5×10^{-05}**
TDE1922	**0.43**	**7.0×10^{-08}**
TDE1938	**0.88**	**1.1×10^{-20}**
TDE1976	**1.11**	**1.0×10^{-15}**
TDE1991	0.10	1.6×10^{-01}
TDE2097	0.01	9.5×10^{-01}
TDE2098	−0.01	9.0×10^{-01}
TDE2135	**0.61**	**4.7×10^{-06}**
TDE2136	−0.06	6.8×10^{-01}
TDE2196	0.07	4.8×10^{-01}
TDE2436	0.05	4.8×10^{-01}
TDE2556	**0.52**	**2.1×10^{-05}**
TDE2560	**0.41**	**2.9×10^{-07}**
TDE2689	0.00	9.6×10^{-01}
TDE2691	**0.83**	**6.4×10^{-15}**
TDE2707	**0.82**	**5.1×10^{-14}**
TDE2711	**0.52**	**1.6×10^{-04}**
TDE2719	**0.20**	**4.9×10^{-02}**
TDE2771	**0.37**	**5.2×10^{-08}**

attP site found in the bacteriophage. The 3′ end of the Met tRNA is also present in the bacteriophage, so that the gene is not disrupted by the integration of the bacteriophage, but instead this part of the gene becomes duplicated in the bacterial chromosome.

The predicted domain functions and structural characteristics of genes TDE1133–TDE1136 indicate that they are equivalent to *R* (lysin), *Rz*, *Rz1* and *S* (holin), which form the phage lysis module in bacteriophage λ. TDE1133 encodes a three-transmembrane-domain protein with a strongly polar N terminus and the two potential Met starting cordons typical of the holin–antiholin proteins (Ramanculov & Young, 2001). The starting codon for TDE1134 appears to be incorrectly annotated in the RefSeq entry for *T. denticola* 35405. If the 5′ end of the coding region is extended for an additional 225 bp, the result is an overlapping configuration with the coding region of TDE1135, with the two predicted proteins occurring in different reading frames of the genome. This unusual configuration is often found for the *Rz*/*Rz1* genes, believed to encode a protein complex that spans the periplasm of Gram-negative bacteria (Summer *et al.*, 2007). Consistent with this hypothesis, the extended version of TDE1134 is predicted to encode a transmembrane protein that has a signal peptide, similar to other Rz1 proteins, although the transmembrane region of predicted Rz1 proteins in other bacteriophages and prophages is at the N terminus, whereas the TDE1134-encoded protein has a C-terminal transmembrane domain. TDE1135 encodes a putative lipoprotein (as does the bacteriophage λ *Rz* gene) that has an N-terminal amino acid sequence matching the conserved motif of spirochaete lipoproteins (Setubal *et al.*, 2006). Protein sequence similarity data place TDE1136 into the broad M23/M37 peptidase families typical of cell wall-degrading enzymes, suggesting that this gene encodes the bacteriophage lysin.

TDE1142 encodes a protein of 2689 amino acids in length that may function as a bacteriophage tape measure protein involved in determining the length of the tail. No tail proteins were observed for φtd1, and the large size of the TDE1142 protein suggests that the tail is very long and possibly easily lost, especially during sample preparation. This protein has significant similarity with other known phage proteins, including the *Bortedella* phage BPP-1 (GI: 41179371), enterobacterial shigella-toxin-converting phages (GI:116222027, 32128229, 9632538, 20065846, 9633467 and 32170884), and putative prophage proteins in *Desulfovibrio piger*, *Campylobacter upsaliensis* and *Photobacterium profundum*.

