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A Mutation in *Flavobacterium psychrophilum tlpB* Inhibits Gliding Motility and Induces Biofilm Formation

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Flavobacterium psychrophilum **is a psychrotrophic, fish-pathogenic bacterium belonging to the *Cytophaga-Flavobacterium-Bacteroides* group. Tn*4351*-induced mutants deficient in gliding motility, growth on iron-depleted media, and extracellular proteolytic activity were isolated. Some of these mutants were affected in only one of these characteristics, whereas others had defects in two or more. FP523, a mutant deficient in all of these properties, was studied further. FP523 had a Tn*4351* insertion in *tlpB* (thiol oxidoreductase-like protein gene), which encodes a 41.4-kDa protein whose sequence does not exhibit high levels of similar to the sequences of proteins having known functions. TlpB has two domains; the N-terminal domains has five transmembrane regions, whereas the C-terminal domains has the Cys-X-X-Cys motif and other conserved motifs characteristic of thiol:disulfide oxidoreductases. Quantitative analysis of the thiol groups of periplasmic proteins revealed that TlpB is required for reduction of these groups. The *tlpB* gene is part of the *fpt* (*F. psychrophilum* thiol oxidoreductase) operon that contains two other genes, *tlpA* and *tpiA*, which encode a thiol:disulfide oxidoreductase and a triosephosphate isomerase, respectively. FP523 exhibited enhanced biofilm formation and decreased virulence and cytotoxicity. Complementation with the *tlpB* loci restored the wild-type phenotype. Gliding motility and biofilm formation appear to be antagonistic properties, which are both affected by TlpB.**

Flavobacterium psychrophilum is a gram-negative bacterium which is a member of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group. This microorganism is the etiological agent of the "cold water disease" that affects salmonids. This disease occurs at temperatures below 12°C and causes significant economic losses for salmonid fish farms worldwide. No vaccines are available; therefore, control of outbreaks is based on fish management strategies and the use of antimicrobial therapy (for a review see reference 38).

The psychrotrophic bacterium *F. psychrophilum* does not grow at temperatures above 20°C and is considered "fastidious" because it is difficult to isolate and culture (33, 38). This has hindered physiological, biochemical, and genetic studies of *F. psychrophilum*, and consequently, our knowledge concerning the mechanism of pathogenesis is limited. Nevertheless, in recent years advances in cultivation (33), diagnosis (11), and experimental infection techniques (14) and in genetic techniques for DNA transfer (3) have been reported.

Some mechanisms involved in the development of the disease have been studied. Adhesion is a prerequisite for colonization, and it has been found that highly virulent strains of *F. psychrophilum* attach more readily to host tissues than less virulent strains attach (39). Furthermore, a specific lectin, which is able to aggregate *F. psychrophilum* cells and erythrocytes, has been related to the adhesion of this bacterium (36). Several reports have correlated the presence of an extracellular proteolytic enzyme with virulence (6). Two *F. psychrophilum* metalloproteases, Fpp1 and Fpp2, have been purified and characterized (46, 47). Both of these proteins have a broad hydrolysis spectrum that includes matrix and muscle proteins. Fpp1 is maximally induced in the presence of calcium levels (10 mM) similar to those present in the fish (46). Recently, several putative virulence factors of *F. psychrophilum* have been identified by subtractive hybridization (50).

Bacterial motility is an important factor for rapid colonization. Motility has been linked to biofilm formation and production of virulence factors in different pathogenic gram-negative bacteria (7, 15, 17, 23, 42). Like many other members of the CFB group, *F. psychrophilum* crawls over surfaces by a process known as gliding motility (38). The mechanism of this form of movement is not known. Genetic analyses of the related bacterium *Flavobacterium johnsoniae* have identified Gld proteins required for gliding (27). Some Gld proteins exhibit sequence similarities to components of ABC transporters (2, 19) and lipoproteins (20, 21, 28, 29). Disruption of the *gld* genes results in a loss of gliding motility and pleiotropic effects, such as resistance to bacteriophages and loss of the ability to hydrolyze chitin (28, 29).

In this study, we isolated Tn*4351*-induced mutants of *F. psychrophilum* that are deficient in proteolytic activity, motility, and iron uptake. One of these mutants, designated FP523, had an insertion in *tlpB*, which encodes a putative thiol:disulfide oxidoreductase. Periplasmic thiol:disulfide oxidoreductases are part of the folding pathway of many secreted proteins (for reviews see references 13 and 37). These enzymes have an active site containing two cysteines in the motif C-X-X-C and an overall tertiary structure known as the thioredoxin-like fold (34). They facilitate the formation of disulfide bridges (Dsb proteins) and are essential for correct folding or assembly of many proteins, including toxins, adhesins, and components of

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the type III secretion system (17, 40, 51, 54). FP523 was deficient in gliding motility, extracellular proteolytic activity, cytotoxicity, and virulence and exhibited an enhanced ability to form biofilms.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Wild-type strain *F. psychrophilum* THC02-90 was used in this study. *Escherichia coli* strains S17-1λ *pir* (48) and BW19851 (32) were used to transfer DNA into *F. psychrophilum*. *E. coli* strains were grown at 37°C in 2× TY medium (10 g of tryptone per liter, 10 g of yeast extract per liter, 5 g of NaCl per liter) with 20 g of agar per liter added for solid medium. *F. psychrophilum* strains were grown at 20°C on solid EAOS medium (33) or at 12°C and 18°C in nutrient broth (NB) (Pronadisa) as previously described (3). To observe colony spreading, *F. psychrophilum* was grown on gliding nutrient agar (GNA), which consisted of half-strength NB containing 7.5 g of agar per liter. To test the extracellular proteolytic activity of *F. psychrophilum* strains, 0.5% (wt/vol) gelatin was added to nutrient agar (NA). NA consisted of NB with 1.5% (wt/vol) agar. To identify mutants which had defects in iron uptake, the iron chelator 2,2′-dipyridyl was added to NA at a final concentration of 50 μM. For selection of *F. psychrophilum* transconjugants or transformants, erythromycin or tetracycline was used at a concentration of 10 μg per ml. For selective growth of *E. coli* strains, antibiotics were added when needed at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; streptomycin, 50 μg/ml; and tetracycline, 15 μg/ml. The plasmids and primers used are listed in Table 1.

Tn*4351* mutagenesis of *F. psychrophilum*. Tn*4351* was introduced into *F. psychrophilum* THC02-90 by conjugation from *E. coli* BW19851, as previously described (3). Erythromycin-resistant colonies of *F. psychrophilum* appeared after 7 to 10 days of incubation at 20°C. The colonies were grown on GNA, NA with gelatin, and NA with 2,2′-dipyridyl to select mutants defective in gliding motility, proteolytic activity, and iron uptake, respectively.

