A flagellar gene cluster from the oral spirochaete Treponema maltophilum

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A flagellar gene cluster from the oral spirochaete Treponema maltophilum ATCC 519391 was cloned. Sequence analysis revealed six putative ORFs, two of which encode the flagellar subunit proteins FlaB2 (286 aa) and FlaB3 (285 aa). Northern blot analysis revealed two flagellin transcripts with the expected size of monocistronic mRNAs. Sequence analysis and primer extension experiments indicated that the transcription of the flaB2 gene is directed by a σ28-like FliaA factor. Using fliaA and fliaA+ Escherichia coli K-12 strains, it was shown that flaB2 expression in E. coli required the σ28 factor using an initiation site identical to that in Treponema maltophilum. Primer extension analysis revealed two transcriptional start sites 5′ of the flaB3 gene, a strong promoter with a σ28-like –10 promoter element and a weak promoter with a putative σ54 promoter consensus sequence. Downstream of flaB3, a putative flid homologue was found, probably encoding the flagellar cap protein of Treponema maltophilum. Flagellin-gene-specific DNA probes hybridized to all 13 Treponema strains investigated, whereas a flid-specific DNA probe only hybridized to Treponema maltophilum, other treponemal group IV isolates and Treponema brennaborense.

Keywords: treponemes, Treponema maltophilum, flagellar filament, flid

INTRODUCTION

Periodontitis is a mixed bacterial infection leading to progressive destruction of connective gingival tissue (Listgarten, 1987; Saglie et al., 1982). The possible aetiologic role of oral treponemes in this process is based on the presence of elevated numbers of these organisms in periodontal lesions (Fiehn, 1989; Listgarten & Levin, 1981; Penn, 1991). The interaction of periodontal bacteria with the host tissue is complex, involving motility, adherence, invasion of deeper tissues and modulation of the immune response (Listgarten, 1987; Loesche, 1993; Saglie et al., 1982). Treponemes possess a variety of putative virulence factors (for a review see Fenna & McBride, 1998), including proteases, haemolysins and adhesins (Dawson & Ellen, 1990; Fenna et al., 1996; Grenier, 1991; Haapasalo et al., 1992; Que & Kuramitsu, 1990; Reijntjens et al., 1986). Motility may also contribute significantly to spirochaete pathogenicity as it has been shown that some treponemes are able to invade tissues (Moter et al., 1998). Motility of treponemes is due to the rotation of the periplasmic flagella between the sheath and the cell cylinder (Berg, 1976; Berg & Turner, 1979; Charon et al., 1992), leading to directional movements within viscous environments (Berg & Turner, 1979; Klitorinos et al., 1993). The flagellum of Treponema pallidum is composed of three core proteins (FlaB1–3) and one sheath protein (FlaA) (Champion et al., 1990; Isaacs & Radolf, 1990; Pallesen & Hindersson, 1989). In most flagellated bacteria, the cap protein (Flid) is involved in flagella assembly and is often located near the flagellin-encoding genes (Arora et al., 1998; Chen & Helmann, 1994; McCarter, 1995). However, although flid homologous sequences have been found in the published sequences of the T. pallidum and Borrelia burgdorferi genomes (TIGR database), no treponeme flid gene has been cloned from this or other species.

Little is known about the regulation of flagellin gene expression in treponemes. In ‘Treponema phagedenis’ a σ28-like promoter consensus sequence was identified

Abbreviations: CBP, calmodulin-binding protein; IP, isoelectric point; UAS, upstream activator sequence.

The GenBank accession number for the sequence reported in this paper is Y18889.
upstream of the \textit{flaB2} gene (Limberger et al., 1992). Similar putative \textit{σ}^B consensus sequences were found next to the \textit{flaB1} and \textit{flaB2} genes of \textit{T. pallidum} (Champion et al., 1990; Pallesen & Hindersson, 1989) as well as in the \textit{flgB} and \textit{flgK} operons of \textit{Treponema denticola}, ‘\textit{T. phagedenis}’ and \textit{T. pallidum} (Heinzerling et al., 1997; Limberger et al., 1996). Although the effect of temperature and viscosity on motility of \textit{T. denticola} was studied recently, little is known about the influence of environmental factors on flagellin gene expression in treponemes (Klitorinos et al., 1993; Ruby & Charon, 1998).

