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Identification and Characterization of the Conjugal Transfer Region of the pCg1 plasmid from Naphthalene-Degrading *Pseudomonas putida* Cg1

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Hybridization and restriction fragment length polymorphism data (K. G. Stuart-Keil, A. M. Hohnstock, K. P. Drees, J. B. Herrick, and E. L. Madsen, Appl. Environ. Microbiol. 64:3633-3640, 1998) have shown that pCg1, a naphthalene catabolic plasmid carried by *Pseudomonas putida* Cg1, is homologous to the archetypal naphthalene catabolic plasmid, pDTG1, in *P. putida* NCIB 9816-4. Sequencing of the latter plasmid allowed PCR primers to be designed for amplifying and sequencing the conjugal transfer region in pCg1. The mating pair formation (*mpf*) gene, *mpfA* encoding the putative precursor of the conjugative pilin subunit from pCg1, was identified along with other *trb*-like *mpf* genes. Sequence comparison revealed that the 10 *mpf* genes in pCg1 and pDTG1 are closely related (61 to 84% identity) in sequence and operon structure to the putative *mpf* genes of catabolic plasmid pWW0 (TOL plasmid of *P. putida*) and pM3 (antibiotic resistance plasmid of *Pseudomonas.* spp). A polar mutation caused by insertional inactivation in *mpfA* of pCg1 and reverse transcriptase PCR analysis of mRNA showed that this *mpf* region was involved in conjugation and was transcribed from a promoter located upstream of an open reading frame adjacent to *mpfA*. *lacZ* transcriptional fusions revealed that *mpf* genes of pCg1 were expressed constitutively both in liquid and on solid media. This expression did not respond to host exposure to naphthalene. Conjugation frequency on semisolid media was consistently 10- to 100-fold higher than that in liquid media. Thus, conjugation of pCg1 in *P. putida* Cg1 was enhanced by expression of genes in the *mpf* region and by surfaces where conditions fostering stable, high-density cell-to-cell contact are manifest.

Horizontal gene transfer (HGT) is the transmission of DNA between organisms of different lineages (5, 28, 53). An understanding of HGT is important because of its implication in the spread of antibiotic resistance genes (9), in the possible exchange of genes between genetically engineered and native microorganisms (10), in interactions between plants and bacteria (61), and in the dissemination of genes involved in pollutant biodegradation (18, 26, 40) and metal resistance (34, 51). It is becoming increasingly apparent that HGT has played an integral role in the evolution of bacterial species (55). One of the mechanisms of HGT is conjugation (28). Bacterial conjugation is a subject of ongoing scientific inquiry that explores how DNA is transmitted from donor to recipient cells via physical contact (31). The conjugation process may involve a combination of DNA rolling-circle replication with a type IV secretion system (32). While the role of pili in establishing cell-cell contact is widely accepted, conveyance of DNA through pili to a recipient cell is still under debate (32).

One of the most intensively studied conjugal transfer systems is the broad-host-range enteric plasmid RP4 (IncPα). RP4 conjugal transfer is encoded by two regions, Tra1 and Tra2, which account for almost half of the 60-kb plasmid DNA (41). The Tra1 region encodes the primase, relaxase, the leader operon, and one protein involved in mating pair formation

(41). Tra2 genes (termed *trb*) code for mating pair formation proteins (29, 30). Many of the Tra2 gene products are homologous to those of the *virB* region of the Ti plasmid of *Agrobacterium* (31). The *mpf* (for mating pair formation; *trb*) gene homologues are believed to encode the mating pair formation apparatus essential for the physical contact and DNA translocation between donor and recipient cells: for instance, the *trbC* gene encodes the precursor of the pilin subunit of the conjugal pilus (17). Conjugal DNA transfer is abolished by inactivation of the pilin precursor gene or of any gene involved in assembling the pilus (14, 17).

Despite their early discovery (e.g., references 11, 24, 47, 54, and 56), very little is known about regulation of conjugal transfer of catabolic plasmids in pseudomonads or about their surface requirement for conjugation. Electron microscopy and conjugation frequency observations suggested that the pili encoded by pWW0 (IncP-9, TOL plasmid of *P. putida* [15]) are thick, flexible, involved in surface-preferred conjugation, and distinct from the rigid pili encoded by IncP-1, IncI, IncW, and IncN plasmids that show surface-obligatory conjugation (4, 54). The plasmids carried by bacteria native to our coal tar-contaminated study site are closely related to the archetypal naphthalene catabolic plasmid pDTG1 (52). This plasmid has recently been sequenced by G. Zylstra (GenBank accession no. AF491307). Several genes in the naphthalene catabolic operon (*nahAc* [iron sulfur protein, large subunit of naphthalene dioxygenase], *nahR* [transcriptional regulator for naphthalene degradation], and intergenic region between *nahR* and *nahG*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
P. putida NCIB 9816-4	Naphthalene degrader containing the pDTG1 plasmid	52
P. putida Cg1	Naphthalene degrader containing the pCg1 plasmid	52
P. putida Cg1CR	pCg1 plasmid-cured derivative of *P. putida* Cg1, Rif^r	52
P. putida KT2440-Tc	TOL plasmid-cured derivative of *P. putida* mt-2 with mini-Tn5 insertion of Tet^r	39
P. putida Cg1pMPF	Derivative of *P. putida* Cg1, insertion of pMPF in pCg1, Kan^r, LacZ$^+$	This study
P. putida Cg1pEM1	Derivative of *P. putida* Cg1, insertion of pEM1 in pCg1, Kan^r, LacZ$^+$	This study
P. putida Cg1pAH7	Derivative of *P. putida* Cg1, insertion of pAH7 in pCg1, Kan^r, LacZ$^+$	This study
E. coli INV-αF′	Host for cloning and maintaining plasmids	Invitrogen
E. coli S17-1 λpir	*trp* Sm^r *recA thi pro hsdM*$^+$ RP4-2-Tc::Mu::Km Tn7λpir; *hsdR* mutant	48
Plasmids		
pCR2.1	Cloning vector, Kan^r, Amp^r	Invitrogen
pVIK110	R6K*oriV*, suicide vector, LacZ translation fusion vector, Kan^r	27
pVIK112	R6K*oriV*, suicide vector, LacZ transcriptional fusion vector, Kan^r	27
pMPF	Internal *mpfA* fragment in pVIK110	This study
pAH7	Putative promoter region of *mpf* genes cloned into pVIK110	This study
pEM1	Putative promoter region of *mpf* genes cloned into pVIK112	This study

