

Development of Genetic Techniques for the Psychrotrophic Fish Pathogen *Flavobacterium psychrophilum*

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Flavobacterium psychrophilum, a member of the *Cytophaga-Flavobacterium-Bacteroides* group, is an important pathogen of salmonid fish. Previous attempts to develop genetic techniques for this fastidious, psychrotrophic bacterium have met with failure. Here we describe the development of techniques for the genetic manipulation of *F. psychrophilum* and the identification of plasmids, selectable markers, a reporter system, and a transposon that function in several isolates of this fish pathogen. The antibiotic resistance genes *ermF*, *cfxA*, and *tetQ* function in *F. psychrophilum*. Cloning vectors based on the *F. psychrophilum* cryptic plasmid pCP1 which carried these selectable markers were introduced by conjugation from *E. coli*, resulting in antibiotic-resistant colonies of *F. psychrophilum*. Conjugative transfer of DNA into *F. psychrophilum* was strain dependent. Efficient transfer was observed for two of the seven strains tested (THC02-90 and THC04-90). *E. coli lacZY* functioned in *F. psychrophilum* when expressed from a pCP1 promoter, allowing its development as a reporter for studies of gene expression. Plasmids isolated from *F. psychrophilum* were efficiently introduced into *F. psychrophilum* by electroporation, but plasmids isolated from *E. coli* were not suitable for transfer by this route, suggesting the presence of a restriction barrier. DNA isolated from *F. psychrophilum* was resistant to digestion by *Sau3AI* and *BamHI*, indicating that a *Sau3AI*-like restriction modification system may constitute part of this barrier. Tn4351 was introduced into *F. psychrophilum* from *E. coli* and transposed with apparent randomness, resulting in erythromycin-resistant colonies. The techniques developed in this study allow for genetic manipulation and analysis of this important fish pathogen.

The gliding bacterium *Flavobacterium psychrophilum* is the causative agent of cold water disease (CWD) in salmonids. Outbreaks of this disease, which may result in up to 50% mortality, are most common in fingerlings and typically occur at water temperatures between 12 and 14°C. CWD has important economic consequences because of the large and growing salmonid aquaculture industry (for a review, see references 7 and 10). No vaccine to prevent CWD is available, and control of the outbreaks currently involves antimicrobial drug administration.

F. psychrophilum is somewhat fastidious, requiring additions such as serum for optimal growth and for efficient colony formation from single cells plated on solid media. The optimum temperature for growth is approximately 18°C, and most strains fail to grow above 20°C. Under optimal conditions, the population doubles in approximately 3 h. These growth properties have hampered studies of *F. psychrophilum* and the development of genetic techniques. Recent advances have been made in methods for cultivation (18) and detection (8, 29, 30, 31) of *F. psychrophilum*, and procedures allowing standardization of experimental infection have been developed (9).

The mechanisms of *F. psychrophilum* pathogenicity are poorly understood. Cells of *F. psychrophilum* produce a number of proteases that may play a role in the disease process (3). A correlation has been established between the presence of specific proteases and virulence (13, 20). The metalloprotease

Fpp1, which may be involved in pathogenicity, has recently been purified and characterized (23). Lipopolysaccharide may also be associated with virulence and has been the subject of several recent studies (6, 12). The lack of genetic techniques for *F. psychrophilum* has hampered further progress toward defining the mechanisms of pathogenesis. The development of methods for genetic manipulation would allow for identification of virulence genes and would facilitate the rational design of vaccine strains.

F. psychrophilum is a member of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group and is very distantly related to organisms with well-developed genetic systems, such as members of the proteobacteria. In general, plasmids, selectable markers, and transposons that function in proteobacteria fail to function in members of the CFB group (16, 21). Methods for genetic manipulation of *Bacteroides* species (21) and of some *Flavobacterium* species (15, 16, 27) have been developed. In spite of the fact that the plasmid used to develop *Flavobacterium* cloning vectors was isolated from *F. psychrophilum* D12, repeated attempts by ourselves and others to demonstrate transfer of these plasmids or other genetic elements into *F. psychrophilum* have been unsuccessful (J. A. Guijarro, unpublished data; M. J. McBride, unpublished data; D. W. Hunnicutt, unpublished data).

