Comparative Genetic Diversity of the *narG*, *nosZ*, and 16S rRNA Genes in Fluorescent Pseudomonads

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The diversity of the membrane-bound nitrate reductase (*narG*) and nitrous oxide reductase (*nosZ*) genes in fluorescent pseudomonads isolated from soil and rhizosphere environments was characterized together with that of the 16S rRNA gene by a PCR-restriction fragment length polymorphism assay. Fragments of 1,008 bp and 1,433 bp were amplified via PCR with primers specific for the *narG* and *nosZ* genes, respectively. The presence of the *narG* and *nosZ* genes in the bacterial strains was confirmed by hybridization of the genomic DNA and the PCR products with the corresponding probes. The ability of the strains to either reduce nitrate or totally dissimilate nitrogen was assessed. Overall, there was a good correspondence between the reductase activities and the presence of the corresponding genes. Distribution in the different ribotypes of strains harboring both the *narG* and *nosZ* genes and of strains missing both genes suggests that these two groups of strains had different evolutionary histories. Both dissimilatory genes showed high polymorphism, with similarity indexes (Jaccard) of between 0.04 and 0.8, whereas those of the 16S rRNA gene only varied from 0.77 to 0.99. No correlation between the similarity indexes of 16S rRNA and dissimilatory genes was seen, suggesting that the evolution rates of ribosomal and functional genes differ. Pairwise comparison of similarity indexes of the *narG* and *nosZ* genes led to the delineation of two types of strains. Within the first type, the similarity indexes of both genes varied in the same range, suggesting that these two genes have followed a similar evolution. Within the second type of strain, the range of variations was higher for the *nosZ* than for the *narG* gene, suggesting that these genes have had a different evolutionary rate.

Denitrification is a microbial process in which oxidized nitrogen compounds are used as alternative electron acceptors for energy production when oxygen is limited. Denitrification consists of four reactions by which nitrate is reduced to dinitrogen by the metalloenzymes nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase. Bacteria capable of denitrification are frequently isolated from soil environments (30). The most common denitrifiers isolated from temperate soils belong to the group of fluorescent *Pseudomonas* spp. (8).

Zones of high denitrifying activity occur predominantly in specific soil microsites (21) and in “activation sites,” such as rhizospheric soil (15). Indeed, the proportion of fluorescent pseudomonads able to reduce nitrates appeared to be significantly higher in the rhizosphere than in the bulk soil. Even more, this proportion increases gradually and significantly in the vicinity of root (4). This observation has been made in the rhizosphere of different plant species cultivated in different soils (5). These results indicate that bacteria able to dissimilate nitrogen are selected in the rhizosphere. Furthermore, the nitrate reductase encoded by *narG* was recently shown to be involved not only in the rhizospheric competitiveness of *Pseudomonas fluorescens* C7R12 but more generally in its saprophytic competence in soil environments (17).

Altogether, these data suggest that enzymatic activities in-

involved in denitrification would be involved in the saprophytic competence of fluorescent pseudomonads in soil environments. The aim of the present study was to compare the genetic diversity of the *narG* and *nosZ* genes to that of 16S ribosomal DNA (rDNA), commonly used as an evolution marker, within a large collection of fluorescent pseudomonads previously isolated from different bulk and rhizospheric soils (13). These two genes were chosen because the enzymes that they encode, nitrate reductase and nitrous oxide reductase, catalyze the first and last steps of denitrification, respectively. Furthermore, the nitrate reductase is known to be involved not only in denitrification but also in another major respiratory process, the dissimilatory reduction of nitrate into ammonia. In addition, the ability of the strains studied to reduce nitrate and/or nitrous oxide was assessed.

MATERIALS AND METHODS

Bacterial strains. Pseudomonad strains used in this work are listed in Table 1. This list includes (i) 26 strains isolated from two soils (Dijon and Châteaurenard, France) and from the rhizosphere of two plant species (*Linum usitatissimum* L., cv. Opaline, and *Lycopersicon esculentum* Mill., cv. H63-5) cultivated in these soils (13, 14), including the *P. lini* type strain (CFBP 5737^T), (ii) four additional type strains, and (iii) two reference strains. Among the 26 strains from Dijon and Châteaurenard soils, 21 came from the selection of one strain from each of the clusters defined previously (13, 14) for the 340 strains isolated. These 21 strains are then expected to be representative of the diversity of the larger bacterial collection (340 strains) from which they are issued.