As shown by epidemiologic analysis of patients suffering from rapidly progressive periodontitis, group IV treponemes have the highest prevalence compared to all other culturable and uncultivable treponemes investigated (Moter et al., 1998). Motility may be a virulence factor of treponemes, enabling active tissue invasion. Recently, we described a novel treponeme, \textit{Treponema maltophilum}, a representative of phylogenetic group IV treponemes (for phylogenetic group determination see Wyss et al., 1996). Here, we report the cloning of a \textit{T. maltophilum} flagellar gene cluster containing two flagellin subunit genes and the \textit{flID} gene. In addition, we report the first characterization of \textit{T. maltophilum} flagellar gene regulation at the molecular level.

**METHODS**

**Bacterial strains, media and plasmids.** Bacterial strains are listed in the legend to Fig. 4. \textit{Treponema} strains were maintained as described previously (Wyss et al., 1996). Cultures were examined by dark field microscopy for motility and typical strain morphology.

\textit{Escherichia coli} XL-1 Blue (Stratagene) and \textit{E. coli} DH5 were used for propagation of recombinant plasmid DNA. Plasmid \textit{pUC19} (Stratagene) was used for subcloning and DNA sequencing. \textit{E. coli} strains were grown in LB medium. Antibiotics used for selection in \textit{E. coli} were ampicillin (100 \(\mu g\) ml\(^{-1}\)), chloramphenicol (34–50 \(\mu g\) ml\(^{-1}\)), tetracycline (12.5 \(\mu g\) ml\(^{-1}\)) and kanamycin (50 \(\mu g\) ml\(^{-1}\)). Recombinant FlaB3 protein was overexpressed in \textit{E. coli} strain

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**Fig. 1.** Restriction map of the \textit{T. maltophilum} \textit{flaB–flID} gene cluster. Coding regions corresponding to ORF1, \textit{flaB2}, \textit{flaB3}, ORF111, \textit{flID} and ORF2 are indicated by large arrows. Small arrows represent the primers used for PCR. P1, P2 and P3; PCR-generated DNA amplicons used as \textit{flaB2}, \textit{flaB3}- and \textit{flID}-specific probes, respectively. Restriction endonuclease sites: Bm, \textit{BamHI}; Ec, \textit{EcoRI}; Hd, \textit{HindIII}; Kp, \textit{KpnI}; Pt, \textit{PstI}; Sl, \textit{SallI}; Sc, \textit{SacII}; Sm, \textit{Smal}.
Table 1. Oligonucleotide primers

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<th>Name</th>
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<td>flaBU</td>
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<td>flaDR1</td>
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BL21 (DE3)/pLys (Stratagene), *E. coli* strain BL21 (DE3)/pLys harbouring plasmid pKH113 was inoculated into LB broth containing chloramphenicol (25 µg ml⁻¹) and tetracycline (30 µg ml⁻¹). The induction of target protein expression was done according to the manufacturer’s instructions (Affinity Protein Expression and Purification System; Stratagene). Chemicals and oligonucleotides were purchased from Gibco-BRL, Boehringer Mannheim, Pharmacia LKB and MWG-Biotech. Preparations of recombinant flagellin proteins of *T. maltophilum* flagellin proteins. Preparations of recombinant flagellin proteins of *T. maltophilum* were done according to the manufacturer’s instructions (Affinity Protein Expression and Purification System; Stratagene). Cells were lysed by sonication and the recombinant protein was purified by a calmodulin affinity column. Samples of eluted protein fractions were checked for purity by SDS-PAGE and recombinant proteins were detected by the Affinity CBP (calmodulin-binding protein) Fusion Protein Detection Kit (Stratagene) (data not shown). Fractions 2 and 3 were denatured with 15% TCA and pelleted by centrifugation (14000 r.p.m. for 10 min in a Labofuge 400R; Hereus). The pellet was resuspended in 200 µl PBS, pH 7.2, and stored at −20 °C.

Preparation of polyclonal monospecific antibodies against *T. maltophilum* flagellin proteins. Rabbits, obtained from a commercial breeder (Hardan Winkelmann, Borchen, Germany), were immunized with 0.2 ml antigenic solution (50 µg recombinant *T. maltophilum* FlaB3 protein; the CBP tag was not removed) mixed with 0.3 ml PBS and 0.5 ml adjuvant (Specol, Institute for Animal Science and Health, Lelystad, The Netherlands) in a total volume of 1 ml. Rabbits were immunized by injection of the antigen solution into or near the popliteal lymph nodes. After 4 weeks booster injections (1 ml) were given into the skin (i.d.) at multiple sites. To reduce cross-reactivity of the resulting antiserum, the polyclonal monospecific antibody was absorbed with sonicated whole-cell extracts of *E. coli* XL1-Blue MRF₉ harbouring plasmid pCAL-n. The non-binding fraction containing the purified anti-FlaB3 antibody was used for Western blot analysis. Western blot analysis showed that this antiserum detected the recombinant and wild-type FlaB2 and FlaB3 proteins (data not shown).

