

## Detection methods for the expression of the dissimilatory copper-containing nitrite reductase gene (*DnirK*) in environmental samples

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### Abstract

In situ assays, based on monoclonal antibodies (mAbs), were developed to study the microbial expression of the bacterial dissimilatory copper-containing nitrite reductase gene (*DnirK*), one of the key enzymes involved in denitrification, in different ecosystems. With a combination of an anti-*DnirK* mAb and phylogenetic oligonucleotide probes, it is possible to bring structural and functional aspects of microbial communities together. To perform a double labelling, yielding a high signal strength for both the oligonucleotide and the antibody, cells have to be labelled with the oligonucleotide first followed by immunostaining. When the labelling sequence was changed, the accessibility for the oligonucleotide was reduced if high amounts of *DnirK* were expressed. Using flow cytometry, it was possible to sort bacterial cells, which were stained by the antibody, from nonlabelled cells. This technique provides means for a detailed analysis of populations, which express *DnirK* genes in the environment, including structural aspects of a community and detailed promoter studies. Using the immunostaining approach, it was possible to identify bacteria, which have the *DnirK* system expressed, in samples from a wastewater sewage treatment plant as well as in samples from the rhizosphere of wheat roots. Furthermore, expression studies using an *Ochrobactrum anthropi* strain were carried out to investigate the correlation between N<sub>2</sub>O production rates and *DnirK* expression in batch cultures, which had been shifted from aerobic to anaerobic conditions. As expected, expression of *DnirK* was the highest during periods with the greatest synthesis rates for N<sub>2</sub>O. However, the amount of expressed enzyme was not reduced in the cells, although the N<sub>2</sub>O production rates dropped in the cultures 12 h after the shift from aerobic to anaerobic conditions.

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### 1. Introduction

Denitrification is the main process, transforming solid N compounds to gaseous N compounds (N<sub>2</sub>O, NO and N<sub>2</sub>), which is indispensable for global N

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cycling. However,  $N_2O$  is a climate-relevant trace gas, which is able to destroy the ozone layer and contributes to the greenhouse effect (for a review, see Conrad, 1996). A good understanding of the conditions, for example, in agricultural soils or in wastewater treatment plants, controlling the denitrification process and the organisms involved could help to balance the N budget and to reduce the  $N_2O$  output by using suitable management conditions. On the other hand, through denitrification, nitrate concentrations in groundwater systems can be significantly reduced. Furthermore, many denitrifying bacteria are able to degrade xenobiotics under anoxic conditions (Zumft, 1997; Van Schie and Young, 1998).

One key enzyme of the denitrification process is the dissimilatory nitrite reductase (Dnir). Two main Dnir types among *Bacteria* and *Archaea* have been described. One group of enzymes harbours heme c and heme  $d_1$  as prosthetical group (DnirS), while the second group carries copper at the active site (DnirK). The latter seems to be more common among different bacterial taxa (Coyne et al., 1989), and the structure of the enzyme appears to be more conserved compared to nirS (Ye et al., 1993).

To investigate microbial functions in general, there are several opportunities: (i) detection of the gene, which provides information about the diversity of microorganisms in a community (e.g. Braker et al., 1998); (ii) detection of the mRNA, which reflects the level of gene expression (Tolker-Nielsen et al., 1997); (iii) detection of the expressed key enzyme to determine the *actual activity* of the sample; and/or (iv) performance of a specific in situ assay.

Denitrification activity of soils has been measured in classical approaches under standardized conditions in the lab. With molecular methods, the genes for nitrite reductase could be amplified and further characterized (Braker et al., 1998). Using these techniques, the potential for denitrification could be determined, however, nothing is known about the actual activity in situ and/or about the organisms involved. Other approaches determine  $N_2O$  levels as an indicator of denitrification activity in situ (Ryden et al., 1978), but the origin of  $N_2O$  (denitrification or nitrification) remains unclear. Furthermore, all of the mentioned approaches do not provide any information about microsites, where denitrification occurs, of a natural sample. In contrast, methods that detect the

corresponding protein in situ (e.g. immunological techniques) are excellent tools to specifically analyse microsites, which are relevant for a specific process and the corresponding organisms. Bartosch et al. (1999), for example, detected nitrifying bacteria in enrichment cultures of a wastewater treatment plant by antibodies, specific for nitrite-oxidoreductase. Also, Lin et al. (1996) used a similar approach to detect nitrogen-fixing cyanobacteria in marine environments. However, no comparable studies exist for the detection of denitrifying bacteria. The reason for this might be the high variability of the nitrite reductase even in strains of the same species. This made an overall detection of active denitrifiers impossible by immunological tools. Using a newly described monoclonal antibody (mAb) (Metz et al., 2002), it was possible to overcome this problem at least to some extent, as the antibody has a broad specificity for bacteria harbouring DnirK.

