APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2009, p. 534–537 Vol. 75, No. 2
0099-2240/09/$08.00+0 doi:10.1128/AEM.01706-08

Characterization of Denitrification Gene Clusters of Soil Bacteria via a Metagenomic Approach▽†

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Received 24 July 2008/Accepted 3 November 2008

We characterized operons encoding enzymes involved in denitrification, a nitrogen-cycling process involved in nitrogen losses and greenhouse gas emission, using a metagenomic approach which combines molecular screening and pyrosequencing. Screening of 77,000 clones from a soil metagenomic library led to the identification and the subsequent characterization of nine denitrification gene clusters.

Denitrification is a microbial respiratory process within the nitrogen cycle responsible for the return of fixed nitrogen to the atmosphere. This process contributes to the emission of N_2O, which is an important greenhouse gas with a global warming potential (ca. 250 times higher than that of carbon dioxide). Denitrifiers, which constitute a taxonomically diverse functional guild with members belonging to more than 60 genera of bacteria and to some archaea and eukaryotes (13), can represent up to 5% of the total soil microbial community (5, 15). However, the study of denitrifying bacteria, like that of others, is hindered by characteristics that can prevent up to 99% of soil bacteria from being cultivated in vitro. The inventory of genes involved in denitrification and the extent of their diversity in bacteria are yet to be fully explored, while characterization of whole denitrification pathways with full-length gene sequences is still restricted to a limited number of denitrifying isolates and a few complete genomes.

New approaches based on the direct extraction of DNA from the natural environment and PCR amplifications can overcome limitations due to bacterial unculturability, but until now their application to denitrification genes has led only to the recovery of partial sequences for some of these genes (12). Our goals in this study were to apply a metagenomic approach (2) characterized by cloning of DNA extracted from soil and screening of metagenomic DNA library clones in order to identify and characterize gene clusters involved in the denitrification process. The soil metagenomic DNA library we used was constructed by Ginolhac et al. (4) with DNA extracted from grassland soil (Montrond, La Batie-Divisin, France) with 35- to 40-kb metagenomic DNA fragments cloned in the pCC1Fos vector and replicated in the *Escherichia coli* EC10 bacterial host. About 77,000 clones were screened by colony hybridization according to the protocol described previously (2). In order to increase the range of retrievable sequences, [^{33}P]dCTP-labeled probes consisted of PCR products obtained from DNA extracted from Montrond soil as templates by using degenerate primers targeting the *nirS*, *nirK*, and *nosZ* denitrification genes encoding the cytochrome cd_1 nitrite reductase, the copper nitrite reductase, and the nitrous oxide reductase, respectively (5, 6, 14). Pyrosequencing (GATC, Konstanz, Germany) was used to sequence DNA from the clones identified as yielding a positive hybridization signal on the membranes (2). Nine recombinant clones were positively identified by hybridization and sequence analysis as carrying genes coding for denitrification functions: four clones contained a *nirS*-like gene, three clones had a *nirK*-like gene, one clone had a *nosZ*-like gene, and one clone contained both *nirK*-like and *nosZ*-like genes (Fig. 1). This number of positive clones is in agreement with the estimated proportion of denitrifiers in the soil bacterial community (between 0.5 and 5%) (5, 15) and the calculation of Leveau (9) that estimated that 57,500 clones with 40-kb metagenomic inserts would be required to recover one gene (99% probability) present in 1% of the soil bacteria, considering an average genome size of 5 Mbp for each soil bacterium. Other genes present in these nine clones are described in Tables S1 to S9 in the supplemental material.

The genetic organization of the *nirS* clusters, with most of the *nir* gene products presumably involved in the heme D_1 biosynthesis (19), was *nirESM-FDGHJN*, *nirSTB---SCFDGHJN*, *nirS-CFD*, and *nirDGHJS* (each hyphen here indicates an inserted gene) on contigs 888, 2303, 2304, and 6254, respectively. Unfortunately, the assembly of a few contigs could not be completed, and the end of the *nirS* cluster is missing for contigs 2304 and 6254. The results show a variable gene organization among bacteria, confirming previous data from isolate analysis, and indicate that these clusters are probably subjected to shuffling either by endogenous gene displacement or by horizontal gene transfer between bacteria (11). Two *nirS* copies were detected in contig 2303 with a 69% similarity, indicating that the original bacterium that provided the DNA fragment contained more than one copy of this gene in its genome. Previous studies

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 14 November 2008.