In order to check if the selected strains are really representative of the strains included in the 21 clusters, all strains (DLRp712, DLRp811, DTE911, DTRp522, and DTRp622) belonging to one cluster (cluster 9) (14) were also characterized. The type strains of the species *P. aeruginosa*, *P. chlororaphis*, *P. fluorescens*, and *P. putida* were included in addition to that of *P. lini*. Finally, two reference

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TABLE 1. Phenotypic and genotypic diversity of the fluorescent pseudomonad strains analyzed in this study

Strain and origin	Other designation	Species and biovar	Reference	Dissimilating abilities ^a			<i>narG</i>			<i>nosZ</i>		
				NDi	NR	TDe	PCR	Hybridization of:		PCR	Hybridization of:	
								Total DNA	PCR products		RFLP genotypes ^b	Total DNA
Dion soil												
CFBP 5760	DTR133	<i>P. chlororaphis</i>	14		+	+	+	+	13 DBHGG	+/- ^c	+	+
CFBP 5735	DLR426	<i>P. lini</i>	14			+	+	+	3 BABBB	+	+	+
CFBP 5736	DLRp214	<i>P. lini</i>	14			+	+	+	4 AABBB	+	+	+
CFBP 5738	DTR335	<i>P. lini</i>	14			+	+	+	1 AAAAA	+	+	+
CFBP 5741	DS131	<i>P. putida</i> bv. A	14	+			-	-		-	-	-
CFBP 5742	DS1026	<i>P. putida</i> bv. A	14	+	+		-	-		-	-	-
CFBP 5743	DLR223	<i>P. putida</i> bv. A	14		+		-	-		-	-	-
CFBP 5744	DLR228	<i>P. putida</i> bv. A	14		+		-	-		-	-	-
CFBP 5745	DLE3216	<i>P. putida</i> bv. A	14		+		-	-		-	-	-
CFBP 5746	DTRp621	<i>P. putida</i> bv. A	14		+		-	-		-	-	-
CFBP 5747	DS824	<i>Pseudomonas</i> sp.	14	+			-	-		-	-	-
DLRp712		<i>P. fluorescens</i> bv. IV	14			+	+	+	9 AAFBC	+	+	+
DLRp811		<i>P. fluorescens</i> bv. IV	14			+	+	+	9 AAFBC	+	+	+
DTE911		<i>P. fluorescens</i> bv. IV	14			+	+	+	9 AAFBC	+	+	+
DTRp522		<i>P. fluorescens</i> bv. IV	14			+	+	+	9 AAFBC	+	+	+
DTRp622		<i>P. fluorescens</i> bv. IV	14			+	+	+	9 AAFBC	+	+	+
Châteauneuf soil												
CFBP 5757	CTR212	<i>P. fluorescens</i> bv. II	13			+	+	+	2 AAEDA	+	+	+
CFBP 5758	CTR1015	<i>P. fluorescens</i> bv. II	13			+	+	+	11 BBDCD	+	+	+
CFBP 5755	CLR711	<i>P. fluorescens</i> bv. VI	13	+			-	-		-	-	-
CFBP 5756	CTRp112	<i>P. fluorescens</i> bv. VI	13		+		-	-		-	-	-
CFBP 5732	CS611	<i>P. lini</i>	13			+	+	+	8 BAFBC	+	+	+
CFBP 5733	CLRp812	<i>P. lini</i>	13			+	+	+	10 BAFBE	+	+	+
CFBP 5734	CLE513	<i>P. lini</i>	13			+	+	+	5 AABEE	+	+	+
CFBP 5739	CS111	<i>P. putida</i> bv. A	13	+			-	-		-	-	-
CFBP 5740	CS413	<i>P. putida</i> bv. A	13	+			-	-		-	-	-
Type strains												
CFBP 2466 ^T	ATCC 10145 ^T	<i>P. aeruginosa</i>				+	+	+	14 EBIHH	+	+	+
CFBP 2132 ^T	ATCC 9446 ^T	<i>P. chlororaphis</i>			+		+	+	13 DBHGG	+/- ^c	+	+
CFBP 2102 ^T	ATCC 13525 ^T	<i>P. fluorescens</i> bv. I		+		-	-	-		-	-	-
CFBP 5737 ^T	DLE411J	<i>P. lini</i>	6			+	+	+	7 AACBC	+	+	+
CFBP 2066 ^T	ATCC 12633 ^T	<i>P. putida</i> bv. A		+		-	-	-		-	-	-
Reference strains												
AK15		<i>P. fluorescens</i>	33			+	+	+	6 ABBA	+	+	+
CFBP 5759	CTR12	<i>P. fluorescens</i> bv. II	7			+	+	+	12 CBGFF	+	+	+
											8 FBBFCE	6 EACEAD

