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Development of Techniques for the Genetic Manipulation of the Gliding Bacterium *Cytophaga johnsonae*

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Cytophaga johnsonae **displays many features that make it an excellent model of bacterial gliding motility. Unfortunately, genetic analyses of *C. johnsonae*, or any related gliding bacteria, were not possible because of a complete lack of selectable markers, cloning vectors, transposons, and convenient methods of gene transfer. As a first step toward a molecular analysis of gliding motility of *C. johnsonae*, we developed these genetic techniques and tools. Common broad-host-range plasmids and transposons did not function in *C. johnsonae*. We identified one *Bacteroides* transposon, Tn*4351*, that could be introduced into *C. johnsonae* on plasmid R751 by conjugation from *Escherichia coli*. Tn*4351* inserted in the *C. johnsonae* genome and conferred erythromycin resistance. Tn*4351* insertions resulted in auxotrophic mutations and motility mutations. We constructed novel plasmids and cosmids for genetic analyses of *C. johnsonae*. These cloning vectors are derived from a small cryptic plasmid (pCP1) that we identified in the fish pathogen *Cytophaga psychrophila* D12. These plasmids contain the *ermF* (erythromycin resistance) gene from Tn*4351* and a variety of features that facilitate propagation and selection in *E. coli* and conjugative transfer from *E. coli* to *C. johnsonae.***

Gliding motility, the movement of cells over surfaces without the aid of flagella, is a trait shared by a large number of bacteria belonging to different phylogenetic branches of the eubacterial tree (39). These include, among others, cytophagas and related bacteria, myxobacteria, lysobacters, cyanobacteria, and beggiatoas. The basis of bacterial gliding motility has been an unsolved biological mystery for over 100 years (54).

Genetic studies of bacterial gliding motility have been limited almost entirely to one species, *Myxococcus xanthus*, primarily because a wide variety of techniques are available for the genetic manipulation of *M. xanthus* (15, 25) but not for other gliding bacteria. Mutants of *M. xanthus* with motility defects have been isolated and analyzed. These analyses have identified a number of genes which are necessary for colony spreading (swarming) or fruiting body formation and have identified the chemotaxis system that controls cell movements in response to stimuli (32, 46). However, despite intensive investigation, the mechanism of myxobacterial gliding motility remains a mystery.

Cytophaga johnsonae is a common soil and aquatic bacterium that exhibits gliding motility (36). It is one species of a large and diverse group of gliding bacteria that belong to the *Cytophaga-Flavobacterium-Bacteroides* subgroup of the eubacteria (28, 38). *C. johnsonae* displays many features which make it an attractive model organism for study of gliding motility. *C. johnsonae* grows rapidly on simple laboratory media (minimal doubling time of approximately 1 h). Cells of *C. johnsonae* also move rapidly over many surfaces. These movements are easily observed in real time without resorting to time lapse videomicroscopy. The cells glide at rates of up to 600 μm/min over glass surfaces and up to 60 μm/min over agar surfaces (36). Biochemical, morphological, and behavioral studies have been performed on *C. johnsonae* (1, 16, 36). Finally, a large collection of *C. johnsonae* mutants with motility defects, including complete loss of motility, have been isolated (17, 36). Despite the obvious advantages of *C. johnsonae* as a model of bacterial gliding motility, relatively little progress has been made toward determining the mechanism of its movement, at least in part because genetic analyses have not been possible (36). Gene transfer of auxotrophic markers by phage transduction was reported (36), but this was of limited value for studies of gliding motility since nonmotile mutants were resistant to infection by the transducing phage. Despite the efforts of several laboratories, no reliable method of gene transfer, selectable markers, transposons, or cloning vectors have been described for *C. johnsonae* or any related gliding bacterium (36, 40).

In this paper, we report that the *Bacteroides* transposon Tn*4351* functions in *C. johnsonae*. Tn*4351* inserts in the *C. johnsonae* genome, confers erythromycin resistance, and causes a variety of mutations. We also report the development of the first *Cytophaga* cloning vectors. These vectors use the replication functions of a cryptic plasmid from the fish pathogen *Cytophaga psychrophila* to replicate in *C. johnsonae*. They also contain the *ermF* gene from Tn*4351* and thus confer erythromycin resistance on *C. johnsonae*. They contain additional features that allow propagation and selection in *Escherichia coli*, packaging by lambda phage heads, and conjugative transfer from *E. coli* into *C. johnsonae*. The plasmids, transposons, and techniques for gene transfer described here are sufficient for a rigorous genetic analysis of *Cytophaga* gliding motility.

Many of the genetic tools that we developed for *C. johnsonae* should also be useful for studies of other members of the *Cytophaga-Flavobacterium* group. This group of bacteria includes a number of animal and human pathogens (3, 6, 8, 9, 30, 31, 45), bacteria capable of degrading complex biomolecules (18, 28, 38), and bacteria that produce novel and potentially useful antibiotics and other bioactive compounds (11, 26, 35, 38). Adaptation of the tools described here should allow genetic analyses of many of these bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The organisms and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown in LB broth (43) at 37°C. *C. johnsonae* strains were grown in CYE medium (10 g of Casitone

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TABLE 1. Organisms, plasmids, and transposons used in this study