Southern blot analysis of Tn*4351* insertions in *F. psychrophilum* mutants. Genomic DNA of the mutants was isolated, digested with XbaI, separated by gel electrophoresis, and transferred to nylon membranes essentially as previously described (3). A DIG DNA labeling and detection kit (Roche, Basel, Switzerland) was used to prepare the probes and to perform hybridization. Two probes were used, a 6.2-kb SalI fragment from pEP4351 containing transposon Tn*4351* and the chloramphenicol acetyltransferase gene (*cat*), which is also present in pEP4351. The *cat* gene was amplified from pIVET8 as a 633-bp PCR product using primers CAT-1 and CAT-2 (Table 1).

Identification of the insertion site of Tn*4351* in FP523 and amplification of the DNA surrounding *F. psychrophilum tlpB* by inverse PCR. Chromosomal DNA of the mutant was digested with HindIII and religated, which resulted in the formation of circular molecules. Tn*4351*-specific pairs of primers (primers 340 and 341 and primers TN-1 and IS4351-F) (Table 1) were used to amplify the sequences adjacent to the insertion site using a Certamp long amplification kit (BIOTOOLS B&M Labs, S.A.). DNA isolated from wild-type strain THC02-90 was digested with XbaI and religated to amplify the DNA surrounding *tlpB* by inverse PCR using primers 523-A and 523-B (Table 1).

Nucleic acid sequencing. Automated fluorescence sequencing was performed at the Oviedo University DNA analysis facility using BigDye 3.1 terminator chemistry with an ABI PRISM 3100 genetic analyzer platform (Applied Biosystems). Sequences were compared to databases by using BLAST from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/). Predictions of protein subcellular localization were made using PSORTb v.2.0 (16). Secondary structure and fold recognition analyses were carried out using the programs PSIPRED (30) and 3D-PSSM (22), respectively.

RT-PCR. Total RNA was obtained from 3-ml late-exponential-phase cultures of wild-type strain THC02-90 and mutant FP523 grown in half-strength NB. RNA was isolated using an RNeasy mini kit (QIAGEN, Valencia, Calif.) and was treated with RNase-free DNase (Promega, Inc.) to eliminate traces of DNA. Reverse transcription (RT)-PCRs were performed using Superscript One-Step with Platinum *Taq* (Invitrogen Life Technologies, Carlsbad, Calif.). Fifteen nanograms of RNA was used in each reaction. Control PCRs using Dynazyme II DNA polymerase (Finnzymes OY, Finland) were performed to determine whether RNA was free of contaminant DNA. The primers used (RT-A, RT-B, RT-C, RT-D, RT-E, RT-F, RT-G, RT-H, RT-I, and RT-J) are listed in Table 1.

Growth curves and determination of the proteolytic activities of THC02-90 and FP523. THC02-90 and FP523 cultures were grown in NB in a Gallemkamp rotary incubator at 250 rpm and 12°C or 18°C. Growth was determined by measuring the absorbance of the cultures at 525 nm. One-milliliter aliquots were removed to assay proteolytic activity. These aliquots were centrifuged at 15,000 × *g* for 5 min, and the supernatants were collected and stored frozen until the proteolytic activity was determined. Proteolytic activity was assayed by using azocasein (Sigma Co.) as a substrate, as previously described (46).

TABLE 1. Plasmids and primers used in this study

Plasmid or primer	Description or sequence[a]	Reference
Plasmids		
pEP4351	*pir*-requiring R6K *oriV*, RP4 *oriT*, Cm^r Tc^r (Em^r), vector for Tn*4351* transfer	9
pCP23	ColE1 *ori*; (pCP1 *ori*), Ap^r (Tc^r), *E. coli-F. psychrophilum* shuttle plasmid	2
pIVET8	*pir*-dependent *ori*R6K, Mob$^+$ Ap^r	25
pGA1	pCP23 containing *tlpB*, Ap^r (Tc^r)	This study
pGA2	pCP23 containing *tlpA*, Ap^r (Tc^r)	This study
pGA3	pCP23 containing *tlpB* and *tlpA*, Ap^r (Tc^r)	This study
Primers		
340	5′ GACTTGGATACCTCACGCC 3′	29
341	5′ TTGGAAATTTTCTGGGAGG 3′	29
TN-1	5′ GGACCTACCTCATAGACAA 3′	This study
IS4351-F	5′ TCAGAGTGAGAGAAAGGG 3′	This study
523-A	5′ TTCCCTGTGTTTCCGCC 3′	This study
523-B	5′ CACCTTAATAGACAGCACTA 3′	This study
tlpB-F	5′ AGCTGGATCCGATGCCAATTATCAAGA TATG 3′	This study
tlpB-R	5′ AGCTCTGCAGAAATATTTAATGCTATAA CGAA 3′	This study
tlpA-F	5′ AGCTGGATCCCTATCGATTATCAAGC 3′	This study
tlpA-R	5′ AGCTCTGCAGGCTCTCATTAGTTTAGGCT 3′	This study
CAT-1	5′ CACTGGATATACCACCG 3′	This study
CAT-2	5′ TGCCACTCATCGCAGTA 3′	This study
RT-A	5′ ACCTTACACAATTAGAATACTAATTTCG 3′	This study
RT-B	5′ GTTAATTAGTGCTTGATAATCGATAGGG 3′	This study
RT-C	5′ TTAGTGGCTCCAGATGCAAGCG 3′	This study
RT-D	5′ CCTGTCTTGTCTATAATAATATATCTGGGA ATCC 3′	This study
RT-E	5′ GTAGCTGGAAACTGGAAGATGA 3′	This study
RT-F	5′ TTATATCCCATTTACGATAACTACAAAA 3′	This study
RT-G	5′ GTTCCAGGTTGCGACCACTGCCGC 3′	This study
RT-H	5′ CGCTTGCATCTGGAGCCACTAATTTGC 3′	This study
RT-I	5′ ATAGCCTTATATAAAGCCATTGAAACCG 3′	This study
RT-J	5′ TTCATGAAAGATAGCTCTTCGTTCCG 3′	This study

[a] Antibiotic resistance phenotypes: Ap^r, ampicillin resistance; Cf^r, cefoxitin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Tc^r, tetracycline resistance. Antibiotic resistance phenotypes and other characteristics in parentheses are expressed by *F. psychrophilum* but not by *E. coli*. BamHI (primers tlpB-F and tlpA-F) and PstI (primers tlpB-R and tlpA-R) recognition sequences added to the primer sequences are underlined.

Confocal laser scanning microscopic observations of colony spreading. Exponential-phase cells of strains THC02-90 and FP523 were spotted onto thin layers of GNA on microscope slides and incubated for 3 days at 20°C. The colonies formed after this incubation were stained with SYTO 9 (Molecular Probes, Eugene, Oreg.) and observed with a Leica TCS-SP2-AOBS confocal laser scanning microscope at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Colonies were also examined by phase-contrast microscopy.

