DNA-Probing for Genes Coding for Denitrification, N_2-Fixation and Nitrification in Bacteria Isolated from Different Soils

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Bacteria isolated from different layers of four soils of the Cologne area were analyzed for denitrifying, nitrifying and N_2-fixing isolates by colony hybridization using gene probes. In the soils tested, the percentage of denitrifying bacteria among the total population isolated was 3–8% (in one case exceptionally 15%) and thus small. Denitrifying bacteria were particularly enriched in the upper layer (depth ~ 5 cm) and were present only in low amounts at 25 cm depth in two gleysol soils. Nitrate apparently did not determine the distribution of denitrifying bacteria in these soils. The potential denitrification activity of different soil layers coincided with the distribution pattern of isolates assessed by DNA-probing. The total number of bacteria and of denitrifying isolates was considerably higher in or at the roots of plants than in the bulk, root-free soil adjacent to the plants. The percentage of the isolated aerobic N_2-fixing bacteria varied between 0–3%, and these bacteria could be isolated mainly from the upper 5 cm layer. A small portion of the isolates hybridized with the probe coding for part of one subunit of ammonia monooxygenase from *Nitrosomonas europaea.* The investigation showed that DNA-probing can provide useful information about the relative distribution of denitrifying and N_2-fixing bacteria in different soils and their layers.

Introduction

Soil microorganisms are essential contributors to the global atmospheric changes of nitrogenous compounds. Estimates indicate that 8–32% of the total nitric oxide and more than 50% of nitrous oxide annually released to the atmosphere come from soils (Skiba *et al.,* 1993). Nitrous oxide is about 180fold more efficient than CO_2 in contributing to the greenhouse effect (Hardy and Eaglesham, 1995), and NO significantly acts in destroying the O_3-layer of the stratosphere in complex reactions (Crutzen, 1979). In soils, nitrous oxide is mainly produced by denitrifying bacteria, whereas the nitrifying microorganisms, particularly the heterotrophic species, are said to be the main source for NO-production (Anderson and Levine, 1986). It is also stated that N_2-fixation by microorganisms grossly compensates the global losses of nitrogen caused by denitrification and nitrification (Postgate, 1982).

Little is known about the distribution of denitrifying, nitrifying and N_2-fixing bacteria in soils, mainly due to the fact that soil structures are inhomogenous and often highly variable. Moreover, only a percentage of soil bacteria can be isolated and grown under laboratory conditions. Physical parameters like temperature, pH, soil texture and moisture determine the activities, and those conditions can hardly be mimicked in the laboratory. Field studies are numerous, but can generally provide little informations about the relative contributions of bacteria in denitrification, nitrification and N_2-fixation. Recently, molecular biological tools have been introduced to obtain additional information on the subject. Although the techniques are not so far developed to assess the distribution of bacteria with these traits in the N-cycle by *in situ* methods, DNA-probing allows to screen for the occurrence of genes in small amounts of cells as recently demonstrated for isolates of the genus *Hyphomicrobium* (Kloos *et al.,* 1995). In a pilot study (Linne von Berg and Bothe, 1992), using fairly large probes with partially unspecific sequences, DNA probing of the bacteria isolated indicated that denitrifying bacteria predominantly live in or at the vicinity of roots, whereas non-denitrifiers were more enriched in the plant-free, bulk soil. For the present study, gene probes have

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been improved. Published sequences were screened for presumptive conserved regions by computer analysis. Oligonucleotide primers of such constant domains were synthesized which allowed to amplify gene segments of 400–850 bp length. Cloning and sequencing of these amplificates verified that segments of genes coding for denitrification enzymes, namely nitrate reductase (*narG*), cytochrome cd_1 (*nirS*) and Cu (*nirK*) containing nitrite reductases and the nitrous oxide reducing enzyme (*nosZ*), for the nitrification enzyme ammonia monooxygenase (*amoA*) and for the nitrogenase reductase (*nifH*) have been obtained. These probes have now been used to screen for the distribution of these genes in bacteria isolated from different layers of four soils in the Cologne area. The data obtained for denitrification show that DNA probing provides reproducible sets of data that largely match with profiles obtained by activity measurements.

Materials and Methods

Culture conditions for reference bacteria

The following microorganisms were grown in 100 ml Erlenmeyer flasks at 30 °C in the following media: *Aquaspirillum*, *Azospirillum*, *Herbaspirillum*, *Rhodospirillum* in AB supplemented with malate or sucrose (for the medium see Bothe *et al.,* 1981); bacteria of the Enterobacteriaceae, *Alcaligenes* and *Pseudomonas* in LB (Sambrook *et al.*, 1989), *Lactobacillus*, *Staphylococcus* in MRS (De Man *et al.,* 1960), *Ochrobactrum* in NB (8 g nutrient broth per liter), cyanobacteria in BG11 (Rippka *et al.,* 1979), *Arthrobacter*, *Bacillus*, *Rhodococcus* in PF (Papen *et al.,* 1989), rhizobia in YEM (Vincent, 1971), *Acetobacter* in LG1 (A. Hartmann, D-Neuherberg, personal communication, see Kloos, 1996), *Rhodobacter* in YPS (Weaver *et al.,* 1975).

Preparation of genomic DNA from the reference cultures

Liquid cultures (50 ml, optical density at 620 nm ~1.5) were centrifuged (10 min, 5 000×*g*). The pellet was suspended in 5 ml 1×TE-buffer (10 mM tris[hydroxymethyl]amino-methane (Sigma)/HCl pH 8, 1 mM EDTA) containing 5 mg/ml lysozyme and incubated (2 h, 37 °C). After adding 1 ml 10% SDS, gently shaking, and incubation (2 h, 68 °C), the lysate was extracted with TE-buffered phenol followed by two extractions with chloroform. 300 µl of 5 M Na-acetate pH 7.0 and 10 ml of 96% ethanol was added to the aqueous phase, mixed and incubated (1 h at −20 °C). After centrifugation (15 min, 12 000×*g*), the pellet was washed with 70% ethanol and dried in a vacuum centrifuge. DNA was subsequently suspended in 100 µl water.