Strain, plasmid, or transposon	Genotype or description	Source or reference(s)
Bacterial strains		
E. coli		
S17-1	*hsdR17* (r_K^- m_K^-)*recA* RP4-2(Tc^r::Mu-Km^r::Tn*7* Str^r)	51
SM10(λ*pir*)	*recA* RP4-2(Tc^r::Mu Km^r) λ*pir*	21, 51
HB101	λ$^-$ *recA13 proA2 leu lacY1 galK2 xyl-5 mtl-1 ara-14* F$^-$ *hsdS20* (r_B^- m_B^+) *supE44 rpsL20*	5
DH5αmcr	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lac*ZΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 deoR thi-1 supE44* λ$^-$ *gyrA96 relA1*	Bethesda Research Laboratories
C. johnsonae		
ATCC 17061	Wild type	ATCC
ATCC 29587	Wild type	ATCC
ATCC 29589	Wild type	ATCC
CR1	Rifampin-resistant mutant of *C. johnsonae* ATCC 17061	This study
CJ6R1	Rifampin-resistant mutant of *C. johnsonae* ATCC 29587	This study
CJ12R1	Rifampin-resistant mutant of *C. johnsonae* ATCC 29589	This study
CJ101-205	Tn*4351*-induced motility mutant of *C. johnsonae* ATCC 17061	This study
CJ101-207	Tn*4351*-induced motility mutant of *C. johnsonae* ATCC 17061	This study
CJ101-229	Tn*4351*-induced motility mutant of *C. johnsonae* ATCC 17061	This study
CJ101-232	Tn*4351*-induced motility mutant of *C. johnsonae* ATCC 17061	This study
C. psychrophila D12	Wild type	J. Pate (from E. J. Ordal)
Plasmids and transposons		
R702	IncP; Tc^r Km^r	20
pVK100	IncP; Tc^r Km^r	27
pPH1JI	IncP; Cm^r	22
pSa4	IncW; Cm^r Km^r	52
pSa152	IncW; Cm^r Km^r	52
pSa322	Helper plasmid for conjugative transfer of pSa152; Ap^r	52
pSUP106	IncQ; Cm^r Tc^r	37
pKT210	IncQ; Cm^r	4
pUTmini-Tn*5* Cm	Ap^r Cm^r	12
pUTmini-Tn*5* Sm	Ap^r Sm^r	12
pCHR83::Tn*5* Cm	Cm^r	44
pRU669::Tn*1725*	IncT; Cm^r Km^r	56
pRU670::Tn*1731*	IncT; Tc^r Km^r	56
pRU664::Tn*1732*	IncP; Ap^r Kn^r Tc^r	56
pMD100::Tn*501*	Km^r Hg^r	7
R751	IncP; Tp^r	33
R751::Tn*4351*Ω4	IncP; Tp^r Em^{ra} Tc^{rb}	49
pNJR5	IncQ; *E. coli-Bacteroides* shuttle vector; Kn^r Em^{ra}	48
pSPORT1	ColE1; Ap^r	GibcoBRL
pLYL03	ColE1; Ap^r Em^{ra}	29
pVOH1	ColE1; Ap^r Cm^r Tc^r Em^{ra}	24
pHC79	ColE1; Ap^r Tc^r; cosmid	23
pCP1	Cryptic plasmid from *C. psychrophila*	This study
pCP10	*Eco*RI-linearized pCP1 in *Eco*RI site of pSPORT1; Ap^r	This study
pCP11	*E. coli-C. johnsonae* shuttle plasmid; Ap^r Em^{ra}	This study
pCP16	pCP11 with *Bam*HI site removed; Ap^r Em^{ra}	This study
pCP17	*E. coli-C. johnsonae* shuttle cosmid; Ap^r Tc^r Em^{ra}	This study

[a] Expressed in *Bacteroides* spp. and in *C. johnsonae* but not in *E. coli.*
[b] Expressed in *E. coli* under aerobic conditions but not in *Bacteroides* spp. or *C. johnsonae.*

per liter, 5 g of yeast extract per liter, and 8 mM $MgSO_4$ in 10 mM Tris buffer [pH 7.6]). Liquid cultures were incubated with shaking at 225 rpm. Solid CYE medium contained 15 g of agar per liter. Higher agar concentrations (20 g/liter) were used in some conjugation experiments, since it was determined that conjugation was more efficient under these conditions. Cultures of *C. johnsonae* were routinely incubated at 30°C. Colony spreading of *C. johnsonae* is inhibited by high concentrations of various nutrients (57) and by incubation temperatures in excess of 29°C. When we wished to observe colony spreading, we incubated *C. johnsonae* at 25°C on AOE agar (5 g of tryptone, 0.5 g of yeast extract, 0.2 g of sodium acetate, 0.2 g of beef extract, and 10 g of agar per liter). *C. psychrophila* cultures were grown in AOE medium with or without agar at 18°C. To select for plasmids or transposons, antibiotics were added at the following concentrations (micrograms per milliliter) unless stated otherwise: erythromycin, 20 or 100; ampicillin, 100; tetracycline, 15; kanamycin, 50; chloramphenicol, 25; and streptomycin, 50.

Isolation of rifampin-resistant mutants. Rifampin-resistant mutants of *C. johnsonae* strains were used in some conjugation experiments to allow for counterselection and elimination of the *E. coli* donor cells. Rifampin-resistant mutants were obtained by plating approximately 10^9 cells on CYE medium containing 25 μg of rifampin per ml.

Conjugation. The donor strains of *E. coli* used for conjugative transfer of plasmids were HB101, S17-1, and SM10(λ*pir*). HB101 was used as the donor for transfer of self-transmissible plasmids such as R751. S17-1 or SM10 was used as the donor for mobilizable but not self-transmissible plasmids such as pCP11. Donor *E. coli* strains containing mobilizable plasmids were grown to mid-exponential phase in LB. Rifampin-resistant *C. johnsonae* was grown to mid-exponential phase in CYE at 30°C. Cells were harvested by centrifugation and mixed together (1:1 ratio), and approximately 5×10^9 cells were spotted on CYE agar. Following overnight incubation at 30°C, cells were scraped off the plates, diluted in CYE or 10 mM Tris (pH 7.3)–8 mM $CaCl_2$ (TC buffer), and plated on CYE agar containing 25 μg of rifampin per ml and the appropriate antibiotic to select for plasmid or transposon transfer. Plates were incubated for 2 to 3 days at 30°C.