In this study, we describe the development of methods for the introduction of DNA into *F. psychrophilum* by conjugation and electroporation. Selectable markers, plasmid cloning vectors, a *lacZY* reporter construct, and a transposon which functions in *F. psychrophilum* are also described, and evidence for a *Sau3AI*-like restriction modification system in *F. psychrophilum* THC02-90 is presented. The availability of these genetic

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Bacterial strains		
<i>E. coli</i>		
S17-1 λ <i>pir</i>	<i>λpir hsdR pro thi</i> ; chromosomal integrated RP4-2 Tc::Mu Km::Tn7	25
BW19851	RP4-2 <i>ter</i> ::Mu-1 <i>kan</i> ::Tn7 integrant; Δ <i>uidA</i> :: <i>pir</i> ⁺ <i>recA1 hsdR17 creB510 endA1 zbf-5 thi</i>	17
<i>F. psychrophilum</i>		
ATCC 49418	Wild type	ATCC ^a
ATCC 49510	Wild type	ATCC
ATCC 49511	Wild type	ATCC
D12	Wild type	15
OSU SH3-81	Wild type	4
OSU THCO2-90	Wild type	4
OSU THCO4-90	Wild type	4
OSU Ch8-80	Wild type	4
FPC 830	Wild type	4
Plasmids^b		
pCP29	ColE1 ori; (pCP1 ori); Ap ^r (Cf ^r Em ^r); <i>E. coli</i> - <i>F. psychrophilum</i> shuttle plasmid	11
pCP23	ColE1 ori (pCP1 ori); Ap ^r (Tc ^r); <i>E. coli</i> - <i>F. psychrophilum</i> shuttle plasmid	1
pIVET8	<i>pir</i> -dependent <i>oriR6K</i> ; Mob ⁺ Ap ^r	14
pCP23- β	6-kb <i>SphI</i> fragment of pIVET8 containing <i>lacZY</i> genes cloned in pCP23	This study
pEP4351	<i>pir</i> -requiring R6K oriV; RP4 oriT; Cm ^r Tc ^r (Em ^r); vector for Tn4351 transfer	5

^a ATCC, American Type Culture Collection, Rockville, Md.

^b Antibiotic resistance phenotypes: ampicillin, Ap^r; cefoxitin, Cf^r; chloramphenicol, Cm^r; erythromycin, Em^r; tetracycline, Tc^r. Antibiotic resistance phenotypes and other features listed in parentheses are those expressed by *F. psychrophilum* but not by *E. coli*.

techniques should allow analysis of the underlying mechanisms of *F. psychrophilum* virulence and facilitate the development of vaccine strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. EAO medium (18) was used, consisting of 5 g of tryptone, 0.5 g of yeast extract, 0.2 g of beef extract, and 0.2 g of sodium acetate per liter, with the pH adjusted to 7.2 to 7.4. EAOS medium was the same as EAO medium except that it was supplemented with 5% horse serum, 10 μ M L-phenylalanine, 10 μ M L-tyrosine, 10 μ M L-tryptophan, 10 μ M *p*-aminobenzoic acid, 10 μ M 4-hydroxybenzoic acid, and 10 μ M 2,3-dihydroxybenzoic acid. For growth on solid medium, 15 g of agar was added per liter of EAOS medium, and cultures were incubated at 20°C. Nutrient broth (NB; Promega) was used for growth of *F. psychrophilum* in liquid, and cultures were incubated at 18°C with shaking at 250 rpm. *Escherichia coli* strains were grown at 37°C in 2 \times TY medium (15 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter), with 20 g of agar per liter added for solid medium. For selection of *F. psychrophilum* transconjugants or transformants, erythromycin, cefoxitin, or tetracycline was used at 10 μ g/ml. For selective growth of *E. coli* strains, antibiotics were used when needed at the following concentrations: ampicillin, 100 μ g/ml; chloramphenicol, 30 μ g/ml; streptomycin, 50 μ g/ml; and tetracycline, 15 μ g/ml.

Manipulation of DNA and nucleic acid sequencing. Standard procedures were used to isolate plasmid and genomic DNA and to clone and analyze DNA fragments (22). Nucleic acid sequencing was performed by the dideoxy nucleotide procedure, using an automated (Applied Biosystems) sequencing system. Sequences were analyzed with MacVector and AssemblyLign software (Accelrys, San Diego, Calif.), and comparisons to database sequences were made by use of the BLAST algorithm (2).