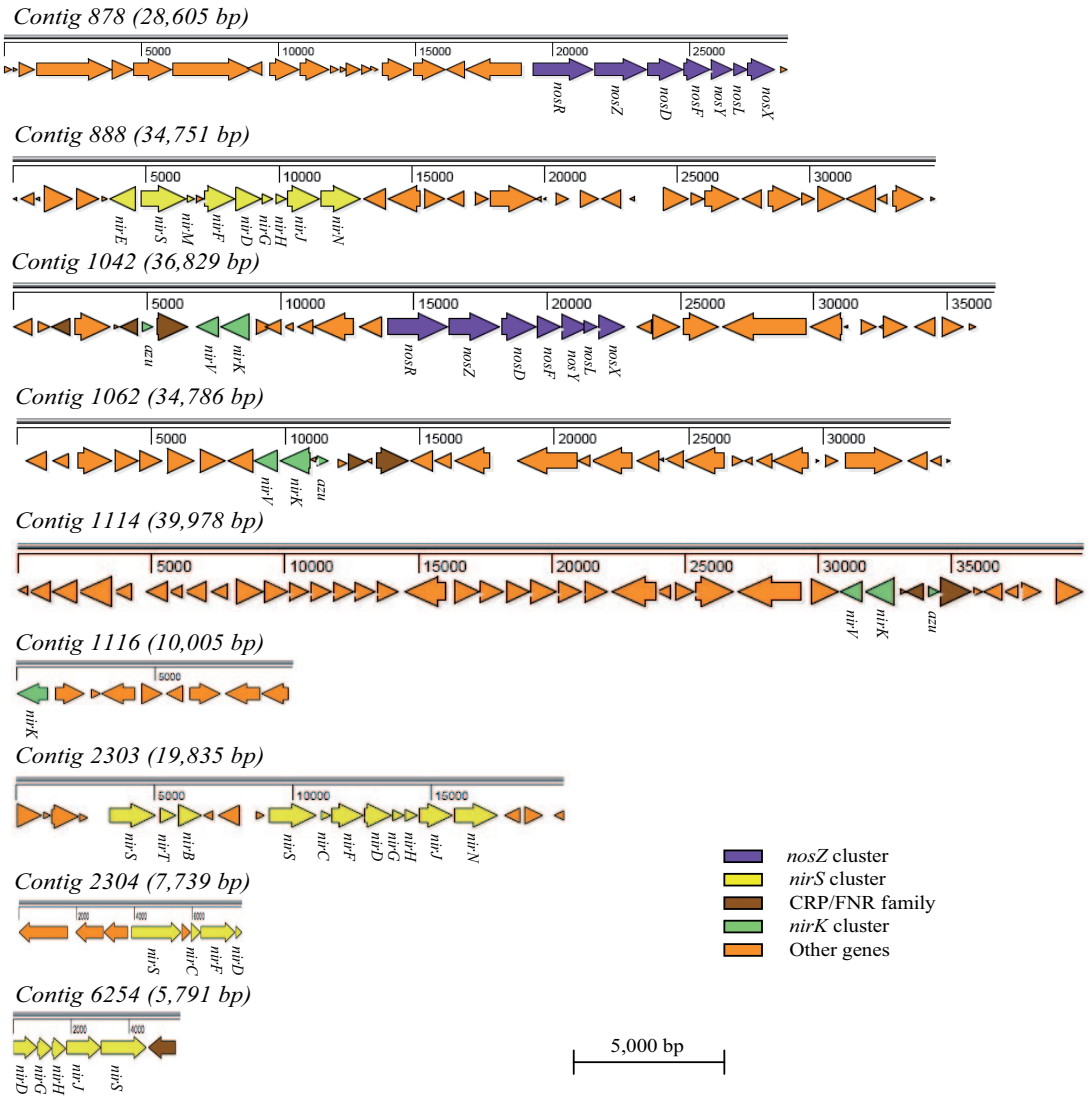

FIG. 1. Physical maps of environmental gene contigs involved in denitrification processes. Shown are the *nosZ* clusters (purple), the *nirS* clusters (yellow), genes from the CRP/FNR family involved in the expression control of the denitrification process (brown), the *nirK* clusters (green), and other genes not directly involved in the denitrification process (orange), which are described in Tables S1 to S9 in the supplemental material.

