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Integration of Heterologous DNA into the Genome of *Paracoccus denitrificans* Is Mediated by a Family of IS*1248*-Related Elements and a Second Type of Integrative Recombination Event

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All members of the IS*1248* family residing in the genome of *Paracoccus denitrificans* have been isolated by using a set of insertion sequence entrapment vectors. The family consists of five closely related members that integrate the entrapment vectors at distinct sites. One of these, IS*1248b*, was sequenced and, except for a single base change, shown to be identical to the previously isolated IS*1248a*. Southern analysis of genomic DNA with labeled IS*1248* revealed different hybridization patterns for different isolates of *P. denitrificans* and *Thiosphaera pantotropha*. No hybridization was observed with DNA from *Thiobacillus versutus* and more distantly related species. From a comparison of the fingerprints it was shown that one of the members of the IS*1248* family found in *P. denitrificans* DSM413 is absent in strain NCIB8944, although they are catalogued in international strain catalogues as identical strains. Furthermore, strains Pd1222 and Pd1235, both derivatives of *P. denitrificans* DSM413, were shown to have different patterns of IS*1248* hybridizing restriction fragments. In 14 of 18 strains, the entrapment vectors used in this study were incorporated into the genome via IS*1248*-mediated cointegrate formation. In the other four strains, the entrapment vectors were shown to be integrated through a different mechanism not involving IS*1248*.

Bacterial insertion sequence (IS) elements are small mobile elements that are widespread amongst many different bacteria (11). Usually, they reside in multiple copies in the genome or, more frequently, on natural plasmids. The plasmid-associated ISs are often part of compound transposons, in which a nontransposable central segment is flanked by two IS elements. Typical characteristics of IS elements are the presence of inverted repeats at their ends and their ability to encode a transposase that recognizes these ends and catalyzes the insertion into new sites. Integration often involves replicative transposition. The insertion is suggested to be preceded by a staggered cleavage of the target site, resulting in the generation of directly repeated duplications of this site at the point of insertion; the length that is duplicated is characteristic to each element. As a result of transposition, genes or operons located in the target DNA might be disrupted, resulting in the loss of the encoded functions. Other mutations induced by these elements involve the switching on of silent genes under control of the IS promoters and deletions or inversions of chromosomal DNA.

In a previous study, it was shown that incorporation of an entrapment vector pLOT1 into the genome of *Paracoccus denitrificans* was the result of IS*1248*-mediated cointegrate formation (27). Analysis of the resulting insertion region demonstrated the presence of two identical copies of IS*1248* flanking the trapped foreign DNA. Southern blotting showed that there are at least four copies of IS*1248* in the genome. One of the members of the family, designated IS*1248a*, has been analyzed in detail (27); it is 830 bp in size, has 13-bp inverted repeats, and contains five overlapping open reading frames (ORFs). IS*1248* from *P. denitrificans* closely resembles IS*869*, which resides on the Ti plasmid of *Agrobacterium tumefaciens* (18). Other members belonging to this group of IS elements include IS*427*, also found in *A. tumefaciens* (7); IS*402* from *Pseudomonas cepacia* (10); IS*myco*, found in *Mycobacterium tuberculosis* (16); IS*1106* from *Neisseria meningitidis* (13); Tn*4811* from *Streptomyces lividans* (4); IS*Rm4* of *Rhizobium meliloti* (23); another previously identified IS found in *R. meliloti* (19); and IS*1031* of *Acetobacter xylinum* (6). IS*myco*, the IS element from *R. meliloti*, and IS*1031* have only single ORFs. These ORFs encode highly basic proteins that show homology with a number of DNA-binding proteins. For this reason, it is believed that these particular ORFs encode the transposase functions of these elements. IS*1248*, IS*869*, IS*427*, and IS*402* have a more complex pattern of ORFs.

The aim of the work described in this paper is to confirm the IS entrapment procedure for the characterization of all the IS*1248* elements and the loci where they reside. Here, it will be shown that the IS*1248* family consists of five members, which were all able to mediate integration of the entrapment vectors. In addition, it will be shown that *P. denitrificans* has a second mechanism for integration of foreign DNA that does not involve IS*1248*. IS*1248* is present in a number of different *P. denitrificans* strains and in *Thiosphaera pantotropha* but not in other related species tested thus far. The use of an IS*1248*-detecting fingerprint method for easy identification and classification of *P. denitrificans* isolates is discussed.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used are listed in Table 1. *Escherichia coli* and *P. denitrificans* strains were grown