Southern hybridization of genomic DNA

Genomic DNA (1–2 µg) was restricted with *Eco*RI (GIBCO Brl, D-Eggenstein), separated in an 0.8% agarose gel and transferred to a nylon$^+$-membrane (Qiagen, D-Hilden). Hybridization was performed at 68 °C with 200–300 ng labeled probe per 100 cm^2 membrane in the mixture 5×SSC, 0.5% blocking reagent, 0.1% Na-laurylsarcosine, 0.002% SDS overnight. Filters were then rinsed 2× at 68 °C with 2×SSC, 0.1% SDS for 10 min. Immunological detection with the non-radioactive hybridization kit (Boehringer Mannheim) followed standard protocols (Kloos *et al.,* 1995). The labeling of the gene probes with digoxigenin has been described (Kloos *et al.,*1995). The oligonucleotide used as universal rRNA probe for eubacteria (Amann *et al.,* 1991) was labeled with the dig-oligonucleotide 3′-end labeling kit (Boehringer Mannheim) substituting dig-dUTP by the dig-labeling mix.

Probes used

For the present study, the following gene segments were amplified by PCR, cloned, sequenced and subsequently used for DNA-probing:

a) *narG* (dissimilatory nitrate reductase, Mopterin binding subunit): a 414 bp amplificate of DNA from *E. coli* K12, showing 100% sequence identity, both on the DNA and the amino acid level, to the published sequence (McPherson *et al.,* 1984; EMBL accession no. X15181).

b) *nirS* (cytochrome cd_1 containing nitrite reductase): a 596 bp segment from *Pseudomonas aeruginosa* DSM 6195, showing 100% identity to the sequence published for the same segment from *P. aeruginosa* NCTC 6750 (Silvestrini *et al.,* 1989; X16452).

c) *nirK* (Cu containing nitrite reductase): a 576 bp segment from *Alcaligenes xylosoxidans* NCIMB 11015, showing 68% identity on the DNA basis and 67% on the amino acid level to the sequence published for *Alcaligenes faecalis* S-6 (Nishiyama *et al.,* 1993; D13155).

d) *nosZ* (N_2O-reductase): a 598 bp segment from *Pseudomonas stutzeri* ZoBell, showing only an identity of 88% (DNA) or of 91% (amino acid basis), respectively, to the sequence published for *nosZ* from the same organism (Viebrock and Zumft, 1988; M 22628) for unknown reasons.

e) *amoA* (C_2H_2-binding subunit of the ammonia monooxygenase): a 820 bp segment from *Nitrosomonas europaea,* showing 95% (DNA), or 98% (amino acid) identity to the published sequence from the same organism (McTavish *et al.,* 1993; L08050).

f) *nifH* (nitrogenase reductase): a 450 bp part of this nitrogenase gene of *Azospirillum brasilense* Sp7, showing 100% identity to the published sequence of the same organism (De Zamaroczy *et al.,* 1989; L08050).

g) a general probe of 16S-rRNA with the sequence 5′-GCTGCCTCCCGTAGGAGT-3′ (Amann *et al.,* 1991), to quantify the release of DNA from the eubacteria lysed.

The details for obtaining the probes, in particular the primers used, have been described in a previous publication (Kloos *et al.*, 1995).

Isolation of bacteria from soil samples

Soil (10 g) was suspended in 10 ml sterile water, stirred for 1 h, and larger particles were removed with a sieve (0.5 μm pore size). The more or less homogenous sieved material was plated in a dilution series from 10^{-2}–10^{-4} onto agar. For each sample to be investigated, the following media and incubation times were used: LB: 10 g tryptone, 5 g yeast extract, 10 g NaCl/l – 1 d; YEM: 10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \times 7\ H_2O$, 0.1 g NaCl, 0.4 g yeast extract/l, pH 6.8–5; heterotrophic MIN: 200 mg $MgSO_4 \times 7\ H_2O$, 100 mg NaCl, 20 mg $Na_2MoO_4 \times 2\ H_2O$, 20 mg $CaCl_2$, 10 mg $MnSO_4 \times H_2O$, 265 mg NH_4Cl, 784 mg $K_2HPO_4 \times 3\ H_2O$, 613 mg KH_2PO_4, 138 mg $FeSO_4 \times 7\ H_2O$, 186 mg EDTA, 2 g malate, 2 g sucrose/l, pH 7.0–5 d. All media were supplemented with 250 mg/l cycloheximide to prevent fungal growth. Plates were incubated at 30 °C. After determining the cell number, colonies were picked and transferred to fresh plates.

Colony hybridization of the bacteria from the soils

Colonies grown on the agar plates in the different media LB, YEM, heterotrophic and autotrophic MIN were transferred to sterile microtiter plates (Greiner, D-Solingen) and grown in 250 μl of the appropriate liquid medium in the wells of the plates as suspension cultures. Triplicate samples were assayed from each soil layer and medium. As each plate contained 96 wells, 288 colonies for each trial were grown up. Of these colonies, 250 (on the average) had both an optical density at 620 nm > 0.1 after growth and hybridized with the 16S-rRNA probe. Therefore, for each medium and soil layer, 250 isolates were analyzed by colony hybridization (Grunstein and Hogness, 1975) with each of the six probes *narG, nirS, nirK, nosZ, amoA,* and *nifH*. The 250 isolates were blotted as 50 μl aliquots onto nylon$^+$-membranes (Qiagen, Hilden) using a vacuum blotting apparatus. Filters were incubated on denaturing solution (0.5 M NaOH, 1.5 M NaCl, 0.1% SDS, 15 min), then on neutralizing solution (1 M Tris/HCl pH 7.5, 1.5 M NaCl, 5 min), and on 2×SSC (0.3 M NaCl, 0.03 M sodium citrate, 15 min) followed by treatment with UV for 2 min. Filters were shaken in washing buffer (3×SSC, 0.1% SDS) at 68 °C overnight, treated with proteinase K (20 μg/ml in 10 mM Tris/HCl buffer, pH 7.0, 1 mM EDTA, 0.1% SDS) at 50 °C for 1 h and then with PMSF (40 μg/ml in water) at room temperature for 5 min. Finally, the filters were rinsed twice with 2×SSC and stored in dryness. The hybridization conditions were the same as for the Southerns.