In the present paper, we describe new techniques which are based on combinations of immunological and molecular tools to detect denitrifying bacteria that express *DnirK* in situ and ex situ. An in situ double labelling technique was developed using the antibody, specific for nirK, 16S rRNA-directed probes and confocal laser scanning microscopy. Furthermore, a cell sorting method was applied to separate antibody-labelled bacteria (which express *DnirK*) from non-labelled organisms. Both methods might be good tools to perform structural and functional analyses of microbial communities in environmental samples.

## 2. Materials and methods

### 2.1. Bacterial strain and incubation conditions for expression studies of *DnirK*

For expression studies, *Ochrobactrum anthropi* DSM 14396<sup>T</sup> (a denitrifying bacterium, isolated from soil; Lebuhn et al., 2000) was grown overnight in NB-medium (5 g/l peptone; 3 g/l meat extract) under aerobic conditions. One milliliter of grown cells was used as an inoculum for the anoxic incubation using 20 ml NB-medium supplemented with potassium nitrate (5 mg/l). For incubation, round bottles with a volume of 100 ml, which were locked by a gas-tight septum, were used. The gas phase was exchanged by

helium to achieve an anaerobic atmosphere. The bottles were incubated without shaking at 30 °C for 35 h. The reduction of N<sub>2</sub>O to N<sub>2</sub> was blocked by acetylene (10% (v/v)). Concentrations of nitrate, nitrite and N<sub>2</sub>O were determined after different incubation times. For each experiment, nine individual bottles were incubated and analysed separately for nitrate, nitrite, N<sub>2</sub>O and DnirK.

## 2.2. Sample material and sample preparation

### 2.2.1. Wastewater treatment plant

Five individual samples, each consisting of approximately 4 ml, were taken from the denitrification basin, 30 cm beneath the water surface, of the sewage treatment plant München II (Dietersheim, Germany) treating municipal wastewaters of the city of Munich and fixed at once with 20 ml of a 4% paraformaldehyde (PFA) as described by Amann et al. (1990).

### 2.2.2. Wheat roots

Wheat grains were sterilized as described by Schlöter et al. (1997) 5 days after germination. Ten seedlings were transferred to a pot filled with 10 kg (dry weight), 2-mm sieved agricultural soil (fine-loamy Dystric Eutrochrept). The water content of the soil was adjusted to 50% of the maximum water holding capacity. After 5 weeks of growth, the roots were dissected and fixed with 20 ml of a 4% paraformaldehyde (PFA).

## 2.3. Measurement of nitrate, nitrite and N<sub>2</sub>O

Nitrate and nitrite concentrations were determined using the Spectroquant<sup>R</sup> system (Merck, Germany) according to the manufacturer's description. Concentrations of N<sub>2</sub>O were measured by gas chromatography (Lofffield et al., 1997).

## 2.4. Simultaneous staining with 16S rRNA-directed oligonucleotide probes and anti-DnirK mAbs

For the double labelling experiment, cells or plant materials were fixed on polylysine-coated glass slides and digested with lysozyme (662 U/μl PBS; 15 min; 22 °C). After two washing steps in PBS, the samples were dehydrated in an increasing series (50%, 80% and 96% (v/v), 60 s each) of ethanol. The material

was then hybridised with a Cy3-labelled oligonucleotide probe EUB 338 according to Amann et al. (1995). The probe EUB338 is complementary to a constant region of the Eubacterial 16S rRNA. Briefly 8-ml buffer (Tris-HCl 20 mM, SDS 0.02%, EDTA 5.0 mM, NaCl 0.9 M, pH 7.4) were mixed on the slide with 1 μl probe (50 μg/ml) and incubated for 2 h at 46 °C in a wet chamber, followed by a washing step in 48 °C warm buffer solution. Finally, the slides were air-dried. The following steps of antibody labelling had to be performed in the presence of 0.9 M NaCl to stabilize the oligonucleotide rRNA complex. Non-specific binding sites were blocked with a 3% serum albumin solution (BSA; in 0.9 M NaCl) for 60 min at 4 °C. The slides were then covered with 20 μl of the anti-DnirK antibody (1 μg/ml), diluted in washing solution ((WS) 0.5% BSA; 0.5% Tween 80; 0.9 M NaCl) and incubated for 2.5 h at 4 °C. The used mAb has a broad specificity for the DnirK of different bacteria (Metz et al., 2002). Unbound antibody was removed by two incubation steps in WS (10 min; 4 °C) followed by an incubation step with the fluorescence-labelled secondary antibody (anti-mouse-FLUOS-Fab-Fragment, Roche Diagnostics, Germany; diluted 1:20 in WS for 120 min; 4 °C). The slides were washed as above. Finally, the slides were rinsed with demineralised water, stained with 10 μl of a 1 μg/ml DAPI (4',6-diamidino-2-phenylindol-dihydrochlorid) solution (Porter and Feig, 1980), air-dried and mounted with an antifading reagent (Citifluor AF1; Citifluor, Great Britain).