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference[b]
E. coli		
S17-1	Sm^r *pro* r$^-$ m$^+$; RP4-2 integrated (Tc::Mu)(Km::Tn*7*)	22
TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q} *lacZ* ΔM15)	20
P. denitrificans		
DSM413		DSM
DSM415		DSM
DSM65	Type strain	DSM
NCIB8944		W. Hamilton, Aberdeen, Scotland
Pd1222	DSM413; Rif^r Spec^r; enhanced conjugation frequencies	8
Pd1235	DSM413; Rif^r Spec^r; enhanced conjugation frequencies, rough	9
PdX11	Pd1222; pLOT1 integrated in IS*1248e* locus	This study
PdX12	Pd1222; pLOT1 integrated in IS*Pd2a* locus	This study
PdX13	Pd1222; pLOT1 integrated in IS*1248a* locus	27
PdX14	Pd1222; pLOT1 integrated in IS*Pd2a* locus	This study
PdX15	Pd1222; pLOT1 integrated in IS*Pd2a* locus	This study
PdX16	Pd1222; pLOT1 integrated in IS*1248a* locus	This study
PdX17	Pd1222; pLOT1 integrated in IS*Pd2a* locus	This study
PdX19	Pd1222; pLOT1 integrated in IS*1248d* locus	This study
PdX112	Pd1222; pLOT1 integrated in IS*1248a* locus	This study
PdX21	Pd1222; pLOT2 integrated in IS*1248a* locus	This study
PdX22	Pd1222; pLOT2 integrated in IS*1248b* locus	This study
PdX23	Pd1222; pLOT2 integrated in IS*1248a* locus	This study
PdX24	Pd1222; pLOT2 integrated in IS*1248b* locus	This study
PdX25	Pd1222; pLOT2 integrated in IS*1248b* locus	This study
PdX26	Pd1222; pLOT2 integrated in IS*1248b* locus	This study
PdX221	Pd1222; pLOT22 integrated in IS*1248a* locus	This study
PdX222	Pd1222; pLOT22 integrated in IS*1248a* locus	This study
PdX223	Pd1222; pLOT22 integrated in IS*1248c* locus	This study
Plasmids		
pRVS3	*oriT* (RK2); Km^r (Tn*903*); pBSII KS derivative	27
pLOT1	*cyo* locus *E. coli*; pRVS3 derivative	27
pLOT2	*cycM* locus *B. japonicum*; pRVS3 derivative	This study
pLOT22	*cycM B. japonicum*; pRVS3 derivative	This study

[a] Sm, streptomycin; Km, kanamycin; Rif, rifampin; Tc, tetracycline; Spec, spectinomycin.
[b] DSM, Deutsche Sammlung von Mikroorganismen Zellkulturen GmbH, Braunschweig, Germany.

aerobically at 34°C in batch cultures with brain heart infusion broth. When necessary, antibiotics were added to final concentrations as follows: 40 μg of rifampin per ml, 25 μg of kanamycin per ml, 25 μg of streptomycin per ml, and 50 μg of ampicillin per ml.

DNA manipulations. General cloning techniques were carried out essentially as described by Maniatis et al. (15). Plasmid DNA was isolated from *E. coli* by the cleared-lysate method (26) and purified by using Qiagen. For rapid screening, plasmid DNA was isolated by the alkaline lysis method (15). Chromosomal DNA of *P. denitrificans* was isolated as described earlier (28). DNA restriction fragments were purified from agarose gels by using GeneClean (Bio 101, Inc., San Diego, Calif.). Digested chromosomal DNA (5 μg per lane) was loaded on 1% agarose gels; after electrophoresis it was denatured and transferred to positively charged nylon membranes (Boehringer Mannheim) according to the method of Southern (24). Southern analysis of chromosomal restriction fragments was done by random-primed DNA labeling of cloned sequences with digoxigenin and subsequent detection of hybrids by an enzyme immunoassay according to the protocol of the manufacturer (Boehringer GmbH, Mannheim, Germany). Conjugations were carried out by streaking cells of donor and recipient strains on brain heart infusion plates; after 1 day of incubation at 30°C, the cells were collected and plated on selective plates. Sequence reactions were performed on single-stranded M13mp18 and M13mp19 clones, using the dye primer and dye terminator cycle kits from ABI, and loaded on an ABI 373A fluorescent sequencer (Applied Biosystems and Perkin-Elmer).

Analyses. The Macintosh computer programs used were DNA Strider 1.0 and GeneWorks 2.2.1. The electronic mail servers of NCBI running the BLAST program (1), the FASTA and BLITZ server at Heidelberg, and the BLOCKS server were used for comparison of sequences with the international databases.