DISCUSSION

T. denticola exists in the oral cavity as part of a polymicrobial biofilm in the relatively protected region of the gingival crevice or periodontal pocket. *T. denticola* tends to inhabit the deeper periodontal pockets and is not an early colonizer of subgingival plaque (Kolenbrander *et al.*, 2002). This habitat is largely protected from the shear forces associated with salivary flow and the effects of mastication. This study focused on the identification of gene products that may be important for the persistence of *T. denticola* in deep biofilms rather than the initial stages of biofilm formation by this bacterium. To achieve this we developed a continuous-culture model system that allowed the simultaneous sampling of biofilm and planktonic cells that were growing in the same environment. Dense, deep biofilms formed slowly on the inner surfaces of the fibrinogen-coated glass tubes used in this study to simulate the low-shear-force environment of the periodontal pocket (Table 1, Fig. 1). A genome-wide transcriptomics approach was used to determine the effects of growth as part of a

Table 7. Changes in mRNA expression of predicted prophage genes in *T. denticola* 35405 between biofilm and planktonic cells

Genes with significant adjusted *P* values are shown in bold type, and those with altered annotation from that in GenBank (accession no. NC_002697.9) are in parentheses.

Gene ID	Size (aa)	Strand	Function	Fold change (log_2)	Adjusted *P* value
TDE1133	101	−	(Holin)	0.06	0.6
TDE1134	159*	−	(Rz1)	0.03	0.7
TDE1135	**116**	−	**(Rz)**	**0.31**	**7.2×10^{-04}**
TDE1136	**168**	−	**(Lysin)**	**0.30**	**2.3×10^{-05}**
TDE1137	227	−	Putative uncharacterized protein	0.11	0.1
TDE1138	226	−	Putative uncharacterized protein	0.09	0.3
TDE1139	352	−	Putative uncharacterized protein	0.02	0.9
TDE1140	371	−	Putative uncharacterized protein	−0.04	0.5
TDE1141	527	−	Tail fibre domain protein	0.06	0.5
TDE1142	**2689**	−	**(Tape measure)**	**0.14**	**0.03**
TDE1143	759	−	Putative uncharacterized protein	0.16	0.1
TDE1144	335	−	Putative uncharacterized protein	0.14	0.3
TDE1145	647	−	Putative uncharacterized protein	0.13	0.09
TDE1146	196	−	Putative uncharacterized protein	0.06	0.5
TDE1147	143	−	Putative uncharacterized protein	0.18	0.1
TDE1148	**127**	−	**Putative uncharacterized protein**	**0.46**	**2.9×10^{-07}**
TDE1149	**320**	−	**Putative uncharacterized protein**	**0.49**	**7.4×10^{-09}**
TDE1150	**280**	−	**Putative uncharacterized protein**	**0.55**	**8.5×10^{-12}**
TDE1151	88	−	Putative uncharacterized protein	−0.06	0.6
TDE1152	560	−	Head-to-tail joining protein, putative	0.11	0.2
TDE1153	198	−	Putative uncharacterized protein	0.15	0.1
TDE1154	81	−	Putative uncharacterized protein	−0.11	0.3
TDE1155	**99**	−	**Putative uncharacterized protein**	**0.21**	**0.02**
TDE1156	104	−	Putative uncharacterized protein	−0.09	0.5
TDE1157	417	−	Phage terminase, large subunit, putative (A)	0.04	0.7
TDE1158	203	−	Phage terminase, small subunit, putative (Nu)	0.04	0.6
TDE1159	**130**	−	**Putative uncharacterized protein**	**0.24**	**3.0×10^{-04}**
TDE1160	474	−	Putative uncharacterized protein	−0.02	0.8
TDE1161	**73**	−	**Putative uncharacterized protein**	**0.18**	**7.8×10^{-03}**
TDE1162	**542**	−	**ParB-like nuclease domain protein**	**0.28**	**6.8×10^{-04}**
TDE1163	**76**	−	**Putative uncharacterized protein**	**1.04**	**2.0×10^{-16}**
TDE1164	**77**	−	**Conserved domain protein**	**0.79**	**2.6×10^{-11}**
TDE1165	**69**	−	**Putative uncharacterized protein**	**1.25**	**7.2×10^{-19}**
TDE1166	**107**	−	**Putative uncharacterized protein**	**1.14**	**1.2×10^{-12}**
TDE1167	**74**	−	**Putative uncharacterized protein**	**0.17**	**3.6×10^{-02}**
TDE1168	**177**	−	**Putative uncharacterized protein**	**1.34**	**1.8×10^{-13}**
TDE1169	81	−	Phage DNA-binding protein, excisionase family	Not determined†	
TDE1170	**120**	+	**DNA-binding protein**	**0.51**	**3.3×10^{-14}**
TDE1171	**267**	+	**Putative uncharacterized protein**	**0.49**	**4.1×10^{-09}**
TDE1172	**64**	+	**Putative uncharacterized protein**	**0.16**	**1.8×10^{-03}**
TDE1173	354	−	Site-specific recombinase, phage integrase family	0.04	0.5