All incubation steps were conducted in a wet chamber. Controls were incubated in washing solution only (instead of the corresponding anti-DnirK mAb). Experiments for expression studies in pure culture were carried out without the first step (hybridisation with EUB 338 probe).

## 2.5. Microscopy

The slides from pure culture experiments were examined by an epifluorescence microscope (Axio-plan, Zeiss, Oberkochen, Germany) using an oil immersion objective (Plan-Neofluar 100 × 1.3) and a 50-W mercury lamp. The staining was visualized by band pass filter 359–371 nm for DAPI, 450–490 nm for FLUOS and 540–552 nm for Cy3 and an excitation long pass filter of 397, 520 and 590 nm, respec-

tively. Photographs were taken with an MC 100 camera (Zeiss) equipped with an EPS 800/1600-film (Kodak, Germany).

Environmental samples were examined by confocal laser scanning microscopy (CLSM). An LSM 410 inverted scanning confocal laser microscope (Zeiss) equipped with three lasers (Ar-ion, UV; Ar-ion, visible; He–Ne) supplying excitation wavelengths at 365, 488 and 543 nm, respectively, was applied to record optical sections using a  $100\times$  oil immersion lense (NA 1.3). Monochrome sequences of images were taken along the optical axis (z-axis) at 0.7- and 1.2- $\mu\text{m}$  increments. Sequentially recorded monochrome images or projections of monochrome z-sequences were assigned to the respective fluorescence color and then merged to a true color red/green/blue (rgb) display. All image combining and processing were performed with the standard software provided by Zeiss.

## 2.6. Fluorescence-activated cell sorting (FACS)

The bacteria were labelled with the mAb as described above, with the only difference being that cells were not fixed on slides, but incubated in Eppendorf cups with the oligonucleotide probe and the antibody. To avoid cell aggregation, the cell suspension was mixed after each step for 10 s. Sorting of the cells was performed with a FACStar<sup>Plus</sup>-Flow-Cytometer (Becton Dickinson, USA) using the same filter sets as described above. Cells were scanned for forward light scatter and antibody-mediated fluorescence intensity. For reproducibility, the experiment was repeated three times.

## 3. Results and discussion

### 3.1. Expression studies

Since the denitrification pathway is inducible, parameters affecting the denitrification activity should also be reflected in the expression of *DnirK* as the key enzyme of this process. To investigate the time course of denitrification and *DnirK* expression in the soil bacterium *O. anthropi* (a denitrifying soil bacterium), flasks with aerobically grown cultures were shifted from aerobic to anaerobic conditions. The reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$  was blocked by acetylene (10% (v/v)).

Concentrations of nitrate, nitrite and  $\text{N}_2\text{O}$  were determined at intervals (Fig. 1). The rate of synthesis of  $\text{N}_2\text{O}$  was calculated as  $\text{N}_2\text{O}$  produced per hour. Nitrate was reduced to a small level after 29 h (data not shown). Simultaneously with the decrease of nitrate content in the flasks, nitrite concentration increased and reached a maximum (0.16 mg/l) 23 h after inoculation with a lag time of 5 h. No more nitrite could be measured in the flasks after 26 h (Fig. 1B).  $\text{N}_2\text{O}$  concentration reached its maximum as expected shortly after the nitrite peak 27 h after inoculation (67 ppm) (Fig. 1C). Both the nitrite and  $\text{N}_2\text{O}$  concentration followed the growth curve ( $\text{OD}_{500}$ ). The highest synthesis rate for  $\text{N}_2\text{O}$  was measured 7 h after inoculation with more than 6 ppm/h. The rate declined towards the stationary phase, despite a sufficient nitrate amount left in the medium (Fig. 1D). At that time, the stationary phase of the culture with reduced physiological activity was reached, as probably the C-sources and consequently the reduction equivalents were exhausted.