Once a selectable cloning vector (pCP11; see below) had been constructed, we optimized our conjugation procedure. In the optimized procedure, cells of the *E.*

coli donor strain and *C. johnsonae* recipient were grown, harvested, mixed, and plated for conjugal transfer as described above except that the conjugation medium contained 20 g of agar per liter. After overnight incubation at 30°C, the cells were scraped off the agar and plated on selective media as described above. In experiments in which erythromycin was the selectable marker, rifampin was omitted from the selective media since 100 μg of erythromycin per ml prevented growth of the *E. coli* donor cells. Although the donor cells carry the plasmid-borne *ermF* gene, this gene is not expressed in *E. coli.*

Electroporation. *C. johnsonae* cells were harvested during exponential growth, washed two times in 10% glycerol at 4°C, and resuspended to a cell density of approximately 10^{11}/ml in 10% glycerol. Approximately 20 ng of plasmid DNA was added to 20 μl of cells. Each mixture was placed in a Bethesda Research Laboratories microelectroporation chamber and pulsed with 1.5 kV (4-kΩ resistance). After electroporation, the cells were transferred to CYE broth and incubated at 30°C for 1.5 h to allow expression of antibiotic resistance. Cells were diluted in CYE broth and plated on CYE agar with the appropriate antibiotic. Colonies were counted after 2 to 3 days of incubation at 30°C.

DNA isolation, gel electrophoresis, blotting, and hybridization procedures. Standard procedures were used to isolate plasmid and genomic DNA, digest with restriction enzymes, separate the fragments by gel electrophoresis, and transfer the fragments to nylon membranes (43). Radiolabeled probes were prepared by using the Prime-a-Gene labeling system (Promega).

Construction of the *C. johnsonae-E. coli* shuttle vectors pCP11 and pCP17. The construction of pCP11 is shown in Fig. 2. In step A, pCPI was linearized with *Eco*RI and ligated with *Eco*RI-cut pSPORT1 to generate pCP10. This provided convenient cloning sites flanking the cytophaga DNA. In step B, pCP10 was cut with *Bam*HI and *Pst*I, and the 3.3-kb fragment containing the *Cytophaga* DNA was purified by gel electrophoresis followed by Geneclean treatment (Bio101 Inc.) and was ligated with pLYL03 that had also been digested with *Bam*HI and *Pst*I, to generate the *C. johnsonae-E. coli* shuttle plasmid pCP11.

The construction of pCP17 is shown in Fig. 4. pCP16 was created by destroying the *Bam*HI site in pCP11. pCP11 was digested with *Bam*HI, treated with Klenow enzyme to fill in the ends, and ligated. pHC79 was digested with *Pvu*II and *Hin*dIII. The 3.4-kb fragment that carries the *cos* site and the tetracycline resistance gene was purified from an agarose gel with Geneclean. The *Hin*dIII site was filled in with Klenow enzyme, and the resulting blunt-ended fragment was ligated with pCP16 that had been cut with *Sma*I and treated with alkaline phosphatase.

Mapping of the *Hin*dIII sites in pCP11. pCP11 was digested with *Bam*HI and *Sph*I, and the 3.5-kb band was purified by agarose gel electrophoresis followed by Geneclean treatment. This fragment was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and then digested with *Kpn*I, resulting in a fragment with one labeled end. This fragment was partially digested with *Hin*dIII. The fragments were separated by gel electrophoresis and visualized by autoradiography.

RESULTS

Many common broad-host-range plasmids and transposons do not function in *C. johnsonae.* We attempted to introduce a wide variety of different broad-host-range plasmids into *C. johnsonae* CR1, CJ6R1, and CJ12R1 by conjugation and electroporation as described in Materials and Methods. The plasmids tested are listed in Table 1 and included members of incompatibility groups P (R702, pVK100, and pPH1JI), Q (pSUP106 and pKT210), and W (pSa4 and pSa152). We did not obtain any antibiotic-resistant colonies as a result of plasmid transfer. Generation of antibiotic-resistant colonies required that a number of independent events occur efficiently. First, the DNA must be transferred into the cells. Second, the DNA must not be immediately degraded by host restriction systems. Third, the antibiotic resistance genes must be expressed, and they must confer antibiotic resistance on the cells. Fourth, the resistance elements must be maintained as the cells multiply. This requires either independent replication of the plasmid or integration of the plasmid into the host genome. Our negative results did not allow us to determine which of these events was lacking.

We also attempted to introduce a number of broad-host-range transposons into the same *C. johnsonae* strains. The transposons tested (Table 1) included mini-Tn*5*cm, mini-Tn*5*sm, Tn*5*cm, Tn*1725*, Tn*1731*, Tn*1732*, and Tn*501*. We did not obtain any antibiotic-resistant colonies as a result of transposon transfer. This failure could have been the result of lack of transfer, degradation of transferred DNA, lack of expression of antibiotic resistance genes, or lack of transposition.