[salicylate hydroxylase]) from the naphthalene catabolic plasmid pCg1 in *P. putida* Cg1 were previously detected with degenerate primer pairs and characterized (20, 43). Sequence analysis showed that these regions were identical to those genes of pDTG1. In this report, putative *mpf* genes (*trb*-type genes) were identified on the naphthalene catabolic plasmids pCg1 and pDTG1. Sequence comparisons showed that the *mpf* genes in pCg1 and pDTG1 are closely related in sequence and operon structure to those in pWW0. Furthermore, the influence of solid surfaces and induction of the *mpf* genes on conjugal transfer of pDTG1-like plasmids were investigated.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *P. putida* NCIB 9816-4 and *P. putida* Cg1 naphthalene-degrading strains have been described elsewhere (20, 42, 43, 52). *P. putida* KT2440-Tc was kindly provided by Niels Kroer (Department of Marine Ecology and Microbiology, National Environmental Research Institute, Roskilde, Denmark). All media and growth condition for *Escherichia coli* strains were prepared as described by Ausubel et al. (1). All *Pseudomonas* strains were grown at 22°C in mineral salts broth (MSB) and other routine media described elsewhere (21, 52). Rifampin (100 μg/ml), tetracycline (15 μg/ml), kanamycin (100 μg/ml), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml) were added to broth or agar plates when necessary.

DNA and plasmid manipulation. Primers used in this study are listed in Table 2. Restriction fragment length polymorphism (RFLP) characterization of the *mpf* regions in pDTG1 and pCg1 was performed by using the primer pairs listed in Table 2. In RFLP analysis, each fragment was digested with *Hae*III and *Hha*I (Promega) and was electrophoresed on a 3% agarose gel (Metaphor agarose; BMA). Colony PCR and cloning for sequencing were carried out by the following procedures: a 10-μl volume of sterile water was added to each PCR tube, and bacterial cells were transferred to the PCR tube with a sterile platinum needle from a freshly grown colony. The cells were lysed (95°C for 5 min) in a PTC-200 thermocycler (MJ Research Inc., Watertown, Mass.). The 50-μl volume reaction mixture in each tube contained each primer (0.5 μM), 1× PCR buffer (Gibco BRL, Gaithersburg, Md.), deoxynucleoside triphosphate (0.2 mM), MgCl$_2$ (1.5 mM), and 1.25 U of *Taq* DNA polymerase (Gibco BRL). A cycling regimen of 94°C for 5 min (one cycle), 94°C for 1 min, 55°C for 30 s, 72°C for 1 min (35 cycles), and 72°C for 5 min (one cycle) was employed. Amplicons were separated by electrophoresis in a 2% agarose gel. Amplified PCR fragments were cloned into the pCR2.1 TA vector (Invitrogen, Carlsbad, Calif.). The constructed plasmids were introduced into *E. coli* INV-αF′ competent cells (Invitrogen). Both directions of the insert fragments were sequenced, and all sequencing was completed on an ABI model 3700 instrument at the Biological Resource Center, Cornell University. The DNA sequences were aligned by using the MegAlign

program, and all sequence comparisons were computed as percent identity by using Clustal (DNASTAR, Madison, Wis.).

Construction of polar mutation in the *mpf* region. The suicide translational fusion vector pVIK110 (27) was used for constructing a polar mutation of *mpf* regions (Fig. 1A). Primer pair mpfA-F/mpfA-R was designed to target an internal region of a putative *mpfA* gene. The amplified 192-bp fragment of the internal *mpfA* fragment gene was cloned into the *Kpn*I-*Sal*I cloning sites of pVIK110, creating the pMPF plasmid (full sequencing of the modification of pVIK110 verified that the sought genetic construct was achieved [Fig. 1A]), which was introduced into *E. coli* S-17-1 λpir. Then conjugation was performed by filter mating with *E. coli* S-17-1 λpir (pMPF) and *P. putida* Cg1 as donor and recipient bacteria, respectively. The transconjugants (*P. putida* Cg1pMPF) were selected on MSB-P medium (MSB plus 0.1% pyruvate) containing kanamycin at room temperature (22°C). Confirmation of transconjugants was conducted by PCR using primers specific to pCg1 or pVIK110. The expected PCR fragment was amplified only in the transconjugant (data not shown).

RT-PCR. Reverse transcriptase PCR (RT-PCR) was performed on total RNA extracted from *P. putida* Cg1. Primers (orf25-F, mpfA-R, and mpfB-R) used for RT-PCR are listed in Table 2. Total RNA was extracted according to the manufacturer's procedure (RNeasy mini kit; Qiagen). The extracted total RNA was treated with DNase I (Gibco BRL) for 15 min at 37°C. DNase I was inactivated with boiling at 70°C for 10 min. Reverse transcription and PCR were performed by using Superscript II RT (Gibco BRL) as follows: the 50-μl volume reaction mixture in each tube contained each primer (1 μM), 2× reaction buffer (Gibco BRL), and 1 μl of RT/*Taq* polymerase mixture (Gibco BRL). A cycling regimen of 50°C for 20 min and 94°C for 2 min (one cycle) for cDNA synthesis and of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min (35 cycles), and 72°C for 5 min (one cycle) for PCR was used. To confirm that cDNA synthesis occurred and that RNA preparation was free of genomic DNA, negative control RT-PCR was performed only with *Taq* polymerase.