Biofilm formation assay. Wild-type strain THC02-90 and the mutant strain were grown on half-strength NB to the mid-exponential phase. The cultures were diluted 1:100 in different concentrations of NB, and then 100 μl of each dilution was inoculated in quadruplicate into wells of a 96-well microtiter polystyrene plate. Wells containing uninoculated medium were used as negative controls. The microtiter plate was incubated at 12°C for 4 days. Following this incubation, the supernatants were discarded, and the wells were washed six times with 200 μl of sterile distilled water. Then 150 μl of a 1% (wt/vol) crystal violet solution was added to each well to stain the cells. After 45 min, the crystal violet solution was removed, the wells were washed six times with 200 μl of sterile distilled water, and 200 μl of 96% (vol/vol) ethanol was added. Biofilm formation was then quantified by measuring the A_{595}. To observe biofilms by confocal laser scanning microscopy, 500 μl of cells was added to each chamber of BD Falcon four-chamber culture slides, incubated for 4 days at 12°C, and stained with SYTO 9 as described above.

Disulfide reductase and Ellman assays and sensitivity to diamide, dithiothreitol (DTT), menadione, and plumbagin. The wild-type strain and the FP523 mutant strain were grown in NB to the late exponential phase and exposed to 0.5

mM diamide for 3 h when appropriate. Then cells were harvested and washed with 50 mM Tris-HCl (pH 8) buffer containing 1 mM EDTA. Cell extracts were prepared by sonication and centrifugation at 25,000 × *g* for 20 min, and disulfide reductase activity was assayed as described by Holmgren (18) using 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) as the substrate. DTNB reductase activity was determined by measuring the increase in A_{412} at room temperature during the initial 5 min of the reaction. The reaction mixture (final volume, 1 ml) contained 50 mM Tris-HCl (pH 8), 1 mM EDTA, 0.2 mM NADPH, 0.2 mM DTNB, and cell extract. In control reactions in which NADPH was omitted there was no significant increase in absorbance. One unit of activity was defined as the amount of protein that oxidized 1 μmol of NADPH per min, calculated using $\Delta A_{412}/(13.6 \times 2)$ (24).

Periplasmic protein samples from cells of the wild-type and mutant strains in the exponential phase of growth were isolated by using the chloroform method described by Ames et al. (4), except that the periplasmic proteins were solubilized with Ellman's buffer (1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride in 50 mM phosphate buffer, pH 7.5). To determine the amount of free thiols groups, a sample containing 0.3 mg of protein was mixed with 0.8 mM (final concentration) DTNB. The absorbance at 412 nm of each sample was determined after 1 min of incubation at room temperature. The assays were carried out in quadruplicate.

The sensitivities of the wild-type and mutant strains to the thiol-specific oxidant and reductant and to a redox cycling compound were determined on plates containing NA using paper disks that were soaked in 100 mM or 1 M solutions of diamide and DTT and 1 mM or 10 mM solutions of metadione and plumbagin. The results were analyzed after 4 days of incubation at 20°C.

Complementation of *tlpB* mutant. To complement FP523, three plasmids based on pCP23 (Table 1) were constructed. The *tlpB* gene was amplified by PCR using a Certamp long amplification kit (BIOTOOLS B&M Labs, S.A.) and primers tlpB-F and tlpB-R (Table 1). BamHI and PstI restriction sites were introduced into the sequences of tlpB-F and tlpB-R, respectively, to clone the PCR product digested with BamHI and PstI into pCP23 that had been digested with the same restriction enzymes. The resulting plasmid was designated pGA1. *tlpA* was amplified using primers tlpA-F and tlpA-R (Table 1), and a DNA fragment which contained *tlpB* and *tlpA* was amplified using primers tlpB-F and tlpA-R (Table 1). Each PCR product was cloned into pCP23 as described above to generate pGA2, which contained *tlpA*, and pGA3, which contained *tlpB* and *tlpA*. For complementation analysis, pGA1, pGA2, and pGA3 were first introduced into *F. psychrophilum* THC02-90 by conjugation. The plasmids were isolated from wild-type *F. psychrophilum* and transferred by electroporation into *F. psychrophilum* FP523 as previously described (3).

Cytopathic effects and 50% lethal dose (LD_{50}) of FP523. The bluegill fry BF2 cell line was used in cytotoxicity studies. Cells were cultured in Eagle minimal essential medium (Sigma Co.) supplemented with 2.2 g $NaHC0_3$ per liter and 10% serum at 15°C. Monolayer cultures of BF2 cells in 24-well plates were inoculated with approximately 10^7 CFU per well of exponential-phase bacterial cultures previously washed with phosphate-buffered saline (PBS). After 48 h of incubation at 15°C, the plates were observed with an inverted microscope.

To determine the LD_{50}, we used rainbow trout (*Oncorhynchus mykiss*) that weighed between 5 and 7 g, which were kept in 60-liter tanks containing dechlorinated water at 12 ± 1°C. *F. psychrophilum* cultures were grown to the exponential phase, harvested by centrifugation, and washed with PBS. Cells were suspended in PBS, and serial dilutions were prepared in PBS. Groups of 10 fish were challenged by intramuscular injection of 0.05 ml of dilutions containing 10^3 to 10^7 cells, and the LD_{50} was calculated by using the method of Reed and Muench (45).

Genetic nomenclature. Open reading frames (ORFs) that code for predicted thiol:disulfide oxidoreductases were designated *tlp* (*t*hioredoxin-*l*ike *p*rotein), followed by a letter. The ORFs that did not exhibit high levels of homology with known genes were designated fpo (*F. psychrophilum* open reading frame), followed by a number.

Nucleotide sequence accession number. The sequence of the *fpt* operon and the surrounding DNA has been deposited in the GenBank database under accession number DQ137800.

RESULTS

Isolation of Tn*4351*-induced mutants. Using plasmid pEP4351 as a Tn*4351* delivery system, approximately 1,600 erythromycin-resistant colonies of *F. psychrophilum* were obtained. Thirteen mutants with defects in motility, proteolytic activity, and/or the ability to grow in the presence of 2,2′-dipyridyl were obtained. Some of these mutants were affected only in one characteristic, whereas others had two or more phenotypic defects. Southern blot analysis of Tn*4351* insertions revealed that most mutants had a single Tn*4351* insertion (data not shown). The pleiotropic mutant FP523, which lacked extracellular proteolytic activity and colony spreading, grew poorly in iron-limited medium, and had a single Tn*4351* insertion (Fig. 1), was chosen for further study.

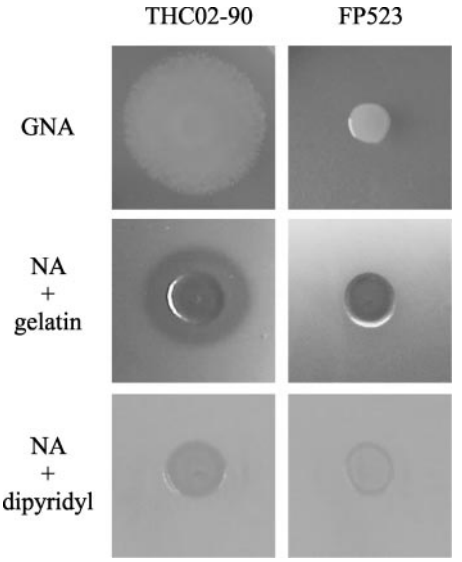

FIG. 1. Phenotypic characteristics of the *F. psychrophilum* FP523 mutant. *F. psychrophilum* wild-type strain THC02-90 exhibits gliding motility in GNA, extracellular proteolytic activity in NA containing 0.5% gelatin, and growth in the presence of 2,2′-dipyridyl. The FP523 mutant lacks gliding motility and extracellular proteolytic activity and exhibits limited growth in the presence of 2,2′-dipyridyl.