Determination of soil moisture, pH, nitrate and nitrite concentrations

Soil moisture was calculated from the dry weight of the soil. Aqueous soil extracts served to determine the pH using a PHM 61 laboratory pH meter electrode (Radiometer, Copenhavn). The concentrations of nitrate (Cataldo *et al.,* 1975) and nitrite (Snell and Snell, 1951) were determined colourimetrically in the same extract.

Parameters of the four soils investigated

These are listed in Table I.

Activity measurements

Fernbach flasks (~7.0 ml) were filled with 5 g soil and covered with gas-tight suba seals. Respiratory activity was determined by O_2-consumption or CO_2-production under aerobic conditions. Denitrification activity was determined by the formation of N_2O under argon supplemented with C_2H_2 (~15% v/v). All incubations were done at 30 °C for 1 d, and the gas chromatographic determinations were performed as before (Kloos *et al.*, 1995).

Results

Use of the gene probes for hybridization with DNA from control organisms

Southern hybridizations were performed with the gene probes for denitrification, nitrification and N_2-fixation (see Materials and Methods) and with DNA from many systematically unrelated bacteria of three subgroups of proteobacteria, Gram-positive bacteria, and cyanobacteria to reveal the range of the probes for recognizing these genes in microorganisms (Table II). Hybridizations with all probes had to be performed at the high stringency of 68 °C and the buffer concentrations given in Materials and Methods to avoid unspecific bands or smear. The nitrogenase probe *nifH* hybridized with DNA from almost all N_2-fixers of unrelated affinities, indicating that the *nifH* probe can serve as a general tool to recognize this trait in bacteria. Likewise, among the denitrification genes, the data obtained from probing with *nosZ* (for N_2O-reductase) and *nirK* (for Cu nitrite reductase) generally matched with known activity profiles in the control organisms. The *nirS* (cytochrome cd_1 nitrite reductase) probe hybridized only with DNA from few bacteria, but not in the case of *A. brasilense* Sp 59b or *Pseudomonas stutzeri* ZoBell which possess this enzyme (Coyne *et al.*, 1989; Jüngst *et al.*, 1991). Likewise, probing with *narG* (nitrate reductase) was more narrow ranged. Signals were obtained with DNA samples from α, β and γ proteobacteria and even from Gram-positive bacteria, but not from azospirilla and rhizobia (Table II). However, taking the signals with the four denitrification probes together, clear and unequivocal signals were obtained with at least one of the probes (and in most cases with more than one) with almost all denitrifying bacteria tested, indicating that the denitrification probes in sum can identify the genetic capability of a bacterium for denitrification. The nitrification probe *amoA* gave distinct signals with DNA from some autotrophic nitrifiers (Limburg, 1996) and from the heterotrophic nitrifying isolate of *Pseudomonas putida* (unpublished results of M. Daum, Garmisch, see Table II). Since autotrophic nitrifying

Table I. Characterization of the four soils investigated.

Parameter	Chorbusch			Worringer Bruch			Biesberg			Hühlesberg		
Soil depth [cm]	5	10	25	5	10	25	5	10	25	5	10	25
Location	L 4906			L 4906			L 5304			L 5506		
Soil type	gleysol alluvial			gleysol recent sediments of the Rhine			rendzina triadic chalk Muschelkalk			rendzina middle devonic "Givet period"		
Vegetation	oak-horn-beam forest			willow-elder forest			chalk meadow			chalk meadow		
pH	4.2	4.3	4.3	5.9	6.2	6.8	8.8	8.8	xxx	8.4	xxx	xxx
Nitrate content	304	245	191	809	50	194	53	244	xxx	8.0	xxx	xxx
[nmol/g dw]	±150	±141	±72	±358	±22	±150	±32	±349	xxx	±3	xxx	xxx
Nitrite content	17	4	3	23	13	24	277	179	xxx	5	xxx	xxx
[nmol/g dw]	±10	±1	±1	±12	±14	±22	±88	±130	xxx	±2	xxx	xxx

dw = dry weight; xxx could not be determined because this layer consisted of stones in this rendzina type soil. Locations: The numbers refer to the maps (1:50000) of the "Landesvermessungsamt Nordrhein-Westfalen", L 4906 = Neuss for Chorbusch, located between Pulheim and Worringen, left side of the Rhine valley and also for Worringer Bruch, close to the village Worringen, L 5304 = Zülpich for Biesberg close to the village Muldenau and L 5506 = Bad Münstereifel for Hühlesberg, close to the village Iversheim. Each soil was analyzed for bacterial content (DNA-probing) at least three times.

Table II. Southern hybridizations of the gene probes with DNA isolated from reference organisms.