*Size predicted from this study.
†No microarray probe present for predicted ORF.

mature biofilm on *T. denticola* gene expression. In the biofilm state there was an upregulation of virulence genes and changes in expression of genes encoding lipoproteins and binding proteins. More significantly, there were simultaneous increases in expression of a range of transposase-like genetic elements, a bacteriophage and a number of TA systems.

The expression of several genes encoding known virulence factors, including cystalysin and the dentilisin complex, was found to be upregulated in the biofilm relative to planktonic cells, raising the possibility that *T. denticola* is more virulent when growing as a biofilm. Cystalysin is a pyridoxal 5′-phosphate-dependent C^{β}-S^{γ} lyase that catalyses the conversion of L-cysteine to pyruvate, ammonia

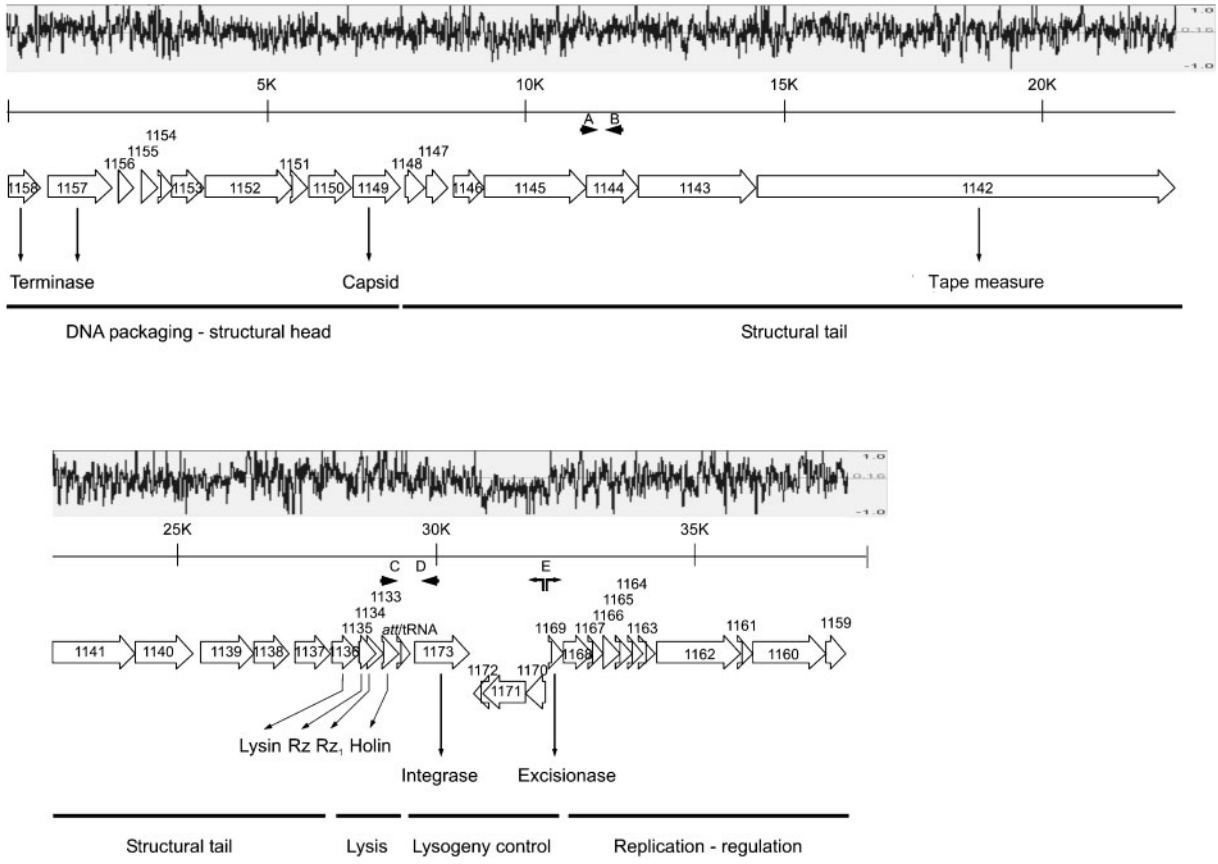

Fig. 2. Genome of bacteriophage φtd1. GC skew (%) shown as top line. A, B, C and D are binding sites for primers TDE1144_R, TDE1144_F, TDE1133_R and TDE1173_F, respectively. E is the location of a putative bidirectional promoter.

and H_2S, and was first described as a 46 kDa haemolysin that was able to agglutinate and lyse erythrocytes (Chu *et al.*, 1994; Krupka *et al.*, 2000). This lysis of red blood cells allows *T. denticola* to access iron-containing molecules such as haem and haemoglobin. Cystalysin synthesis is increased under iron-deprived conditions, suggesting its role in iron acquisition. However, gene expression of several other putative iron/haem-related proteins was