^a NDI, Nondissimilators; NR, nitrate reducers; TDe, total denitrifiers.^b Numbers designate the *narG* or *nosZ* type, and letters designate the patterns revealed with the restriction enzymes *Ava*II, *Bam*HI, *Nla*III, *Nci*I, and *Hin*II, respectively (*narG*), or *Ava*II, *Nor*I, *Eco*RI, *Hin*II, *Hin*dIII, and *Hin*cII, respectively (*nosZ*).^c +/ -, too little PCR product to allow PCR-restriction fragment length polymorphism (RFLP) analysis.

strains, *P. fluorescens* CFBP 5759 and AK15, were included in the present study because information on their nitrogen dissimilating activities and corresponding genes was available (9, 17, 25, 26).

Test for nitrogen dissimilation. For preparation of inocula, strains were cultured in 10 ml of Luria broth (LB) medium (16). After 24 h of incubation, bacterial cells were collected by centrifugation at $8,000 \times g$ for 20 min. The cells were resuspended in LB medium supplemented with KNO_3 (1 mM) to obtain an absorbance at 580 nm of 2. For each strain, 2 ml of culture was aseptically introduced into 20-ml Vacutainer (Becton Dickinson) test tubes with a syringe through a rubber stopper. Nitrate reducers were revealed by the presence of NO_2^- in the culture, determined colorimetrically with the Griess Illosway reagent and by the absence of significant amounts of N_2O when grown in tubes in which the atmosphere had been evacuated and replaced with a 90:10 $\text{He-C}_2\text{H}_2$ mixture to ensure anaerobiosis and avoid any reduction of N_2O to N_2 (34). Denitrifiers able to reduce N_2O were considered total denitrifiers and determined on the basis of the absence of N_2O accumulation when grown in tubes in which the atmosphere had been evacuated and replaced with helium to ensure anaerobiosis. Nitrous oxide concentration was measured with an MTI high-speed micro-gas chromatograph equipped with a catharometer detector (SRA Instruments). Strains unable to dissimilate NO_3^- in anaerobiosis were considered nondissimilators.

PCR amplification. Amino acid sequences derived from the *narG* genes of *P. aeruginosa* PAO1 and *P. fluorescens* CFBP 5759 and AK15 (accession nos. Y15252, AF197465, and U71398, respectively) were aligned, and conserved regions were used for the design by back-translation of the PCR primers narGf (5'-GA[C/T]ATGCA[C/T]CC[A/C/G/T]TT-3') and narGr (5'-A[C/T]CCA[A/G]TC[A/G]TT[A/G]TC-3'). Amino acid sequences derived from the *nosZ* genes of *P. aeruginosa* PAO1, *P. fluorescens* CFBP 5759, *P. denitrificans*, *Rhizobium meliloti*, *Bradyrhizobium japonicum*, and *Ralstonia eutropha* (accession nos. X65277, AF197468, X74792, U47133, AJ002531, and X65278, respectively) were aligned, and conserved regions were used for the design by back-translation of the PCR primers nosZf (5'-AACGACAAG[G/A/T][C/T]CAA-3') and nosZr (5'-A[G/T][G/C]GC[A/G]TGGCAGAA-3'). The primers narGr plus nosZf or nosZr were used to amplify a fragment of *narG* (1,008 bp) and a fragment of *nosZ* (1,433 bp), respectively. The 16S rRNA gene (rDNA) was amplified with primer pair fd1 and rd1 (31).

PCR amplifications were carried out with cell suspensions obtained as described previously (14). PCR amplifications of *narG* and *nosZ* were performed in a total volume of 100 μl , by mixing 5- μl aliquots of cell suspension with 0.5 μM each primer, 50 μM each dATP, dCTP, dGTP, and dTTP, 4 U of *Taq* DNA polymerase (Q-BIOgene, Illkirch, France), and PCR buffer (10 mM Tris-HCl [pH 9.0 at 25°C], 50 mM KCl, 1.5 mM MgCl_2 , 0.1% Triton X-100, 0.2 mg of bovine serum albumin per ml). The PCR mix for 16S rDNA was similar to those for *narG* and *nosZ* except for the primer (0.2 μM) and the deoxynucleotide triphosphate (40 μM each) concentrations. A negative control for amplification was prepared with 5 μl of H_2O instead of 5 μl of cell suspension.