As expected, the expression of *DnirK* would be highest at the times with the greatest synthesis rates for  $\text{N}_2\text{O}$ . Cells were labelled with the anti-*DnirK* mAb and a secondary fluorescence-labelled antibody. A maximum level of *DnirK* could be shown by immunostaining with the anti-*DnirK* mAb between 8 and 20 h after inoculation (Fig. 2). A correlation between the  $\text{N}_2\text{O}$  production rate and the immunofluorescence signal is visible (compare Figs. 1 and 2) until 8 h after inoculation. In contrast, there is no clear correlation between enzyme amount and  $\text{N}_2\text{O}$  production in the stationary phase. Although a good fluorescence signal is visible, indicating high amounts of expressed enzyme, the  $\text{N}_2\text{O}$  production rates are low. The time course of *DnirK* expression following anaerobiosis is in good agreement with the results found in natural environments. Dendooven and Anderson (1994), for example, determined via inhibition studies with chloramphenicol the repression of *DnirK* between 4 and 8 h after oxygen deprivation.

Further physiological studies also indicate that *Dnir* expression is induced under low oxygen pressure and in the presence of N-oxides. Baumann et al. (1996) showed for *dnirS* in continuous cultures of *Pseudomonas denitrificans* after a change from aerobic to anaerobic conditions that the corresponding mRNA reached a maximum after 5 h, subsequently

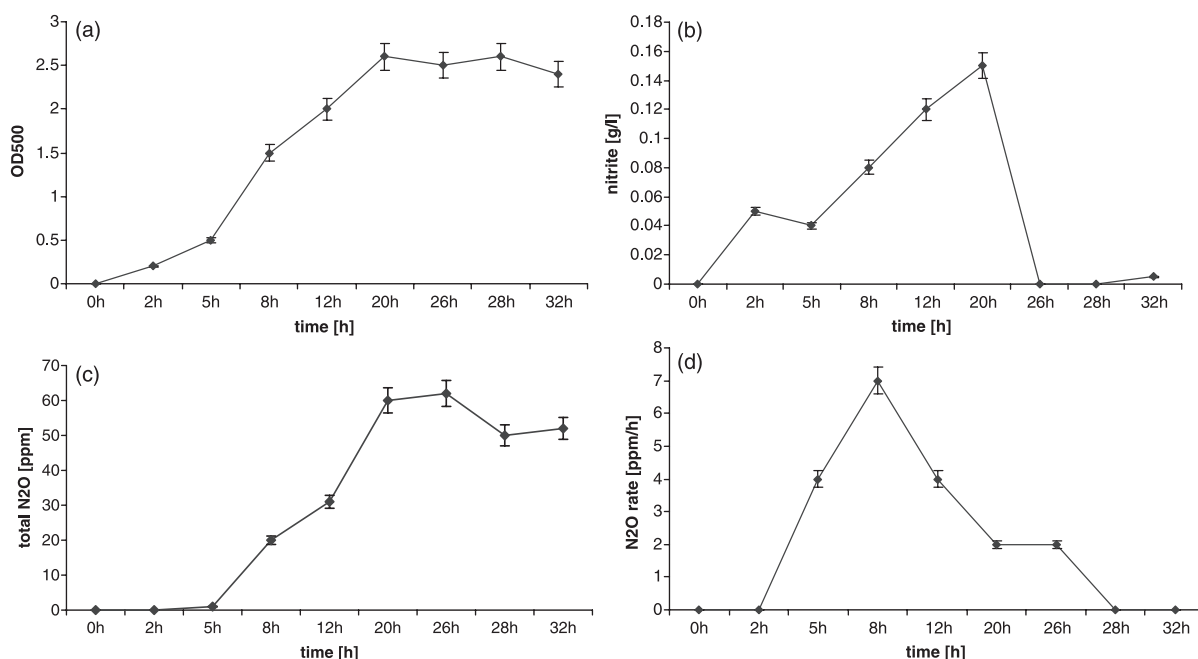


Fig. 1. Concentration respectively of production rate of nitrite (b) and N<sub>2</sub>O (c and d) in the medium after shift of an *O. anthropi* culture to anoxic conditions; OD<sub>500</sub> values were taken as a measure for cell density (a). The values are means from nine replicates. The bar indicates the standard error.

dropping and stabilizing at a reduced level, while *dnirS* reached a maximum gradually over 30 h. In a batch culture of *Pseudomonas stutzeri*, Hartig and Zumft (1999) demonstrated the transcription for *dnirS* following a shift from aerobic to anaerobic conditions and addition of nitrate or nitrite to a maximal induction after 15 min.