The *Bacteroides-E. coli* shuttle vector pNJR5 does not function in *C. johnsonae*. The broad-host-range plasmids and transposons that we tested function in a wide variety of bacteria from the proteobacteria group (55). *C. johnsonae* is not closely related to the proteobacteria, so it was not surprising that these plasmids and transposons did not function in *C. johnsonae*. The closest relatives of *C. johnsonae* for which genetic techniques have been developed are members of the genus *Bacteroides* (14, 42). We attempted to introduce the *Bacteroides-E. coli* shuttle vector pNJR5 into *C. johnsonae*. pNJR5 contains the erythromycin resistance gene *ermF* from the *Bacteroides* transposon Tn*4400* and two origins of replication, one from the cryptic *Bacteroides fragilis* plasmid pB8-51 and one from the IncQ broad-host-range plasmid RSF1010 (48). We introduced pNJR5 into *C. johnsonae* by conjugation from *E. coli* S17-1 and by electroporation. No stable antibiotic-resistant colonies were obtained.

Mutagenesis of *C. johnsonae* with Tn*4351*. Tn*4351* is a transposon that was first identified in *B. fragilis* (50). Tn*4351* carries the *ermF* gene, which confers erythromycin resistance on *B. fragilis*, and the *tetX* gene, which confers tetracycline resistance on *E. coli* but not on *B. fragilis*. Tn*4351* mutagenesis has been used to generate mutations in *Bacteroides* spp. (2, 10, 41, 49, 53). We introduced Tn*4351* into *C. johnsonae* on the broad-host-range plasmid R751 by conjugation from *E. coli* HB101. Erythromycin-resistant colonies of *C. johnsonae* appeared after 2 to 3 days of incubation at 30°C. The frequency of erythromycin-resistant transconjugants per recipient cell was 1.6×10^{-8} for CR1, 3.5×10^{-6} for CR6R1, and 2.4×10^{-8} for CR12R1. No free plasmid DNA was found in the transconjugants (data not shown), indicating that the IncP plasmid R751, which replicates stably in many proteobacteria, was not maintained by *C. johnsonae* but instead functioned as a suicide vector. Genomic DNA from 10 representative erythromycin-resistant transconjugants was transferred to nylon membranes by colony blotting. The DNA from each colony hybridized with pVOH1, indicating that Tn*4351* was present in each strain (data not shown). Five of the samples also hybridized to R751 sequences. Free plasmid was not found in any of these strains, which indicates that R751 had integrated into the *C. johnsonae* genome along with Tn*4351*. In control experiments, neither pVOH1 nor R751 hybridized to DNA from wild-type cells of *C. johnsonae.*

Tn*4351* insertions caused a variety of mutations in *C. johnsonae*. Among our Tn*4351*-induced erythromycin-resistant colonies, we found auxotrophic mutants, mutants with motility defects, and mutants which lacked the characteristic yellow-orange pigment of *C. johnsonae*. Nonspreading colonies (motility mutants) were observed at a frequency of approximately 1% among transposon-mutagenized cells. We have isolated 35 nonspreading mutants with different motility defects.

Southern blot analyses of Tn*4351* insertions in the *C. johnsonae* genome. Figure 1 shows the results of Southern blot analyses for four Tn*4351* mutants with defects in colony spreading (CJ101-205, CJ101-207, CJ101-229, and CJ101-232). Genomic DNA was digested with *Hin*dIII or *Eco*RV, separated by agarose gel electrophoresis, transferred to nylon membranes, and probed with radiolabeled R751 to detect vector sequences (Fig. 1B) or with radiolabeled pVOH1 to detect Tn*4351* sequences (Fig. 1C). Lanes 1 contain genomic DNA from *C. johnsonae* ATCC 17061 digested with *Hin*dIII. Neither pVOH1 nor R751 hybridized to these samples.

CJ101-207 and CJ101-232 apparently have simple insertions of Tn*4351* in their genomes. Genomic DNA from these strains did not hybridize with R751 (Fig. 1B, lanes 3, 5, 7, and 9), which indicates that R751 was not inserted in the genome

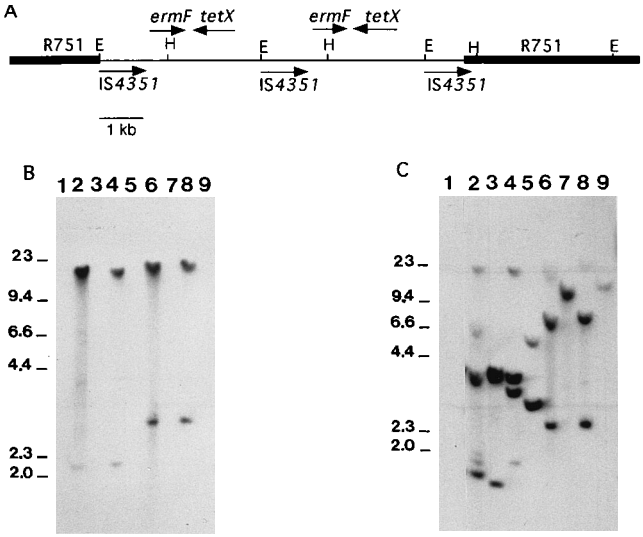

FIG. 1. Analyses of Tn*4351* insertions in *C. johnsonae*. (A) Partial map of R751::Tn*4351*Ω4 constructed from published data (49). Restriction enzyme sites: E, *Eco*RI; H, *Hin*dIII. R751::Tn*4351*Ω4 contains a tandem insertion of Tn*4351* as indicated. (B and C) Autoradiograms of Southern blots of four different *C. johnsonae* erythromycin-resistant transconjugants. Chromosomal DNA was digested with *Hin*dIII (lanes 1 to 5) and *Eco*RV (lanes 6 to 9). Lanes 1 contain chromosomal DNA from wild-type cells (no transposon). The Tn*4351* mutants shown are CJ101-205 (lanes 2 and 6) CJ101-207 (lanes 3 and 7), CJ101-229 (lanes 4 and 8), and CJ101-232 (lanes 5 and 9). Southern blots were hybridized with labeled R751 to detect vector sequences (B) or with labeled pVOH1 to detect Tn*4351* sequences (C).