Cloning of a flagellar gene cluster exhibiting two flagellum subunit genes and the fliD operon of *T. maltophilum*. Based upon published gene sequences encoding the conserved N- and C-terminal regions of flagellin proteins we designed a primer pair, flaBU/FlaBR, to amplify *T. maltophilum* flagellin genes directly from the chromosome by PCR. The sequences and positions of all primers used are shown in Table 1 and Fig. 1, respectively. We obtained two PCR products of 0.7 and 1.9 kb. As shown by sequence analysis the 1.9 kb fragment contained the major coding regions of the flaB and fliB genes (Fig. 1, pKH107). After cloning and sequencing of the PCR products we designed two primer pairs, flaBU/promoter and flaBU1/promoter, to amplify the 5’ and 3’ adjacent regions of the cloned DNA fragment directly from the chromosome by PCR-mediated chromosome walking (PCR). *T. maltophilum* chromosomal DNA was cut with SacII and religated prior to PCR. Resulting amplicons were cloned into vector pUC19 giving plasmids pKH108 and pKH135 (Fig. 1). The complete flaB2 and flaB3 genes were amplified using primer pairs flaBU4/flaBU4 or flaBU4/flaBR3 and cloned in pUC19, resulting in plasmids pKH123 and pKH112, respectively. This...
procedure was performed three times in independent experiments. To avoid PCR-generated errors three clones of each PCR product were sequenced. The flaB3 and flaB2 genes were then subcloned in the expression vector pCAL-n (Stratagene), resulting in plasmids pKH124 and pKH113 (Fig. 1), respectively. The same cloning strategy was used to clone the flfI gene of T. maltophilum using primer pairs flaBu6/flaBr6 and flaBu10/flaBr10, resulting in plasmids pKH121 (not shown) and pKH129 (Fig. 1), respectively. The flfI gene was then amplified from chromosomal DNA using primers flaDu1/flaDR1, cloned, sequenced and subcloned into vector pCAL-n, resulting in plasmid pKH137 (Fig. 1).

**SDS-PAGE and Western blotting.** Whole-cell extracts of *Treponema* spp. and recombinant *E. coli* strains were analysed by SDS-PAGE and Western blotting. SDS-PAGE and Western blotting were carried out as described elsewhere (Laemmli, 1970; Towbin *et al.*, 1979; van Die *et al.*, 1984). Briefly, treponemal strains were grown anaerobically in OMIZ Patt medium (10 ml) at 37°C until late exponential phase and harvested by centrifugation. The pellet was then resuspended in 200 μl ddH₂O. Equal amounts of cells were mixed with 25 μl loading buffer, incubated at 95°C for 5 min and loaded onto an SDS (9%)-polyacrylamide gel. Recombinant *E. coli* K-12 strains were grown either on agar plates or in Luria-Broth medium, each containing 100 μg ampicillin ml⁻¹, at 37°C overnight. *E. coli* cells were resuspended in 25 μl loading buffer and loaded onto an SDS (9%)-polyacrylamide gel.

**Southern hybridization.** SacII-digested chromosomal DNA from various *Treponema* strains was electrophoresed in a 1% (w/v) agarose gel. The DNA fragments were transferred to nylon membranes (Boehringer Mannheim) as described by Southern (1975). The 395 (P1), 822 (P2) and 795 bp (P3) PCR fragments (P1–3; see Fig. 1) were used as *T. maltophilum* flaB2-, flaB3- and flfI-D-specific probes, respectively. The probes were labelled and detected by using the non-radioactive enhanced chemiluminescence detection kit (ECL; Amersham). Hybridization was done as described by Heuner *et al.* (1995).