### 3.2. Simultaneous labelling of bacteria with a 16S rRNA-directed oligonucleotide probe and the anti-DnirK mAb

Using the described protocol, it was possible to combine the immunostaining with a phylogenetic oligonucleotide staining. Fig. 3 shows cells from *O. anthropi* after simultaneous labelling with an anti-DnirK mAb and 16S rRNA-directed oligonucleotide probe Eub338. It is clearly visible that most of the DAPI-stained cells were detected by immunostaining as well as by 16S rRNA-directed probes. As incubation was in a batch experiment and not a chemostat, it is obvious that not all cells have the same physiological status, resulting in a not complete conformity of

DAPI, fluorescence in situ hybridisation and antibody signal. The ring-like immunostaining is clearly visible, indicating the localization of DnirK in the periplasm.

The following steps are of central importance for a successful double labelling. (i) The cells had to be permeabilized by lysozyme to permit diffusion of the antibody into the periplasm. Treatment with paraformaldehyde alone, as described by Bartosch et al. (1999) for the nitrite-oxidizing enzyme system of *Nitrospira*, *Nitrospina* and *Nitrosococcus* species, did not result in any fluorescence signal. The immunofluorescence signal corresponded to the location of the copper nitrite reductase in the periplasm. (ii) It was not successful to follow the protocol of ABmus et al. (1997) in which the antibody labelling was carried out first. Oligonucleotide-mediated fluorescence could only be observed when the labelling procedure for the antibody was performed after the labelling with the probe. Possibly, the complex of anti-DnirK mAb and the secondary antibody, which is formed in the periplasm, hindered the permeability for the 16S rRNA probe into the cell. (iii) Furthermore, all steps for the antibody labelling



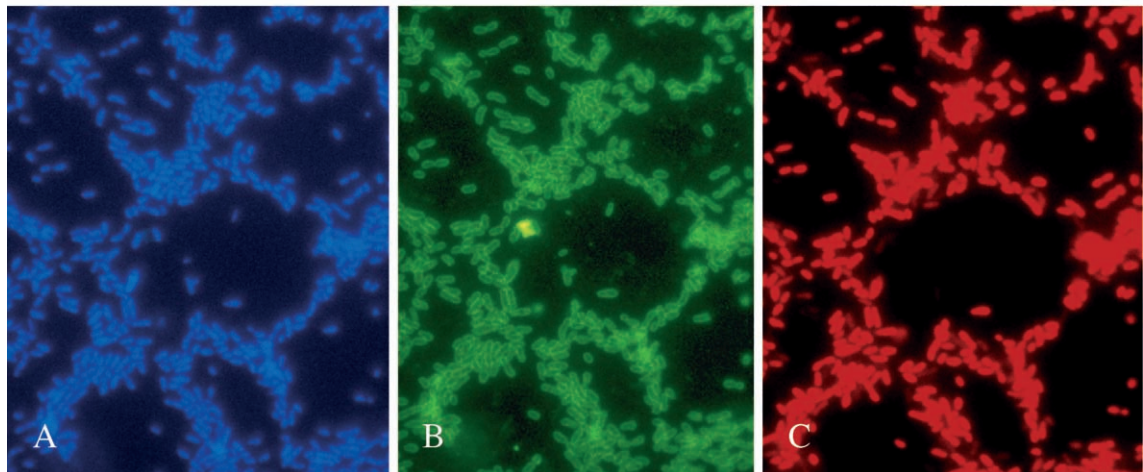
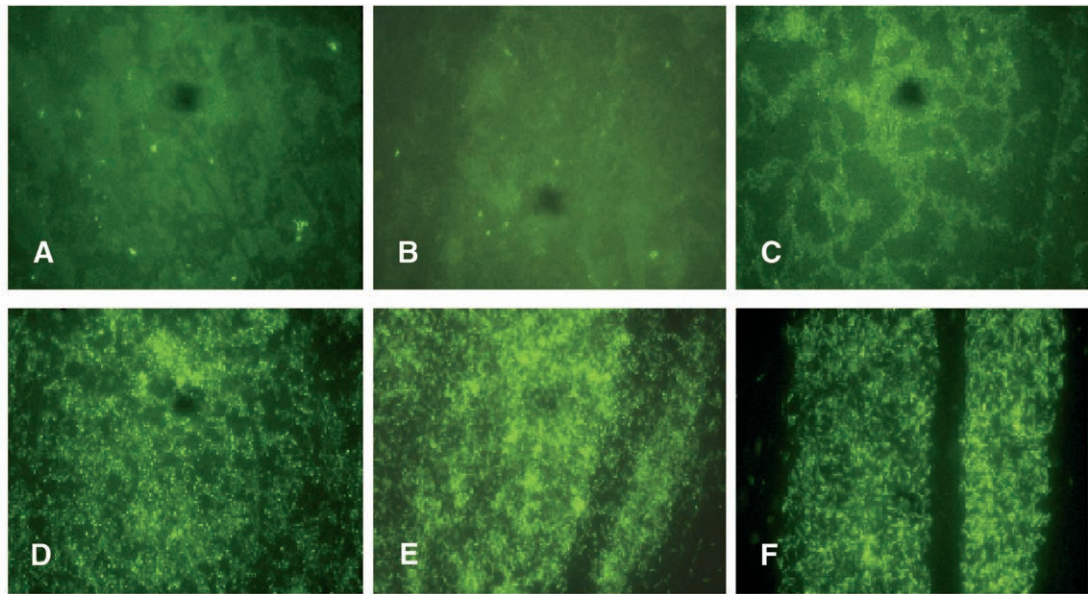