Construction of *mpfA-lacZ* fusions. Two independent reporter fusions were constructed and used, i.e., pEM1 and pAH7. The suicide transcriptional fusion vector pVIK112 (27) was used for constructing an *mpfA-lacZ* transcriptional fusion reporter system (Fig. 1B). The PCR fragment containing parA-F and mpfA-R2 (Table 2) was cloned into TA cloning vector pCR2.1. *Eco*RI-digested fragments were cloned into the *Eco*RI site of pVIK112, creating plasmid pEM1. In addition, the suicide translational fusion vector pVIK110 was used for constructing a translational fusion reporter system. For this, the PCR fragment with parA-F and mpfA-R2 was cloned into pVIK110, creating pAH7 (analogous to pEM1 in Fig. 1B), after digestion with *Kpn*I and *Sal*I restriction enzymes. The orientation of the fragments was confirmed by sequencing the cloned region in the pEM1 and pAH7 plasmids. Then the same cloning and transformation procedures described for creating the polar mutation in the *mpf* region (above) were applied in constructing *P. putida* Cg1pEM1 (*P. putida* Cg1 containing *mpfA-lacZ* transcriptional fusion on pCg1) and *P. putida* Cg1pAH7 (*P. putida* Cg1 containing *mpfA-lacZ* translational fusion on pCg1).

β-Galactosidase activity assay. β-Galactosidase activity was measured by using a microtiter plate modification (49) of the original method of Miller (35).

TABLE 2. Primers used in this study to amplify and analyze portions of a 26.8-kb region of naphthalene catabolic plasmids pDTG1 and pCg1[a]

Primer name	Sequence (5′→3′)	Corresponding region of 26.886 kb
Primers used for cloning and sequencing		
mpfA-F[b]	CCC GGT ACC CAA ACC CTG CAA AGC GTA ATC	21145–21125
mpfA-R[b]	CCC GTC GAC GGC CAT GCT CTT CAA ACC ACT	20972–20992
orf25-F	ATG CAG AGC CTA GAC AAA AGA G	21521–21500
mpfB-R	TCA ACG AGG CGC ATA ACT TCT	20474–20494
parA-F[b]	GGG GGT ACC CAT ATA TAG GGT TTA CTT TTC	22102–22082
mpfA-R2[b]	CCC GTC GAC GTC CAA CTT TGG AAG TTT	21205–21222
tra-F[b]	CGC GAA TTC GTC AAA GTC TCA ATA CAC AAC ACG	2683–2706
traB-R[b]	CGC GGT ACC AAT CGA CTT CTC TTT TAT GC	3316–3297
Primers used for RFLP analysis		
493F	CGC GTG CCG AAG TGG TC	493–509
1272R	TGG TCG GGC AGA TGA TGG AAG ATT	1272–1249
1629F	TTG TTG AAG ACC ACC AGA ATC C	1629–1650
2437R	TGA GTC AAA GGC AGC GAA AAT ACG	2437–2414
4122F	GGA CTC CGA GGG CTA CTT	4122–4138
5108R	TCC GAC GAT TAA CTA TTT GAC	5108–5088
6779F	CGA CAG AAG AAC CAG GAA CTA AAA	6779–6802
7510R	TGT AAG ACC TTG GGC GCT GTG GAC	7510–7487
9422F	TCT TTG GCC ATG TCC TAA TCC TCT	9422–9445
10591R	GCT CGC GCT ATG GTT TCT AAG TTG	10591–10568
12475F	ACG CGG CCA GGT TTA CGA ATA CA	12475–12497
13114R	GCG CGG GTG CCT CCA ACT CC	13114–13095
17460F	GTG TTG ACC AGT TCC TCG TAA T	17460–17481
18239R	TCA TGC AGA TCC GCC GTA AAA	18239–18219
19699F	GCT CAG CTC GCA GTA TTC CT	19699–19718
20391R	GAG TTG GTG GTC AGC GAG ATG	20391–20371

[a] Nucleotide positions 1 and 26886 correspond to the extreme left and right, respectively, of Fig. 2. The *mpf* gene cluster (Fig. 2) is bounded by positions 8794 and 21232.
[b] Restriction enzyme sites, used for cloning purposes, are underlined; when restriction enzyme sites were inserted into a primer, three additional nucleotides were added to the 5′ end.

β-Galactosidase activities were measured by using *o*-nitrophenyl β-D-galactopyranoside (10 mg of it per ml in 0.1 M KPO$_4$, pH 7.0) as a substrate. MSB-N (MSB plus 0.1% naphthalene crystals) and MSB-G (MSB plus 10 mM glucose) were used as the minimal medium.

Conjugation frequency. *P. putida* Cg1pEM1 served as the donor and *P. putida* KT2440-Tc (39) served as the recipient in standard filter mating as described elsewhere (52). Donor cells ($\approx 10^7$ to 10^9, grown overnight in Luria-Bertani [LB] broth, in MSB-G and in MSB-N amended with 100 μg of kanamycin/ml) were
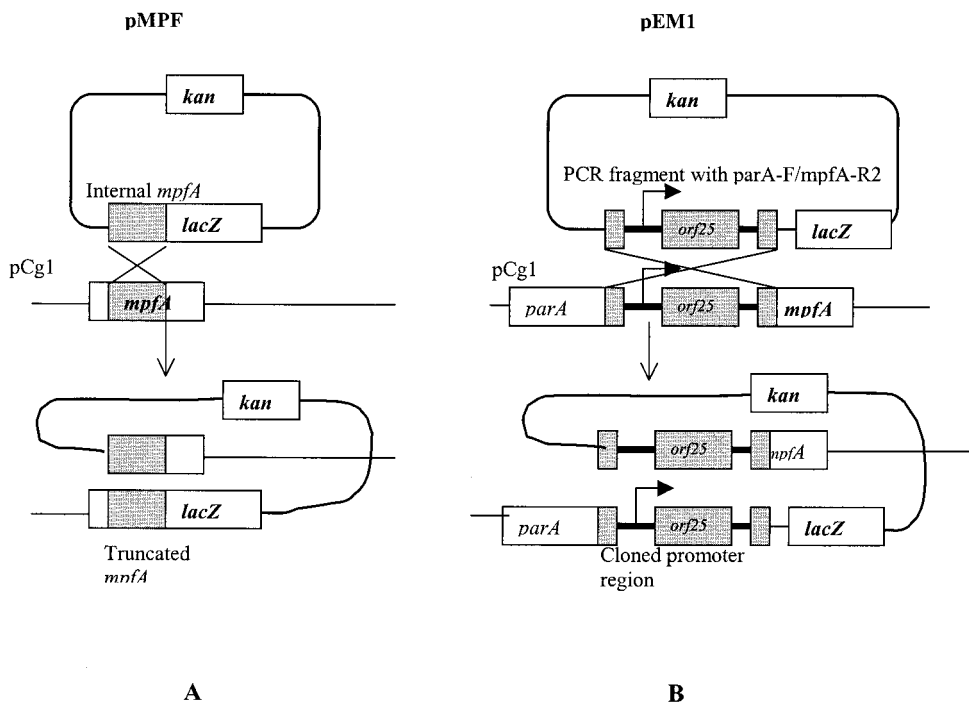