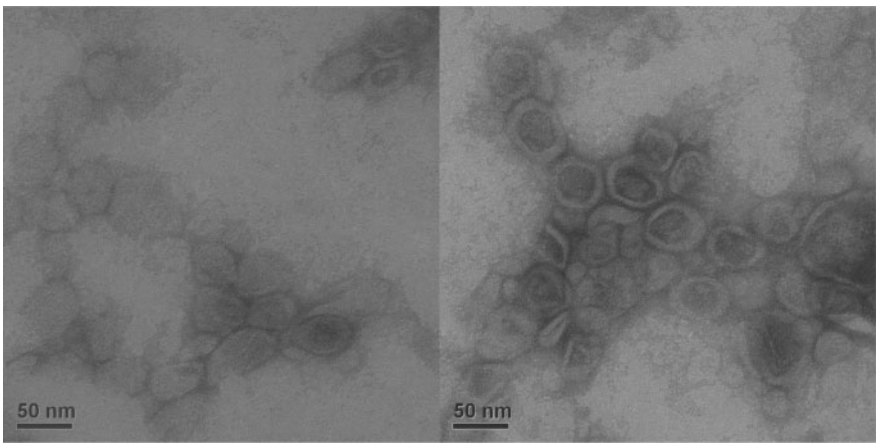

Fig. 3. Two representative transmission electron microscopy images of *T. denticola* bacteriophage φtd1 harvested from biofilm cultures. Bars, 50 nm.

downregulated in the biofilm (TDE0748, TDE2056; Table 3), suggesting that iron was not a limiting micronutrient in the biofilm. Haem was present at a relatively high concentration in the growth medium.

Lipoproteins are often involved in virulence and are thought to play a key role in the inflammatory response of host cells, which in turn contributes to the tissue damage associated with chronic periodontitis (Rosen *et al.*, 1999). Dentilisin is an immunogenic, outer-membrane lipoprotein complex that is the primary mediator of fibrinogen binding by *T. denticola*. It is capable of preventing blood clot formation, has cytopathic properties and is implicated in penetration of epithelial layers (Bamford *et al.*, 2007; Fenno *et al.*, 1998; Grenier *et al.*, 1990; Uitto *et al.*, 1995). All three genes encoding the dentilisin complex were significantly upregulated in biofilm cells, with two genes being upregulated by more than 1.5-fold. Lipoproteins are the most abundant membrane proteins found in spirochaetes and *T. denticola* 35405 is predicted to have 166 lipoproteins, the highest number for any sequenced spirochaete (Setubal *et al.*, 2006). Several lipoproteins were found to have significant changes in expression levels between the planktonic and biofilm cells (Tables 2 and 3).