Fig. 2. Expression of *DnirK* in *O. anthropi* after shift from aerobic to anaerobic conditions; the immunofluorescence staining of nirK was performed by a DnirK specific mAb and a FLUOS-coupled secondary antibody; Epifluorescence image with  $40\times$  magnification (A: 1 min after shift from aerobic to anaerobic conditions; B: 2 h after shift; C: 5 h after shift; D: 8 h after shift; E: 12 h after shift; F: 20 h after shift).

Fig. 3. Labelling of an anaerobic cultivated *O. anthropi* strain in a nitrate-containing medium with a DnirK specific mAb and FLUOS-coupled secondary antibody (B), a Cy3-labelled rRNA-directed probe (Eub 338) (C) and DAPI (A). The three pictures show identical section.

had to be carried out at the optimal salt concentration for the hybridisation (0.9 M NaCl) with 16S rRNA-directed oligonucleotides to stabilize the complex of

the probe with its target molecule, otherwise, no probe-mediated signal was visible. The probe-mediated signal was reduced along with the reduction of the salt

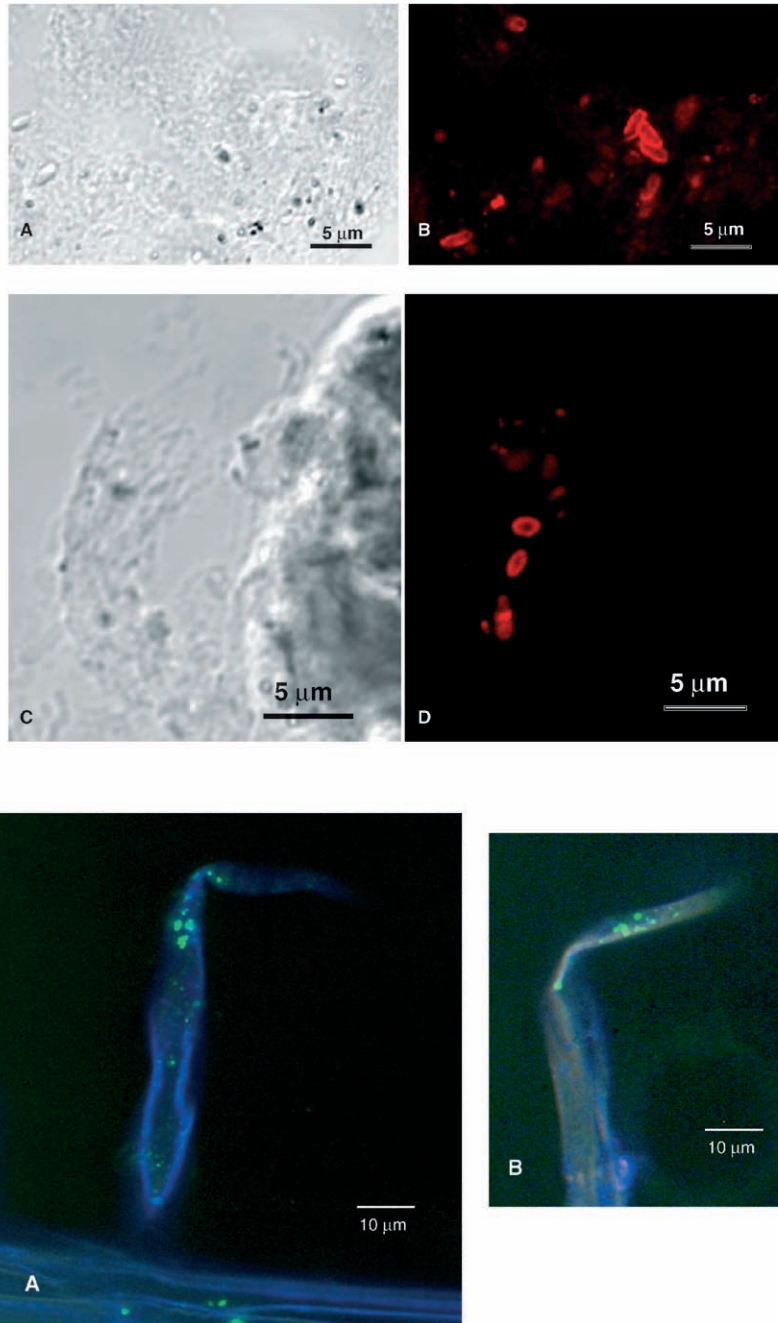


Fig. 4. Expression of DnrK in samples of the denitrification basin from a sewage plant. The cells were stained with a DnrK specific antibody and an ALEXA<sub>546</sub>-coupled secondary antibody. (A and C) Phase contrast image; (B and D) CLSM image; magnification: 1000 ×.

Fig. 5. Expression of DnrK in the rhizosphere of 5-week-old wheat seedlings. The bacteria were stained with a DnrK specific mAb and a FLUOS-coupled secondary antibody (green); CLSM image; magnification: 1000 ×; for counterstaining of the root, calcofluor white was used (blue fluorescence); (A) and (B) show different root pieces.