FIG. 1. Construction of pMPF and pEM1 via Campbell-type homologous recombination with the pCg1 plasmid. The internal regions are depicted as a gray box. pMPF was designed to create a polar mutation (knockout) of *mpfA*. pEM1 reported transcriptional activity of the *mpfA* operon.
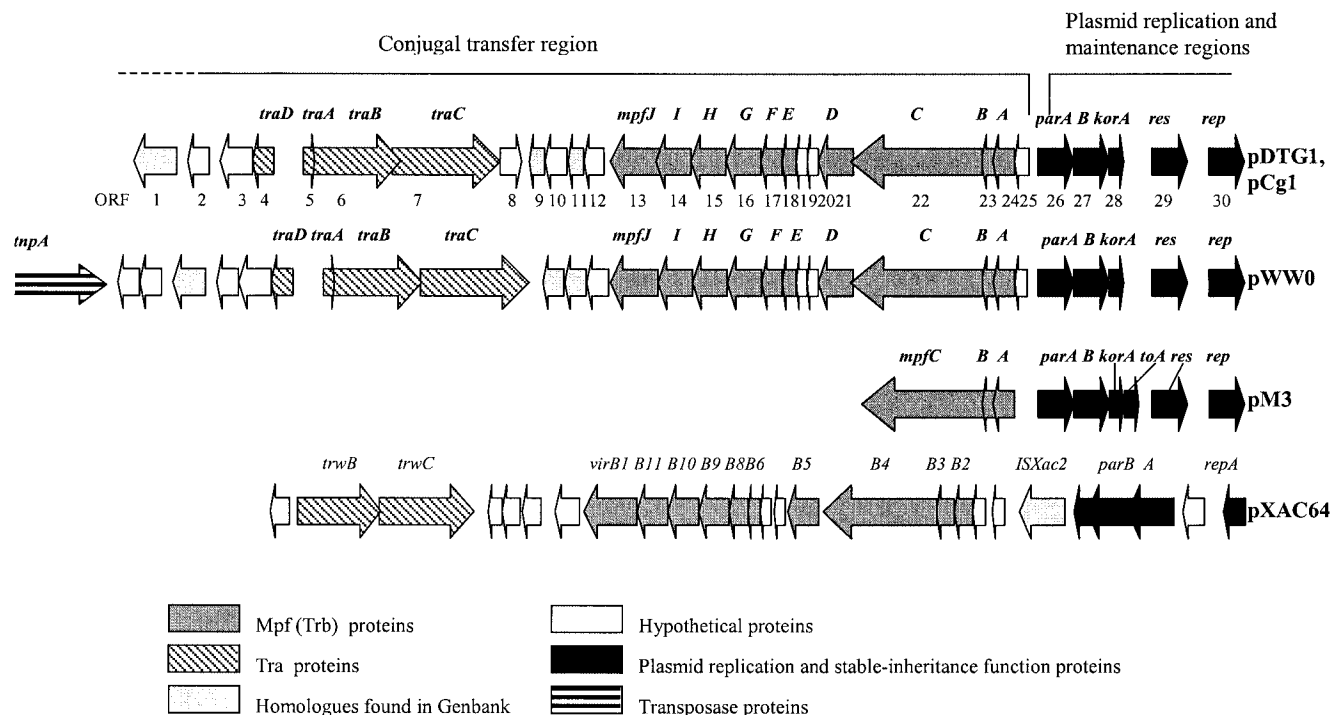
FIG. 2. Comparison of the gene order in mating pair formation (*mpf*) and *tra* gene clusters in ~30-kb regions of five related plasmids.

harvested and washed with phosphate-buffered saline (PBS) (1). To the same tube, ≈10^7 to 10^9 recipient cells grown overnight in LB broth were added and harvested by centrifugation. For solid medium conjugation, cells were resuspended in 50 μl of PBS, collected on a sterile filter (0.2-μm pore size, 25-mm diameter; Millipore, Bedford, Mass.), and placed on an LB or MSB-G plate. The plate contents were incubated for 1 to 24 h, agar side down, at room temperature. After mating, filters were removed and vortexed in 1 ml of PBS for 30 s to disperse the cells. For liquid medium conjugation, cells were resuspended with 50 μl of LB or MSB-G broth and were incubated at room temperature. After mating for 1 to 24 h, serial dilutions were prepared and transconjugants were enumerated on LB plates amended with kanamycin and tetracycline. Donor cells only were enumerated on LB plates amended with kanamycin, and recipient cells only were enumerated on LB plates amended with tetracycline. Donors were also plated on LB amended with kanamycin and tetracycline to account for spontaneous tetracycline resistance, while recipients were also plated onto LB amended with kanamycin and tetracycline to account for potential experimental errors. No spontaneous mutants of donor or recipient cells appeared on the selective media during the entire conjugation assays.

Nucleotide sequence accession number. The nucleotide sequences of the entire pDTG1 plasmid of *P. putida* NCIB 9816-4 have been deposited by G. Zylstra (Rutgers University) in GenBank under accession no AF491307. The nucleotide-numbering system in this paper differs from that of the GenBank submission. Nucleotides 1 to 28886 described here correspond to nucleotides 61380 to 83042 and 1 to 5223 in GenBank. The nucleotide sequences of pCg1 covering *traA* (634 bp) and *orf25-mpfB* (1,629 bp) have been deposited in GenBank under accession numbers AY249146 and AY249147.