Amplifications were conducted in a thermal cycler GeneAmp PCR System 9600 (Perkin Elmer Applied Biosystems, Foster City, Calif.) with an initial denaturation step (3 min at 95°C) followed by 35 cycles of 1 min at 94°C, 1 min at 50°C for *narG* and 55°C for *nosZ* and 16S rDNA, 1 min for *narG* and 2 min for *nosZ* and 16S rDNA at 72°C, and a final extension of 3 min at 72°C. Aliquots (5 μl) of each PCR product were analyzed by electrophoresis in 0.8% agarose gels stained with ethidium bromide and photographed under UV illumination with Polaroid type 665 positive-negative film.

Extraction and digestion of total genomic DNA. For each strain, extraction and purification of the total genomic DNA were performed with previously described methods (2). Aliquots of 1 μg of total genomic DNA were digested overnight in 10 μl with 30 U of *EcoRI* (Roche Diagnostic, Meylan, France). This enzyme was chosen because there is only one site of attack in the *narG* sequence and no site in the *nosZ* sequence of *P. fluorescens* CFBP 5759, both used as probes for hybridization.

Hybridization of total genomic DNA and PCR products. *narG* and *nosZ* probes were prepared from PCR products of strain *P. fluorescens* CFBP 5759 obtained as described above. *narG* and *nosZ* PCR products, corresponding to 1,008-bp and 1,433-bp DNA fragments, respectively, were purified after electrophoresis on a 0.8% agarose gel with a Qiaex II gel extraction kit (Qiagen S.A., Courtaboeuf, France). The purified fragments were analyzed in a 0.8% agarose gel and quantified by comparison with the molecular mass markers Smart Ladder (Eurogentec, Seraing, Belgium). One hundred nanograms of each fragment was labeled for 20 h with digoxigenin with a DIG-High Prime kit (Roche Diagnostic), which theoretically produces 1,500 ng of labeled DNA.

The restriction fragments of total genomic DNA were separated by electrophoresis in 0.8% agarose gels in TAE buffer (40 mM Tris-HCl [pH 7.9], 4 mM

sodium acetate, and 1 mM EDTA). DNA was transferred from the gel to a positively charged nylon membrane (Pall Gelman Laboratory, Ann Arbor, Mich.) with the VacuGene XL apparatus (Pharmacia Biotech, Orsay, France) according to the manufacturer's instructions. The DNA was fixed to the membrane by baking at 120°C for 30 min. Southern blots were hybridized successively with the *narG* and *nosZ* probes. Hybridization was performed according to the manufacturer's instructions with a probe concentration of 25 ng per ml of hybridization solution. Hybridized fragments were revealed with the DIG luminescent detection kit (Roche Diagnostic).

The PCR products of *narG* and *nosZ* were hybridized with the corresponding probes defined above to confirm that these products correspond to the targeted sequences. Aliquots of 1 μl of *narG* PCR product or 5 μl of *nosZ* PCR product were analyzed by electrophoresis in TAE buffer. PCR products were then transferred from the gel to a positively charged nylon membrane (Pall Gelman Laboratory, Ann Arbor, Mich.) and hybridized as described above.

Restriction fragment length polymorphism assay of *narG*, *nosZ*, and 16S rDNA. Discriminant restriction enzymes for *narG* and *nosZ* were selected from comparison of the sequences previously used to design the PCR primers. Aliquots (5 μl) of PCR products were digested overnight with 5 U of the following restriction endonucleases: *AvaII*, *BamHI*, *HinfI*, *NciI*, and *NlaIII* for *narG*; *AvaII*, *EcoRI*, *HincII*, *HindIII*, *HinfI*, and *NarI* for *nosZ*; and the 13 endonucleases described by Laguerre et al. (12) for 16S rDNA. The restriction fragments were separated by electrophoresis at 100 V in TAE buffer with 4% Nusieve 3:1 agarose (FMC, Rockland, Maine) on gels containing 0.4 μg of ethidium bromide per ml. Gels were photographed under UV illumination with Polaroid type 665 positive-negative film.