Sequencing and analysis of the DNA surrounding the site of Tn*4351* insertion in FP523. The site of Tn*4351* insertion and the surrounding DNA in FP523 were analyzed (Fig. 2A). The transposon was inserted in a gene designated *tlpB*. *tlpB* is 1,101 nucleotides long and codes for a predicted protein consisting of 367 amino acids with a molecular mass of 41.4 kDa (Fig. 2B). The amino acid sequence of TlpB does not exhibit high levels of homology with proteins having known functions. TlpB exhibits sequence similarity with the hypothetical protein Chut02002298 of *Cytophaga hutchinsonii* (GenBank accession number ZP_00308793; 26% identity over 362 amino acids). *C. hutchinsonii* is a member of the CFB group, and like *F. psychrophilum*, it exhibits gliding motility. Sequence analysis revealed that TlpB has two distinguishable domains: an N-terminal domain (positions 1 to 174) that has five transmembrane regions (Fig. 2B), which strongly suggests that the protein is membrane anchored, and a C-terminal domain (positions 174 to 367). The carboxy-terminal portion of *F. psychrophilum* TlpB has a putative thioredoxin-like domain which includes the Cys-X-X-Cys motif (C_{260}-D-H-C_{263}) of thiol:disulfide oxidoreductases, as well as a highly conserved proline (P_{227}) that is present in the redox proteins. Secondary structure and fold recognition analyses suggested that the folding of the TlpB C-terminal domain is similar to the folding of the thiol:

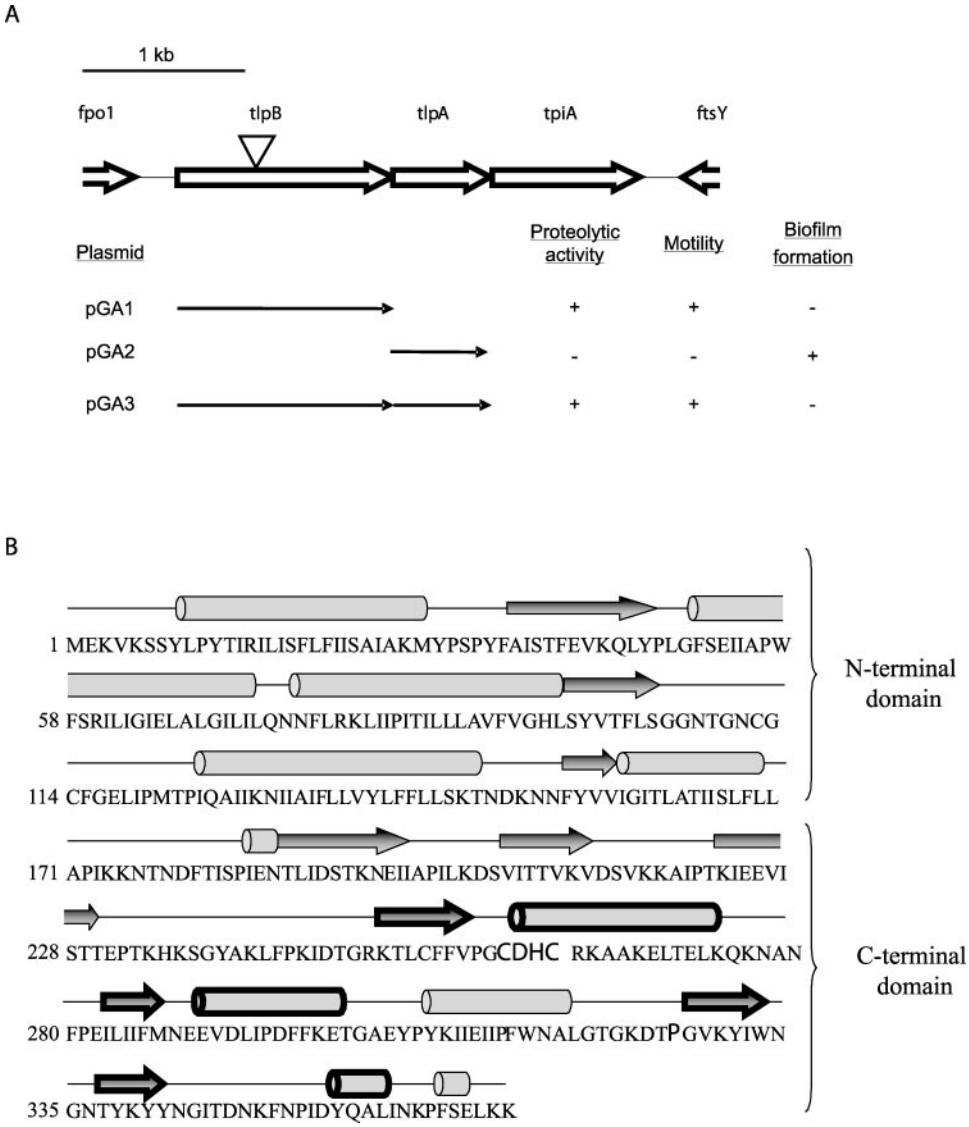

FIG. 2. (A) Genetic organization of the *fpt* operon of *F. psychrophilum* THC02-90. FP523 has a single Tn*4351* transposon insertion (indicated by a triangle) at nucleotide 469 of the *tlpB* gene. Results of complementation of FP523 with pGA1, pGA2, and pGA3 are shown below the map. (B) TlpB amino acid sequence and secondary structure. β-Sheet strands are indicated by arrows, and α-helices are indicated by cylinders. The five α-helices in the N-terminal domain correspond to the five transmembrane regions. The β-sheets and α-helices that are suggested to form part of the thioredoxin fold are indicated by thick lines. The conserved thiol:disulfide oxidoreductase motifs C_{260}-D-H-C_{263} and P_{227} in the C-terminal domain of the protein are indicated by larger type.

disulfide oxidoreductase ResA from *Bacillus subtilis*, a protein which is involved in cytochrome *c* synthesis (10). These molecules have the thioredoxin fold characteristic of this family of proteins (26).