during transposition of Tn*4351*. There are no *Eco*RV restriction sites within Tn*4351*. Digestion with *Eco*RV should produce a single large band that hybridizes with pVOH1 if a simple insertion has occurred. Digestion of genomic DNA from both CJ101-207 and CJ101-232 with *Eco*RV resulted in single bands of greater than 10 kb each (Fig. 1C, lanes 7 and 9). There is one *Hin*dIII site within Tn*4351*. Digestion with *Hin*dIII should give rise to two bands of variable size if a simple insertion of one copy of Tn*4351* has occurred or three bands, one of which is 3.8 kb in size, if the transposon inserted as a tandem duplication as occurs on the donor plasmid (Fig. 1A). Digestion of CJ101-232 DNA with *Hin*dIII resulted in two bands, indicative of a simple insertion of a single copy of the transposon (Fig. 1C, lane 5). Digestion of CJ101-207 DNA with *Hin*dIII resulted in what appear to be three bands, one of which migrates at 3.8 kb (Fig. 1C, lane 3). This mutant apparently carries an insertion of the tandem duplication of Tn*4351*.

CJ101-205 and CJ101-229 apparently have more complicated insertions of Tn*4351*. Genomic DNA from both strains hybridized with R751 (Fig. 1B, lanes 2, 4, 6, and 8), which indicates that R751 was inserted in the genome during transposition of Tn*4351*. Digestion of genomic DNA from both CJ101-205 and CJ101-229 with *Eco*RV and hybridization with pVOH1 resulted in two bands in both cases (Fig. 1C, lanes 6 and 8). This could occur if R751 was integrated into the chromosome with a copy of Tn*4351* at one end and a copy of IS*4351* at the other end. Digestion of CJ101-205 or CJ101-229 DNA with *Hin*dIII and hybridization with pVOH1 resulted in multiple bands in both cases (Fig. 1C, lanes 2 and 4). This result also indicates that some type of complicated insertion event has occurred.

We have analyzed 16 mutants, including the 4 indicated above. Seven of these had simple insertions of Tn*4351*. Each of these seven mutants exhibited different restriction digest patterns, indicating that Tn*4351* had inserted at different sites on the *C. johnsonae* chromosome (data not shown).

Identification of a small cryptic plasmid in *C. psychrophila* D12. Since we had identified a selectable marker (the *ermF* gene) and an efficient method of gene transfer (conjugation from *E. coli*), we next set out to develop cloning vectors for *C. johnsonae*. Forty-six strains of *Cytophaga* spp. and related gliding bacteria were screened for cryptic plasmids to use in the development of cloning vectors. One strain (*C. psychrophila* D12) contained a 3.5-kb cryptic plasmid that we called pCP1. pCP1 contained one *Eco*RI restriction site, one *Pvu*II site, four *Ssp*I sites, and five *Hin*dIII sites (Fig. 2). The *Eco*RI and *Pvu*II sites were mapped by standard procedures (43). The *Hin*dIII sites were mapped following cloning of pCP1 into pLYL03 to generate pCP11 as described below and in Materials and Methods. pCP1 was not cut by the following restriction enzymes: *Bam*HI, *Bgl*II, *Bcl*I, *Bsp*hI, *Cla*I, *Eco*RV, *Kas*I, *Kpn*I, *Mlu*I, *Nhe*I, *Nsi*I, *Pst*I, *Sal*I, *Sca*I, *Sma*I, *Sst*I, *Stu*I, *Sph*I, *Xba*I, *Xho*I, and *Xmn*I. All enzymes were tested on pCP1 isolated from *C. psychrophila* and also on the cloned pCP1 DNA present in pCP11 as isolated from *E. coli* S17-1.

Construction of a *C. johnsonae-E. coli* shuttle vector. *C. psychrophila* is closely related to *C. johnsonae* on the basis of 16S RNA phylogenies (34), which suggested the possibility that pCP1 would function in *C. johnsonae*. Our strategy to construct a cloning vector for *C. johnsonae* was to use the replication functions of pCP1 and the selective markers and other features of pLYL03. pLYL03 is a pUC19-based suicide vector for *Bacteroides* spp. that contains two selectable markers (29). The β-lactamase gene confers ampicillin resistance on *E. coli*, while the *ermF* gene confers erythromycin resistance on *Bacteroides* spp. but not on *E. coli*. pLYL03 also contains an origin of transfer to allow conjugative transfer from *E. coli* S17-1.

We constructed the *C. johnsonae-E. coli* shuttle vector pCP11 as outlined in Fig. 2. pCP1 was linearized with *Eco*RI and ligated into *Eco*RI-digested pSPORT1, generating pCP10. This allowed us to propagate the *C. psychrophila* plasmid DNA in *E. coli* to obtain large quantities and also introduced convenient restriction sites flanking the *Cytophaga* DNA. pCP10 does not contain any selectable markers that function in *C. johnsonae*. pCP10 was digested with *Bam*HI and *Pst*I, and the 3.5-kb fragment was ligated with pLYL03 that had been digested with the same enzymes. The resulting plasmid, pCP11, was transformed into *E. coli* S17-1 and transferred by conjugation into *C. johnsonae*. pCP11 was stably maintained in *E. coli* (conferring ampicillin resistance) and in *C. johnsonae* (conferring erythromycin resistance). pCP11 plasmid DNA was isolated from *E. coli* and from transconjugants of three strains of *C. johnsonae* (Fig. 3). Efficiencies of conjugative transfer from *E. coli* S17-1 into the various *C. johnsonae* strains were 3×10^{-5} (CR1), 1×10^{-3} (CJ6R1), and 2×10^{-8} (CJ12R1) transconjugants per recipient cell. We attempted to introduce pLYL03 into *C. johnsonae* by conjugation but obtained no erythromycin-resistant colonies. This result suggests that pCP1 replication functions are required for the maintenance of pCP11 in *C. johnsonae.*