**DNA techniques, PCR and nucleotide sequencing analysis.** Purification of plasmid DNA, DNA cloning and hybridization procedures were performed according to standard protocols (Sambrook *et al.*, 1989; Thuring *et al.*, 1975). PCR was carried out by the method of Saiki *et al.* (1988), using a Thermocycler TRIO-Thermoblock (Biometra) and AmpliTaq polymerase (Perkin Elmer). A standard amplification protocol was used: initial denaturation step of 3 min at 95°C followed by 30 cycles of 95°C for 60 s, 55°C for 75 s and 72°C for 60 s and a final step of 5 min at 72°C. The annealing temperature was modified for each primer pair. PCR products were cloned in pUC19, using the Sure Clone Ligation Kit (Pharmacia). Transformation was done by electroporation using a Bio-Rad gene pulser. Electroporation of *E. coli* strains was carried out at 2 kV, 200 and 25 μF.

Sequencing of plasmid DNA was done on both strands with IR-dye-labelled primers as described by Choi *et al.* (1994) and Sanger *et al.* (1977). Sequences were analysed using the program Husar 4.0 (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Homology searches were conducted against the GenBank, EMBL, EMBPRO and SWISS-PROT databases, using the FASTA program. Sequence similarities and homologies were calculated by the GAP program [Genetics Computer Group (GCG) package, Husar 4.0]. Multiple alignments were accomplished by using the CLUSTAL program (GCG package, Husar 4.0).

**RNA isolation and Northern (RNA) blot analysis.** *T. maltophilum* total RNA was purified using an RNA isolation kit (Boehringer Mannheim). Bacteria were grown anaerobically in OMIZ-PAT at 37°C for 22, 30, 52 or 76 h (OD₆₀₀ 0.036, 0.045, 0.2 and 0.22, respectively), representing the exponential, late exponential, stationary and late stationary growth phases, respectively. Bacteria were harvested by centrifugation and RNA was isolated as described by the manufacturer. RNA was stored at −80°C. Total RNA (10 μg) was mixed with formaldehyde loading buffer and electrophoresed in 1% (w/v) agarose/formaldehyde gels. The DNA was then transferred to nylon membranes by capillary blotting. DNA probes were labelled by using the non-radioactive enhanced chemiluminescence detection kit (ECL; Amersham). Hybridization with specific DNA probes was done as described by the manufacturer. The hybridization temperature was 42°C.

**Primer extension.** Primer extension analysis was carried out with IR-dye-labelled primers flaBu7 and flaB3PE on an automated DNA sequencer (LI-COR-DNA 4000; MWG-Biotech). Each primer (4 pmol) was annealed in a thermocycler to 10 μg RNA in a volume of 10 μl H₂O (RNase-free) by heating at 90°C for 2 min and subsequent cooling to 30°C within a period of 30 min. Extension was done in a total volume of 37 μl at 42°C for 90 min as described by Ausubel *et al.* (1987). Nucleic acids were precipitated by ethanol. The pellet was dissolved in equal volumes (5 μl each) of H₂O and formamide loading buffer. Samples were boiled for 2 min and aliquots (3 or 6 μl) were applied to sequencing gels. Sequencing reactions were done using the same primers used for the primer extension analysis.

**Construction of pflaB–lacZ fusions and β-galactosidase activity assay.** Plasmid pKH14 was constructed by cloning the 3.6 kb BamHII–SalI DNA fragment containing the promoterless *lacZ* gene of pDN19lac (Totten & Lory, 1990) into the low-copy-number vector pMMB207 (Morales *et al.*, 1991). This plasmid was used to generate *T. maltophilum* flagellar promoter (pflaB)–*lacZ* gene fusions in a similar way to that described by Heuner *et al.* (1997, 1999). Therefore, the flaB2 and flaB3 promoter-containing regions were amplified by PCR using primer pairs flaBu7/flaBr7 (pflaB2, 335 bp) and flaB3PU/flaB3PR (pflaB3, 354 bp). Resulting amplicons were first cloned into plasmid pUC19 to confirm the promoter sequence by DNA sequencing. PCR-generated *SpbHI* and BamHI restriction sites were then used to clone the flagellin promoters upstream of the promoterless *lacZ* gene of pKH14, resulting in plasmids pKH126 (pflaB2–*lacZ* fusion) and pKH128 (pflaB3–*lacZ* fusion). The σ⁷ factor consensus sequence of the flaA gene of *Legionella pneumophila* is recognized by the σ⁷ factor of *E. coli* strain YK410 (Heuner *et al.*, 1997). Therefore, plasmid pKH12 containing the flagellin promoter of *L. pneumophila* fused to the *lacZ* gene of pKH14 (Heuner *et al.*, 1999) was used as a positive control in β-galactosidase activity assays.

*E. coli* strains grown to late exponential phase were adjusted to an OD₆₀₀ of 1.5. β-Galactosidase measurements were done as described by Miller (1972).