concentration (not shown). The protocol for double labelling was not applicable for several mAbs described to be specific against DnirK (Metz et al., 2002) because the increased salt conditions needed to stabilize the complex between the 16S rRNA oligonucleotide probe hindered the formation of the antibody nirK complex.

### 3.3. Immunodetection of DnirK in bacteria from environmental samples

#### 3.3.1. Investigations in a wastewater treatment plant

Samples from the denitrification basin of a wastewater treatment plant were investigated for the presence of DnirK by fluorescence staining with an anti-DnirK mAb. DnirK-induced cells could be detected in the surrounding of the flocs (Fig. 4A and C). However, it was also possible to detect labelled cells inside of aggregates (Fig. 4B and D). The bacteria were rod-shaped or coccoid and arranged in groups or chains, and also single cells were to be stained. The localization of DnirK in the periplasm was clearly reflected by the ring-like fluorescence label.

#### 3.3.2. Investigations in the rhizosphere of wheat

Roots of 6-week-old wheat seedlings were investigated for the presence of denitrifying bacteria in the rhizosphere. In both pictures (Fig. 5A and B), the antibody label, indicating the expression of the DnirK gene complex, was visible. These results are in good agreement with the results of other authors, who described the rhizosphere as a hot spot of high metabolic activity and also the compartment with the highest denitrification rates in soil. Knowles et al. (1982) reported a 514-fold increase of denitrifying bacteria as compared to root-free soil compartments. Killham (1994) could show that this increased poten-

tial for denitrification activity led to increased  $N_2O$  production, which declines with increasing distance from the root.