Conjugal transfer of DNA from *E. coli* to *F. psychrophilum*. *E. coli* S17-1 λ *pir* was used for conjugative transfer of pCP29, pCP23, and pCP23- β into *F. psychrophilum*, and *E. coli* BW19851 was used to transfer pEP4351. Donor *E. coli* strains were grown to mid-log phase in 2 \times TY medium, and recipient *F. psychrophilum* strains were grown to mid-log phase in NB. *F. psychrophilum* cells (10 ml) were harvested by centrifugation, washed twice with TM buffer, consisting of 20 mM Tris-HCl and 20 mM MgSO₄ with the pH adjusted to 7.2, and suspended in 50 μ l of TM buffer. *E. coli* cells (10 ml) were harvested by centrifugation, washed twice with EAO medium, and suspended in 50 μ l of EAO medium. Cells of *F. psychrophilum* and *E. coli* were mixed together (approximately 10⁸ cells of each strain), spotted onto EAOS agar, and incubated at 20°C for 48 h to allow

conjugative transfer of DNA. After conjugation, cells were scraped off the plates, diluted in 1 ml of EAO medium, and plated on EAOS agar containing the appropriate antibiotic (cefoxitin, erythromycin, or tetracycline) to select for plasmid or transposon transfer and to eliminate *E. coli* donor cells. Plates were incubated at 20°C for 4 to 7 days. A separate counterselection to eliminate *E. coli* was not needed, since *cfxA*, *ermF*, and *tetQ* are not expressed in *E. coli*. To calculate the frequencies of conjugative transfer per recipient, cells were plated on EAOS agar without antibiotics to determine the number of surviving *F. psychrophilum* cells.

Electroporation. *F. psychrophilum* cells were harvested during exponential growth, washed three times with ice-cold double-distilled water, and resuspended to a density of approximately 10¹¹ cells/ml in double-distilled water. Approximately 200 ng of pCP29 was added to 40 μ l of cells. For the optimized procedure, each mixture was placed in a Bio-Rad 0.2-cm pulser cuvette and pulsed with a Bio-Rad gene pulser, with a field strength of 10 kV/cm, resistance of 400 Ω , and capacitance of 25 μ F. DNA isolated from *F. psychrophilum* and *E. coli* strain S17-1 λ *pir* was used to electroporate *F. psychrophilum* cells. After electroporation, the cells were transferred to NB and incubated at 18°C, with shaking at 250 rpm, for 3 h to allow expression of antibiotic resistance. Afterwards, cells were diluted in NB and plated on EAOS agar with the appropriate antibiotic. Colonies were counted after 4 to 5 days of incubation at 20°C.

Southern blot analysis of Tn4351 insertions. Tn4351 was introduced into *F. psychrophilum* THC02-90 by conjugation, as described above. Genomic DNA from erythromycin-resistant transconjugants was isolated, digested with *XbaI*, separated by gel electrophoresis, and transferred to nylon membranes essentially as described previously (22). The DIG DNA labeling and detection kit (Roche) was used to prepare probes and to perform hybridization. Two probes were used, a 6.2-kb *SaI* fragment from pEP4351 containing the transposon Tn4351 and the chloramphenicol acetyltransferase gene (*cat*), which is also present on pEP4351. The *cat* gene was amplified as a 633-bp PCR product from pIVET8 by use of the primers CAT-1 (CACTGGATATACCACCG) and CAT-2 (TGCCAATCATC GCAGTA).

In vitro β -galactosidase determination. The *SphI* fragment of pIVET8 (14) containing the *lacZY* genes was inserted into the *SphI* site of pCP23 downstream of the ORF1 promoter to generate pCP23- β . pCP23- β was introduced by conjugation into *F. psychrophilum* THC02-90, and tetracycline-resistant transconjugants (THC02-90- β) were obtained. As a control, the THC02-90 strain containing the pCP23 plasmid (THC02-90-p) was used. For detection of β -galactosidase activity on the plates, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) was added at 50 μ g/ml. NB was used to detect the production of β -galactosidase. Cells from 1 ml of a culture which had been incubated for 48 h were collected by

centrifugation at $12,000 \times g$ for 5 min, suspended in buffer Z (19), and disrupted by sonication. β -Galactosidase activity was measured as previously described (19).

Nucleotide sequence accession number. The sequence of pCP1 which is reported in this paper has been deposited in the GenBank database under accession number AY277637.