**RESULTS**

**Cloning of the *T. maltophilum* flagellar filament genes and the flfI gene cluster.**

*T. maltophilum* flaB2 and flaB3 genes were cloned directly from the chromosome of *T. maltophilum* by

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**RESULTS**

**Cloning of the *T. maltophilum* flagellar filament genes and the flfI gene cluster.**

*T. maltophilum* flaB2 and flaB3 genes were cloned directly from the chromosome of *T. maltophilum* by
PCR as described above. The complete chromosomal region of T. maltophilum encoding the flaB2, flaB3 and fliD region was obtained by rPCR. The flaB2-, flaB3- and fliD-encoding DNA sequences were subcloned in vector pCAL-n, resulting in plasmids pKH113, pKH124 and pKH137, respectively (Fig. 1). Purified recombinant FlaB3 protein was used to generate a monospecific polyclonal rabbit anti-FlaB3 antiserum. Western blot analysis showed that this antiserum detected the recombinant and wild-type FlaB2 and FlaB3 proteins (data not shown).

Southern blot analysis of T. maltophilum chromosomal DNA digested with various restriction enzymes using flaB2-, flaB3- and fliD-specific DNA probes (see Fig. 1, P1–3) indicated that flaB2 cross-hybridizes with the flaB3 gene and that T. maltophilum may contain a third flaB gene located approximately 3–5 kb upstream of flaB2. These experiments suggest that T. maltophilum contains only one copy of the fliD gene (data not shown).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Identity (%)*</th>
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<tr>
<td></td>
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<tr>
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<td>T. pallidum flaB2</td>
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<tr>
<td>T. pallidum flaB3</td>
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<td>T. phagedenis flaB2</td>
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<tr>
<td>T. maltophilum flaB2</td>
<td>100.0 (100.0)</td>
</tr>
<tr>
<td>T. maltophilum flaB3</td>
<td>67.0 (79.5)</td>
</tr>
</tbody>
</table>

*Similarity of flagellin proteins is shown in parentheses.

The T. maltophilum flaB2 exhibited 79.5% similarity to its own FlaB3 protein, but 82% similarity to T. pallidum FlaB3, whereas FlaB2 exhibited 86 and 84% similarity to T. pallidum FlaB2 and FlaB1, respectively, as summarized in Table 2. Based upon these similarities, the T. maltophilum flagellar filament genes were named flaB2 and flaB3.

Downstream of flaB3, additional ORFs are present, one of which encodes a protein of 111 aa (ORF111) exhibiting 32 and 30% similarity to the ORF99 and Flg proteins of Bacillus subtilis and Pseudomonas aeruginosa, respectively. The putative protein has a theoretical mass of 12 kDa and an IP of 9.78, containing no cysteine, histidine or tryptophan residues.

Downstream of ORF111 another ORF encodes a T. maltophilum FliD (putative flagellar cap protein) homologue, possessing 673 aa with a theoretical mass of 73.8 kDa. It showed the highest similarity (54%) throughout the sequence to the FliD protein of T. pallidum (data not shown) and 48, 46 and 44% similarity to the FliD proteins of Borrelia burgdorferi, Bacillus subtilis and E. coli, respectively. Southern hybridization using a fliD-specific probe (P3; see Fig. 1) revealed fliD homologue genes in other group IV treponemes and Treponema brennaborense, but in no other treponeme investigated so far (data not shown).

The 3’ end of an additional ORF located downstream of flaB3 (named ORF2), is missing. The truncated gene encodes a hypothetical protein with 43% similarity to the hypothetical protein TP0873 (200 aa) of T. pallidum (sequence from the TIGR database).

Transcriptional analysis

The size of the flaB transcripts were determined by Northern blot analysis with total RNA prepared from T. maltophilum cells grown to exponential, late exponential, stationary and late stationary growth phases. By using probes P1 (385 bp, flaB2) and P2 (822 bp, flaB3) corresponding to the FlaB2 and FlaB3 coding regions,
two specific transcripts of about 1·2 and 1·4 kb were detected (Fig. 2a, lane 2). While the flaB3-specific DNA probe predominantly detected the 1·2 kb transcript (Fig. 2a, lane 4), probe P1 recognized both transcripts (Fig. 2a, lane 2). The length of the 1·2 kb transcript corresponds to the coding regions of flaB2 and flaB3, indicating that flaB genes are transcribed as monocistronic units. Northern blot analysis revealed that the flaB2/3 genes are transcribed during exponential growth (Fig. 2b, lanes 2 and 3), but significantly fewer transcripts are detectable in the late stationary growth phase (Fig. 2b, lane 5).