Strain	Denitrification				Nitrification	Nitrogen-fixation
	narG	*nirS*	*nirK*	*nosZ*	*amoA*	*nifH*
Proteobacteria						
α-subdivision						
Acetobacter diazotrophicus PAL 5	−		+		−	+
Azospirillum amazonense Y-1	−	−	−	−		+
A. brasilense Sp 7	−	+	−	+		+
A. halopraeferens AU4	−	−	−	+		+
A. irakense KA3	−	−	+	−		+
A. lipoferum Sp 59b	−	−	−	+		+
Hyphomicrobium hollandicum KB-677	+	−	−	−	−	−
H. zavarzinii ZV-622	+	−	+	+	−	+
Ochrobactrum anthropi LMG 3331	+	−	+	−	−	−
O. anthropi LMG 3333	+	−	+	+	−	−
Azorhizobium caulinodans ORS 572	+	−	−	−		+
Bradyrhizobium japonicum DES 122	−	−	−	−		−
Rhizobium leguminosarum LMG 8897	−	−		−		+
R. meliloti 21015	−	−	+	+		+
R. phaseoli CIAT 883	−	−	−	−		+
R. trifolii ANV843	−	−	−	−		+
Rhodobacter capsulatus B10	−					+
Rhodospirillum rubrum S1	−	−	−	−		+
β-subdivision						
Aquaspirillum autotrophicum SA32	−	−	−	+	+	
Alcaligenes eutrophus H16	+	+	−	+	−	−
A. faecalis A15	+	+	−	+	−	+
A- faecaöos	−	−	+	+	−	−
A. xylosoxidans NCIMB 11015	+	−	+	+	−	−
Herbaspirillum seropaedica HZ78	−	−	+	−		+
γ-subdivision						
Enterobacter agglomerans 339	+	−	−	−	−	+
E. cloacae NCIMB 11463	+	−	−	−	−	+
Escherichia coli K 12	+	−	−	−	−	−
Klebsiella aerogenes DSM 681	+	−	−	−	−	−
K. oxytoca DSM 3539	+	−	−	−	−	+
Pseudomonas aeruginosa DSM 6195	+	+	−	+	−	−
P. aeruginosa DSM 50071	+	+		+	−	−
P. aureofaciens NCIMB 9030	−	−	+	−	−	−
P. putida	−	−		−	+	−
P. stutzeri ZoBell	+	−	−	+	−	−
Gram-positive bacteria						
high GC						
Arthrobacter sp 312		−	−		−	−
Micrococcus varians LTH 1430	−	−	−	−		
Rhodococcus rhodocrus	+	−	−		−	−
low GC						
Bacillus cereus		−	−		−	−
Lactobacillus farciminis LTH 1136	−	−	−	−		
Staphylococcus carnosus LTH 55	−	−	−	−		
Cyanobacteria						
Anabaena azotica	−	−	−	−	−	+
Anabaena cylindrica	−	−	−	−	−	+
Anabaena variabilis ATCC 29413	−	−	−	−	−	+
Anacystis nidulans 1402-1	−	−	−	−	−	−
Anacystis nidulans PCC 7942	−	−	−	−	−	−
Plectonema boryanum	−	−	−	−	−	+
Synechococcus sp 27144	−	−	−	−	−	−

Southern hybridization was performed with 1−2 μg genomic DNA, restricted with *Eco*RI. Hybridization conditions were described in Materials and Methods. + indicates that a distinct hybridization band was found, − no hybridization signal, a free space was left where hybridization was not performed.

bacteria can be grown under laboratory conditions only slowly, hybridizations were not systematically performed with the *amoA* probe and DNA from diverse nitrifying laboratory strains. Consequently, the hybridization data with this gene probe do not allow conclusion about the distribution in soil layers, but possibly give access to new groups of nitrifying bacteria.

Isolation of bacteria from different soil types of the Cologne area

Prior to the hybridization experiments with the gene probes, bacteria had to be isolated from four typical but rather different soil types of the Cologne area characterized in Table I. Samples were taken from 5, 10 and 25 cm depth in each soil (however, not possible in the rendzinas), extracted and plated on agar in the three different media LB, YEM and heterotrophic MIN to enrich a broad spectrum of bacteria. As expected, the number of cfu on such plates was largely dependent on the medium and the incubation time, as documented for the acid soil "Chorbusch" (Table III). After 1 d, colony numbers were highest and grossly the same in LB and YEM, and the counts for the cfu increased 2–5 fold by incubating for 5–7 d. In contrast, only few cfu were detected in the heterotrophic MIN after 1 d which increased by two orders of magnitude by incubating for 5–7 d. Visually, the colonies grown in the heterotrophic MIN were largely different from each other and also from those grown on LB and YEM. Initially, the enrichment was also tried in an autotrophic mineral medium. After 7 d, this treatment resulted in the formation of few small and more or less uniformous colonies which grew at best poorly in suspension cultures. Consequently, such isolates could not be taken for the hybridization experiments.

The number of cfu varied not more than twofold when replica platings from the same soil layer were counted (Table III). It should be noted that colony numbers were consistently highest in the upper layer (5 cm) with the three enrichment media LB, YEM and heterotrophic MIN. These counts decreased 2–5 fold in 10 cm depths. Further down at 25 cm the counts were only slightly lower than at the 10 cm layer. The pattern for the other three soils was similar as for the acid Chorbusch soil (Kloos, 1996).

Colony hybridizations of the bacteria isolated from the acid soil using the gene probes for denitrification, nitrification and N_2-fixation

Nearly 100% of the colonies from the plates could be grown in the LB medium in the microtiter plates, whereas 83% and 84% grew up in YEM and heterotrophic MIN, respectively. Hy-

Table III. The number bacteria isolated from the acid soil "Chorbusch" and grown in three different media.