Construction of pCP17, a *C. johnsonae-E. coli* shuttle cosmid. To make pCP11 more useful for complementation analyses in *C. johnsonae*, we introduced a fragment of cosmid pHC79, containing the pBR322 tetracycline resistance gene and the lambda *cos* site, to generate the *C. johnsonae-E. coli* shuttle cosmid pCP17 (Fig. 4). pCP17 has all of the features of pCP11, but the added feature of the λ *cos* site allows for efficient construction of libraries of *Cytophaga* DNA in *E. coli*. pCP17 libraries carrying large inserts of *C. johnsonae* DNA are

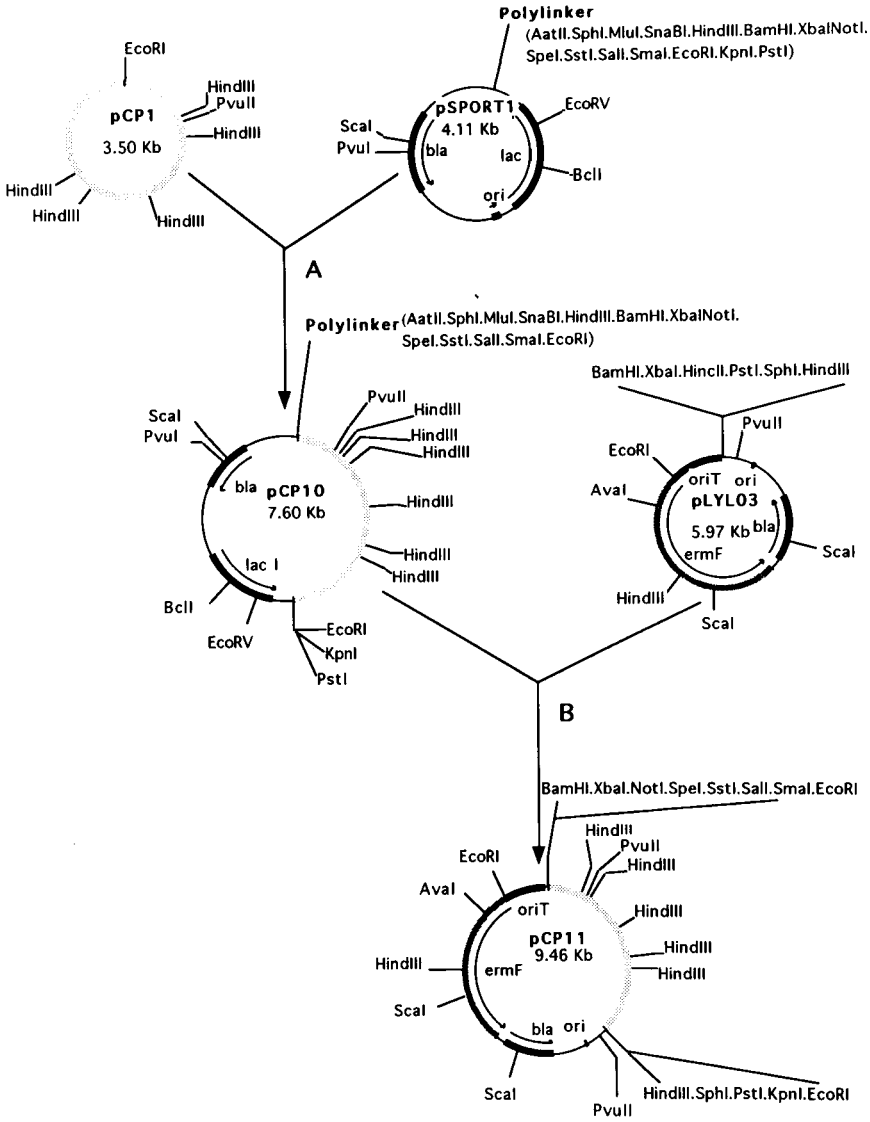

FIG. 2. Construction of the *C. johnsonae-E. coli* shuttle plasmid pCP11. Letters refer to individual steps as described in Materials and Methods.

easily introduced into *E. coli* following in vitro packaging in λ phage heads. These cosmids may then be transferred to *C. johnsonae* by conjugation. In preliminary experiments, we have used pCP17 to generate a library of *C. johnsonae* DNA and have isolated a cosmid clone that complements a nonmotile mutant of *C. johnsonae* (data not shown).

Transfer of plasmids into *C. johnsonae* by electroporation. The plasmids mentioned above can be transferred from *E. coli* into *C. johnsonae* by conjugation. For some experiments, it may be more convenient to directly introduce plasmid DNA into *C. johnsonae* without the additional step of propagation in *E. coli*. Electroporation is a general method to introduce DNA into many different types of bacterial cells (19). We determined the optimal conditions for electroporation of pCP11 into *C. johnsonae* ATCC 17061. Plasmid DNA was isolated from CR1, and cells were prepared for electroporation as described in Materials and Methods. Under optimal conditions (1.5 kV, 10 kV/cm), 2.7×10^5 erythromycin-resistant transformants were obtained per μg of DNA (Table 2).