For each gene, each strain was assigned a composite type defined by the combination of the patterns obtained with the different restriction endonucleases. The computer program NTSYS (27) was used to estimate the relationships among *narG*, *nosZ*, and 16S rDNA types from the fragments obtained with the different restriction endonucleases. The *narG*, *nosZ*, and 16S rDNA types were compared by pairwise coefficients of similarity calculated by the Jaccard similarity coefficient (28). For each gene, the matrix of similarities was displayed as a dendrogram with the UPGMA (unweighted pair group method arithmetic) algorithm (28). For each possible combination, *narG*/16S rDNA, *nosZ*/16S rDNA, and *narG*/*nosZ*, the level of correlation between the genes was determined with pairwise similarities between strains.

RESULTS

Abilities to dissimilate nitrate and nitrogenous oxide. The abilities of the type strains to dissimilate nitrate and nitrogenous oxide were in agreement with previous reports (6, 20, 29). *P. aeruginosa* CFBP 2466^T and *P. lini* CFBP 5737^T were total denitrifiers, *P. chlororaphis* CFBP 2132^T was a nitrate reducer, and *P. fluorescens* CFBP 2102^T and *P. putida* CFBP 2066^T were both nondissimilators (Table 1).

The 28 strains isolated from soil environments, including the two reference strains AK15 and CFBP 5759, were either total denitrifiers (57.1%), nitrate reducers (14.3%), or nondissimilators (28.6%). Strains of *P. fluorescens* were total denitrifiers (81.8%) or nondissimilators (18.2%), whereas strains of *P. putida* were nitrate reducers (37.5%) or nondissimilators (62.5%). All *P. lini* strains were total denitrifiers, and the two strains of *P. chlororaphis* were nitrate reducers.

Detection of *narG* and *nosZ*. Primers narGf and narGr permitted the amplification of a single DNA fragment of about 1,000 bp for 19 of the 32 strains analyzed (Table 1). A positive hybridization was recorded for all the PCR products. Additionally, hybridization of the genomic DNA with this probe gave a positive signal for the 19 strains.

Primers nosZf and nosZr permitted the amplification of a single DNA fragment of about 1,450 bp for 19 of the 32 strains analyzed. A positive hybridization was recorded for all the PCR products. Additionally, hybridization of the genomic DNA with this probe gave a positive signal in the 19 strains.

FIG. 1. Dendrogram showing the relationships between the fluorescent *Pseudomonas* strains based on variation in restriction sites in the *narG* gene. The pairwise coefficients of similarity (Jaccard) were clustered with the UPGMA algorithm of NTSYS-pc. Numbers 1 through 14 indicate the 14 *narG* types.

Despite optimization of PCR conditions (data not shown), the quantities of PCR products for both *P. chlororaphis* strains were too low to allow restriction fragment length polymorphism analysis of their *nosZ* genes but high enough to hybridize with the *nosZ* probe.

Analysis of restriction fragment length polymorphisms of *narG* and *nosZ*. The PCR products of the *narG* genes were digested with each of the five restriction enzymes *Ava*II, *Bam*HI, *Hinf*I, *Nci*I, and *Nla*III for the 19 strains that gave a positive amplification with the *narGf* and *narGr* primers. Depending on the restriction enzyme, two to nine distinct restriction patterns were resolved. A total of 69 different restriction fragments were obtained, all of them being polymorphic. The 19 strains analyzed with the five restriction enzymes gave 14 different combinations of patterns representing 14 *narG* types (Table 1). The level of similarity among the *narG* types ranged from 0.05 to 0.79, indicating a high polymorphism of *narG* among the strains of fluorescent pseudomonads tested (Fig. 1).

P. aeruginosa CFBP 2466^T was the most distant from the

others and was thus used to root the dendrogram. According to their level of similarity, strains could be clustered in two groups. The first group was made up of strains showing a level of similarity at least equal to 0.43; this group included all the strains of *P. lini* (CFBP 5738, CFBP 5735, CFBP 5736, CFBP 5734, CFBP 5737^T, CFBP 5732, and CFBP 5733) and seven strains of *P. fluorescens* (CFBP 5757, AK15, DLRp712, DLRp811, DTRp522, DTRp622, and DTE911). The second was made up of strains showing a level of similarity at least equal to 0.21; this second group included the two strains of *P. chlororaphis* (CFBP 2132^T and CFBP 5760) and two strains of *P. fluorescens* (CFBP 5758 and CFBP 5759).