The start codon of *tlpA* is located six nucleotides downstream of the *tlpB* stop codon (Fig. 2A). TlpA consists of 165 amino acids, exhibits sequence similarity with thiol:disulfide oxidoreductase, and has the Cys-X-X-Cys motif (data not shown). It exhibits identity to the TlpA protein (31% identity over 132 amino acids; GenBank accession number NP_811658) of *Bacteroides thetaiotaomicron* VPI-54 and to the thiol:disulfide oxidoreductase family of proteins, such as COG0526 (30% identity over 169 amino acids; GenBank accession number ZP_00310634) of *C. hutchinsonii*. Downstream of *tlpA* (Fig. 2A) is *tpiA*, whose start codon overlaps the *tlpA* stop codon. *tpiA* encodes a predicted triosephosphate isomerase of the glycolytic pathway. The amino acid sequence encoded by *F. psychrophilum tpiA* is similar to the sequences of triosephosphate isomerases of many microorganisms, such as *Porphyromonas gingivalis* W83 (51% identity over 248 amino acids; GenBank accession number AAQ65807). A 19-bp inverted repeat sequence (TTTAAAAAAGTAAAACCACCCGAGACGAGTGGTTTTACTTTTTTAAA) that is 11 bp downstream of the stop codon of *tpiA* may function in transcription termination. Downstream of *tpiA* and oriented in the opposite direction is the predicted *ftsY* gene (Fig. 2A). The TAA stop codon of *ftsY* is located 225 nucleotides from the *tpiA* stop codon. We do not have the complete coding region of *F. psychrophilum ftsY*, but the translated partial amino acid sequence exhibits sim-

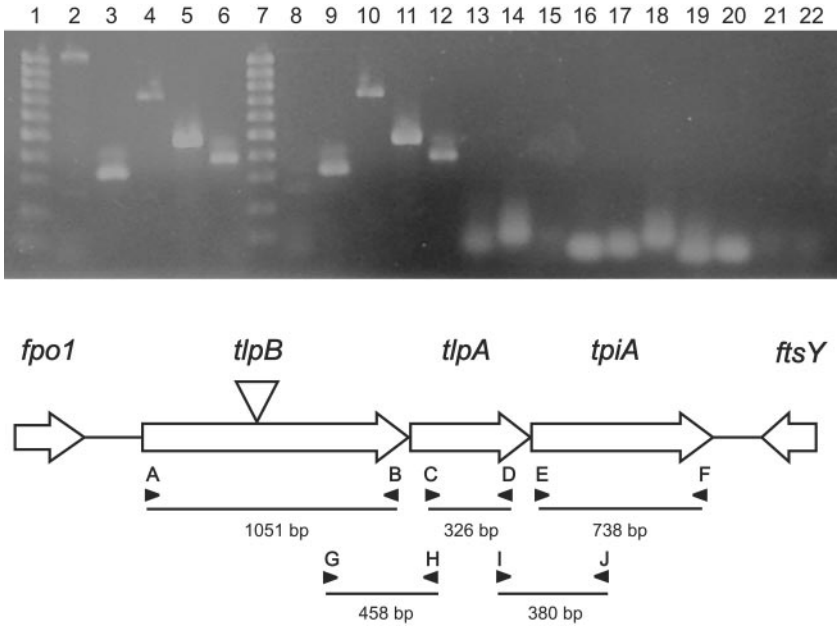

FIG. 3. RT-PCR analysis of the *fpt* operon in the RNA of *F. psychrophilum* THC02-90 and FP523. PCRs were performed with 15 ng of RNA from *F. psychrophilum* strains using primers RT-A, RT-B, RT-C, RT-D, RT-E, RT-F, RT-G, RT-H, RT-I, and RT-J. As a control for DNA contamination, PCRs were also performed with the primers used for RT-PCR analyses and *Taq* polymerase. Lanes 1 and 7, 100-bp molecular marker; lanes 2 to 6, RT-PCRs performed with THC02-90 RNA; lanes 8 to 12, RT-PCRs performed with FP523 RNA; lanes 13 to 22, PCRs performed without reverse transcription as negative controls. The following primers were used: RT-A and RT-B for lanes 2, 8, 13, and 18; RT-C and RT-D for lanes 3, 9, 14, and 19; RT-E and RT-F for lanes 4, 10, 15, and 20; RT-G and RT-H for lanes 5, 11, 16, and 21; and RT-I and RT-J for lanes 6, 12, 17, and 22. The map of the *fpt* operon shows the localization of the primers used for RT-PCR analysis and the lengths of the amplicons obtained.

ilarity to FtsY proteins of *P. gingivalis* W83 (86% identity over 73 amino acids; GenBank accession number NP_904493.1) and other microorganisms. FtsY is a component of the signal recognition particle complex that is involved in targeting and integration of inner membrane proteins. fpo1 is upstream of *tlpB*. The stop codon of fpo1 is 217 nucleotides upstream of the *tlpB* start codon (Fig. 2A). The sequence of the protein encoded by fpo1 is not similar to the sequences of proteins having known functions.

RT-PCR analysis of the *tlpB*, *tlpA*, and *tpiA* loci. RT-PCR studies of *F. psychrophilum* THC02-90 indicated that transcripts corresponding to the *tlpB*, *tlpA*, and *tpiA* genes were present (Fig. 3, lanes 2, 3, and 4). However, as expected, a *tlpB* transcript was not obtained for FP523 (Fig. 3, lane 8). To determine if the three loci were transcribed as a single mRNA, as suggested by sequence analysis, RT-PCR using primers complementary to the end of a locus and the beginning of the next locus was carried out (Fig. 3). As shown in Fig. 3, lanes 5 and 11, amplicons that were the expected size were generated between *tlpB-tlpA* (458 bp) and *tlpA-tpiA* (380 bp) (Fig. 3, lanes 6 and 12), indicating that the three loci are transcribed as a polycistronic mRNA. The operon was designated the *fpt* operon. Although *fpt* appears to be an operon, the Tn*4351* insertion in *tlpB* did not eliminate transcription of *tlpA* and *tpiA* (Fig. 3, lanes 9 and 10). Transcription of *tlpA* and *tpiA* in FP523 is probably driven by a promoter in Tn*4351*. Tn*4351* has promoters reading out of each end and often causes nonpolar mutations in *Bacteroides fragilis* and *F. johnsoniae* (20, 49).

Phenotypic characterization of the FP523 mutant. To further characterize FP523, different phenotypic characteristics were studied. FP523 had a longer lag phase and a slightly lower growth rate than the wild type, and the final cell density was not as high (Fig. 4A). FP523 cultures exhibited basal levels of proteolytic activity that were much lower than the levels observed for the wild type (Fig. 4A). These experiments were conducted at 12°C, but similar results were obtained at 18°C.

Colonies formed in GNA by wild-type strain THC02-90 and mutant FP523 were examined by confocal laser scanning microscopy and phase-contrast microscopy. The cells on the edges of FP523 colonies were perpendicular to the colony radius (Fig. 4B). In contrast, on the edges of THC02-90 colonies we observed flares of cells that were roughly parallel to the colony radius, which resulted in colony spreading (Fig. 4B).