T. denticola 35405 possesses 35 genes encoding proteins belonging to a conserved protein family of unknown function with some similarity to known transposases. Proteins belonging to this family are found in relatively few bacterial genomes, many of which are pathogens, including members of the genera *Leptospira*, *Clostridium*, *Salmonella* and *Yersinia*. The genomes of many of these bacteria contain a high number of these genes. Interestingly *T. denticola* 35405 has the highest number of any sequenced bacterium and many of these genes were upregulated in the biofilm. These putative transposases may be involved in chromosomal rearrangement using internal genetic elements or even horizontal gene transfer. They may also play a role in the regulation of other differentially expressed genes under conditions of biofilm growth.

The co-ordinated multicellular-like behaviour of biofilms is a key component of their survival (Webb *et al.*, 2003). There is a growing body of evidence suggesting that TA systems are involved in programmed cell death and/or growth inhibition, and influence biofilm formation and persistence (Fineran *et al.*, 2009; Gerdes *et al.*, 2005; Kim *et al.*, 2009). TA systems consist of both a toxin, which poisons the cell by inhibiting essential cell components, and an antitoxin, which counteracts the toxin (Yamaguchi & Inouye, 2009). A variety of modes of action have been demonstrated for the protein toxins of TA systems, including inhibition of DNA gyrase, degradation of mRNA and damage to bacterial membranes. At least 33 TA systems are predicted to occur in *T. denticola* 35405, none of which has been functionally characterized. The large number of these systems found in *T. denticola* 35405 and their increased levels of expression in mature biofilm suggest that they play a role in biofilm persistence and/or bacteriophage resistance. The role of the TA systems in programmed cell death and bacteriostasis is consistent with the attempted survival of a small subpopulation of cells during stressful conditions, which may be induced by lack of nutrients or lytic bacteriophage infection.

The transcript expression analysis led to the discovery of a functional temperate bacteriophage in *T. denticola* 35405, which we have designated φtd1. Although bacteriophage-like particles have been observed in association with various members of the phylum Spirochaetes, including *Treponema phagedenis* (Demirkan *et al.*, 2006), and functional bacteriophages have been isolated and characterized from *Leptospira*, *Brachyspira* and *Borrelia* (Eggers & Samuels, 1999; Saint Girons *et al.*, 1990; Humphrey *et al.*, 1995, 1997), this, to our knowledge, is the first functional bacteriophage isolated from *T. denticola*.

Bacteriophages may contribute to the survival and/or virulence of their host cells, and bacterial cell death and lysis are thought to play critical roles in biofilm formation. Other studies have recently demonstrated a high level of bacteriophage release in biofilms (Resch *et al.*, 2005; Rice *et al.*, 2009). The resulting lysis of cells may provide nutrients and allow the persistence of neighbouring cells. Lysogenic bacteriophages are also known to cause changes to the immunogenic properties of the host cell, known as 'cell surface conversion' (Uetake, 1979).

Some lysogenic bacteriophages are also capable of transferring virulence genes through horizontal gene transfer. Approximately half of the predicted φtd1 bacteriophage proteins had no significant sequence similarities to any other protein (Table 7). BLAST and domain analyses indicated that the remaining bacteriophage genes have been acquired from a variety of bacteria, including several pathogenic species such as *Yersinia pestis*, and also from other bacteriophages. In addition, φtd1 appears to have acquired a conserved spirochaete lipoprotein signal sequence, which should allow the bacteriophage to target this protein to the membrane of the host bacterium.

This study indicates that there is a higher potential for genetic mobility in *T. denticola* when cultured as a biofilm. In addition to the potential for bacteriophage-based horizontal gene transfer, the upregulation of a large number of putative transposases may also allow *T. denticola* to acquire new genes, which might be a particularly useful strategy when in a mixed-species biofilm. The high density of cells within a biofilm provides an ideal environment for genetic exchange, and the diversity provided by acquisition of new genes could allow the survival of some members of the population under changed conditions (Boles *et al.*, 2004). In support of this, 31 and 58 *T. denticola* predicted proteins have higher levels of sequence similarity with Archaea and Eukaryota proteins, respectively, than they do with any other eubacterial species, suggesting that horizontal gene transfer has occurred frequently in this strain.

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