Nucleotide sequence accession number. The nucleotide sequence of IS*1248b* and the region located downstream has been assigned GenBank accession number U08856.

RESULTS

Isolation of the members of the IS*1248* family. An entrapment vector is a vector which cannot replicate in the second host and therefore must be integrated into a replicating genome in order to be maintained. In the absence of homology, such integration often occurs via the action of ISs or other transposable elements. In order to isolate and analyze the different members of the IS*1248* family, three entrapment vectors were constructed from pRVS3. One of these, pLOT1 (27), is equipped with a 4.5-kb *Kpn*I fragment covering a large part of the *E. coli cyo* locus (5). The second vector, pLOT2, contains a 3-kb chromosomal fragment from *Bradyrhizobium japonicum* that contains the *cycM* gene and flanking regions (3). The third vector, pLOT22, is a smaller derivative of pLOT2 and contains only the *cycM* gene. Introduction of these various sequences, differing largely in G+C content (*E. coli*, 45%; *B. japonicum*, 68%) was done to provide diverse target sequences for transposition of IS*1248* members. The entrapment vectors were introduced into the mobilizing strain *E. coli* S17-1 and transferred to *P. denitrificans* Pd1222. Kanamycin-resistant strains were isolated at frequencies of between 10^{-6} and 10^{-7}, irrespective of the entrapment vector. Nine (PdX11 to -19), six (PdX21 to -26), and three (PdX221 to -223) exconjugant

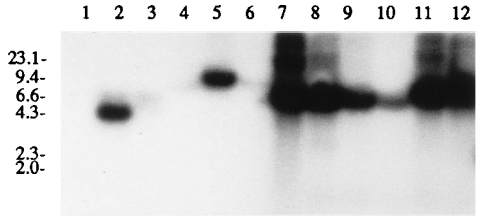

FIG. 1. Southern analysis of *Eco*RI-digested plasmids recovered from the genomes of different integrant strains. Lanes 1 to 3: pXSP12, -13, and -14, respectively. Lanes 4 to 6: pXSS12, -13, and -14, respectively. Lanes 7 to 9: pXSP21, -22, and -23, respectively. Lanes 10 to 12: pXSS21, -22, and -23, respectively. The numbers of the plasmids refer to the numbers of the PdX strains from which they were isolated. The 0.3-kb *Hin*dIII-*Sph*I fragment of IS*1248a* was used as a probe. In each lane, 500 ng of plasmid DNA was loaded, except for lane 10, in which only 100 ng was loaded. The smear in lanes 7 to 12 is the result of incomplete digestion. The positions of the markers are indicated by their sizes (in kilobases).

strains were isolated and characterized after introduction of pLOT1, pLOT2, and pLOT22, respectively.

Characterization of the integration regions. Chromosomal DNAs of the 18 strains were completely digested with the appropriate enzymes (*Eco*RI, *Pst*I, *Sph*I, and *Sst*I) to recover different portions of the entrapment vectors and flanking DNA. After ligation to circularize these fragments, the DNA was used to transform competent *E. coli* TG1. The replicon and drug resistance of the entrapment vector were used to provide for replication and selection in the *E. coli* host. In this way, several different plasmids (pXEI [*Eco*RI], pXPS [*Pst*I], pXSP [*Sph*I], and pXSS [*Sst*I], plasmids with numbers the same as those of the insertion strain from which they were isolated) were obtained from each original strain. Southern analysis revealed that the plasmids recovered from 14 of the 18 strains contained a sequence that hybridized with IS*1248a* DNA. Surprisingly, no hybridization was observed with the plasmids from the remaining four strains. Representative results are presented in Fig. 1. Restriction sites on the plasmids were mapped to determine the positions of the two copies of IS*1248* and flanking DNA from the insertion site. From these data and from the Southern analyses of the wild-type and integrant strains (see below), physical maps of the regions that contain the IS*1248* elements were deduced (Fig. 2). For seven strains (PdX13, -16, -112, -21, -23, -221, and -222), the maps of the DNA flanking the integration region were similar, showing that plasmid integration involved the previously described IS*1248a* (27). In addition, four new and different maps were deduced, demonstrating the presence of four other copies of IS*1248* (IS*1248b*, *-c*, *-d*, and *-e*) in the *P. denitrificans* genome. IS*1248b* was isolated from four strains (PdX22, -24, -25, and -26), each of which arose from independent insertion events. In all four clones, the insertion site in the plasmid DNA was mapped at a similar position. IS*1248c*, *-d*, and *-e* were isolated from single strains PdX223, -19, and -11, respectively. In all cases, an *Sph*I site was mapped within each copy of IS*1248* at the same relative position. Southern blotting of genomic DNA from the wild-type and integrant strains revealed that the number and sizes of IS-containing restriction fragments observed before and after integration of the vectors were in agreement with the corresponding physical maps. Depending on the number and nature of the restriction enzyme sites on the entrapment vector, insertion of a plasmid led to the loss of one band and the generation of one or more new bands visible with at least one restriction enzyme digestion. Typical examples of these blots are presented in Fig. 3. Table 2 presents the frequency of transposition and insertion site locations of the IS*1248* elements. It should be noted that the 5-kb fragment in the *Eco*RI digest of the wild-type strain is a doublet.

