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SHORT COMMUNICATION

NirK and *nirS* Nitrite reductase genes from non-agricultural forest soil bacteria in Thailand

Monnat Theerachat · Chompunuch Virunanon · Suphang Chulalaksananukul · Nusara Sinbuathong · Warawut Chulalaksananukul

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Abstract The genetic heterogeneity of the nitrite reductase gene (*nirK* and *nirS)* fragments from denitrifying prokaryotes in a non-agricultural forest soil in Thailand was investigated using soil samples from the Plant Germplasm-Royal Initiation Project area in Kanchanaburi Province, Thailand. Soil bacteria were screened for denitrification activity and 13 (from 211) positive isolates were obtained and further evaluated for their ability to reduce nitrate and to accumulate or reduce nitrite. Three species with potentially previously unreported denitrifying activities were recorded. Analysis of the partial *nirK* and *nirS* sequences of these 13 strains revealed a diverse sequence heterogeneity in these two genes within the same environment and even potentially within the same host species, the potential existence of lateral gene transfer and the first record of both *nirK* and *nirS* homologues in one bacterial species. Finally, isolates of two species of bacteria (*Corynebacterium propinquum* and *Micrococcus lylae*) are recorded as denitrifiers for the first time.

Keywords Denitrifying bacteria · Genetic diversity · Nitrite reductase gene · Nitrite reductase ability

M. Theerachat · C. Virunanon · W. Chulalaksananukul (✉)
Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
e-mail: warawut.C@chula.ac.th

S. Chulalaksananukul
Department of Chemical Engineering, Faculty of Engineering, Mahidol University, Salaya Campus, Nakornpathom 73170, Thailand

N. Sinbuathong
Scientific Equipment Center, Kasetsart University Research and Development Institute, Kasetsart University, Bangkok 10900, Thailand

Introduction

Denitrification is an anaerobic process, occuring only in low-oxygen or anoxic environments and consists of four reaction steps by which nitrate is reduced to dinitrogen gas sequentially by the four metalloenzyme classes of nitrate reductases, nitrite reductases, nitric oxide reductases and nitrous oxide reductases. The nitrite reductase is the key enzyme of this respiratory process since it catalyses the reduction of soluble nitrite into gas (Zumft 1997). Two types of nitrite reductase, that differ in terms of their structure and prosthetic metal, have been characterized: a copper nitrite reductase (EC 1.7.99.3) encoded by the *nirK* gene and a cytochrome cd_1-nitite reductase (EC 1.7.2.1) encoded by the *nirS* gene that is the key enzyme and forms the distinguishing feature between denitrifiers and nitrate reducers (Henry et al. 2004).

Denitrifying microorganisms span a wide range of taxonomic groups including over 50 different genera and are not defined by a close phylogenetic relationship. Thus a phylogenetic approach involving simply the species identification, such as by the 16S rRNA gene, to detect denitrifying microorganisms in the environment is not suitable (Santoro et al. 2006; Heylen et al. 2006) Therefore, an alternative approach has been to amplify denitrifying bacteria *nir* gene fragments by PCR amplification. To this end, degenerate *nirK* and *nirS* primers have been applied to identify denitrifiers, and to study the denitrification diversity and community structures in various environments (Braker et al. 2000; Priemé et al. 2002; Rich et al. 2003; Wang and Skipper 2004; Santoro et al. 2006; Oakley et al. 2007).

However, the research into variation and genetic analysis of the nitrite reductase genes of denitrifying bacteria in Thailand is limited, despite the diverse geochemical soil

arrays and generally high but fragmented biodiversity in this region. In this study, the relationship between the nitrite reductase encoding genes (*nirK* and *nirS* homologs) and the in vitro ability to reduce nitrate was investigated by screening soil bacteria collected from the Plant Germplasm-Royal Initiation Project at Kanchanaburi Province, Thailand, for their denitrifying ability and then assaying for the presence of *nirK* and *nirS* homologs by PCR with degenerate primers.