reported the presence of multiple copies of *nirS* in "*Magnetospirillum magneticum*," "*Dechloromonas aromatica*," and *Thiobacillus denitrificans* that also exhibited a significant level of divergence within the same genome (3, 8).

In three out of four *nirK*-containing clones (partial gene sequence in contig 1116), a *nirV*-like gene was located at a position linked to the *nirK* gene, as previously observed for several cultivated denitrifiers. The frequent proximity of these two genes on the genome supports the hypothesis of an involvement of a *nirV* gene product in nitrite reduction (7, 11). In addition to *nirV*, the *azu* gene encoding a pseudoazurin electron carrier, the principal electron donor to the copper nitrite reductase (19), was identified 2,503 bp downstream of the *nirK* gene in contig 1042 and 233 bp and 1,220 bp upstream of *nirK* in contigs 1062 and 1114, respectively, but with the transcription direction opposite of that of the *nirK* gene.

The two *nos* clusters identified in our study contained the *nosRZDFYLX* genes, with *nosR* encoding a membrane-bound regulatory protein, *nosZ* encoding the catalytic subunit of the multicopper nitrous oxide reductase, *nosDFY* encoding a putative copper insertion complex, *nosL* encoding a putative outer membrane protein, and *nosX* encoding a periplasmic component (1, 17, 20). In contrast to the organization of the *nirS* cluster, the organization of the *nosRZDFYL* genes observed in our study was identical to that of most cultivated denitrifiers, which indicates a high level of synteny. Interestingly, the *nosX* gene was located downstream of *nosL* for both *nos* contigs. This is commonly observed in *Alphaproteobacteria* but not in other proteobacteria (11). In contig 1042, the *nos* genes were located ca. 7,500 bp upstream of the *nirK* gene. Genetic linkage of the *nir* and *nos* genes has also been observed in *Brucella melitensis* and *Bradyrhizobium japonicum*

TABLE 1. Sequences similar to *nirS*, *nirK*, and *nosZ* found in cultivated microorganisms based on the application of the BLASTN program to the NCBI database

Environmental gene (contig)	Accession no.	Description of cultivated microorganism	BLASTN score	BLASTN query coverage (%)	E value	Sequence identity (%)
nosZ (878)	CP000301.1	*Rhodopseudomonas palustris* BisB18	708	87	0.0	75
nirS (888)	AM260480.1	*Ralstonia eutropha* H16 chromosome 2	876	85	0.0	77
nirK (1042)	CP000390.1	*Mesorhizobium* sp. strain BNC1	745	86	0.0	80
nosZ (1042)	AE006469.1	*Sinorhizobium meliloti* 1021 plasmid pSymA	1738	91	0.0	84
nirK (1062)	BX572606.1	*Rhodopseudomonas palustris* CGA009	656	86	0.0	79
nirK (1114)	BA000040.2	*Bradyrhizobium japonicum* USDA 110	784	86	0.0	81
nirK (1116)	CU234118.1	*Bradyrhizobium* sp. strain ORS278	865	86	0.0	82
nirS (2303a)	CR555306.1	*Azoarcus* sp. strain EbN1	1260	92	0.0	81
nirS (2303b)	CP000089.1	"*Dechloromonas aromatica*" RCB	1230	87	0.0	81
nirS (2304)	CP001013.1	*Leptothrix cholodnii* SP-6	1509	89	0.0	84
nirS (6254)	CP001013.1	*Leptothrix cholodnii* SP-6	1003	92	0.0	79

USDA110, suggesting that denitrification gene islands are not rare in soil bacteria. Although the *nor* genes encoding the nitric oxide reductase enzyme were located in the vicinity of the *nir* genes in several cultivated denitrifiers (11), such linkage was not confirmed in our study.