One important factor for bacterial colonization is the development of biofilms. The ability of *F. psychrophilum* to form biofilms was not known until this study. In fact, the wild-type strain was not able to form biofilms under the culture conditions assayed (data not shown). However, there were clearly biofilms on polystyrene plastic wells after incubation of FP523 in half-strength NB (Fig. 5A). This mutant was not able to form biofilms when it was incubated in undiluted NB. This result revealed that the formation of biofilms depends on the concentration of nutrients. FP523 adherent cells in the biofilms were observed by confocal microscopy (Fig. 5B). Few wild-type cells were found in the glass slide cultures (Fig. 5B), but a well-developed biofilm was formed during culture of the FP523 mutant (Fig. 5B). Wild-type cells of related species, such as *Flavobacterium columnare* and *F. johnsoniae*, were not able to

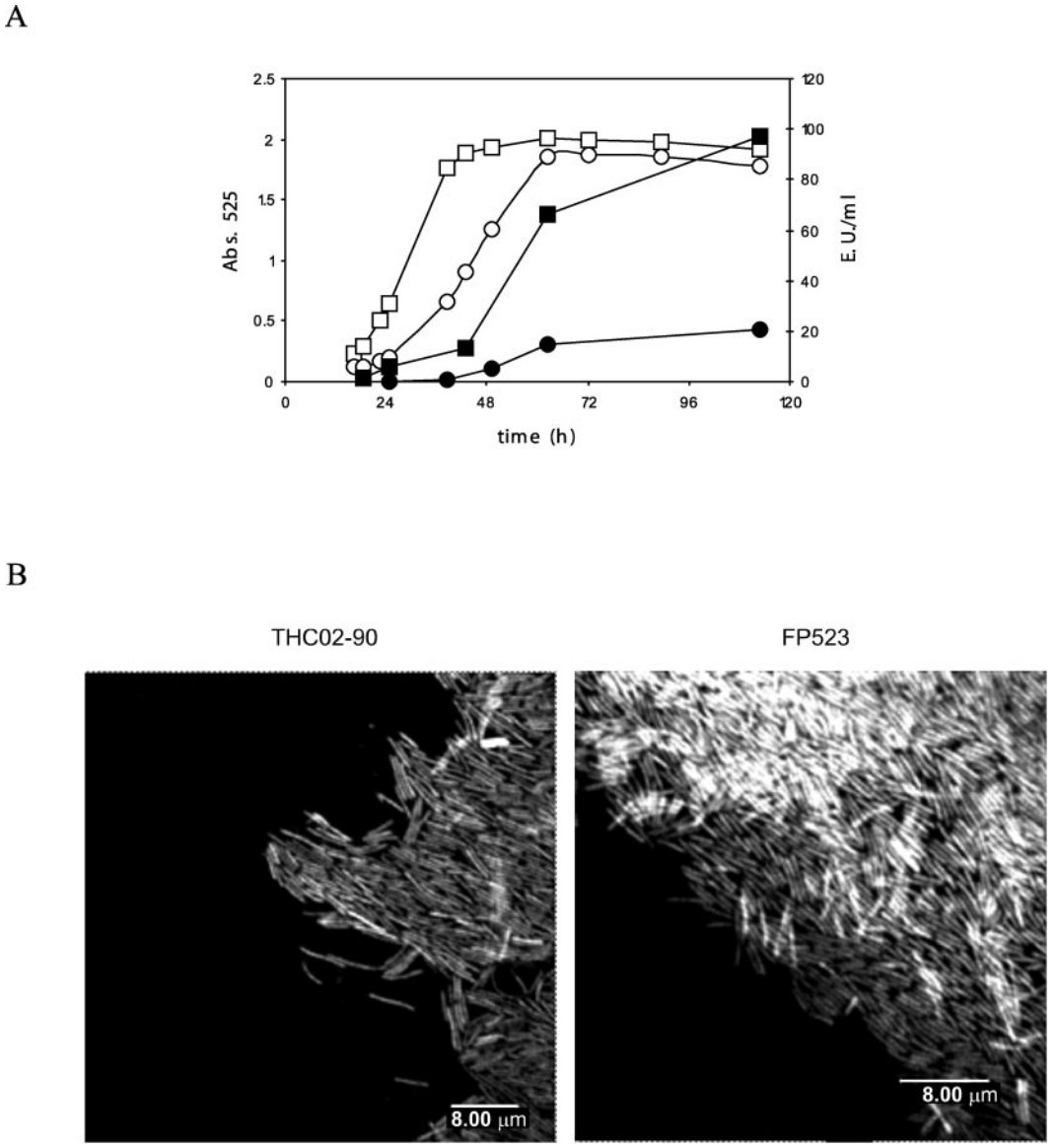

FIG. 4. (A) Growth and extracellular proteolytic activity of *F. psychrophilum* strains THC02-90 and FP523. The bacteria were grown at 12°C in NB, and growth was monitored by determining the absorbance at 525 nm (Abs. 525). The extracellular caseinolytic activity in cell-free supernatants was determined with azocasein as described by Secades et al. (46). □, THC02-90 growth; ○, FP523 growth; ■, THC02-90 proteolytic activity; ●, FP523 proteolytic activity. E.U., enzyme units. (B) Cell orientation at the periphery of colonies of *F. psychrophilum* strains THC02-90 and FP523. Exponential-phase cells were spotted on glass microscope slides that were previously covered with a thin layer of GNA. After 3 days of incubation at 20°C, colonies were stained with SYTO 9 and visualized by confocal laser scanning microscopy. Wild-type strain THC02-90 cells exhibited a pattern in which the long axis of the cells was oriented in the same direction as colony spreading. FP523 did not exhibit this cell disposition, as the entire colony front was composed of cells that formed a retaining wall-like structure with the cells transversally oriented.

form biofilms under the same culture conditions (data not shown).

Inactivation of the *tlpB* gene did not have any significant effect on the sensitivity to the thiol-specific oxidant diamide or on the sensitivity to menadione and plumbagin, two superoxidizing agents (data not shown). This is consistent with the similar levels of disulfide reductase activity determined for the wild type (0.00391 U/mg protein) and the FP523 strain (0.00413 U/mg protein) after induction with diamide. These results showed that the general redox state was not altered by the mutation. On the other hand, the amount of free thiol groups of periplasmic proteins was about 22% higher in the wild type than in the mutant strain (Fig. 6A), and there was also a clear difference between the sensitivities of the strains to DTT (Fig. 6B). These data strongly suggest that TlpB works as a periplasmic reductase.

Complementation of *tlpB* mutant. Plasmids pGA1, pGA2, and pGA3 were constructed as described in Materials and Methods to complement the FP523 mutant. Introduction of pGA1, which contained *tlpB*, into the FP523 mutant restored gliding motility and the extracellular proteolytic activity (70% of the wild-type proteolytic activity) (Fig. 2A). In addition,