Depth [cm]	Indubation-time [d]	Cell number [10^5 cfu/g dry weight] LB	YEM	MIN heterotrophic	MIN autotrophic
5	1	23.5 ± 27.3	16.7 ± 6.7	0.0 ± 0.8	<0.1 ± 0.0
	5	65.2 ± 34.5	51.5 ± 18.3	112.9 ± 22.6	17.7 ± 6.7
	7	–	–	91.8 ± 18.0	24.7 ± 16.5
10	1	8.7 ± 2.7	6.6 ± 2.6	<0.2 ± 0.2	<0.1 ± 0.0
	5	18.5 ± 8.1	24.0 ± 13.3	44.0 ± 35.4	9.7 ± 6.2
	7	–	30.1 ± 3.8	38.4 ± 12.0	19.4 ± 11.0
25	1	7.2 ± 4.6	4.7 ± 2.2	<0.1 ± 0.1	<0.1 ± 0.0
	5	9.8 ± 3.4	17.8 ± 10.5	31.0 ± 21.2	6.1 ± 3.1
	7	–	21.9 ± 13.6	13.4 ± 4.9	14.3 ± 5.9

The soil extract was diluted in a series from 10^{-2}–10^{-4} and 100 µl of each were plated onto agar with the different media. The cfu on the agar plates were counted after 1, 5 and 7 d and the cell number was determined from the average values of the dilution series. The data represent average values and their standard deviations from three different soil samples taken.

bridizations with the 16S r-RNA probe gave distinct signals with the isolates grown in LB in almost all cases (~90%), but only 80% with the YEM grown cells and 62% with the bacteria of the heterotrophic MIN medium (Fig. 1). The isolates grown up to an O. D. > 0.1 at 620 nm were taken for colony hybridization. Of these, the colonies hybridizing with the probes for denitrification from all media varied between 3–8% (Fig. 1) and maximally reached 15% in one case (Fig. 4). The scores with the *nifH* probe were even lower (~3%; Figs. 1 and 4). This general pattern showed only few exceptions with the different probes as tested in detail for the acid soil (Chorbusch). The *narG* probe gave twofold higher counts with YEM than with LB grown cells. The *nifH* probe did not hybridize with DNA isolates grown in LB, but did with those from YEM and from heterotrophic MIN. These more specific cases did, however, not play a role when the counts obtained with the cells from the three media were combined for comparing the distribution of denitrifying and of aerobically growing N_2-fixing bacteria in the different soil layers.

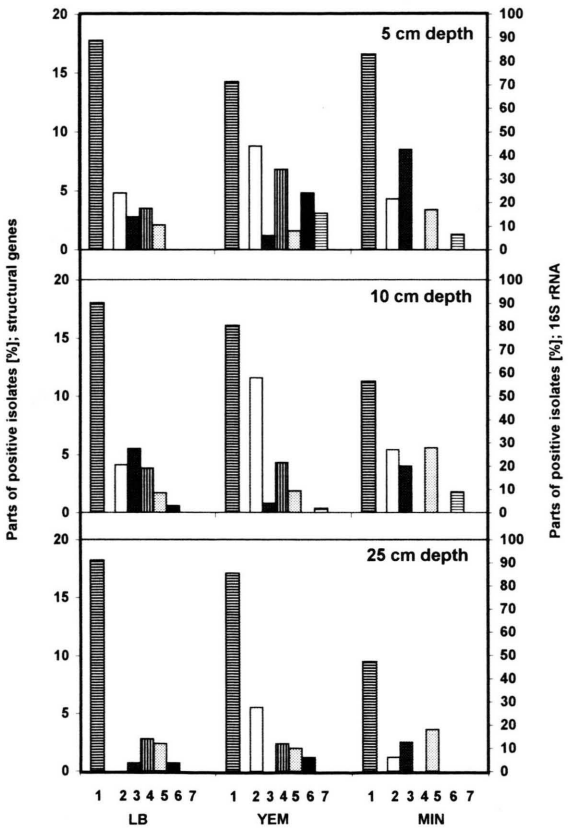

Fig. 1. The percentage of denitrifiying, nitrifying and N_2-fixing bacteria among the total population from the acid soil (Chorbusch) enriched in three different media. Bacteria were isolated from the a) 5 cm, b) 10 cm, c) 25 cm layer of the Chorbusch soil and grown on agar plates. Isolates were inoculated in microtiter plates in the media LB, YEM and MIN and analysed by colony hybridization using the probes: 1 = 16S rRNA, 2 = *narG*, 3 = *nirS*, 4 = *nirK*, 5 = *nosZ*, 6 = *amoA*, 7 = *nifH*. 100% refers to the total amount of bacteria which grew (OD 620 nm > 0.1) in one medium. For details see Materials and Methods.

In the acid soil (Chorbusch), in parallel with the higher number of isolates, the scores for the denitrifying bacteria were always highest in the upper layer (5 cm depth), irrespectively of the gene probe used (*narG, nirS, nirK* and *nosZ*) and independently of the enrichment medium. These scores decreased in parallel with the depth of the soil as documented for the sum of the bacteria isolated in the three media (Fig. 2a). In addition, a relative enrichment of bacteria, determined as percentage of denitrifying bacteria among the total population, was also observed in the upper zone (Fig. 2b). This result is somewhat unexpected since the concentration of nitrate was only approximately 1/3 lower in 25 cm than in 5 cm (Table I). Hybridizations with the *nifH* probe also indicated that such bacteria mainly occurred in the upper zone, although the positive scores were significantly lower than with the denitrification gene probes (Fig. 2a). A relative enrichment in the upper layer was seen for the *nifH* positive isolates (Fig. 2b). In sum, the percentage of bacteria having denitrification and N_2-fixation genes of the total population of isolates in the different layers of this acid soil and of the others (see Fig. 4) was fairly small. Some positive scores, particularly in the upper zone, were also obtained with the *amoA* probe (Fig. 2).