The PCR products of the *nosZ* genes were digested with each of the six restriction enzymes *Ava*II, *Eco*RI, *Hinc*II, *Hind*III, *Hinf*I, and *Nar*I for the 17 strains that gave a clear positive amplification with the *nosZf* and *nosZr* primers. Depending on the restriction enzyme, three to seven distinct restriction patterns were resolved. A total of 57 different restriction fragments were obtained, all of them being polymorphic.

FIG. 2. Dendrogram showing the relationships between the fluorescent *Pseudomonas* strains based on variation in restriction sites in the *nosZ* gene. The pairwise coefficients of similarity (Jaccard) were clustered with the UPGMA algorithm of NTSYS-pc. Numbers 1 to 11 indicate the 11 *nosZ* types.

The 17 strains analyzed with the six restriction enzymes gave 11 different combinations of patterns representing nine *nosZ* types (Table 1). The level of similarity among the *nosZ* types ranged from 0.04 to 0.88, indicating a high polymorphism of *nosZ* among the strains of fluorescent pseudomonads tested (Fig. 2).

P. fluorescens CFBP 5758 was the most distant from the others and was thus used to root the dendrogram. *P. aeruginosa* CFBP 2466^T was also distant from the others. The other strains could be clustered in two groups. The first was made up of strains showing a similarity level at least equal to 0.35; this group included five strains of *P. lini* (CFBP 5732, CFBP 5733, CFBP 5734, CFBP 5737^T, and CFBP 5738) and two strains of *P. fluorescens* (CFBP 5757 and CFBP 5759). The second group was made up of strains showing a similarity level at least equal to 0.40; this group included two strains of *P. lini* (CFBP 5735 and CFBP 5736) and six strains of *P. fluorescens* (AK15, DLRp712, DLRp811, DTE911, DTRp522, and DTRp622).

Analysis of restriction fragment length polymorphisms of

16S rDNA. The combined patterns obtained with 13 restriction endonucleases gave 15 distinct ribotypes among the 32 strains analyzed (Fig. 3). The ribotypes of most reference and type strains were published previously (12). The level of similarity among the ribotypes ranged from 0.77 to 0.99, indicating a low polymorphism of 16S rDNA among the strains of fluorescent pseudomonads tested. The 16S rDNA type Aer, including the *P. aeruginosa* type strain (CFBP 2466^T), was the most distant from the others and was thus used to root the dendrogram.

The 16S rDNA type PuA, including *P. putida* type strain (CFBP 2066^T), was also separated from all the other ribotypes and was especially distant from all the other strains of *P. putida* (CFBP 5739, CFBP 5740, CFBP 5741, CFBP 5742, CFBP 5743, CFBP 5744, CFBP 5745, and CFBP 5746). These strains were distributed, together with three *P. fluorescens* strains, including the corresponding type strain (CFBP 2102^T, CFBP 5755, and CFBP 5756), in a group of five ribotypes (FI, D6, C1, C5, and D7) showing a level of similarity at least equal to 0.96. A second group of eight ribotypes showing a level of similarity

FIG. 3. Dendrogram showing the relationships between the 16S rDNA types. The pairwise coefficients of similarity (Jaccard) were clustered with the UPGMA algorithm of NTSYS-pc. *P. lini* CFBP 5732 and CFBP 5733 were identified by the double C3/C2 16S rDNA type described previously (13). Bacterial strains underlined were shown to harbor both the *narG* and *nosZ* genes; bacterial strains not underlined were shown to harbor neither the *narG* gene nor the *nosZ* gene.

at least equal to 0.95 could be delineated. This group included the two strains of *P. chlororaphis* (CFBP 2132^T and CFBP 5760) which belonged to same ribotype (Chl), the seven strains of *P. lini* (CFBP 5732, CFBP 5733, CFBP 5734, CFBP 5735, CFBP 5736, CFBP 5737^T, and CFBP 5738) belonging to the D8, D3, C2, and C3/C2 ribotypes, and finally the remaining strains of *P. fluorescens* (CFBP 5757, DLRp712, DLRp811, DTE911, DTRp522, DTRp622, AK15, CFBP 5758, and CFBP 5759) belonging to the C8, C3, A1, and C9 ribotypes. Although strains of *P. fluorescens*, *P. lini*, and *P. putida* were distributed in different ribotypes, only two ribotypes included strains of two different species: (i) the D6 ribotype was found in one *Pseudomonas* sp. strain and one *P. putida* strain and (ii) the C3 ribotype was found in *P. fluorescens* strains and two *P. lini* strains identified by the double C3/C2 type (13).