Sequence analysis of IS*1248b*. In a previous study, IS*1248a* was sequenced and characterized (27). In this study, IS*1248b* was sequenced and compared with IS*1248a*. The starting ma-

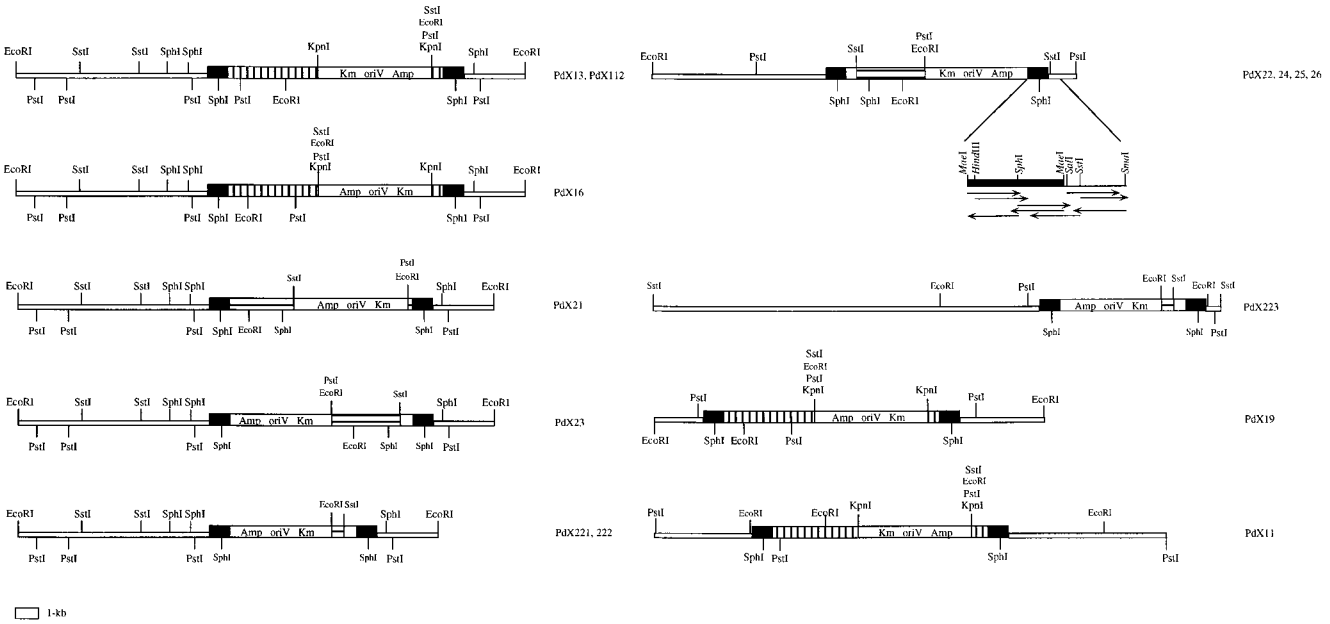

FIG. 2. Physical maps of *P. denitrificans* genomic loci that represent insertion of pRVS3 derivatives pLOT1 (strains PdX11, -13, -16, and -19), pLOT2 (strains PdX21 to -26), and pLOT22 (PdX221 to -223). Black bars represent copies of IS*1248*. The *E. coli cyo* locus in pLOT1 is indicated by a bar with vertical lines; the *cycM* locus (pLOT2) and the derivative of it (pLOT22) are indicated by bars with horizontal lines. Plasmid pRVS3 itself is indicated by the white bar in between these elements. The locations of the replicon (*oriV*) and selectable markers (Amp, ampicillin resistance; Km, kanamycin resistance) are shown. The sequencing strategy for IS*1248b* is shown under the map of PdX22.