To determine the promoters of T. maltophilum flagellin genes, we mapped the transcriptional start site of the chromosomal flagellin genes by primer extension (Fig. 3). These experiments revealed an adenine residue as the transcriptional start site of flaB2 (Fig. 3b, lane 5). These experiments revealed an adenine residue of the chromosomal flagellin genes by primer extension and to determine the promoters of flaB2, lane 5).

Fig. 2. Northern blot analysis of flaB gene transcription. (a) Northern blot analysis showing the presence of different flaB transcripts. (b) Northern blot analysis showing growth-phase-dependent flaB transcription. RNA was extracted from T. maltophilum grown anaerobically for 30 (a, lanes 2 and 4; b, lane 3), 22 (b, lane 2), 52 (b, lane 4) or 76 h (b, lane 5) at 37 °C. PCR-generated flaB2, flaB3 or flaB2–flaB3 fragments, corresponding to flaB2, flaB3 or flaB2–flaB3 coding regions, respectively, were used as probes. PCR-generated DNA probes were used as positive controls in Northern blot analysis (a, lanes 1/3, flaB2-specific probe; b, lane 1, 1·9 kb flaB2–flaB3-specific probe).

GG-N$_{10}$-GC invariants and one putative upstream activator sequence (UAS, TGT-N$_{7}$-ACA) are found near the −100 region of the promoter (data not shown). Primer extension products were identified in the early and late exponential growth phases (Fig. 3b, lanes 1 and 2).

Promoter expression studies of T. maltophilum flagellin genes were performed in E. coli strain YK410 and strain YK4104, the isogenic σ$_{28}$ factor (fla$^{-}$) mutant strain of strain YK410 (Chen & Helmann, 1992). Strains YK410 and YK4104 transformed with pKH126, harbouring the T. maltophilum flaB2 promoter–lacZ gene fusion, or with pKH128, containing the flaB3 promoter–lacZ gene fusion, were used for reporter gene expression studies. Reporter gene activity (β-galactosidase) was measured in the late exponential growth phase. E. coli strains harbouring pKH12, containing the L. pneumophila σ$_{28}$ flaA promoter–lacZ gene fusion, and pKH14, containing the promoterless lacZ gene were used as positive and negative controls, respectively. As shown in Table 3 the lacZ gene containing the pflaB2 promoter of T. maltophilum is expressed in the E. coli wild-type strain, but not in the flaA mutant. Primer extension analysis with total RNA isolated during these experiments revealed the same transcriptional start site in E. coli wild-type as in T. maltophilum (Fig. 3a, lane 4). No signal was seen in the flaA mutant strain (Fig. 3a, lane 5), indicating that the T. maltophilum σ$_{28}$-like element is recognized by the σ$_{28}$ factor of E. coli. However, one should note that expression is approximately sevenfold lower than for the L. pneumophila pflaA–lacZ fusion (Table 3). No significant β-galactosidase activity was seen in E. coli strains harbouring the pflaB3–lacZ gene fusion, indicating that this promoter element is not active in E. coli.
The *T. maltophilum* flaB and fliD gene cluster

![Image](90x406 to 183x712)

![Image](220x406 to 308x712)

![Image](90x324 to 358x387)

Fig. 3. Primer extension experiments to map the transcriptional start site of the *T. maltophilum* flaB2 (a, using primer flaBR7) and flaB3 (b, using primer flaB3) genes. Transcription start sites are indicated by arrows. Total RNA was isolated from *T. maltophilum* cultures grown to exponential (lane 1), late exponential (2) and stationary phase (3). Total RNA from *E. coli* strains YK410 (wild-type) and YK4104 (*fliA* mutant) harbouring plasmid pKH126 (pflaB2–lacZ fusion) was isolated (lanes 4 and 5). Lanes designated G, A, T and C represent DNA sequencing ladders. (c) Positions of the transcription start sites deduced from primer extension analysis. Putative promoter elements are underlined and transcriptional start sites are indicated by arrows.