Relationships among polymorphisms of *narG*, *nosZ*, and

16S rDNA. For each combination of gene types, *narG*/16S rDNA (Fig. 4A), *nosZ*/16S rDNA (Fig. 4B), and *nosZ/narG* (Fig. 4C), the level of correlation was determined by pairwise similarities between strains.

Comparison of functional genes (*narG* and *nosZ*) with 16S rDNA indicated no correlation between the levels of similarities of *narG* and 16S rDNA or between those of *nosZ* and 16S rDNA. Two major types of relation could still be identified. The first one, corresponding to the type strain of *P. aeruginosa* compared to the other strains harboring *narG* and *nosZ*, appeared to present both a low level of similarity of their 16S rDNA (0.7453 to 0.7788) and functional genes *narG* (0 to 0.1111) (Fig. 4A) and *nosZ* (0.0741 to 0.3478) (Fig. 4B). The second type, corresponding to the other pairwise comparisons, in which strains appeared to present a high level of similarity of their 16S rDNA (0.9341 to 1) together with a high range of

FIG. 4. Correlation between pairwise coefficients of similarity (Jaccard) of the *narG* gene and 16S rDNA (A), the *nosZ* gene and 16S rDNA (B), and the *narG* and *nosZ* genes (C). Equation of linear regressions 1 and 2 were $y = 1.1492x + 0.2117$ and $y = 0.2059x + 0.0368$, respectively. Linear regressions 1 and 2 were calculated with the pairwise coefficients of similarity represented by triangles and diamonds, respectively.

similarity of their *narG* (0.0714 to 1) and *nosZ* (0 to 0.8824) genes.

Comparison of the two functional genes, *narG* and *nosZ*, allowed us to identify two different groups among the pairwise

comparisons (Fig. 4C). For each of these two groups, a linear regression between the levels of similarity of the two genes in the different strains was calculated. The slope values, obtained after calculation of the equations of the two regressions, were

equal to 1.15 (regression 1) and 0.21 (regression 2), and their correlation coefficients were equal to 0.87 and 0.45, respectively. All species having *narG* and *nosZ* genes were represented in these two groups.

DISCUSSION

The diversity of two nitrogen dissimilatory genes (*narG* and *nosZ*) was characterized in a collection of fluorescent pseudomonads together with that of the 16S rRNA gene. The strains analyzed were chosen as being representative of a larger collection by selecting one strain from each of the phenotypic clusters defined previously (13). The validity of this strategy was checked by showing that all the strains belonging to a given cluster belonged to the same ribotype (C3), the same *narG* type (9), and the same *nosZ* type (9).

The ability of the strains studied to reduce nitrate and nitrous oxides was assessed. A large majority of the strains analyzed (71.4%) expressed the ability to reduce nitrate, and among them, 78.2% were able to reduce nitrate into dinitrogen. The ability to only reduce nitrate in nitrite was present at a low frequency within our collection of soil-borne pseudomonads (28.6%). Overall, the ability to express the dissimilatory activities was well related to the presence of the corresponding genes (*narG* and *nosZ*). Most of the strains harbored both genes, a few of them harbored only the *narG* gene, and none of them harbored only the *nosZ* gene. Our experimental procedures did not enable us to check the absence of nitrous oxide reduction by strains missing the *nosZ* gene. Nitrate reductase activity was recorded in some strains missing the *narG* gene (*P. putida* CFBP 5744, CFBP 5745, and CFBP 5746), suggesting the presence of another enzyme performing nitrate reduction, such as the periplasmic nitrate reductase previously described in other fluorescent pseudomonads (3, 24). In contrast, the two strains of *P. chlororaphis* were not able to reduce nitrous into dinitrogen despite a positive hybridization signal of total DNA and PCR products with the *nosZ* probe. However, the quantity of these PCR products was very low, suggesting either that the primers were not specific enough, that other sequences than *nosZ* were amplified, or that the corresponding genes were not functional.