The metagenomic pyrosequencing approach also detected several genes encoding one-component transcriptional regulators belonging to the superfamily of cyclic AMP receptor protein (CRP)-like proteins and fumarate and nitrate reductase regulatory protein (FNR)-like proteins (Fig. 1) in the vicinity of the denitrification genes. CRP/FNR-like proteins have been established as major transcriptional factors controlling expression of the denitrification process in response to oxygen and nitric oxide presence (16, 18). Putative DNA binding sites of CRP/FNR-like proteins, which consist of inverted and repeated sequences of nucleotides (TTGATNNNATCAA), were identified in the promoter regions of (i) *nosR* on contigs 878 and 1042, (ii) *nirS* on contigs 2303, 2304, and 888, and (iii) *nirK* on contig 1114. CRP/FNR boxes were also found in the promoter regions of genes encoding a nitrate/nitrite antiporter and cytochrome oxidase assembly factor in contig 888 and encoding Fnr protein in contig 1042. Presence of FNR/CRP-like proteins near the denitrification genes and presence of Fnr boxes in their promoter regions support an oxygen-dependent regulation of the denitrification process in the corresponding host strains as commonly observed in cultivated strains (11).

Phylogenetic analysis of the *nirS*, *nirK*, and *nosZ* catalytic subunits revealed that the *nirK* and *nosZ* sequences obtained in this study were related to the *nirK* or *nosZ* gene from *Alphaproteobacteria* (up to 84% identity) (Table 1) (see Fig. S1 and S2 in the supplemental material). In addition, gene organization in contigs 1042 and 1062 with the *nosX* gene downstream of the *nosL* gene is similar to that found in denitrifier isolates classified in the subclass of the *Alphaproteobacteria*. Accordingly, assigning contigs to their respective phylogenetic groups using the PhyloPythia software (10) showed that contigs 878, 1042, 1062, 1114, and 1116 were related to *Alphaproteobacteria*. The four *nirS* sequences identified in this study were phylogenetically related to the *nirS* sequences from *Betaproteobacteria* (Table 1) (see Fig. S3 in the supplemental material). However, phylogenetic affiliation of the full contigs with the PhyloPythia software revealed that contig 6254 was affiliated with *Gammaproteobacteria* while the three others were affiliated with *Betaproteobacteria*. This underlined the difficulty of phylogenetic affiliation of the denitrification genes due to the lack of congruence between the denitrification genes and 16S rRNA trees as previously reported by Jones et al. (8).

Our results highlight the potential of the metagenomic approach (2) combined with molecular screening and pyrosequencing to broaden our knowledge of genetic organization and diversity of gene clusters or operons that are distributed in soil microorganisms far beyond the small proportion of cultivable bacteria. Systematic sequencing of the entire soil metagenomic DNA still remains difficult; therefore, an intermediate step of screening a recombinant clone library, such as the hybridization method used in this study, is useful in order to reduce the number of clones to be sequenced. The use of a probe consisting of mixed PCR products allowed us to detect denitrification genes from metagenomic DNA with percentage identities as low as 75% to known genes (Table 1). Use of functional screening in future studies could help detect denitrification genes that would not be detected by hybridization because of their sequence divergence.

Nucleotide sequence accession numbers. Sequences obtained and annotated in this study have been deposited in GenBank under the accession numbers EU910852 to EU910860.

This work was supported in part by the Programme National de Recherches sur les Organismes Génétiquement Modifiés from the Agence Nationale de la Recherche for the project Ploben (grant ANR-05-POGM-004-01), the Rhône-Alpes Region, the Bureau des Ressources Génétiques and the Agence Française de Sécurité Sanitaire de l'Environnement et du Travail for the projects AntiReSol EST-2006/1/44 and Gestions Biologique et Sociale de la Dispersion des Résistances aux Antibiotiques EST-2007-1.

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