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**Table 3.** *T. maltophilum* flaB promoter activity in *E. coli*

Results were obtained in three independent experiments.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Promoter*</th>
<th>β-Galactosidase activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>YK410 (wt)</td>
</tr>
<tr>
<td>pKH126</td>
<td>Tm flaB2</td>
<td>39.8 ± 1.13</td>
</tr>
<tr>
<td>pKH128</td>
<td>Tm flaB3</td>
<td>2.4 ± 1.9</td>
</tr>
<tr>
<td>pKH12</td>
<td>Lp flaA</td>
<td>296.1 ± 14.3</td>
</tr>
<tr>
<td>pKH14</td>
<td>None</td>
<td>2.3 ± 0.26</td>
</tr>
</tbody>
</table>


†β-Galactosidase activity is given in Miller Units (Miller, 1972). wt, Wild-type; YK4104, isogenic *E. coli* strain of YK410 defective for σ70 factor expression.

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**Distribution and expression of flagellin genes in various treponeme species**

The results of Western blot analysis of whole-cell proteins from various treponemal strains with the *T. maltophilum* FlaB3-specific antiserum is shown in Fig. 4. Immunoblot analysis of all treponeme isolates investigated so far showed two to three protein bands of approximately 31–35 kDa, indicating the conserved structure of treponeme FlaB proteins. For *’T. phagedenis’* and in *T. denticola* CD-1 Western blot analysis showed only one band reacting with the antiserum (Fig. 4, lanes 4 and 5), but Southern blot analysis revealed the presence of more than one flagellar gene in these strains. Southern blot analysis using *T. maltophilum* flaB primer extension products were detected in these strains (data not shown).
maltophilum flaB2- and flaB3-specific DNA probes exhibited positive bands in all strains tested (data not shown).

**DISCUSSION**

The **flaB** gene cluster

In this paper we have reported the sequence and expression analysis of a flagellar gene cluster cloned by PCR-mediated chromosomal walking of the oral spirochaete *T. maltophilum*. Six putative ORFs were identified. Two ORFs, 861 and 858 bp in length encode the FlaB2 and FlaB3 proteins, respectively. The apparent sizes in SDS-PAGE analysis seemed too high compared to the calculated molecular masses of both proteins: 31.3 and 30.7 kDa, respectively. This may be explained by post-translational modification of the FlaB proteins, e.g. by glycosylation of *T. maltophilum* FlaB proteins as reported by Wyss (1998). Furthermore, flagellar proteins are known to migrate irregularly in SDS-PAGE (Simon et al., 1977).

While only two flagellins were detected by immunoblotting, the presence of a third flagellar gene was shown by Southern blot analysis (data not shown). This has also been shown for *T. pallidum* (Champion et al., 1990; Pallesen & Hindersson, 1989). The cloned genes were named flaB2 and flaB3 according to their similarity to the respective *T. pallidum* genes. The similarity of *T. maltophilum* FlaB2 to *T. pallidum* FlaB2 is higher (86%) than the similarity to its own FlaB3 protein (79.5%). This is also the case for FlaB proteins of other spirochaetes (Charon et al., 1992; Ruby et al., 1997).

Little is known about the mechanisms of flagellin gene expression in treponemes, but it has been suggested that differential gene regulation is required for optimal motility in varying environments (Alm et al., 1993; Belas et al., 1986; Wassenaar et al., 1994). Northern blot analysis revealed the presence of monocistronic flaB transcripts (1.2 and 1.4 kb) in *T. maltophilum*. The 1.4 kb transcript seen in Northern blot analysis may represent the mRNA of the flaB1 gene. In *T. pallidum* a similar flaB1–flaB3 gene arrangement was found (Champion et al., 1990) and these authors speculated about the existence of a polycistronic mRNA, but no Northern blot or primer extension analyses were performed.

We showed that the flaB2 gene of *T. maltophilum* contains a σ28-like promoter sequence and primer extension analysis revealed that it acts as a promoter for flaB2 gene expression. Using flaA/flaA+E. coli strains we showed that an identical initiation site was used in both *E. coli* and *T. maltophilum*. This strongly suggests that the *T. maltophilum* σ28 promoter is recognized by the σ28 factor of *E. coli*. The significant reduction of promoter activity compared to the *L. pneumophila* flaA σ28 promoter may be explained by a variation in length of the spacer region (Dombroski et al., 1996; Mazouni et al., 1998). The *T. maltophilum* spacer contains 16 instead of 15 nt typically found in the *L. pneumophila* flaA promoter or other σ28 consensus sequences (Fig. 5a). σ28-factor-dependent expression was also shown for the ‘*T. phagedenis*’ flaB2 gene (Limberger et al., 1992) and the flaB and fliK operons of *T. denticola* and ‘*T. phagedenis*’ (Heinzerling et al., 1997; Limberger et al., 1996). In contrast, motility operons in *B. burgdorferi* are transcribed from σ28 promoters (Ge et al., 1997).