RESULTS

Conjugative transfer of plasmids into *F. psychrophilum*. pCP29 was selected as a test plasmid to determine the optimal conditions for conjugative transfer of DNA into *F. psychrophilum*. Despite the fact that previous attempts to transfer pCP29 and other plasmids into this bacterium had all failed, we predicted that *F. psychrophilum* would support replication of pCP29 once barriers to transfer were overcome because this plasmid is derived from the cryptic *F. psychrophilum* D12 plasmid pCP1 (11, 15). pCP29 carries two antibiotic resistance genes (*cfxA* and *ermF*) that function in *Flavobacterium johnsoniae*, *Bacteroides thetaiotaomicron*, and many other members of the CFB group, so it seemed likely that pCP29 would confer erythromycin and cefoxitin resistance to *F. psychrophilum*. *F. psychrophilum* THC02-90, which does not carry pCP1, was chosen as the recipient strain to avoid problems of plasmid incompatibility. pCP29 was transferred by conjugation from *E. coli* to *F. psychrophilum* THC02-90, and cefoxitin- and erythromycin-resistant colonies arose after 4 to 5 days of incubation at 20°C. pCP29 plasmid DNA was isolated from the transconjugants, and phenotypic analysis and PCR analysis using specific *F. psychrophilum* primers (8) confirmed that the transconjugants were *F. psychrophilum*. A number of variables were tested to determine the optimal conditions for conjugative transfer. Recipient cells harvested during the early exponential phase of growth (optical density at 525 nm, 0.3 to 0.5) resulted in the highest frequencies of conjugation (1.3×10^{-5} to 1.7×10^{-5} antibiotic-resistant colonies per recipient cell). Cells harvested during stationary phase exhibited much lower rates of conjugative transfer (5.9×10^{-7} to 8.3×10^{-7} antibiotic-resistant colonies per recipient cell). The length of mating time (24, 48, or 72 h) was tested, and the maximum number of transconjugants per recipient cell was obtained with 48 h of incubation. We expected that conjugative transfer from *E. coli* would not be efficient at the low temperatures required for cultivation of *F. psychrophilum* and therefore used the highest temperature that *F. psychrophilum* would easily tolerate (20°C). Reduction of the temperature of incubation during mating to 18 and 15°C resulted in a reduction of 10- and 100-fold, respectively, in the number of antibiotic-resistant transconjugants. With control experiments, we found that conjugation of pCP29 from *E. coli* into *F. johnsoniae*, whose optimum growth temperature is 30°C, was also reduced 10-fold when conjugation was performed at 20°C instead of 30°C. *cfxA* and *ermF* functioned equally well as selectable markers in *F. psychrophilum*, since similar numbers of transconjugants were obtained with either cefoxitin or erythromycin as the selective agent. *tetQ* conferred tetracycline resistance on *F. psychrophilum*, but colonies did not appear until after 5 to 7 days of incubation when the *tetQ*-containing plasmid, pCP23, was transferred, and conjugative transfer frequencies were low (2.44×10^{-8} to 3.33×10^{-8} antibiotic-resistant colonies per recipient cell). Plasmids were also transferred by conjugation

into several other strains of *F. psychrophilum*. pCP29 was transferred by conjugation into *F. psychrophilum* THC04-90 (6×10^{-6} colonies per recipient cell) and, less efficiently, into *F. psychrophilum* SH3-81. Despite repeated attempts, no transconjugants were obtained with *F. psychrophilum* strains ATCC 49418, ATCC 49510, ATCC 49511, Ch8-80, D12, and FPC 830.

Electroporation of *F. psychrophilum*. A procedure was developed to allow for the direct introduction of DNA into *F. psychrophilum* cells by electroporation. pCP29 isolated from *F. psychrophilum* THC02-90 was used to optimize this procedure. Electrocompetent cells of *F. psychrophilum* were prepared by washing with either 10% glycerol or distilled water. Cells produced by either procedure were equally competent. The voltage (2.5 to 12.5 kV/cm) and resistance (200 to 1,000 Ω) were varied to optimize transfer. The optimal conditions for transfer were 7.5 to 12.5 kV/cm and 200 to 600 Ω . The highest number of transformants (2×10^6 to 2.6×10^6 transformants/ μ g of DNA) was obtained when the electrical parameters were 400 Ω , 10 kV/cm, and 25 μ F and cefoxitin was used as the selective agent. The antibiotic used for selection had a marked effect on the efficiency of electrotransformation. When erythromycin was used instead of cefoxitin, only 4×10^4 to 5.3×10^4 colonies/ μ g of DNA were obtained.