Measurements of potential activities

To have an estimate for the potential activities of the soil investigated, samples of the different layers were taken to the laboratory and assayed for O_2-consumption and CO_2-release in air (potential respiratory activity) and for N_2O-formation under argon in the presence of C_2H_2 (estimate for the denitrification rate). These activities proceeded linearly till 18 h. The rates for respiratory

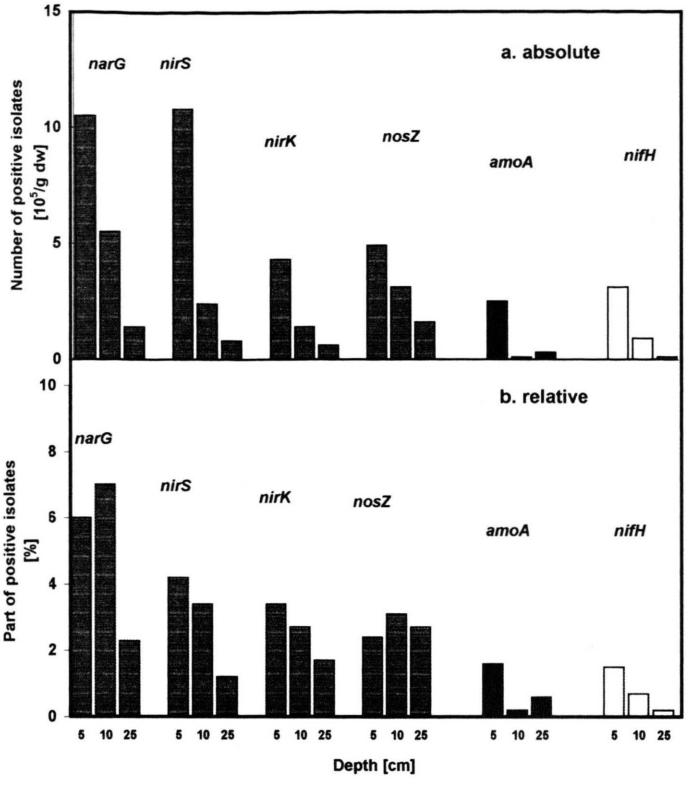

Fig. 2. The distribution of denitrifying, nitrifying and N_2-fixing bacteria in the three layers of the acid soil (Chorbusch). a) Absolute number of hybridizing isolates determined as colony forming units (cfu) in sum on all three media taken. b) Same data given as relative amount of bacteria with the special trait in the N-cycle. 100% refers to the total number of isolates which grew in the three media (maximal values). The gene probes are defined in Fig. 1 or Materials and Methods, d.w. = dry weight.

CO_2-release and O_2-consumption were essentially the same and were highest in the upper layer and lowest at 25 cm (Fig. 3). N_2O-formation activity showed the same activity profile in the three soil layers (Fig. 3). For the assays, the soil samples were not supplemented with nitrate or any other addition. Thus this pattern of the potential activity paralleled the distribution of denitrifying bacteria isolated from the layers of this soil.

Influence of plant roots on the distribution of bacteria

Bacteria isolated from the roots and the surrounding soil of three plants (*Oxalis acetosella, Deschampsia cespitosa, Galeobdolon luteum*) typically occurring in the forest "Chorbusch" were probed for the presence of isolates with denitrification and N_2-fixation genes. Bacteria from the roots of two individuals of each plant were analyzed, and the data obtained grossly matched with each other (Table IV). Extracts from roots of the plants were obtained by rinsing with tap water and by subsequently grinding them. The pH-value and

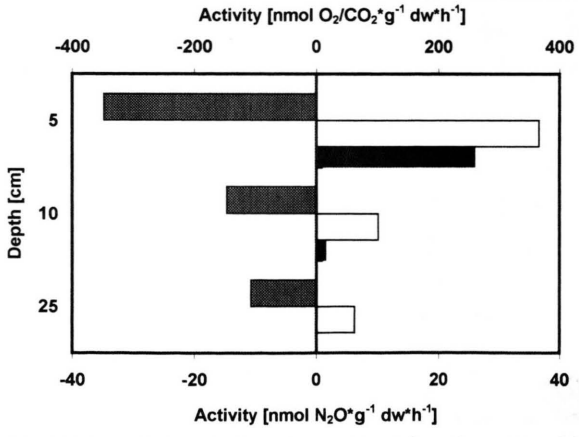

Fig. 3. Potential activities of samples taken from the different layers of the acid soil (Chorbusch). Soil samples were taken from 5, 10 and 25 cm depth. Activities were determined in 5 g samples assayed in 7.0 ml Fernbach flasks. ■ CO_2-release, aerobically assayed, □ O_2-uptake, aerobically assayed, ■ N_2O-formation in the presence of C_2H_2, under anaerobic conditions.

the water content significantly differed between the plants and also between the bulk, root free soil adjacent to them. Root extracts of *G. luteum* had

Table IV. Distribution of bacteria in plant roots of three selected plants and in the bulk, root-free soil at "Chorbusch".

Parameter	*O. acetosella*		*D. cespitosa*		*G. luteum*	
	Roots	Soil around the roots	Roots	Soil around the roots	Roots	Soil around the roots
Dry weight	29.9	76.7	63.3	87.0	50.5	79.5
[% of fresh weight]	*24.7*	*78.7*	*75.8*	*86.2*	*39.4*	*75.6*
pH-Value	4.3	6.0	5.4	5.3	6.2	5.7
(in the water extract)	*4.0*	*5.9*	*5.3*	*5.9*	*5.7*	*5.8*
Nitrate content	15.8	0.2	12.3	0.1	22.5	0.4
[µmol/g dry weight]	*13.1*	*0.2*	*8.4*	*0.1*	*43.7*	*0.4*
Nitrite content	0.12	0.0	0.03	0.0	0.25	0.0
[µmol/g dry weight]	*0.07*	*0.0*	*0.01*	*0.0*	*0.14*	*0.0*
Cell number						
[cfu $\times 10^5$/g dry weight]						
in LB (after 1 d)	84	9.0	36	10	246	13
	92	*7.5*	*26*	*17*	*72*	*17*
in YEM (after 5 d)	468	42	223	28	822	49
	276	*37*	*207*	*38*	*746*	*29*
in heterotrophic MIN	502	59	336	20	1121	39
(after 5 d)	*219*	*55*	*247*	*39*	*827*	*21*
Maximal number of bacteria hybridizing						
[cfu $\times 10^5$/g dry weight]						
with *narG*	36.3	0.8	22.4	0.3	30.4	2.4
	31.8	*4.4*	*0.3*	*5.1*	*11.2*	*9.7*
with *nirS*	0.0	0.8	0.0	0.2	35.6	0.1
	13.4	*0.7*	*0.0*	*0.0*	*77.6*	*0.7*
with *nirK*	73.7	5.2	29.9	0.2	46.4	2.4
	44.2	*2.3*	*24.8*	*2.1*	*101.5*	*3.7*
with *nosZ*	31.6	11.4	19.3	0.9	135	2.7
	41.5	*9.5*	*24.3*	*0.8*	*158*	*0.7*
with 1 of these 4 probes	148	17	52	2.5	248	6.5
	135	*15*	*35*	*6.5*	*270*	*14.5*
with *amoA*	0	0	0	0.5	29.2	1.1
with *nifH*	2.2	0	2.5	0.9	27.3	0