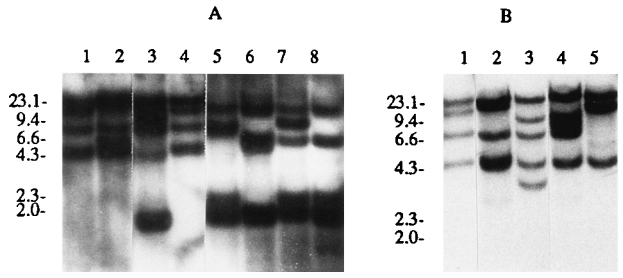

FIG. 3. (A) Southern analysis of *Eco*RI-digested (lanes 1 to 4) and *Pst*I-digested (lanes 5 to 8) chromosomal DNA of *P. denitrificans* PdX22 (lanes 1 and 5), PdX23 (lanes 2 and 6), PdX223 (lanes 3 and 7), and their parent Pd1222 (lanes 4 and 8). Hybridization was performed with the probe described in the legend to Fig. 1. The positions of the markers are indicated by their sizes (in kilobases). (B) Southern analysis of *Eco*RI-digested chromosomal DNA from strains Pd1222 (lane 1), PdX112 (lane 2), PdX21 (lane 3), PdX16 (lane 4), and PdX19 (lane 5). Hybridization was performed with the probe described in the legend to Fig. 1. The positions of the markers are indicated by their sizes (in kilobases). IS*1248b*-mediated integration of pLOT2 into the genome of strain PdX22 results in the appearance of two new IS-containing *Eco*RI fragments of 15 and 22 kb, respectively (panel A, lane 1). The original 5-kb *Pst*I fragment with IS*1248b* (panel A, lane 8) is replaced with two new fragments of 7 and 7.5 kb, respectively (panel A, lane 5). IS*1248a*-mediated integration of both pLOT1 and pLOT2 in strains PdX23 (panel A, lanes 2 and 6), PdX112 (panel B, lane 2), PdX21 (panel B, lane 3), and PdX16 (panel B, lane 4) is demonstrated. In all these cases, the original 12-kb *Eco*RI fragment containing IS*1248a* has disappeared and two new IS-containing fragments were observed. In addition, the original 2.3-kb *Pst*I fragment containing IS*1248a* (panel A, lane 8) appears as two new fragments of 5.2 and 6 kb after integration of pLOT2 (panel A, lane 6). IS*1248c*-mediated insertion of pLOT22 in PdX223 (panel A, lanes 3 and 7) results in the loss of one of the original 5-kb *Eco*RI fragments and in the appearance of fragments of 10 and 1.8 kb. Note that the second 5-kb *Eco*RI fragment of the doublet, bearing IS*1248e*, is not affected by the integration process. Furthermore, the original 1.9-kb *Pst*I fragment in PdX223 is extended to 8.3 kb upon integration of the 6.4-kb hybrid (panel A, lane 7). Since pLOT22 has no internal *Pst*I sites, the two copies of IS*1248c* are not separated in this integrant strain. IS*1248d*-mediated insertion of pLOT1 results in the loss of the original 7-kb *Eco*RI fragment (panel B, lane 5).

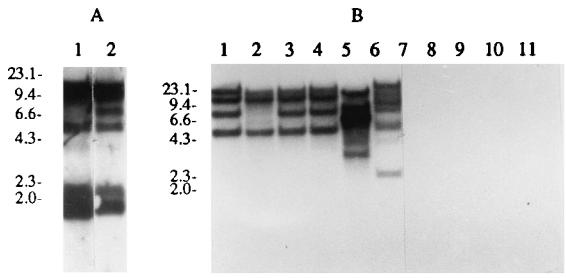

FIG. 4. (A) Southern analysis of *Pst*I-digested chromosomal DNA from strains Pd1222 (lane 1) and Pd1235 (lane 2). Hybridization was performed with the probe described in the legend to Fig. 1. The positions of the markers are indicated by their sizes (in kilobases). (B) Southern analysis of *Eco*RI-digested chromosomal DNA from *P. denitrificans* Pd1222 (lane 1), NCIB8944 (lane 2), DSM413 (lane 3), DSM415 (lane 4), DSM65^T (lane 5), *T. pantotropha* (lane 6), *T. versutus* (lane 7), *A. caulinodans* (lane 8), *A. eutrophus* (lane 9), *B. japonicum* (lane 10), and *M. organophilum* XX (lane 11). The results were obtained from a single electrophoresis and blotting experiment, after which the data mentioned here were combined. Hybridization was performed with the probe described in the legend to Fig. 1. The positions of the markers are indicated by their sizes (in kilobases).