DISCUSSION

Gliding motility (active movement over surfaces without the aid of flagella) is a trait shared by many bacteria belonging to different evolutionary branches of the eubacterial phylogenetic tree (39). The mechanism(s) of bacterial gliding motility is not known, and it is not even known whether gliding bacteria from different branches of the eubacterial phylogenetic tree employ similar or fundamentally different mechanisms to move over surfaces. Until now, genetic analyses of gliding motility have been confined primarily to the myxobacterium *M. xanthus. C. johnsonae* has several advantages over *M. xanthus* as a model of bacterial gliding motility. *C. johnsonae* (doubling time of about 1 h) grows more rapidly than *M. xanthus* (doubling time of about 3 to 4 h). *C. johnsonae* cells also glide 10 to 100 times more rapidly than *M. xanthus* cells (36). Biochemical and behavioral studies have been performed on *C. johnsonae*, and many completely nonmotile mutants have been isolated, but further genetic analyses of *C. johnsonae* gliding motility have

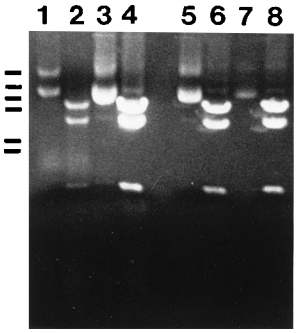

FIG. 3. Restriction analysis of pCP11 isolated from *E. coli* and *C. johnsonae*. pCP11 was isolated from each strain and digested with *Eco*RI, and fragments were separated by gel electrophoresis. Lanes 1, 3, 5, and 7 contain uncut plasmid; lanes 2, 4, 6, and 8 contain plasmid digested with *Eco*RI. Lanes 1 and 2, plasmid from *E. coli* S17-1; lanes 3 and 4, plasmid from CR1; lanes 5 and 6, plasmid from CJ6R1; lanes 7 and 8, plasmid from CJ12R1.

not been possible (36). Studies of *C. johnsonae* gliding motility have been limited by the lack of genetic tools. Until now, no reliable methods of gene transfer, convenient selectable markers, cloning vectors, or transposons were available for *C. johnsonae* or related gliding bacteria. In this study, we have identified and developed genetic tools for *C. johnsonae.*

A wide variety of broad-host-range plasmids and transposons were tested for the ability to function in *C. johnsonae*. None of these conferred antibiotic resistance on *C. johnsonae*. In retrospect, it is clear that transfer of these plasmids and transposons into *C. johnsonae* probably occurred efficiently. Other plasmids containing IncP origins of transfer (pCP11 and R751) were efficiently transferred by conjugation from *E. coli*. It is likely that the broad-host-range plasmids failed to confer

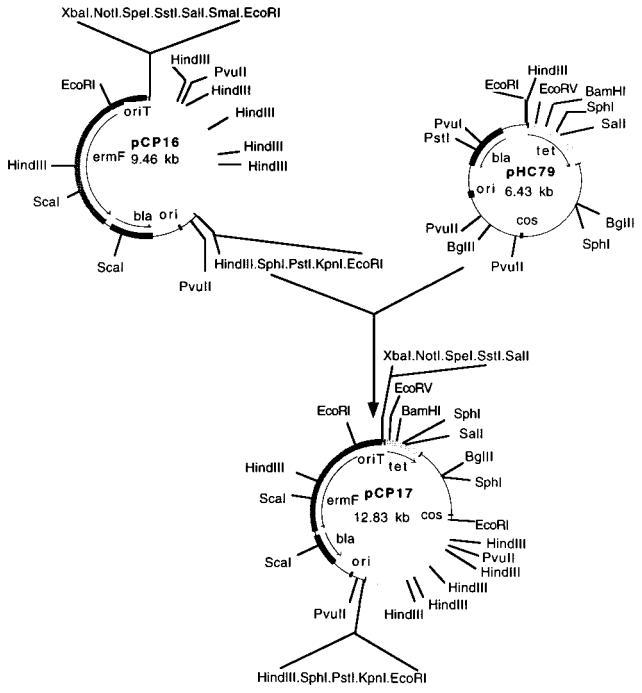

FIG. 4. Construction of the *C. johnsonae-E. coli* shuttle cosmid pCP17. pCP16 was generated from pCP11 by destroying the *Bam*HI site as described in Materials and Methods. pCP17 was constructed from pHC79 and pCP16 as described in Materials and Methods.

TABLE 2. Transformation of *C. johnsonae* with pCP11 by electroporation[a]

Voltage (kV)	kV/cm	No. of erythromycin-resistant colonies/μg of DNA
0	0	0
1.0	6.7	1.1×10^5
1.5	10.0	2.7×10^5
2.0	13.3	1.2×10^5
2.4	16.0	5.2×10^4

[a] Cells of *C. johnsonae* ATCC 17061 were prepared for electroporation as described in Materials and Methods. Approximately 18 ng of plasmid DNA was added to 20 μl of cells at a density of approximately 10^{11} cells per ml. Each mixture was placed in a Bethesda Research Laboratories microelectroporation chamber and pulsed with the voltage indicated.

antibiotic resistance or failed to be propagated. This accounts for the difficulty observed by others in developing genetic techniques for *C. johnsonae* using broad-host-range plasmids (36, 40).

The *Bacteroides* transposon Tn*4351* did function in *C. johnsonae*. This transposon conferred erythromycin resistance on *C. johnsonae* and was found integrated into the host genome after conjugation. It is likely that transposition accounts for at least some of these insertions since many of the insertions contain Tn*4351* sequences but no vector sequences, and Tn*4351* insertions occur at many different sites in the *C. johnsonae* genome, as indicated by Southern blot analyses and by the different phenotypes of Tn*4351*-induced mutants. Tn*4351* should be useful for generating tagged mutations in *C. johnsonae* that can be readily cloned in *E. coli*. Tn*4351* contains two antibiotic resistance genes; *ermF* is expressed in *C. johnsonae* but not in *E. coli*, while *tetX* is expressed in *E. coli* but not in *C. johnsonae*. Fragments of *C. johnsonae* chromosomal DNA containing Tn*4351* insertions can thus be cloned in *E. coli* by using tetracycline resistance as a selective marker. The pattern of expression of the antibiotic resistance genes of Tn*4351* in *C. johnsonae* is similar to what has been previously observed for *Bacteroides* spp. (49).