RESULTS

Identification and sequence analysis of *mpf* regions from plasmid pCg1. The map of a 26.8-kb portion of pDTG1/pCg1 shows that it has *tra* and *trb* (referred to henceforth as *mpf*) regions (Fig. 2). To confirm and extend prior measures (43, 52) of homology between pDTG1 and pCg1, we conducted RFLP analysis of PCR-amplified *tra* and *mpf* regions by using primers listed in Table 2 (primers 493F/1272R [780-bp product], 1629F/2437R [809-bp product], 4122F/5108R [987-bp prod-

uct], 6779F/7510R [732-bp product], 9422F/10591R [1,170-bp product], 12475F/13114R [639-bp product], 17460F/18239R [780-bp product]; and 19699F/20391R [693-bp product]). Over a ~20-kb region both plasmids showed indistinguishable patterns (data not shown). Sequence comparison of the fragments amplified with primer pairs (tra-F/traB-R, parA-F/mpfA-R2, and orf25-F/mpfB-R; ~2.5 kb) confirmed that in these regions pCg1 was 100% identical to pDTG1.

As shown in Fig. 2 and Table 3, there is considerable similarity in both the order and sequence of many open reading frames in the putative transfer regions in pCg1, pDTG1, pWW0 (TOL plasmid in *P. putida*; IncP-9 incompatibility group [15]), and pM3 (IncP-9 incompatibility group; found in 16 isolates of *P. putida* species originating from farmland soil in Belarus, from industrial site soil in Belarus and Azerbaijan, and from sewage from a pharmaceutical plant in Belarus [16]). Nine of the 10 genes in the *mpf* cluster in pDTG1/pCg1 have their closest homologue (65 to 87% identity) in pWW0. The closest match to the remaining gene (*mpfA*) was in pM3 (73% identity). Two other matches between pDTG1/pCg1 and pM3 homologues were also high (70 and 77% identity). Figure 2 further reveals that there are four *tra*-like genes shared by pWW0 and pDTG1/pCg1. The *oriT* region of pWW0 was identified between the *traD* and *traA* genes (15). The putative *oriT* site of pDTG1/pCg1 is also present between *traD* and *traA* genes, and its sequence is 100% identical to the *oriT* site of pWW0.

An extended comparison of *mpfA*-related proteins (Fig. 3) confirms close relatedness between pDTG1/pCg1 and pM3/pWW0 (~72% identity) and distant relatedness (11.6% identity) to well-characterized *trbC* genes of broad-host-range enteric plasmids IncPα (RP4) and IncPβ (R751). The matrix in Fig. 3 also shows a moderate match between MpfA and VirB2

TABLE 3. Summary of computer-based nucleotide sequence analyses of open reading frames in the conjugal transfer regions in pDTG1/pCg1 plasmids

ORF[a]	Gene[b]	aa[f] length	Mol mass (kDa)	pI	BLAST result[c]	Degree of relatedness (aa pDTG1/aa match, % aa identity)[c]	Motif found in GenBank
orf1	*hyp*	261	28.8	6.22	Hyp of pWW0	261/263, 77	
					Mpr (Zinc metalloproteinase) of IncN plasmid R64	261/263, 41	
orf2	*hyp*	128	13.8	6.67	Hyp of pWW0	128/130, 67	
orf3	*hyp*	239	26.5	5.33	Hyp of pWW0	239/239, 73	
orf4	*traD*	151	16.7	8.90	TraD of pWW0	151/151, 60	
orf5	*traA*	72	7.9	4.48	TraA of pWW0	72/127, 56	
orf6	*traB*	518	58.3	6.97	TraB of pWW0	518/516, 86	
					TrwB of pXAC64	518/524, 65	
orf7	*traC*	981	109.0	9.46	TraC of pWW0	981/978, 80	Viral helicase I
					TrwC of pXAC64	981/991, 63	
orf8	*hyp*	193	21.9	10.03	Hyp (ORF7) of pWW0	193/244, 40	
orf9	*nuc*	212	22.9	9.09	Putative nuclease (ORF188) of pWW0	212/210, 60	
orf10	*hyp*	160	17.7	6.51	Putative nuclease (ORF188) of pWW0	160/210, 23	
					Hyp (XACb0057) of pXAC64	160/395, 23	
orf11	*nuc*	201	21.6	8.39	Putative nuclease (ORF189) of pWW0	201/185, 73	
					Endonuclease of IncN Plasmid R64	201/177, 48	
orf12	*hyp*	109	11.7	8.47	Hyp of pXAC64	109/111, 68	
orf13	*mpfJ*	311	33.6	7.21	MpfJ of pWW0	311/315, 65	Transglycosylase (SLT domain[d])
					VirB1 of pXAC64	311/292, 35	
orf14	*mpfI*	342	38.3	6.13	Mpf of pWW0	342/343, 81	GSPII[e] (bacterial type II secretion)
					VirB11 of pXAC64	342/340, 44	
orf15	*mpfH*	427	45.5	5.54	MpfH of pWW0	427/421, 65	
					VirB10 of p XAC64	427/406, 40	
orf16	*mpfG*	263	29.1	9.04	MpfG of pWW0	263/261, 87	
					VirB9 of pXAC64	263/260, 52	
orf17	*mpfF*	221	25.4	8.90	MpfF of pWW0	221/226, 66	
					VirB8 of pXAC64	221/224, 36	
orf18	*mpfE*	287	31.1	5.30	MpfE of pWW0	287/287, 73	
					VirB6 of pXAC64	287/288, 43	
orf19	*hyp*	134	14.2	8.28	Hyp of pWW0	134/130, 67	
orf20	*hyp*	70	7.9	9.60	Hyp of pWW0	70/70, 73	
orf21	*mpfD*	229	24.9	5.46	MpfD of pWW0	229/229, 84	
					VirB5 of pXAC64	229/220, 55	
orf22	*mpfC*	894	101.7	5.79	MpfC of pWW0	894/894, 75	
					TrbE of pM3	894/339, 70	
					VirB4 of pXAC64	894/877, 56	
orf23	*mpfB*	97	10.8	9.84	MpfB of pWW0	97/98, 77	
					TrbD of pM3	97/98, 77	
					VirB3 of pXAC64	97/99, 47	
orf24	mpfA	153	16.8	10.01	TrbC of pM3	153/153, 73	
					MpfA of pWW0	153/153, 71	
					VirB2 of pXAC64	153/125, 27	
orf25	*hyp*	90	10.0	6.55	Hyp of pWW0	90/115, 81	
					Hyp of pXAC64	90/118, 43	