The presence in the bacteria of the dissimilatory *narG* and *nosZ* genes and the diversity of these genes were analyzed in relation to the ribotypes of the bacterial strains. 16S rDNA is commonly used for diversity analyses of bacterial populations (19), especially of fluorescent pseudomonads (11, 12, 13). 16S rDNA is also considered a genetic marker for phylogeny studies in bacteria (32) and has been applied by several authors when studying the phylogeny of functional genes in bacteria (10). In contrast to functional genes, which are able to accumulate mutations without being impaired (e.g., variation of the third position of the codon), the level of acceptable variation in the 16S rRNA gene (not lethal) is low due to the functionality in this gene, which is strongly related to its structure. Consequently, the 16S rRNA gene has an evolutionary rate significantly lower than that of functional genes (0.02 and 0.7 to 0.8 per 10^6 years, respectively) (18).

The distribution of strains among the different ribotypes was clearly related to the presence of the *narG* and *nosZ* genes. Indeed, the *narG* and *nosZ* genes were never detected in the

strains belonging to the ribotypes included in the first cluster of the 16S rDNA dendrogram. In contrast, both *narG* and *nosZ* were detected in all strains belonging to the ribotypes included in the second cluster of the 16S rDNA dendrogram. This observation suggests that the strains with *narG* and *nosZ* genes have an evolutionary history different from that of those harboring neither of the dissimilatory genes.

Polymorphism analysis of the two denitrifying genes indicated clearly the wide range of similarities within the bacterial strains tested. Indeed, the similarity of the *narG* and *nosZ* genes varied from 0.05 to 0.79 and from 0.04 to 0.88, respectively. As expected, this range of similarities was a lot lower than that of the 16S rRNA gene. This observation suggests that the evolution rates of ribosomal and functional genes differ. This is in agreement with the estimations made by Ochman and Wilson (18). No correlation was found between the similarity indexes of 16S rDNA and denitrifying genes (*narG* or *nosZ*). In contrast, Philippot (22) reported a congruence between the 16S rDNA and *narG* genes when analyzing denitrifying genes of strains belonging to different genera in the major microbial groups. The lack of congruence between 16S rDNA and the dissimilatory genes recorded in the present study could be related to the strains analyzed, which belong to species which are closely related (fluorescent *Pseudomonas* spp., oxidase positive) and not well defined (1), with ribotypes showing a high level of similarity (12).

Since *narG* and *nosZ* genes are known to be organized in two distinct operons (22, 35) and to encode enzymes contributing to nitrogen dissimilation, a similar rate of evolution of these two functional genes could be expected. However, the pairwise comparison of similarity indexes of *narG* and *nosZ* in the different strains leads to the identification of two different groups of strains. These groups were distributed along two linear regressions showing different slopes (1.15 and 0.21). The slope value close to 1 in the first linear regression indicates that the similarity indexes of the *narG* and *nosZ* genes harbored by the corresponding strains varied within the same range. This observation suggests that these two genes have followed a similar evolution and therefore have a similar molecular clock. In the second group of strains, the slope value of the linear regression was lower than 1, indicating that the range of diversity of the *nosZ* gene is higher than that of the *narG* gene. This suggests that the two genes had different evolutionary rates.

Altogether, our data suggest that in the strains belonging to the first group (slope equal to 1.15), the *narG* and *nosZ* genes were present together in more ancestral strains than in the strains belonging to the second group (slope equal to 0.21). According to Petri and Imhoff (22) and to Zumft (35), the respiratory nitrate reductase gene had a long evolutionary history and already played a key function in energy metabolism during preoxic times. To our knowledge, bacteria harboring only the *nosZ* gene have never been described. These observations, taken together, support the hypothesis that the acquisition by bacteria of nitrous oxide reduction is probably a more recent event than that of the ability to reduce nitrate. This would suggest that strains harboring both genes were favored in soil environments.

This hypothesis is supported by the significantly higher frequency among soil-borne pseudomonads of strains which are total denitrifiers (57.1%) compared to those which are nitrate

reducers (14.3%), this difference having already been stressed in our previous work (4). This hypothesis is also supported by the observations recently made in our group indicating that the fluorescent pseudomonads showing the best rhizosphere competence are total denitrifiers (S. Delorme, T. Corberand, Y. Dessaux, L. Gardan, J.-M. Meyer, J. Raaijmakers, and P. Lemanceau, unpublished data). Altogether, these observations suggest that the ability to reduce nitrate to molecular nitrogen confers a selective advantage to fluorescent pseudomonads in soil environments.

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