Materials and methods

Denitrifying bacteria isolation and nitrite-nitrate reducing ability test

Soil samples (500 g) were collected at a depth of 15 cm below the undisturbed surface. Soil bacteria were screened and then the isolation of denitrifying bacteria was performed under anaerobic conditions by plating soil bacteria after dilution onto nutrient agar (Schalau) at 30°C. All bacterial isolates were transferred separately into individual Durham tubes containing nitrate broth and incubated at 30°C for 14 days in an anaerobic chamber (Bactron/Sheldon, US). Samples from each culture were collected at day 0 and 7, respectively. The nitrate reduction ability was analysed by using the alpha-naphthylamine method (Beishir 1996). The likely species identification of all denitrifying bacteria was performed using the API test kit according to the manufacturer's conditions (BioMerieux). Then denitrifying bacteria were cultivated in nutrient broth at 30°C for 18–24 h. One ml of cell suspension was transferred to 150 ml of nitrate broth and incubated in an anaerobic chamber at 30°C with shaking at 200 rev/min for 7 days. Nitrate and nitrite levels were measured at day 0 and 7 using a nitrate–nitrogen and nitrite–nitrogen test kit (HACH, USA), respectively, as per the manufacturer's instructions.

DNA extraction, PCR amplification, cloning, sequencing and phylogenetic analysis

Genomic DNA was extracted from the cultured bacteria as described by Ausubel et al. (2002). Fragments of the *nirK* and *nirS* genes were amplified using the primer pairs nirK1F-nirK5R for *nirK* and nirS1F-nirS6R for *nirS* (Braker et al. 1998) in a final volume of 50 µl containing 5 µl of 10× PCR buffer 200 µM of deoxyribonucleoside triphosphate 1.0 U of *Taq* polymerase 1 µM of both primers, 400 ng of bovine serum albumin μl^{-1} and 10–100 ng of extracted gDNA. PCR conditions consisted of 1 min at 95°C, followed by a 1 min primer-annealing step and 1 min at 72°C. After 35 cycles, a final 7 min incubation at 72°C was performed. Expected amplicons were cloned using the PCR-Script™ Amp Cloning Kit (Stratagene, USA), and transformant selection was performed according to the manufacturer's instructions. Selected transformants were then screened for the correct plasmid inserts by nested PCR, using two sets of primers (Braker et al. 1998). Plasmids were extracted from transformants that contained the desired inserts by the Fast Plasmid™ Mini kit (Eppendorf, USA). DNA sequencing was performed commercially by pyrosequencing at Macrogen, Korea.

For each of the two gene fragments, the sequences were "blasted" and aligned by the National Center for

Table 1 Species identification, PCR amplification of nitrite reductase genes, and the percent nitrate reduction by day 7 of culture of the 13 selected isolates of denitrifying bacteria

Isolate	Organism	Nitrite reductase gene	% Nitrate reduction by day 7	Amount of nitrate reduction (mg/l)	Amount of nitrite accumulated (mg/l)
C22-18	*Agrobacterium radiobacter*	*nirK*	22.41	65	156.0
C23-5	*Agrobacterium radiobacter*	*nirK, nirS*	46.55	135	81.1
C23-15	*Agrobacterium radiobacter*	*nirK*	29.31	85	141.0
C32-7	*Agrobacterium radiobacter*	*nirK*	19.64	55	173.8
C32-13	*Agrobacterium radiobacter*	*nirK*	2.5	7	158.0
C33-1	*Agrobacterium radiobacter*	*nirK*	21.43	60	173.3
C22-14	*Burkholderia capacia*	–	58.93	165	75.0
A31-18	*Corynebacterium propinquum*	–	97.32	171.5	1.18
C32-5	*Micrococcus lylae*	*nirS*	34.48	100	137.5
C32-6	*Micrococcus lylae*	*nirS*	56.90	165	147.2
B11-3	*Pseudomonas aeruginosa*	*nirS*	99.14	287.5	0.2
C22-5	*Pseudomonas aeruginosa*	–	62.5	175	61.2
C22-24	*Pseudomonas stutzeri*	–	67.24	195	6.95
Control			0.00	ND	ND

Biotechnology Information BLASTN program and the ClustalX programmes, respectively. Phylogenetic analysis was performed with a neighbor-joining algorithm and distance calculation according to Jukes and Cantor using PAUP4.0*b. *Neisseria gonorrhoeae aniA* gene (accession no. M97926) and the *nir*N gene from *Pseudomonas aeruginosa* (accession no. D84475) were used as out groups for *nir*K and *nir*S, respectively. The tree topology was evaluated by bootstrap analysis using 1,000 replicates. ClustalX programs, respectively. Phylogenetic analysis was performed with a neighbor-joining algorithm and distance calculation according to Jukes and Cantor using PAUP4.0*b.