[a] See Fig. 2 for position of open reading frames (ORFs) in the conjugal transfer regions.
[b] *hyp*, hypothetical.
[c] 1:1 Clustal alignment between the two proteins over the entire sequences.
[d] Soluble lytic domain.
[e] General secretory pathway II.
[f] aa, amino acid.

of pXAC64 of *Xanthomonas axonopodis* pv. *citri*. (8) and pTiC58 of *Agrobacterium tumefaciens*. Relatedness is confirmed by sequence comparison between structural genes adjacent to *mpfA* and corresponding genes in VirbB (pXAC64) and Trb (pM3) systems (Table 3; Fig. 2).

Insertional inactivation of the *mpfA* gene. The *mpfA* gene is predicted to encode the precursor of the pilin subunit of the conjugal pilus based on homology with other known pilin subunit genes. To test the hypothesis that *mpfA* of pCg1 is critical for conjugation, perhaps playing the role of other *trbC*-type products, we disrupted it by using Campbell-type single-cross-over recombination (Fig. 1A). The insertional inactivation used was designed to cause a polar mutation and disrupt other genes downstream of *mpfA* as well, should expression of these be controlled by a shared promoter. A standard filter mating was performed between *P. putida* Cg1pMPF and *P. putida* Cg1CR (52). Conjugation was not detected. The number of transconjugants and the number of spontaneous rifampin-resistant colonies per donor were equal (10^{-7}). In contrast, the positive control filter mating performed between wild-type *P. putida* Cg1 donor and *P. putida* Cg1CR recipient yielded 10^{-3} transconjugants per donor and 10^{-8} spontaneous rifampin re-

Percent Identity

2	3	4	5	6	7	8		
72.5	71.2	12.0	19.8	27.2	11.6	12.3	1	MpfA in pDTG1,pCg1
	92.8	12.0	18.2	25.6	11.6	10.5	2	MpfA in pM3 (IncP-9)
		12.0	18.2	24.8	11.6	10.5	3	MpfA in pWW0 (IncP-9)
			15.7	14.4	33.8	33.1	4	TrbC in pTiC58
				17.4	19.8	16.5	5	VirB2 in pTiC58
					17.6	15.2	6	VirB2 in pXAC64
						67.1	7	TrbC in RP4 (IncPα)
							8	TrbC in R751 (IncPβ)

FIG. 3. Similarity matrix comparing percent identity scores for complete pilin subunit proteins encoded on seven plasmids. The scores were calculated by using the MEGALIGN program (LASER-GENE package; DNASTAR Inc.).

sistance colonies per donor (22). This experiment showed that the *mpf* region of pCg1 plasmid is essential for conjugation.

Detection of *mpf* gene transcripts by RT-PCR. Computer analysis (Neural Network Promoter Prediction; http://www.fruitfly.org/seq_tools/promoter.html) showed that there is one putative promoter region upstream of *mpfA* and *orf25* (hypothetical protein) (Fig. 2 and 4). To analyze expression of the *mpf* region, we extracted DNA-free total RNA from *P. putida* Cg1 grown in MSB liquid medium with naphthalene as the carbon source (MSB-N) or in LB broth. The extracted RNA was used as the template for RT reactions. Subsequently, the cDNA template was amplified with PCR primers yielding products corresponding to two intercistronic regions, i.e., *orf25-mpfA* and *orf25-mpfA-mpfB* (Fig. 4). Total RNA extracted from naphthalene-grown cells and LB broth-grown cells both showed

A

B

FIG. 5. Use of pEM1, containing a P*mpfA-lacZ* fusion, to examine the influence of medium composition and growth phase on expression of conjugal transfer genes in *P. putida* Cg1. Expression of *mpfA* during growth in three different liquid media (A) and after 1 day of growth on agar (B). Labels for media appear in panel B. Each treatment was replicated three times. Error bars show standard deviation. β-galactosidase activities are expressed in Miller units. OD600, optical density at 600 nm.

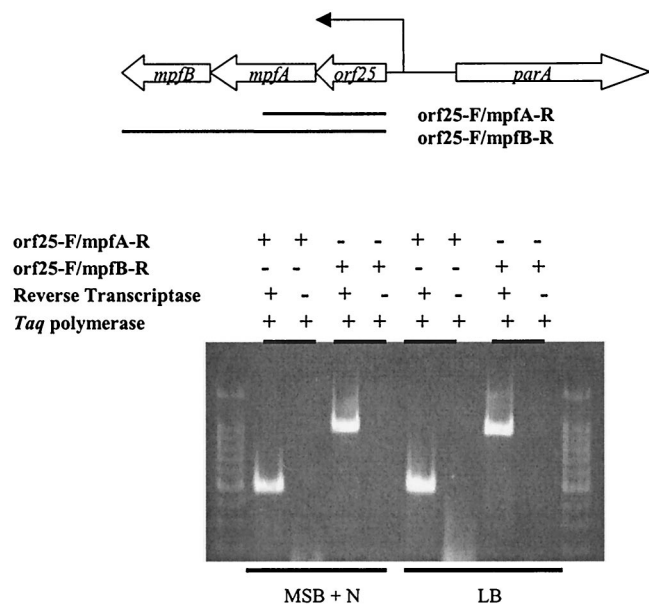| orf25-F/mpfA-R | + | + | - | - | + | + | - | - |
orf25-F/mpfB-R	-	-	+	+	-	-	+	+
Reverse Transcriptase	+	-	+	-	+	-	+	-
Taq polymerase	+	+	+	+	+	+	+	+