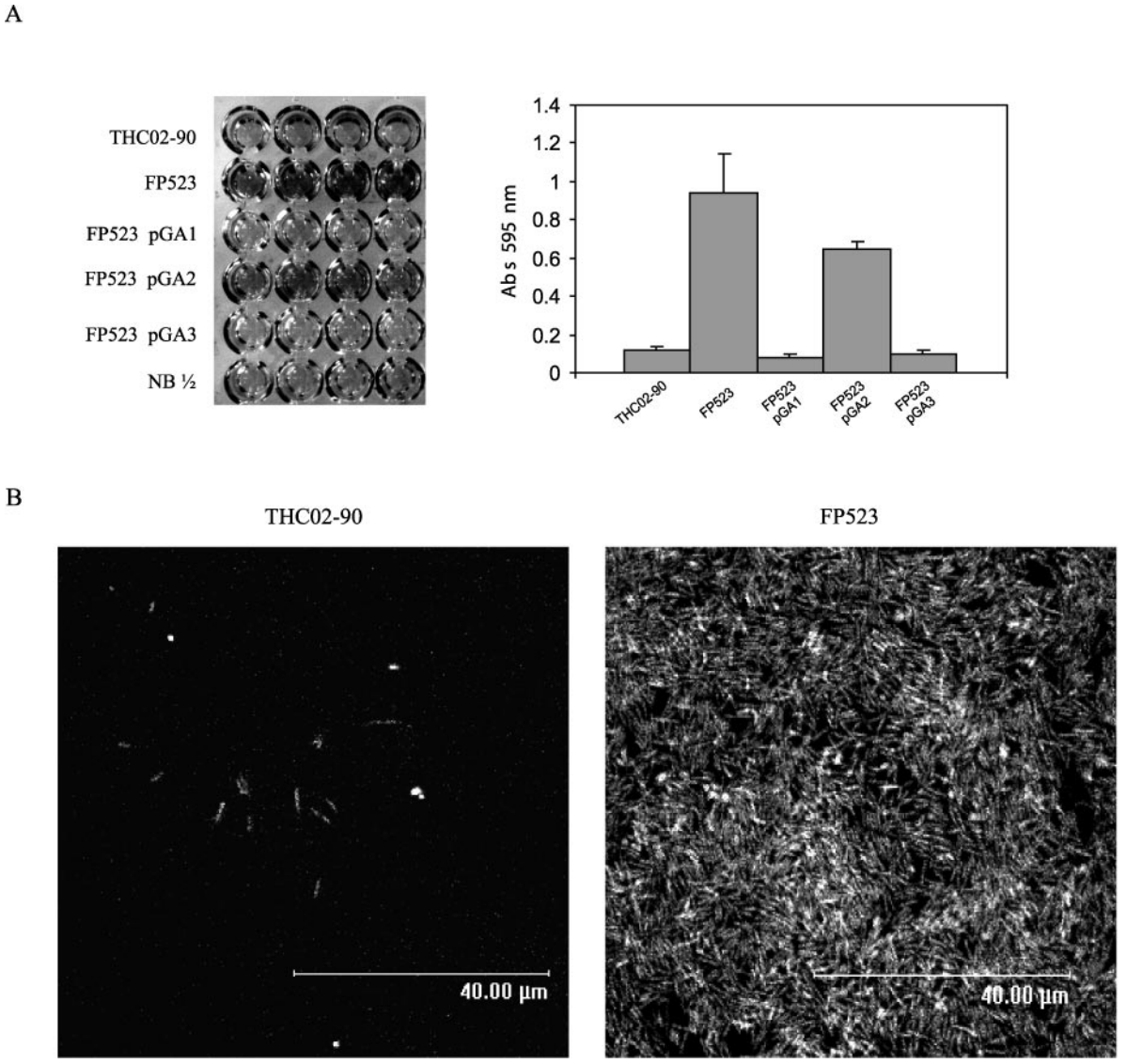

FIG. 5. (A) Biofilm formation by *F. psychrophilum* THC02-90, FP523, and complemented FP523, as determined in stained microtiter wells. Microtiter wells containing half-strength NB cultures of the bacterial strains were incubated for 4 days and stained with 0.1% crystal violet (left panel). The four wells for each strain are replicates. The amount of biofilm formed was determined by ethanol solubilization of crystal violet and measuring the absorbance at 525 nm (Abs 525 nm) of the resulting solution (right panel). The error bars indicate standard deviations. (B) Biofilm formation by cells of wild-type strain THC02-90 and mutant FP523. *F. psychrophilum* cells were incubated on glass slides in half-strength NB for 4 days at 12°C and stained with SYTO 9.

80% recovery of the proteolytic activity and recovery of the gliding motility phenotype and the level of periplasmic thiol groups (Fig. 6A) were observed when plasmid pGA3, which contained the *tlpB* and *tlpA* loci, was used in the complementation analysis. In contrast, plasmid pGA2, which contained only *tlpA*, did not complement FP523. The complemented FP523 strains containing pGA1 and pGA3 were like the wild type in that they were not able to attach efficiently to a surface (Fig. 2A). The results of quantification of biofilm formation by the crystal violet assay are shown in Fig. 5A. As expected, the FP523 mutant containing plasmid pGA1 or pGA3 did not form a biofilm (Fig. 2A and 5A). Thus, disruption of the *tlpB* gene resulted in loss of motility and promoted biofilm formation. All these findings indicate that *tlpB* is involved in the biofilm formation pathway, in addition to gliding motility, suggesting that these two cellular processes are in some way antagonistic in *F. psychrophilum*.

Cytopathic effects and LD_{50}. In order to determine the cytotoxicity for fish culture cells, wild-type *F. psychrophilum* strain THC02-90 and the FP523 mutant were incubated with bluegill sunfish BF2 cells, a fibroblastic cell line. There was a clear difference in the cytopathic effects on BF2 cells exposed to similar concentrations of the wild-type and FP523 mutant strains. Addition of wild-type *F. psychrophilum* cells resulted in lysis of the BF2 cells, whereas addition of cells of the *tlpB* mutant had no effect on the sunfish cells (data not shown).

Rainbow trout were infected with wild-type and FP523 mutant cells to determine the effect of the mutation on virulence.

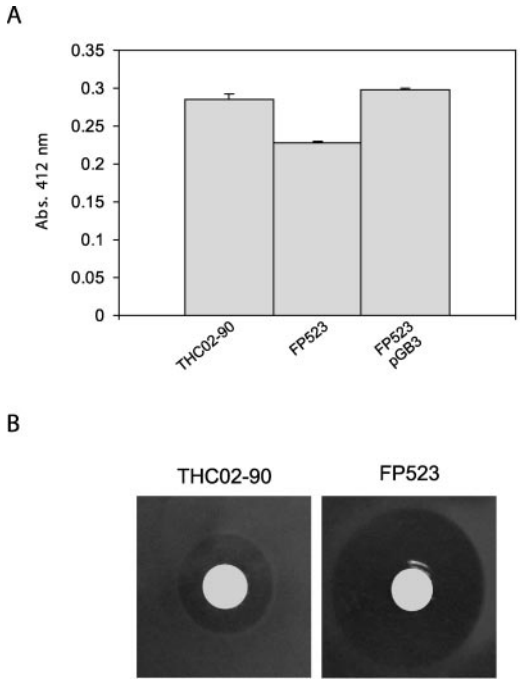

FIG. 6. (A) Levels of reduced periplasmic proteins in *F. psychrophilum* wild-type strain THC02-90, mutant FP523, and FP523 complemented with plasmid pGA3 (containing *tlpB* and *tlpA* loci). Periplasmic proteins isolated from cultures in the late exponential growth phase were mixed with Ellman's reagent at a final concentration of 0.8 mM, and the absorbance at 412 nm (Abs. 412 nm) was determined. The error bars indicate standard deviations. (B) Bioassays showing the DTT sensitivity of *F. psychrophilum* THC02-90 and FP523. Paper disks were soaked in 100 mM DTT.