Sequence analysis of pCP1. The 3,407-bp nucleotide sequence of pCP1 was determined in order to enhance the usefulness of cloning vectors such as pCP29 and pCP23, which were derived from this plasmid. pCP1 contains four open reading frames: *repA*, ORF1, ORF2, and ORF3 (Fig. 1). RepA is similar to the *Reimerella antatipestifer* plasmid replication protein 1 from pCFC1 (49% identity over 317 amino acids; GenBank accession number AAC27555) and is probably involved in plasmid replication. The functions of the other open reading frames are not known. ORF1 and ORF2 are apparently not needed for replication in *F. psychrophilum* or *F. johnsoniae*, since ORF1 was disrupted during construction of pCP11 (15) and pCP29 (11) (Fig. 1) and ORF1 and ORF2 were disrupted during construction of pCP23 (1) (Fig. 1). Upstream of *repA* is a 21-bp sequence, AAACCTTTCTTTTCGCTTATAA, that is tandemly repeated 4.7 times and may be involved in plasmid replication.

Development of *lacZY* as a transcriptional reporter for *F. psychrophilum*. The ORF1 promoter reads toward the multiple cloning site of pCP23 (Fig. 1). Promoterless *lacZY* was cloned into the multiple cloning site to test this promoter and to determine whether *lacZ* could be used as a reporter of gene expression for *F. psychrophilum*. Green tetracycline-resistant transconjugants appeared after 7 days of incubation (Fig. 2). Expression of *lacZ* from this promoter was measured under various conditions as a first test of its usefulness as a reporter of *F. psychrophilum* gene expression. The expression level was similar for cultures grown at 12 and 18°C and for cultures grown at pHs 6, 6.5, 7, and 7.5. In contrast, the addition of 1 to 5 mM CaCl₂ resulted in decreased expression.

Transposition of Tn4351 in *F. psychrophilum*. Tn4351 was introduced into *F. psychrophilum* THC02-90 on pEP4351 by conjugation, and erythromycin-resistant transconjugants were obtained at a frequency of 0.8×10^{-8} to 1.8×10^{-8} per recipient cell. PCR and Southern blot analysis of transconjugants verified that Tn4351 had been transferred (Fig. 3). When

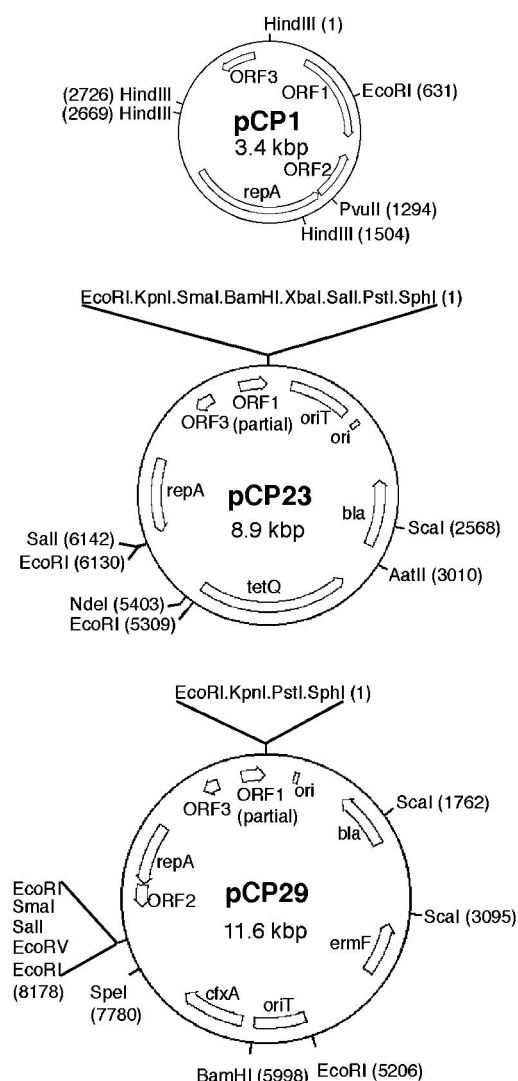


FIG. 1. Maps of the plasmids pCP1, pCP23, and pCP29. Numbers in parentheses indicate map positions in nucleotides. The relative sizes of the maps do not correspond exactly to the sizes of the plasmids. Abbreviations: *oriT*, origin of transfer for conjugation; *ori*, ColE1 origin of replication, which functions in *E. coli* but not in *F. psychrophilum*; *bla*, β -lactamase, which confers resistance to ampicillin to *E. coli* but not to *F. psychrophilum*. The exact site of the pCP1 origin of replication, which functions in *F. psychrophilum*, has not been determined but is probably between ORF3 and *repA*.