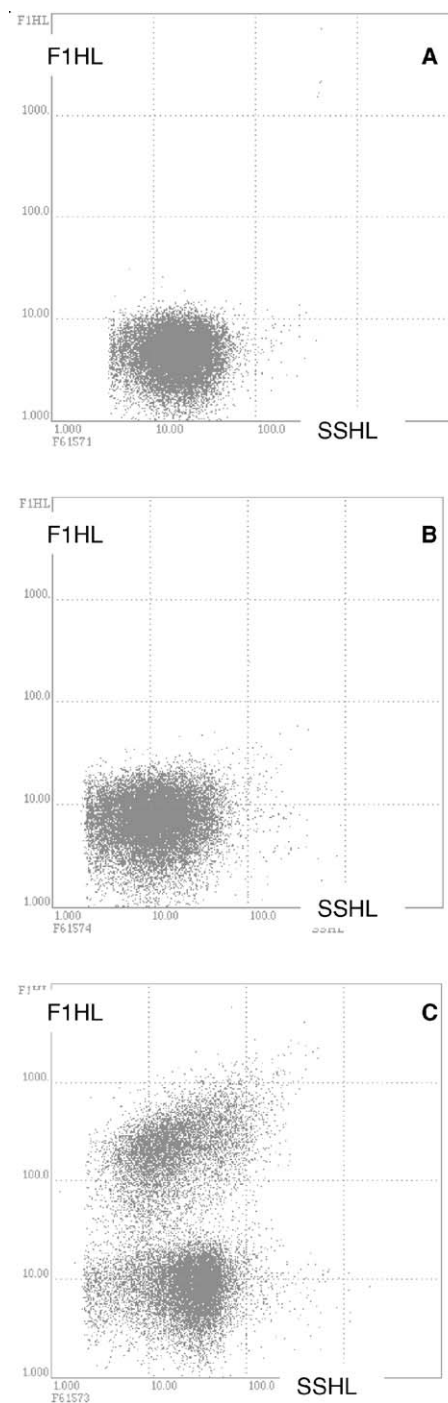


Fig. 6. FACS analysis of an anaerobic cultivated *O. anthropi* strain in a nitrate-containing medium. As a control aerobic, cultivated *O. anthropi* cells were used. Cells were stained with a DnirK specific mAb and a secondary FLUOS-coupled antibody. Dot plot of side scatter (SSH), representing the size of the measured objects) versus forward scatter (F1HL, representing the antibody-mediated fluorescence) in relative units. Each spot represents 20,000 analysed events. (A) Unlabelled control; (B) staining only with the FLUOS-coupled secondary antibody; (C) staining with an anti-DnirK mAb and a FLUOS-coupled secondary antibody.



### 3.4. Fluorescence-activated cell sorting (FACS) bacteria labelled with an anti-DnirK mAb

To achieve enrichment of bacteria, which express the *DnirK* system from microbial communities, immunolabelled cells were separated by flow cytometry. A batch culture of *O. anthropi* was grown aerobically and shifted to anoxic conditions to induce expression of the denitrifying enzyme system. The bacteria were labelled with an anti-DnirK mAb and a fluorescein-conjugated secondary antibody. During the sorting process with the FACS, the side scatter and the antibody-mediated fluorescence were determined for each individual bacterial cell. Unlabelled cells and cells only labelled with the secondary antibody were used as controls. In Fig. 6, dot plots of the sorting process are shown. There was no detectable background, caused by noncellular particles in the unlabelled sample (Fig. 6A). In addition, treatment with the secondary antibody alone did not lead to nonspecific fluorescence (Fig. 6B). Labelling with the anti-DnirK mAb and the secondary antibody, in contrast, resulted in two clearly separated populations of *DnirK*-induced and *DnirK*-noninduced cells (Fig. 6C). Thus, it can be concluded that the antibody-mediated fluorescence signal is strong enough to sort bacteria, which have expressed *DnirK* by flow cytometry.

Taking into account the results of Porter et al. (1993) and Bach (1996), it can be expected that sorting of denitrifying bacteria with the developed anti-DnirK mAbs in an environmental sample may be practicable. Porter et al. (1993) were successful in retrieving *Staphylococcus aureus* after immunofluorescence staining from a bacterial consortium in which the bacterium represented only 0.4% of the total. In environmental samples, it was possible to recover the bacterium from the indigenous flora at a proportion of 1% of the whole bacterial community. In cell mixture, an enhancement of up to 90% could be achieved, and in the environmental sample, it was feasible to enrich for *S. aureus* up to 70%. Bach (1996) determined a quantity of potentially denitrifying bacteria in an upper soil layer of  $4.6 \times 10^6$ /g dried soil as compared to the entire heterotrophic bacteria  $4.9 \times 10^6$ /g to  $5.3 \times 10^7$ /g dried soil by the most probable number method.

The separated denitrifying cells can be used for further phylogenetical analysis to allow us to learn more about the members and diversity of the denitrifying community of a given habitat.

## 4. Conclusion

The results indicate that the developed antibody-based techniques provide a good tool to study microbial in situ activities in ecological systems. The detection of the key enzyme in denitrification is a direct proof at the expression level and therefore represents a strong hint for the presence of denitrifying activity. However, it must be taken into account that not much is known about the in situ stability of periplasmic proteins. It is uncertain how long enzymes remain functional, even when the corresponding process had stopped due to the lack of substrate. First hints about a high stability of DnirK could be given by the expression studies, which were described in this paper. Therefore, transcriptional mRNA-based research should be complemented by identification of the protein with immunological techniques.

By applying the techniques described here, it is possible to bring structural aspects of a microbial community together with functional aspects. Labelled bacteria could be sorted by FACS and further characterized by phylogenetic studies to gain an insight on the diversity of the denitrifying community of a sample. This might be useful for practical application to make wastewater treatment plant more efficient. Also, in the field of agriculture, where nitrogen turnover is of central importance, information about the key players in the denitrification pathway will be very useful to improve, for example, the fertilization strategies.

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