The soil of the forest "Chorbusch" dominated by *Quercus robur* and *Carpinus betulus* was analyzed for the occurrence of denitrifying, nitrifying and N_2-fixing bacteria. The roots of three typical weeds *Oxalis acetosella, Deschampsia cespitosa* and *Galeobdolon luteum* and the soil (a core of 20 x 20 cm and 10 cm depth) surrounding these plants were analyzed for the bacterial content. Data refer to g dry weight of soil or roots, respectively, and represent the sum of bacteria which could be grown up in the three media LB, YEM and heterotrophic MIN (= maximal number of bacteria). Two different individuals for each plants and their surrounding soils were analyzed in each case, the first line (in straight letters) is for one individal, and the second line (in italics) for the other. Samples were analyzed in October 1995.

the highest nitrate content (Table IV). With all plants tested, the absolute number of bacteria in such extracts was much higher than in the bulk, plant free soil, probably due to the better supply with organic carbon. The root extracts of *G. luteum* had the highest bacterial counts, whereas the numbers were roughly the same with the other two plants. In parallel with the absolute number, the positive scores with the denitrification probes were consistently higher in the roots than in the soil. In contrast to the absolute numbers, the percentage of bacteria with the denitrification genes was not enhanced in the roots, even not in the case of *G. luteum*. Thus denitrifying bacteria were not selectively enriched in the vicinity of the roots in this soil. The roots and the bulk soil contained rel-

atively few bacteria with positive scores with the *nifH* and *amoA* probe (Table IV).

The distribution of bacteria in three other soil types

Of the other three locations tested, the neutral soil "Worringer Bruch" (a gleysol) had the highest nitrate content, probably due to the sediments deposited with the periodical flooding of this soil by water from the Rhine surroundings. Denitrifying organisms could have a selective advantage in this soil under water-logged conditions. Indeed, the number of bacteria showing hybridization with the *nirK* and *nosZ*, but not with the *narG* and *nirS* probe, varied between 10–15% among the total population of bacteria (Fig. 4) and was thus highest in all soils tested. Thus bacteria with these genetic traits appeared to be enriched particularly in this soil. Also at this location, the highest number of N_2-fixing bacteria was isolated from the upper 5 cm layer (both as absolute counts and as relative percentage among the total amount of bacteria isolated). Of the two basic soils tested (both rendzinas), the one at Hühlesberg had relatively low numbers of denitrifying bacteria (Fig. 4). Percentages slightly higher than at other soils were obtained with the *amoA* and *nifH* probes at Biesberg (Fig. 4). Investigations at further sites have to be performed to see whether basicity of the soil, indeed, favours the growth of N_2-fixing bacteria and of microorganisms which show hybridizations with the *amoA* probe.

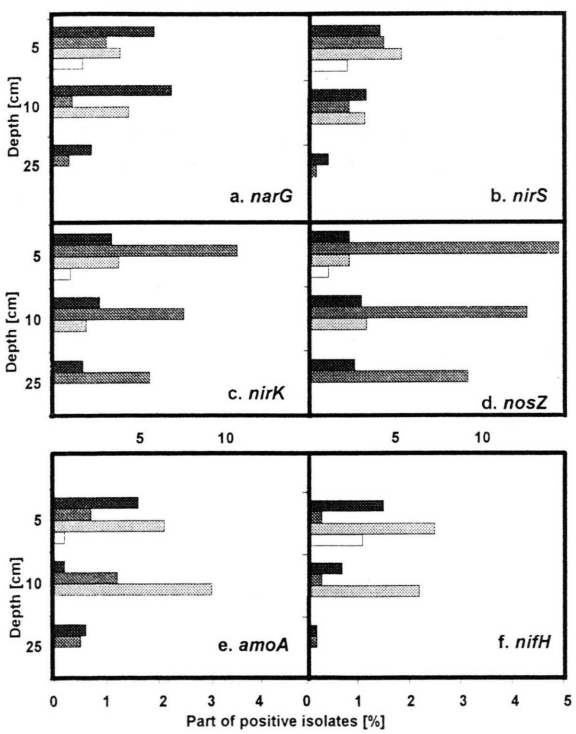

Fig. 4. The distribution of denitrifying, nitrifying and N_2-fixing bacteria in the layers of four different soils of the Cologne area. ■ Chorbusch (acid), ■ Worringer Bruch (neutral), ■ Biesberg (basic), und □ Hühlesberg (basic). 100% represents the total number of isolates which could grow in the three media LB, YEM or MIN.