terial was strain PdX22, which has pLOT2 integrated. Genomic DNA from this strain was isolated and restricted with *Pst*I. After ligation to circularize the fragments, they were transferred to competent *E. coli* TG1 cells. The plasmid from the resulting transformants was designated pXPS22. Restriction fragments of this plasmid were cloned into M13mp18 and M13mp19 and sequenced (see Materials and Methods). The strategy is presented under the map of PdX22 shown in Fig. 2. Analysis of the IS*1248b* sequence revealed that it was identical to the IS*1248a* sequence, except for a single base change at position 466 of the IS element (results not shown). Where IS*1248a* has an adenine residue at this position, IS*1248b* has a thymine residue. As a consequence, the coding strands of ORFs 1 and 2 of IS*1248b* predict a leucine residue and a tryptophan residue at this position, respectively, instead of a glutamine residue and an arginine residue as in IS*1248a*. In addition, the sequences of the flanking direct repeats were different. IS*1248b* is flanked by 5′-CTAG-3′ direct repeats at both ends of the sequence. This sequence is also located upstream from IS*1248a*, but the downstream one was shown to be 5′-CTAA-3′.

Identification of different isolates of *P. denitrificans*. In order to study the occurrence of IS*1248* in different isolates of *P. denitrificans* and related species, chromosomal restriction fragments from these strains were analyzed with IS*1248* as a probe. The strains used in this study were *P. denitrificans* Pd1222, Pd1235, NCIB8944, DSM413, DSM415, and DSM65^T and the more- and less-related species *T. pantotropha*, *Thiobacillus versutus*, *Azorhizobium caulinodans*, *Alcaligenes eutrophus*, *B. japonicum*, and *Methanobacterium organophilum* XX. Pd1235 and Pd1222 are both restriction-negative derivatives of DSM413 (8, 9). Pd1235 colonies are rough, while colonies of Pd1222 and its parent strain DSM413 are smooth. A comparison of IS-containing *Pst*I fragments from both strains revealed that Pd1235 contains an extra copy of IS*1248*, which is located on a 7-kb fragment (Fig. 4A). Patterns of IS-containing *Eco*RI fragments of the other strains are shown in Fig. 4B. Hybrid-

TABLE 2. Characteristics of IS*1248*- and INTPd-mediated insertion of DNA into the genome of *P. denitrificans*

Element	Size (kb) of IS/INT-containing fragments		Integration characteristics		
	*Pst*I	*Eco*RI	No.[a]	PdX strain(s)	Target region
IS*1248a*	2.3	12.0	7	-13, -112	*E. coli cyoA*
				-16	*E. coli cyoE*
				-21	*B. japonicum cycM*
				-23, -221, -222	*oriT*
IS*1248b*	5.5	20.0	4	-22, -24, -25, -26	*oriT*
IS*1248c*	1.9	5.0	1	-223	*oriT*
IS*1248d*	2.0	7.0	1	-19	*E. coli cyoE*
IS*1248e*	12.0	5.0	1	-11	*E. coli cyoA*
INT*Pd1a*	5.5	>23.0	4	-12, -14, -15, -17	*E. coli cyoB*
INT*Pd1b*	>23	7.0			

[a] The number of strains isolated in which the element concerned mediated integration.

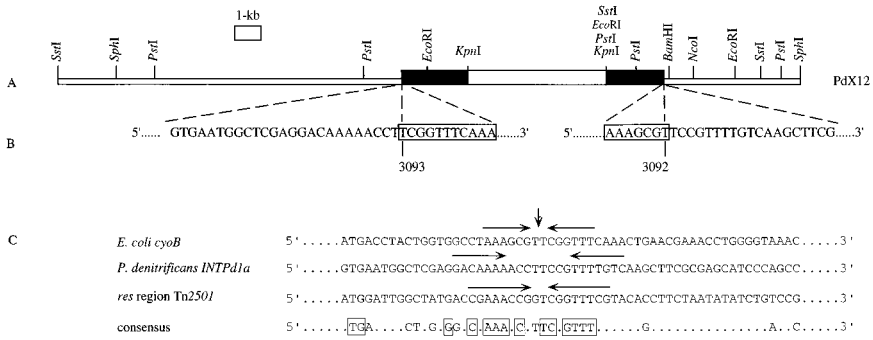

FIG. 5. (A) Physical map of the integration region of pLOT1 integrant strain PdX12. Vector pRVS3 (white bar) is flanked by the two portions of the *E. coli cyo* gene cluster (black bars). (B) Nucleotide sequences near the junctions of the integration site in PdX12. The integration site in the *E. coli cyoB* sequence (boxed) is located in between positions 3092 and 3093 of the published sequence (5). (C) Alignment of the *P. denitrificans* insertion region, the target region in the *E. coli cyoB* gene, and the *res* region of Tn*2501*. The vertical arrow shows the position at which recombination occurs, and the horizontal arrows represent inverted repeats. The consensus sequence shows identical residues of the *E. coli cyoB* and INT*Pd1a* sequences. The boxed residues are identical for the three sequences.