The properties of Tn*4351* have been well characterized in *Bacteroides* spp. (42). Tn*4351* is a composite transposon. The individual IS*4351* elements at the ends of the transposon can transpose on their own, or they can allow transposition of the entire transposon. Introduction of Tn*4351* into *Bacteroides* species on R751 results in transposition of Tn*4351* into the *Bacteroides* chromosome and also results in integration of R751 in about half of the transconjugants. Most of the cointegrates contain R751 flanked by a complete copy of Tn*4351* at one end and a copy of IS*4351* at the other end (2, 49, 53). We observed the same types of events when Tn*4351* on R751 was introduced into *C. johnsonae*. In *Bacteroides* species, both simple insertions and the more complicated events that lead to cointegration result in mutations (42, 49). Tn*4351* has been used to genetically analyze *Bacteroides thetaiotaomicron* starch utilization, chondroitin sulfate utilization, and ability to colonize the mouse intestine (2, 10, 41). Both simple insertions and cointegrations also result in mutations in *C. johnsonae*. We have observed motility, pigmentation, and auxotrophic mutants of *C. johnsonae* that contain simple Tn*4351* insertions or the more complicated cointegrates.

The identification of a selectable marker (*ermF*) and method of gene transfer (conjugation) allowed us to rigorously test whether broad-host-range plasmids would replicate in *C. johnsonae*. We introduced several broad-host-range plasmids that carry the *ermF* gene into *C. johnsonae*. pNJR5 carries

ermF and has IncQ replication functions as well as a *Bacteroides* origin of replication. pNJR5 did not confer erythromycin resistance on *C. johnsonae*, presumably because neither the IncQ nor the *Bacteroides* replication region allowed stable replication and maintenance of this plasmid in *C. johnsonae*. R751::Tn*4351*Ω4, which carries Tn*4351* and has an IncP origin of replication, was also not maintained as free plasmid in *C. johnsonae*. As mentioned above, erythromycin-resistant colonies did arise when Tn*4351* integrated into the host genome.

We also developed cloning vectors for *C. johnsonae* by using a small cryptic plasmid (pCP1) that we identified in the fish pathogen *C. psychrophila* D12. pCP11 contains the *ermF* gene, which confers erythromycin resistance on *C. johnsonae*, a β-lactamase gene which confers ampicillin resistance on *E. coli*, two origins of replication allowing the plasmid to replicate in both *C. johnsonae* and *E. coli*, and an origin of transfer to allow conjugative transfer from *E. coli* into *C. johnsonae*. pCP17 contains the same features plus an additional antibiotic resistance marker (tetracycline resistance) and the λ *cos* site to allow in vitro packaging in λ phage heads for efficient delivery of large cloned fragments into *E. coli. Cytophaga* DNA is most conveniently cloned into the *Bam*HI site of pCP17. Clones in *E. coli* are screened for tetracycline sensitivity to confirm that an insert has disrupted the tetracycline resistance gene. We have recently used pCP17 to generate a library of *C. johnsonae* DNA. From this library, we found one clone that complemented a nonmotile mutant of *C. johnsonae* (data not shown). This indicates that pCP17 will be useful for identifying and cloning genes involved in *C. johnsonae* gliding motility.

In addition to cloning vectors and transposons that function in *C. johnsonae*, we have also identified a suicide vector, pLYL03 (29), that carries the erythromycin resistance gene but which apparently does not replicate in *C. johnsonae*. pLYL03 should be useful for insertional mutagenesis and for construction of partial diploids for single-copy complementation of *C. johnsonae* mutants.

The genetic tools and techniques described above are sufficient for many genetic experiments with *C. johnsonae*. However, when one is transferring plasmids between *C. johnsonae* strains, it is sometimes inconvenient to use *E. coli* as an intermediary. To simplify these transfers, we optimized conditions for electroporation of *C. johnsonae*. Plasmid DNA isolated from one strain of *C. johnsonae* can be transferred to a second strain with an efficiency of approximately 3×10^5 transformants per μg of DNA. This compares favorably with electroporation results for many other bacteria (19). We have recently introduced pCP17 carrying a putative gliding motility gene into several nonmotile mutants by electroporation and restored gliding motility (data not shown).

Genetic analyses of *Cytophaga* gliding motility have not been possible until now. The results presented in this paper provide the first evidence of gene transfer and transposon mutagenesis in a cytophaga. They also describe the first cloning vectors for any cytophaga. With these tools we can now begin a rigorous genetic analysis of *Cytophaga* gliding motility.

C. johnsonae is one representative of a large and diverse assemblage of bacteria belonging to the *Cytophaga-Flavobacterium-Bacteroides* subphylum of the eubacterial tree. Within this group, genetic techniques were previously available only for members of the genus *Bacteroides* and related bacteria in the genera *Prevotella* (47) and *Porphyromonas* (13). Cytophagas and flavobacteria are important as pathogens of animals and humans (3, 6, 8, 9, 30, 31, 45), as agents of biodegradation (18, 28, 38), and as producers of novel antibiotics and antitumor drugs (11, 26, 35, 38). We have recently demonstrated that some of the genetic tools described above function in a number of bacteria belonging to the *Cytophaga-Flavobacterium* group (data not shown). This opens the possibility of genetic analyses for the many important species of *Cytophaga*, *Flavobacterium*, and related genera.

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