Discussion

The gene probes developed for all steps of denitrification (with the exception of NO-reduction) and for N_2-fixation recognize a broad range of the corresponding genes in Gram-negative and other bacteria (Table II). *NifH* can serve as a general probe, in line with the fact that the sequences for this protein are very similar in the different organisms (Ruvkun and Ausubel, 1980). Among the denitrification genes, *nosZ* (for N_2O-reductase) and *nirK* (for Cu nitrite reductase) hybridized with DNA of a broad spectrum of bacteria, whereas *nirS* (for cytochrome cd_1 nitrite reductase) from *Pseudomonas aeruginosa* gave positive signals with DNA from some bacteria, but unexpectedly not from *Pseudomonas stutzeri* ZoBell and several others known to have this gene (Zumft, 1992). Other probes of this gene available in the laboratory (Kloos, 1996) were also developed from the C-terminal region and showed specificities, as also observed by others with similar gene probes (Smith and Tiedje, 1992; Ward *et al.*, 1993) or with antisera of cytochrome cd_1 containing nitrite reductase (Coyne *et al.*, 1989; Ward *et al.*, 1993). It has been reported (De Boer *et al.*, 1994) that the N-terminal region of this gene may offer better perspectives to develop a more broad-range gene probe for future experiments. *NarG* occurs in bacteria that perform denitrification and nitrate ammonification and is therefore less suitable for probing denitrification. It was advantageous in the present study to have more than one gene probe for denitrification since hybridization with one gene probe might underestimate the percentage of denitrifying bacteria within a population (Fig 4). However, around 20–25% of the bacteria gave

only faint hybridization signals which may or not have been denitrifiers. Thus the method employed does not allow the quantification of the total population of denitrifying bacteria in soils. However, it permits an assessment of the relative abundance of denitrifying, as well as N_2-fixing bacteria *among* different soils and their layers.

In the course of the present study, a gene probe for nitrification (*amoA*) has also been developed. The *amoA* gene is strongly conserved in ammonia oxidizing bacteria and shows sequence similarity to the *pmo* gene of methane oxidizers (Holmes *et al.,* 1995). The media and the culture conditions were not optimized for the growth of nitrifying bacteria. Therefore, the hybridization data obtained with this probe do not permit conclusions about the distribution of ammonia oxidizing bacteria in different soil layers to be drawn. Screening with this probe may lead to the identification of new nitrifiers or methane oxidizing bacteria. Recent findings (Moir *et al.,* 1996) suggest that the ammonia monooxygenase is not only restricted to autotrophic nitrifying bacteria.

Apart from the fact that the gene probes used lack absolute broadness, the present approach can be criticized for other reasons also. The percentage of bacteria which can be isolated and grown in media is unknown. Bacteria may too tightly adhere to be removed from soil particles or may be destroyed by the isolation procedure (Lindahl and Bakken, 1995). Soils are often inhomogenous, and the population of bacteria in soils may vary within short distances (Parkin, 1990). The colonies grown in the three media were assayed independently from each other and represent maximal values, since individuals may have been grown in more than one medium. For the minimal values (sum of the bacteria grown in the three media divided by three), the data were roughly 50% lower, but the differences in the relative distributions of bacteria between different soils or soil layers were the same (Hüsgen, 1996; Kloos, 1996). Testing of soil samples in the laboratory for maximal potential activities (Fig. 4) does not reflect the *in situ* situation, but gave clear-cut differences between the different layers, and the data obtained by these activity measurements surprisingly well matched with the profiles obtained from the DNA-probing.

Despite all the obstacles, the investigation is a start to characterize the soil microflora for structural genes by specific probes. Other methods, e.g. total extraction of the DNA from soil samples, are probably not quantitative also and are therefore similarly problematic to assess the distribution of bacteria with the special traits in the N-cycle in the soils (Smith and Tiedje, 1992; Ward *et al.*, 1993). In addition, the message which results from studies on the DNA recovery from soils (Zhou *et al.*, 1996) is totally different from the information obtained by the colony hybridizations with the specific probes used here. This latter approach gave reproducible comparisons between the *relative* distributions of bacteria in soil layers. In all soils, the highest scores were found in the upper zone. This is to be expected since the supply with organic carbon might be best there. The more surprising result is that denitrifiers, both in absolute numbers, and as percentage of the total population, were highest in the upper zone in all soils despite the fact that the soils were still rich in nitrate in the 25 cm zone. A similar distribution of denitrifying bacteria has also been described in sedimental horizons (Joergensen, 1989). The nitrate content was stated not to determine the distribution of denitrifying bacteria (Tiedje, 1988), although it was recently reported to have an influence on the composition of the nitrate-reducing and denitrifying community (Nijburg and Laanbroek, 1997). Unless anaerobic microsites exist in the upper soil layer, denitrification may not represent a selective advantage for bacteria in such ecosystems. Such a statement may also be suspected from the findings that the percentage of denitrifying bacteria is only ~5% of the total population of bacteria isolated. Values for denitrifying bacteria ($0.6-1.5\times10^5$/g soil fresh weight) reported by others (Martin *et al.,* 1988) are similar to ours.

The situation may be different under waterlogged conditions where denitrification may enable bacteria to survive. In the soil where waterlogging regularly occurs (Worringer Bruch), the percentage of denitrifying bacteria, determined with the *nosZ* and *nirK* probes, was significantly increased. In such a soil, the high water content may limit the O_2-availability which may govern the distribution of denitrifying bacteria, as suggested earlier (Smith, 1990). Plant respiration may lower the concentration of the gas in or at the roots, and an increase in the absolute number (this study) and also a relative enrichment (Linne von

Berg and Bothe, 1992) was seen for denitrifying bacteria in the vicinity of roots. In addition, plants can greatly influence the composition of the nitrate-reducing and denitrifying community (Nijburg and Laanbroek, 1997). The distribution in the different horizons (highest number in the upper 5 cm, lower at 25 cm) was observed also for N_2-fixing isolates in the two gleysol soils. Bacteria which showed positive DNA-hybridizations with the *amoA* probe appeared to be particularly enriched in the upper 5 cm layer of the rendzina of the Biesberg. It remains to be shown whether this is a special case of a fertile chalk soil which carries a particular rich plant community.

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