Ten days after infection the calculated LD_{50}s of the mutant and wild-type strains were 8.37×10^6 and 1.03×10^5, respectively. The approximately 80-fold difference between the two strains indicates that disruption of *tlpB* results in attenuation of *F. psychrophilum* virulence.

DISCUSSION

In spite of the importance of cold water disease and the interest in *F. psychrophilum*, there are many aspects of this bacterium that are still not known. In the present study, genetic techniques that were recently developed for manipulation of *F. psychrophilum* (3) were used to obtain a set of mutants with mutations in extracellular proteolytic activities, gliding motility, and iron dependence. Some mutants were affected in only one of the phenotypic characteristics, whereas in others there were two or more phenotypic alterations. Gliding motility mutants of *F. johnsoniae* which exhibit pleiotropic effects have been isolated previously (28, 29). These mutants were unable to degrade chitin and were resistant to phage infection (27). One the pleiotropic mutants of *F. psychrophilum*, FP523, was chosen for further study. This mutant had defects in extracellular proteolytic activity, gliding motility, and growth in iron-deprived medium.

The gene disrupted in the FP523 pleiotropic mutant, *tlpB*, encodes a putative membrane-anchored thiol:disulfide oxidoreductase. Such proteins form disulfide bonds in unfolded proteins, reduce disulfide bonds to dithiols, or reduce for isomerization and reoxidation when undesirable disulfide bonds are formed. Oxidative formation of disulfide bonds is necessary for correct folding and assembly of secreted proteins in prokaryotes. The thiol:disulfide oxidoreductases have a Cys-X-X-Cys motif in the active site. The two cysteines that are present in this motif react with cysteines or cystines in the target molecules (13, 37). TlpB has the motif C_{260}-D-H-C_{263} and a proline at position 227 which is highly conserved in redox proteins (26). Thiol:disulfide oxidoreductases exhibit very low primary sequence similarity, but most of them have the same overall tertiary structure, known as the thioredoxin-like fold (26).

Mutations in *E. coli* genes that code for thiol:disulfide oxidoreductases lead to pleiotropic effects. For example, *dsbA* mutations have severe effects on flagellar motility, resistance to reduced DTT and benzylpenicillin, infection with phage M13, sensitivity to Cd^{2+}, Zn^{2+}, and Hg^{2+}, and other processes in which proteins need disulfide bonds in order to function. Similar results were obtained for *Pseudomonas aeruginosa* (17). Therefore, the effects of disruption of *tlpB* on the growth rate, growth in iron-deprived conditions, extracellular proteolytic activity, gliding motility, and virulence are consistent with the effects found in other bacterial mutants with mutations in genes encoding thiol:disulfide oxidoreductases. Perhaps the most surprising effect of the mutation in *tlpB* is activation of biofilm formation. Until now, biofilm formation had not been described for members of the genus *Flavobacterium*. The most interesting finding is that when the FP523 mutant was complemented in *trans* with the *tlpB* or *tlpB-tlpA* genes, it exhibited proteolytic activity and gliding motility and simultaneously lost the capacity to produce biofilms. Gliding motility and biofilm formation may be mutually exclusive in *F. psychrophilum*, or TlpB may indirectly affect motility and biofilm formation in different ways. To our knowledge, antagonism between motility and biofilm formation has not been reported previously, although flagellum-dependent motility has been related to adhesion, biofilm formation, and colonization in *P. aeruginosa* (7, 17), *Vibrio cholerae* (15), *Vibrio vulnificus* (23), *E. coli* (42), and *Caulobacter crescentus* (12).

The results of the disulfide reductase assay, together with the results obtained using compounds such as diamide, menadione, and plumbagin, indicate that TlpB is not involved in maintaining the redox state of the cytoplasm. However, the increase in sensitivity to DTT and the decrease in the number of thiol groups of periplasmic proteins in the mutated strain strongly suggest that TlpB plays a reducing role in the periplasm. Thus, as a putative inner membrane protein, TlpB is functionally similar to the DsbD proteins of *E. coli* and other bacteria (8, 13, 35, 41). This protein seems to play a role in folding secreted proteins, and when it is absent, there are pleiotropic effects, like those that occur in different *dsb* mutants of several bacteria (13, 17, 35, 37, 40, 51, 54). In the FP523 mutant, biofilm formation could be the result of a stress response induced by misfolding of proteins in the periplasmic space, as occurred with activation of the heat shock system in the *E. coli dsbA* and *dsbC* mutants (31, 44). Different stress response regulators are involved in triggering biofilm formation in *E. coli*, *Staphyloccus aureus*, *P. aeruginosa*, and *Streptococcus mutans* (1, 43, 52, 53). Therefore, external conditions or a specific genetic background of the bacteria with alterations in

the function of a subset of proteins could generate a stress response and induce biofilm formation. In spite of its capacity to form biofilms, the FP523 mutant was less virulent than the wild-type strain. FP523 has simultaneously altered putative virulence factors, such as extracellular proteolytic activities, the iron uptake system, motility, etc. Thus, the balance of the multiple effects caused by the mutation on the different putative virulence factors seems to result in attenuation of the mutant both in in vivo experiments and in the toxic effect on cell cultures.

tlpA, which is immediately downstream of *tlpB*, codes for another predicted thiol:disulfide oxidoreductase. In this case, the level of similarity of TlpA with the thiol:disulfide oxidoreductase family of proteins is higher. TlpA has the thioredoxin motif C_{65}-G-D-C_{68} and the conserved proline (P_{133}). Moreover, it also has the thioredoxin-like fold. We do not have a *tlpA* mutant, so the exact function of this gene is not known. *tlpA* is expressed in FP523, presumably from a Tn*4351* promoter. This result, together with the complementation data, indicates that the phenotypes described in this paper are probably not the result of effects on *tlpA* expression. However, introduction of both *tlpB* and *tlpA* into cells of FP523 resulted in a 10% increase in the proteolytic activity compared with the results obtained when only *tlpB* was used. This result could have been due to nonspecific additive enzyme activity or to a simple genetic dose effect for the presence of the plasmid harboring multiple copies of the genes in the cell. The third locus of the *fpt* operon, the putative triosephosphate isomerase-encoding gene *tpiA*, is involved mainly in the glycolysis pathway. However, a triosephosphate isomerase gene was specifically expressed in *S. aureus* during biofilm formation (5), indicating that there is some relationship between this enzyme and biofilm development.

In conclusion, here we describe isolation of mutants of *F. psychrophilum*. One of these mutants, the FP523 mutant, was affected in the *tlpB* gene encoding a novel protein which has a putative thiol:disulfide oxidoreductase domain, which is necessary for efficient gliding motility of the bacterium. Disruption of the *tlpB* gene led to biofilm formation. This gene is part of the *fpt* operon, and studies to define the roles of this operon and the importance and relationship of the different loci in the behavior of this bacterium have been started. Finally, clear attenuation of the virulence of the FP523 mutant was observed. This result, together with the lack of cytopathic effects of the mutant on BF2 cells, shows the importance of the *tlpB* gene in the pathogenic process.

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