Results and discussion

Denitrification bacteria and the nitrate–nitrite reduction ability

From the 211 soil bacteria isolates, 13 were found to be capable of denitrification and were then evaluated for the ability to reduce nitrate, and to accumulate or reduce nitrite according to the method described by Wistreich and Lechtman (1988). These 13 bacterial isolates with denitrifying activity were classified as six species by the API test results (Table 1). API kit was selected primarily to

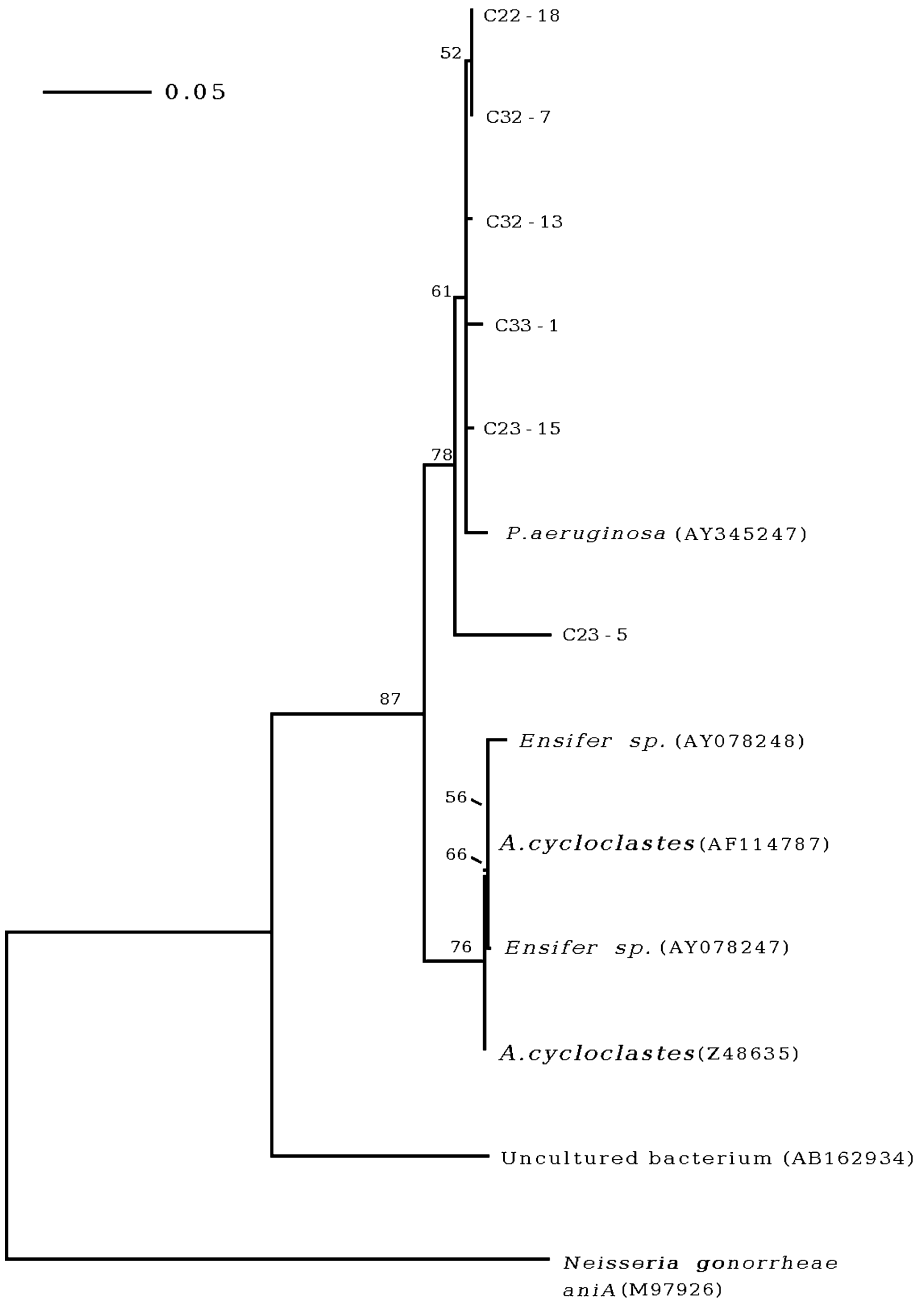

Fig. 1 A rooted neighbor-joining phylogram of the partial *nirK* sequences. The *scale bar* indicates the expected number of changes per sequence position. For each