The *T. maltophilum* flaB3 gene contains two initiation sites (P1 and P2). Upstream of the major initiation site, P1, a σ28-like promoter element lacking an obvious −35 element was found. A putative σ28-like promoter element was identified upstream of P2 (Fig. 5b). Flagellin genes under the control of different sigma factors were also reported for *P. aeruginosa*, *Helicobacter pylori* and *Campylobacter coli* (Alm et al., 1993; Guerry et al., 1991; Josenhans et al., 1995). The *T. maltophilum* flaB3

![Fig. 4. Western blot analysis using the anti-FlaB3 antibody. Equal amounts of whole-cell extracts of different treponemal strains were loaded onto the polyacrylamide gel. Lanes: 1, molecular mass standard (Gibco-BRL); 2, ‘*T. vincentii*’ ATCC 35580; 3, ‘*T. vincentii*’ RitzA; 4, ‘*T. phagedenis*’ biotype Reiter (provided by B. Wilcke, Ludwigs-Maximilians-Universität München, Germany); 5, *T. denticola* CD-1; 6, *T. denticola* ATCC 35405; 7, *T. maltophilum* ATCC 51940; 8, *T. lecitholyticum* ATCC 70032; 9, *T. brennaborense* DSM 12168 (Schrank et al., 1999); 10, *T. socranskii* subsp. *socranskii* ATCC 35536; 11, *T. socranskii* subsp. *buccale* ATCC 35534; 12, *T. pectinovorum* ATCC 33768; 13, *T. maltophilum* ATCC 51940; 14, *T. maltophilum* ATCC 51941. All strains investigated were oral isolates except ‘*T. phagedenis*’ and *T. brennaborense.*](https://www.microbiologyresearch.org/content/pic/10/9/4/504.png)
The *T. maltophilum* flaB and fliD gene cluster

This study

Pallesen & Hindersson (1989)
Champion et al. (1990)
Limberger et al. (1992)
Heuner et al. (1995)
Limberger et al. (1996)
Ankarloo et al. (1996)
Guerry et al. (1991)
Suerbaum et al. (1993)

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The *fliD* gene cluster

In *T. maltophilum* we identified an ORF encoding a putative filament cap protein (FlID) homologue. The deduced FlID protein exhibited 54% similarity to FlID of *T. pallidum* and 44–48% similarity to the FlID proteins of *Borrelia burgdorferi*, *Bacillus subtilis* and *E. coli*. However, the *fliD* gene seems to be less conserved than the flagellin genes as shown by Southern blot analysis. In general the filament cap protein prevents the free release of flagellin and promotes their polymerization onto growing filament tips (Homma et al., 1986; Ikeda et al., 1985). The *fliD* operons of *P. aeruginosa*, *Bacillus subtilis* and *Vibrio parahaemolyticus* consist of a small ORF followed by the *fliDST* genes (Arora et al., 1998; Chen & Helmann, 1994; McCarter, 1995; McGee et al., 1996). The *fliDST* genes are involved in negative regulation of FlgM (anti-σ28 factor) export (Yokoseki et al., 1996). In *T. maltophilum* a similar gene arrangement may be present downstream of the two *flaB* genes.

Upstream of *fliD* we identified an ORF of 111 aa exhibiting low similarities (32 and 30%) at the protein level to the ORF99 and FlaG proteins of *Bacillus subtilis* and *P. aeruginosa*, respectively, but the function of these proteins is not known. Comparison of the cloned fla operon of *T. maltophilum* with sequences retrieved from the genomic database (TIGR) of *T. pallidum* revealed a similar flaB–fliD cluster arrangement, except that no ORF111 homologue is present in the *T. pallidum* strain sequenced. It has yet to be shown whether the proteins encoded by the genes of the putative *T. maltophilum* *fliD* operon possess a similar function to the *fliDST* genes of other bacteria.

Moter et al. (1998) described the invasion of treponemes into deep tissue of inflamed digital dermatitis lesions. It
is therefore conceivable that motility constitutes an important virulence factor in these bacteria. Hence, the molecular characterization of treponemal motility, e.g. by generating flaB and flaD mutants, may be of great help in elucidating their role in the pathogenesis of chronic human periodontitis and related infections in cattle.

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REFERENCES


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The T. maltophilum flaB and fliD gene cluster