analyzed by miniprep, none of the transconjugants carried pEP4351 as a free plasmid, indicating that as expected, pEP4351 did not replicate in *F. psychrophilum*. Some transconjugants carried only Tn4351 (Fig. 3B and C, lanes 3 to 6, 11, and 13), whereas others had the delivery vector integrated into the chromosome with Tn4351 (Fig. 3B and C, lanes 7 to 10 and 12). Insertions of plasmid DNA into the chromosome, which may be the result of homologous recombination after transposition of Tn4351, have been described previously for *B. thetaiotaomicron*, *F. johnsoniae*, and related bacteria (15, 24). The frequencies of such insertions are strain dependent. Most of the mutants that did not carry vector DNA had single insertions of Tn4351, but the presence of three bands in lane 6 of



FIG. 2. Expression of *lacZ* from the ORF1 promoter of pCP1. Transconjugants of *F. psychrophilum* containing the *lacZY* genes inserted into the *SphI* site of pCP23 downstream of the ORF1 promoter were grown on EAOS agar containing X-Gal. 1, *F. psychrophilum* THC02-90-p; 2, *F. psychrophilum* THC02-90- β .

Fig. 3B suggests that multiple insertions may also occur. Tn4351 inserted at different locations in the chromosome (Fig. 3) and will probably be useful for mutagenesis of *F. psychrophilum*.

Evidence for a *Sau3AI*-like restriction modification system in *F. psychrophilum*. Electroporation of pCP29 extracted from *E. coli* into *F. psychrophilum* resulted in no antibiotic-resistant transformants. We suspected that a restriction barrier was responsible for the lack of transformants. One indication of the presence of a restriction modification system is modification of DNA such that it resists digestion by the restriction enzyme produced by that strain. DNA propagated in *F. psychrophilum* was resistant to digestion by *Sau3AI* (Fig. 4, lanes 5 and 9) and *BamHI* (Fig. 4, lanes 3 and 7). Both enzymes cleave DNA that contains the core sequence GATC and are inhibited by methylation of cytosine in this sequence. *MboI*, which recognizes the same sequence as *Sau3AI* but is not inhibited by cytosine methylation, digested *F. psychrophilum* chromosomal DNA (Fig. 4, lane 4) and pCP29 DNA isolated from *F. psychrophilum* (Fig. 4, lane 8). In contrast, when pCP29 was extracted from *E. coli* S17-1 λ *pir*, it was susceptible to digestion by *BamHI* (Fig. 4, lane 11) and *Sau3AI* (Fig. 4, lane 13) but was resistant to *MboI* (Fig. 4, lane 12), an enzyme that fails to digest DNA that has been methylated on adenine residues by the *dam* system. *F. psychrophilum* DNA appears to be modified, and it is likely that this modification is part of a restriction modification system. This may account for some of the difficulties encountered when introducing DNA into *F. psychrophilum* by electroporation. Others have observed that conjugative transfer is generally less sensitive to the presence of restriction systems in the recipient strain (21), which may account for the success of conjugative transfer of DNA into *F. psychrophilum*.

DISCUSSION

F. psychrophilum, *Flavobacterium columnare*, and *Flavobacterium branchiophilum* are important fish pathogens. Techniques to genetically manipulate some members of the genus *Flavobacterium* have been developed (15, 16, 27), but despite the efforts of several laboratories, the fish pathogenic flavobacteria have resisted attempts of gene transfer. In this study, techniques that allow for the genetic manipulation of several strains of *F. psychrophilum* were developed, opening up the possibility of genetic analysis of pathogenesis by members of this genus.