MSB + N   LB

FIG. 4. RT-PCR analysis assay for detecting pCg1 plasmid gene expression (mRNA transcripts) in *P. putida* Cg1. Diagram at top shows construct used to probe for *mpf* gene expression. Middle diagram shows four pairs of treatments that verify transcript detection. Bottom gel image shows that the *mpf* genes were expressed in cells grown in LB and MSB-N media.
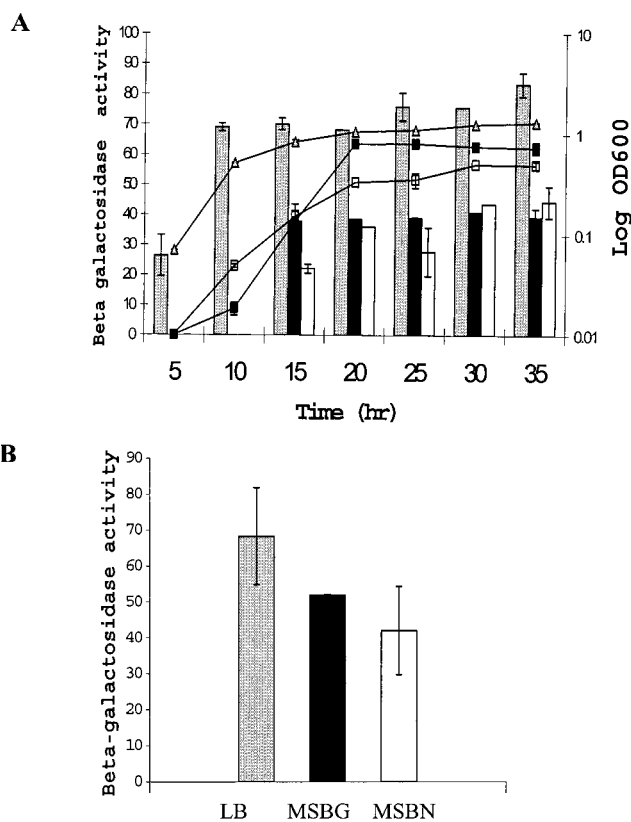
amplified products of the anticipated sizes (Fig. 4). Negative control treatments that omitted RT did not yield any amplified products. This result (Fig. 4) indicated that expression of the *mpf* region was not linked to naphthalene utilization and that *orf25*, *mpfA*, and *mpfB* are cotranscribed.

Expression of P*mpfA-lacZ* reporter construct in *P. putida* Cg1. For quantification of expression of the *mpfA* gene, we constructed an *mpfA* promoter transcriptional fusion to *lacZ* (P*mpfA-lacZ*). This construct, pEM1 (Fig. 1B), contained the fragment extending from a portion of partial *parA* through *orf25* to just downstream of the ATG start site of the *mpfA* gene (+28 nucleotides). We believe that this construct contained the promoter region of the *mpf* operon based on computer analysis and operon structure. We measured β-galactosidase activity in *P. putida* Cg1pEM1 (22°C) in cells harvested from both liquid (Fig. 5A) and solid (Fig. 5B) preparations of three media. Promoter activity of P*mpfA-lacZ* was increased when cells were grown in rich medium (LB). The level of β-galactosidase activity of cells grown in LB liquid medium was roughly two times higher than that of cells grown in MSB-N and MSB-G during the exponential and stationary phases (Fig. 5A). The β-galactosidase activity measured by using cells grown in MSB-G liquid medium was similar to the β-galactosidase activity of cells in MSB-N liquid medium (Fig. 5A). Thus, this

TABLE 4. Influence of mating time, medium, and solid surface on conjugal transfer frequencies of pCg1 between
P. putida Cg1 (host) and *P. putida* KT2440-Tc (recipient)

Donor and recipient density in mating mix		Mating duration (h)	Donor medium	Conjugation frequency[a]	
Donor	Recipient			Solid	Liquid
1.5×10^8 (1.30)[b]	2.7×10^8 (1.50)	1	LB	1.5×10^{-6}	BD[c]
3.6×10^8 (0.98)	3.8×10^8 (1.30)	3	LB	2.7×10^{-7}	1.3×10^{-9}
3.2×10^7 (0.61)	7.4×10^7 (0.90)	3	LB	4.4×10^{-5}	4.6×10^{-7}
2.3×10^8 (1.10)	3.8×10^8 (1.30)	6	LB	1.3×10^{-4}	3.9×10^{-6}
1.9×10^8 (0.90)	1.9×10^9 (0.70)	24	LB	1.9×10^{-4}	1.1×10^{-5}
2.0×10^7 (1.10)	2.7×10^8 (1.50)	1	MSB-G	BD	BD
1.8×10^8 (0.99)	5.8×10^8 (1.05)	3	MSB-G	BD	BD
9.8×10^8 (0.95)	6.0×10^6 (0.70)	6	MSB-G	3.7×10^{-7}	BD
2.4×10^8 (1.05)	6.0×10^6 (0.70)	6	MSB-N	3.9×10^{-6}	BD

[a] Frequencies are expressed as the number of transconjugants per donor cell (transconjugant/donor ratio).
[b] Measurement of optical density at 600 nm is given in parentheses.
[c] BD, below detection limit.

expression was not linked to naphthalene catabolic function of the pCg1 plasmid. The expression of *mpf* genes in the agar media was induced to levels similar to those in liquid media (Fig. 5B). The RT-PCR assay (that produced the data in Fig. 4) and the P*mpfA*-*lacZ* reporter assays showed that the *mpf* genes of pCg1 were constitutively expressed even in the absence of naphthalene and were highly expressed both in liquid and on semisolid media.

Because we recognize that the level of transcription is not the same as the level of expression, we attempted to monitor the promoter activity of the *mpf* region by using a translational fusion construct in *P. putida* Cg1pAH7. However, that construct failed to express β-galactosidase activity in any condition. The expression of β-galactosidase did not occur on semisolid or liquid media in the presence of recipient cells (22). An explanation for failure to detect β-galactosidase activity in *P. putida* Cg1pAH7 is not straightforward. It is possible that the constructed translational fusion suffered from unstable transcripts or impaired initiation of translation (44).