node, bootstrap values greater than 50% are shown. The GenBank accession number is indicated in *brackets* after the name of the organism. The sequence of *aniA* from *Neisseria gonorrhoeae* (accession no. M97926) was used as the out group to root the phylogram

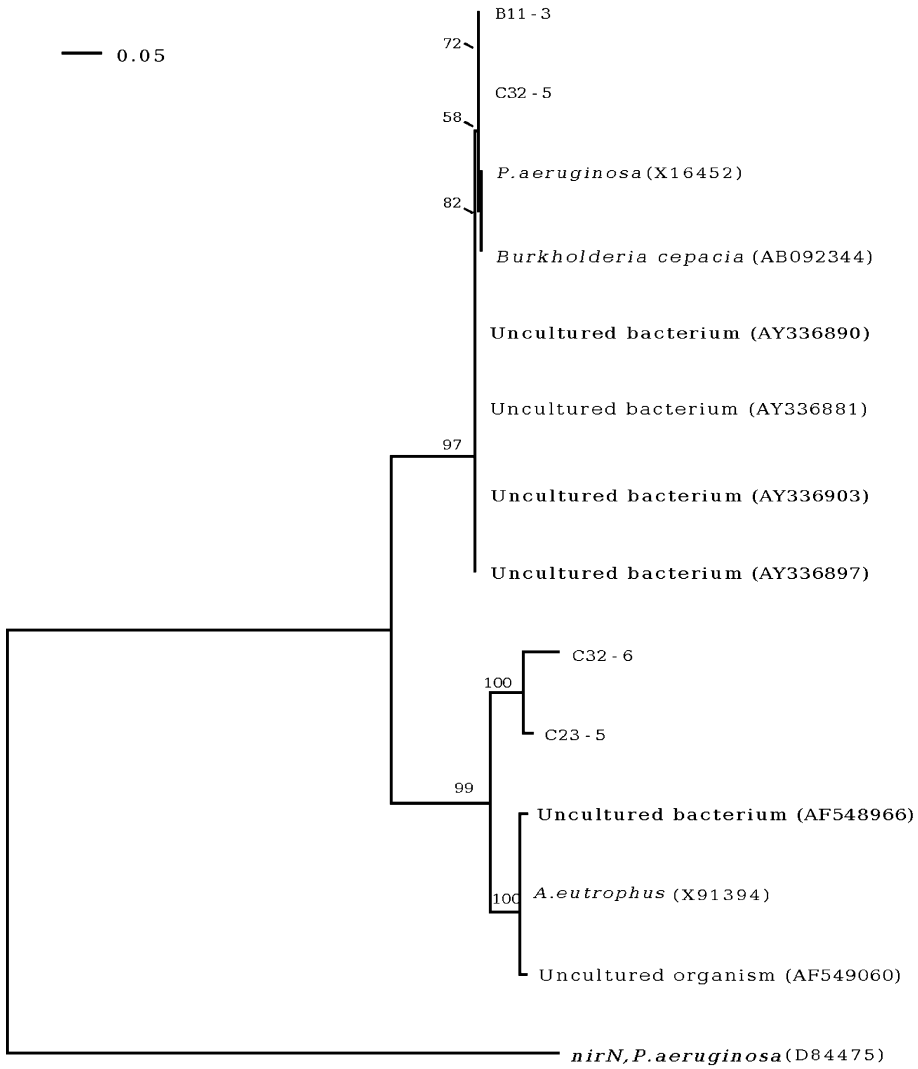

Fig. 2 A rooted neighbor-joining phylogram of the partial *nirS* sequences. The *scale bar* indicates the expected number of changes per sequence position. For each node, bootstrap values greater than 50% are shown. The GenBank accession number is indicated in *brackets* after the name of the organism. The sequence of *nirN* from *Pseudomonas aeruginosa* was used as the out-group to root the phylogram