DNA was introduced into *F. psychrophilum* by conjugation. Plasmids based on pCP1 replicated in *F. psychrophilum*, and

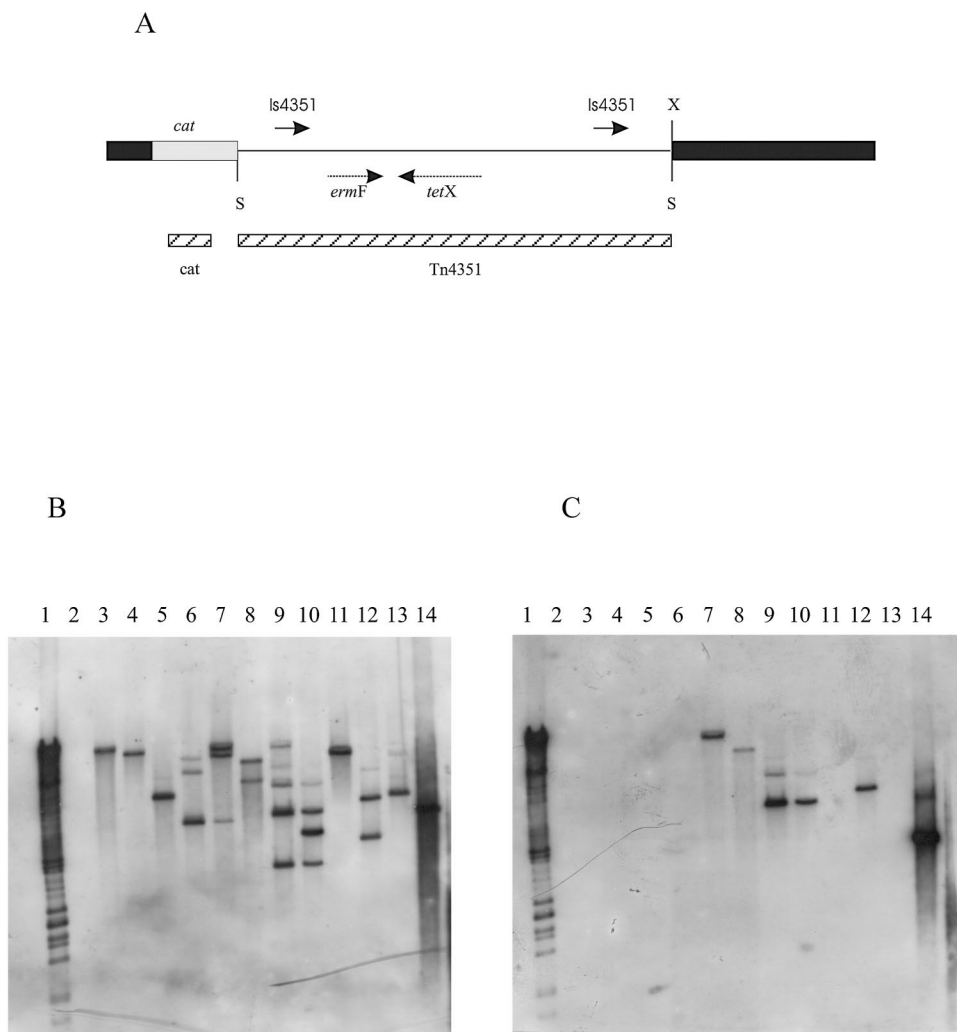


FIG. 3. Southern blot hybridization of *F. psychrophilum* transconjugants carrying Tn4351. (A) Restriction map of pEP4351 showing the probes used for Southern blot hybridization. *cat*, a 633-bp fragment containing the structural part of the *cat* gene; Tn4351, the 6.2-kb *SalI* fragment containing the Tn4351 transposon. S, *SalI*; X, *XbaI*. (B and C) Chromosomal DNA from the transconjugants was digested with *XbaI* and Southern blot hybridization was carried out with the Tn4351 (B) and *cat* (C) probes. Lanes 3 to 13, different isolated transconjugants; lanes 2, DNA from the wild-type strain; lanes 14, pEP4351 DNA digested with *SalI*; lanes 1, λ DNA digested with *PstI*.

the antibiotic resistance genes *cfxA*, *ermF*, and *tetQ* conferred cefoxitin resistance, erythromycin resistance, and tetracycline resistance, respectively. Conjugal transfer was strain dependent and was observed for three of the seven strains tested. *F. psychrophilum* THC02-90 and THC04-90, which were the best recipients for conjugal transfer, do not harbor any of the cryptic plasmids that are present in many strains (4). Since pCP1 was isolated from *F. psychrophilum* D12, it is possible that plasmid incompatibility accounts for some of the difficulties encountered when transferring pCP29 into some strains of *F. psychrophilum*. Another reason that *F. psychrophilum* may have resisted attempts of gene transfer until now is its inability to tolerate temperatures above 20°C. *E. coli* cells incubated at low temperatures exhibit decreased levels of conjugation (26). Low temperatures could alter the expression of genes involved in conjugation or the lipid composition of cell membranes (for

a review, see reference 28), resulting in a decreased efficiency of conjugative transfer.