Conjugation frequency of the pCg1 plasmid of *P. putida* Cg1. Because the reporter assay above indicated that the expression of *mpf* region is constitutive and is higher in rich (LB) media than in minimal (MSB-G or MSB-N) media, we predicted that the conjugation frequency would be higher in LB-grown cells if the expression of the *mpf* region is the rate-limiting factor that governs conjugation frequency. We completed conjugation assays examining the influence of medium and other key variables (mating duration and the presence of a solid surface) on conjugation frequency (Table 4). Comparison of columns 5 and 6 shows that conjugal transfer of pCg1 was consistently higher (~100-fold) on solid media. The pattern within the first five lines of Table 4 (in which donor medium was held constant but mating time increased) indicates that conjugal transfer was roughly proportional to the duration of cell-cell contact. However, extensive incubation of donor and recipient cells beyond 6 (to 24) h did not increase the conjugation frequency. In addition, the trend in assays in which donor cells were prepared in minimal salt media (last four entries [Table 4]) indicates that a longer period was required for successful mating than for donor cells grown on LB broth. Data in Table 4 indicate that several factors, in addition to expression of the *mpf* region, influence conjugation frequency.

DISCUSSION

Among the motivations for this study was a desire to understand the mechanism by which naturally occurring microbial populations at a coal tar-contaminated field site (2, 20, 23, 33) adapt to introduced organic substrates. Previously it has been shown that Cg1-like plasmids, involved in horizontal transfer of naphthalene catabolic genes between bacteria at the study site, are homologous to the archetypal naphthalene catabolic plasmid pDTG1 from *P. putida* NCIB 9816-4 (despite some variability in overall plasmid sizes [e.g., pDTG1, 81 kb; and pCg1, 86 kb {52}]). This led us to investigate further how pCg1 conjugation occurs and is regulated. The entire pDTG1 plasmid has been sequenced (GenBank accession no. AF491307). In this study we have shown by using RFLP and sequencing analysis that the conjugal transfer regions in pCg1 and pDTG1 are closely related in sequence and operon structure to the putative *mpf* genes of broad-host-range plasmids pWW0 and pM3. The incompatibility group of pDTG1 and pCg1 has not been determined. Nonetheless, we feel it is prudent to deem these plasmids "IncP-9-like" because pWW0 and pM3 and the closely related plasmid from *P. putida* NCIB 9816-3 all belong to the IncP-9 group (15, 52, 56). The *mpf* genes of IncP-9 plasmids along with *vir* genes of pXAC64 share distinct sequence identity and operon structure; thus, evolutionary divergence from other plasmids is suggested.

We recognize that complex networks of transcriptional and translational regulatory systems often control plasmid conjugation (57–60). For example, in the *trb* operon (the genes for mating pair formation start with *trbB*) of plasmid RK2, it is thought that translational control on *trbB* transcripts is exerted by the *trbA* promoter (responsible for steady-state transcription of the *trb* operon as well as *trbA*). Furthermore, repression of the *trbB* promoter is exerted by both KorB and TrbA proteins (25, 36, 58, 60). Thus, it is likely that conjugal transfer genes in enteric IncPα plasmids (e.g., RK2 and RP4) are controlled by a network of regulatory elements (regulators and DNA sequences) that reduce the metabolic burden on the bacterial host. It is very probable that a regulatory system unlike that of the IncPα system operates in pDTG1/pCg1. Our data suggest that the *mpf* region of pCg1 is not controlled at the level of transcription because transcription was constitutive

in the promoter-probing construct in *P. putida* Cg1pEM1 (Fig. 5).

It is notable that, although transcription of the *mpf* region of pCg1 occurs constitutively, conjugation frequency is low (Table 4). To place this frequency in perspective, the TOL plasmid of *P. putida* mt-2 (also thought to be naturally derepressed for conjugation) transfers at 2.5×10^{-1} transconjugants per donor h^{-1} (4). Consistent with data reported here on the key role of the *mpfA* pilin subunit in pCg1 conjugation, the thick, flexible conjugative pili encoded by the TOL plasmid (IncP-9) in *P. putida* mt-2 were found to be important for plasmid transfer (4). Interestingly, conjugation frequency of the TOL plasmid depends on the identity of the host cell. The number of transconjugants per donor cell dropped 1,000-fold when the host was altered from *P. putida* mt-2 to *P. putida* S388 (4). We have similar observations for conjugation of pDTG1/pCg1-like plasmids: the conjugation frequency of pCg1 in *P. putida* Cg1 after overnight mating on the filter is 10^{-3} to 10^{-4} (transconjugants per donor), yet conjugation is often below the detection limit (10^{-8}) in the case of pDTG1 in *P. putida* NCIB 9816-4 (20, 43, 52). We conclude then (presuming that any sequence differences between pDTG1 and pCg1 are immaterial) that conjugation of pDTG1/pCg1-like plasmids is highly dependent upon the host in which the plasmids reside.

Systematic observation of relationships between solid surfaces and plasmid transfer has led to the proposal of three categories of conjugation (3, 4, 15): universal mating type (equal conjugation frequency on solid and in liquid medium; e.g., IncK, IncFII, and IncH1), surface-obligatory mating type (conjugation frequency is >2,000 times higher on a solid surface; e.g., IncP-1, IncI, IncW, and IncN), and the solid-preferred type (conjugation frequency is 45 to 450 times higher on a solid surface; e.g., IncP-7 and IncP-9). Data presented here clearly place pCg1 in the latter class (Table 4). Surface-preferred conjugation of pCg1 may be the result of IncP-9-type pili and related, stable mating pair formation on a semisolid surface, where high-density cell-to-cell contact is fostered. Solid surface-preferred conjugation is also observed in the Ti plasmids of *Agrobacterium* (13, 45). It has been thought that this surface, preference might result from the thick, flexible morphology of pili (12) or from biofilm formation that might increase conjugation frequency with quorum-sensing molecules (13, 45). Based on the observations above and discussion by others (4, 6, 7, 19, 37–39, 45, 46, 50), conjugation frequency is confirmed to be governed by many factors, including cell density, surfaces, growth rate, incubation time, media, pH, and moisture. Clearly the *mpfA* and/or cotranscribed genes in the conjugal transfer region of pCg1 are necessary but not sufficient for successful conjugation.

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