ization was observed with the *P. denitrificans* isolates and *T. pantotropha* but not with the other species tested. The IS pattern of Pd1222 was identical to that of DSM413 and DSM415. The IS fingerprint of strain DSM65, which is the *P. denitrificans* type strain originally isolated by Beijerinck and Minkman (2), was quite different from those of the other isolates. The IS fingerprint of NCIB8944 lacks the IS*1248*-containing 7-kb *Eco*RI fragment. However, NCIB8944 and DSM413 are catalogued as identical strains and should therefore give identical IS fingerprints, too. Surprisingly, IS*1248*-like elements were also observed in the genome of *T. pantotropha*. The fingerprint shows the presence of at least five IS-containing fragments, two of which are comparable in size to fragments of the DSM413 fingerprint. No hybridization was observed for the other organisms tested.

A second mechanism involved in integration of foreign DNA. Vectors containing *Sst*I (pXSS12 and -14) or *Sph*I (pXSP12 and -14) fragments were recovered from the genomes of integrant strains PdX12 and -14, respectively. Southern analysis revealed that these plasmids did not contain IS*1248* (Fig. 1). Restriction enzyme analysis revealed that pXSS12 and pXSS14 were identical, as were pXSP12 and pXSP14 (data not shown). A physical map of the region in which pLOT1 had been integrated is presented in Fig. 5. From the data, it was deduced that pLOT1 was integrated at a site within the *E. coli cyoB* gene. A 1-kb *Bam*HI-*Nco*I fragment of pXSP12, located downstream of the junction, was isolated and used as a probe for Southern analysis of the recovered plasmids (results not shown). As expected, hybridization was observed with pXSP12 and -14. No hybridization was observed with pXSS12 and -14, which contain only the genomic DNA located upstream of the junction. When the same probe was used, Southern analysis of *Eco*RI-restricted genomic DNA revealed the presence of two cross-reacting fragments in the wild-type strain with apparent sizes of 7 and >23 kb (Fig. 6). In the lanes of the integrant strains, the high-molecular-weight species has disappeared and, instead, a 4.5-kb fragment was visualized, just as expected from the constructed map. *Sst*I-digested DNA revealed one strong hybridizing band in the lane of the wild-type strain. In agreement with the restriction map of PdX12, a fragment of 5.5 kb appeared in the lane of this integrant strain. In this case, the large band does not disappear completely, since it is a doublet encompassing both the hybridizing fragments. The DNA sequence at the integration site revealed that the target zone on the vector is located in the *E. coli cyoB* gene (Fig. 5B). Integration occurred in between the nucleotides corresponding to positions 3092 and 3093 of the published sequence (5). Sequence comparison revealed a high similarity between the *E. coli* target region and the *P. denitrificans* region of insertion (Fig. 5C). In both regions, a central dinucleotide 5′-TT-3′ is flanked by the sequences 5′-GGNCNAAANCN-3′ and 5′-CNGTTT-3′, respectively, which are parts of inverted repeats. A similar pattern of sequences is found in the *res* region of Tn*2501*, a cryptic transposon of the Tn*3* family (11, 12). The results confirm that integration of DNA into the genome of strains PdX12 and -14 was the result of a second type of integrative recombination differing from the one mediated by IS*1248*.

DISCUSSION

The results presented in this paper demonstrate that *P. denitrificans* has the potential to integrate heterologous DNA into its genome by two different mechanisms, one of which involves a family of IS*1248*-related elements that mediates cointegration of host and target DNA and the second of which involves an integrative recombination event that mediates integration of the target DNA in a different manner.