identify genus in our samples since it can be used for general purposes for genus identification in vary groups of environmental bacteria since the originally widely used 16S rDNA primers may not be suitable for specific or some small groups of bacteria (see comment in Wang and Qian 2009). Three of the isolates have previously been identified as denitrifiers; *Agrobacterium radiobacter*, *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* (Braker et al. 1998; Hallin and Lindgren 1999; Wang and Skipper 2004). The other three isolates, *Burkholderia cepacia*, *Corynebacterium propinquum* and *Micrococcus lylae* are thus identified as potentially new species of denitrifiers in this study, although alternative means of species identification for conformation, such as phylogenetic analysis of the 16S rRNA gene, should be performed. *Agrobacterium radiobacter* was the dominant species of the denitrifying bacteria from the collected soil, in as much as 6/13 isolates were from this species (Table 1). For the ability to reduce both nitrate and nitrite, two isolates were found to reduce nitrate rapidly (*Pseudomonas aeruginosa*, B11-3 and *Corynebacterium propinquum*, A31-18) and one isolate could do it but slowly (*Burkholderia cepacia,* C22-14). These three isolates did not show any accumulation of nitrite, while the others ten isolates were found to lead to nitrite accumulation and also to reduce nitrate slowly.

DNA extraction, PCR amplification, cloning, sequencing and phylogenetic analysis

The thirteen isolates of denitrifying bacteria were then screened for the presence of the cd_1- and Cu-*nir* genes (*nirS* and *nirK*, respectively) by PCR amplification using degenerate primers as outlined under the methods section. Five isolates yielded only *nirK* amplicons (all *Agrobacterium radiobacter*) while three isolates produced only a *nirS* amplicon. One isolate (*Agrobacterium radiobacter* isolate C23-5) apparently had both *nirK* and *nirS* genes, and four isolates failed to produce an amplicon for either of the two

genes (Table 1). For those isolates producing amplicons of the expected size for nirK1F-nirK5R (514 bp) and nirS1F-nirS6R (890 bp), the amplicons were cloned and sequenced.

The partial consensus *nirK* and *nirS* nucleotide sequences from all isolates were aligned with the existent *nirK* and *nirS* sequences of denitrifying bacteria in the GenBank database. For *nirK*, this revealed 92–98% nucleotide sequence identity to the *nirK* of *Pseudomonas aeruginosa* (accession no. AY345247), whilst the *nirS* sequences revealed 92–99% sequence identity, confirming their likely correct identity as *nirK* and *nirS* gene homologues. However, to confirm gene function and structure, complete sequence analysis and protein expression will be performed next. Phylogenetic analysis separated the five isolates which contained *nirK* into one group, as expected given that they are the same bacterial species (*A. radiobacter*) from the same region (Fig. 1). However, this clade also included *P. aeruginosa*, suggesting either a highly conserved gene fragment (homoplasy from insufficient informative characters) or recent lateral gene transfer. Interestingly, the *nirK* sequence of isolate C23-5 which showed a lower sequence identity (92%) to the others in this group, although still in the same closely related group as the other *A. radiobacter* and *P. aeruginosa*, was placed distal to these in its own subclade (Fig. 2). Indeed, this close phylogenetic distance for the *nirK* homologs between *A. radiobacter* and *P. aeruginosa* is not supported by the *nirS* sequences (Fig. 2), although it is not possible to infer lateral gene transfer over artifacts from homoplasy. The *nirS* sequences separated into two main groups. The first consisted of isolates B11-3 (*Pseudomonas aeruginosa*) and C32-5 (*Micrococcus lylae*), along with existent sequences in the database from uncultured bacteria, *B. cepacia* and *P. aeruginosa*. The second well separated group consisted of the published sequences plus a different *Micrococcus lylae* isolate (C32-6) and a sole *Agrobacterium radiobacter* isolate (C23-5). The two isolates of *Micrococcus lylae* segregated into the two different well separated groups, again suggesting potential lateral gene transfer but this needs further work for confirmation including molecular species confirmation.

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