The conditions for the introduction of DNA into *F. psychrophilum* cells by electroporation were also determined. Antibiotic-resistant colonies arose when plasmid DNA isolated from *F. psychrophilum* was introduced into cells of *F. psychrophilum*, but DNA isolated from *E. coli* was not suitable for transfer by electroporation. A restriction barrier is likely to be responsible for our inability to introduce DNA isolated from *E. coli* into *F. psychrophilum* by electroporation. The resistance of *F. psychrophilum* DNA to digestion by *Sau3AI* and *BamHI* suggests that a *Sau3AI*-like restriction modification system may constitute at least part of the restriction barrier. The generation of a strain of *F. psychrophilum* with a restriction enzyme deficiency or the use of an *E. coli* strain containing the modification enzyme may remove the barrier to transfer of

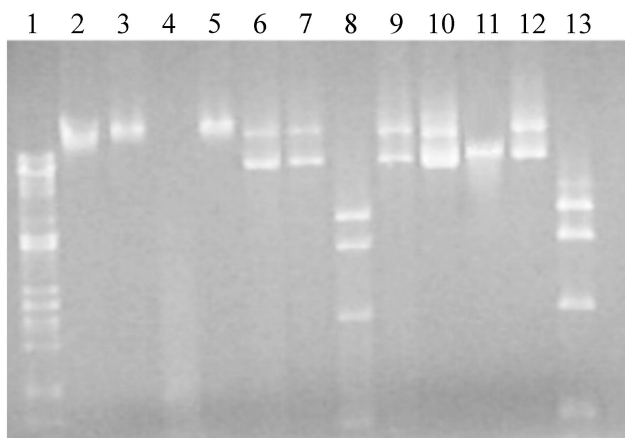


FIG. 4. Restriction analysis of genomic DNA and of pCP29 isolated from *E. coli* λ pir and *F. psychrophilum* THCO2-90. Genomic or plasmid DNA was obtained as described in the text and was digested with different restriction enzymes. Lanes 2 to 5, *F. psychrophilum* genomic DNA; lanes 6 to 9, pCP29 isolated from *F. psychrophilum*; lanes 10 to 13, pCP29 isolated from *E. coli*. DNA was digested with *Bam*HI (lanes 3, 7, and 11), *Mbo*I (lanes 4, 8, and 12), or *Sau*3AI (lanes 5, 9, and 13). No restriction enzymes were added to the samples in lanes 2, 6, and 10. Lane 1 contained λ DNA digested with *Pst*I as a reference.

DNA by electroporation. Until then, conjugation will likely be a more reliable technique for the transfer of DNA from *E. coli* into *F. psychrophilum*.

Tn4351 was demonstrated to function in *F. psychrophilum*, inserting into the chromosome and conferring resistance to erythromycin. Tn4351 has been used for mutagenesis of a number of other members of the CFB group (15, 16, 21, 24) and is likely to be a useful genetic tool for mutagenesis and analysis of *F. psychrophilum* genes.

With the tools described above, a foreign gene (*E. coli lacZ*) was introduced into *F. psychrophilum* and expressed, apparently from the ORF1 promoter of pCP23. The results indicate that *lacZ* may be used in *F. psychrophilum* as a reporter of gene expression. They also suggest that the ORF1 promoter may be used to express genes for complementation analyses of *F. psychrophilum* mutants. The presence of calcium in the culture medium resulted in decreased levels of β -galactosidase activity from this construct. Calcium has previously been implicated in the control of expression of Fpp1 protease, a putative virulence factor of *F. psychrophilum* (23). Growth of *F. psychrophilum* is also sensitive to the level of calcium in the medium (P. Secades and J. A. Guijarro, unpublished data).

F. psychrophilum and the related bacteria *F. columnare* and *F. branchiophilum* are important pathogens of fish. The development of methods to genetically manipulate *F. psychrophilum* should allow for new approaches for the identification of virulence factors and determination of the mechanisms of pathogenesis and could result in the development of vaccine strains to prevent CWD. The results of this study may also aid the development of similar genetic systems for the other flavobacterial fish pathogens.

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