IS-promoted cointegrate formation may occur by several mechanisms. In the case of the IS*1248*-vector integrated structure, cointegrate formation most probably is the result of transpositional cointegration, which results in the integration of the suicide vector in the host genome flanked by identical copies of the transposed IS responsible. Alternatively, the cointegrate structure could be due to an IS element transposed to the vector, followed by reciprocal recombination between the newly transposed copy and another homologous IS. We like to

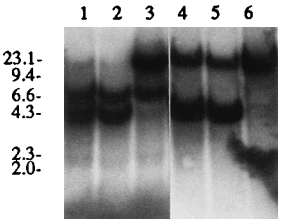

FIG. 6. Southern analysis of *Eco*RI-digested (lanes 1 to 3) and *Sst*I-digested (lanes 4 to 6) chromosomal DNA from strains PdX12 (lanes 1 and 4), PdX14 (lanes 2 and 5), and Pd1222 (lanes 3 and 6). A 1-kb *Bam*HI-*Nco*I fragment located downstream of the integration site in PdX12 was used as a probe. The positions of the markers are indicated by their sizes (in kilobases).

favor the first explanation, since in a previous study it was shown that the DNA sequences of both copies of IS*1248a* that flanked the integrated entrapment vector were indeed identical.

The IS*1248* family of *P. denitrificans* consists of five members, each of which was shown to mediate insertion of foreign DNA, although with different frequencies. IS*1248*-mediated cointegration has already been described for IS*1248a* (27), and the results described here confirm that the other members act in the same way. The different members appear to be closely related, as judged by physical mapping of the integration regions, by Southern blotting, and by the fact that all contain an *Sph*I restriction site at a similar position. Sequence analyses of IS*1248a* and *-b* even show that at least these two members are nearly identical, differing from each other in only one base of 830.

The members of the IS*1248* family appear to insert the foreign DNA at preferred regions. In this study, only four distinct regions on the different entrapment vectors were attacked, two in the *E. coli cyo* locus, one in the *oriT* region, and one downstream from the *B. japonicum cycM* gene. Moreover, in the entrapment vector containing the *E. coli* DNA, transposition to the *oriT* region did not occur. These observations strongly suggest that transposition of IS*1248* may show regional specificity. A pronounced feature of *E. coli* DNA is the high A+T content, which may play a role in a local attraction of these elements. This mechanism has been suggested for a number of different IS elements (11).

Southern analysis of genomic DNA from different sources revealed that IS*1248* is present in different *P. denitrificans* isolates and in *T. pantotropha* but not in *T. versutus* and other more- and less-related species. A close relatedness between *P. denitrificans* and *T. pantotropha* has recently been shown from studies of our group and other groups, indicating that a number of respiratory genes as well as the 16S RNA sequences of the two organisms are highly homologous (14, 21, 25). The studies here corroborate that *P. denitrificans* and *T. pantotropha* are indeed phylogenetically closely related. *T. versutus* is also phylogenetically closely related to *P. denitrificans* and *T. pantotropha*; all three belong to the α-3 cluster of purple bacteria (29). The fact that *T. versutus* does not contain IS*1248* suggests that the evolution of the IS*1248* family within the *Paracoccus* genus occurred after the branching of *Paracoccus* spp. and *T. versutus* from a common ancestor. If so, this assumption would support the idea of horizontal DNA transfer.

The IS fingerprints of strains Pd1222, DSM413 (the parent of Pd1222), and DSM415 are similar, while that of strain DSM65^T deviates. This finding is in agreement with taxonomic studies that showed that DSM413 and DSM415 are closely related and that DSM65^T is a rather distinct member of the *Paracoccus* genus (17). An interesting result came from a comparison of the fingerprints of DSM413 and NCIB8944. According to the available international strain catalogues, these strains should be the same. However, the IS fingerprints were not identical. If the two strains indeed originate from the same isolate, the difference in fingerprints should be attributed to the isolation of a genetically altered strain after the distribution of the particular isolate. The same is true for strain Pd1235. Just like Pd1222, this strain is a genetically accessible derivative of DSM413. However, the IS fingerprint of Pd1235 shows an IS*1248*-containing fragment that is not present in DSM413. Therefore, analysis of the IS*1248* fingerprint of *P. denitrificans* strains is a rapid and specific method, not only for identification and classification but also to check the strains after cultivation under selective conditions.

Apart from the IS*1248*-mediated integration mechanism, *P. denitrificans* has a second mechanism involved in the integration of heterologous DNA into its genome. The result of the latter type of integration is different from that observed for IS*1248*, in that the integrated DNA is not flanked by two identical sequences. Furthermore, the DNA sequences of the donor backbone and the target DNA at the integration site were found to be similar and to resemble the *res* site found in transposons belonging to the Tn*3* family (11, 12). These *res* sites are an essential part of the transposon-mediated site-specific recombination system involved in cointegrate resolution. According to the results of Southern blotting, at least two copies of this integrative element are present in the genome of *P. denitrificans*. The characteristics of this type of element are currently under investigation, in order to provide insight into the mechanism.

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