

# Development and Characterization of Murine Monoclonal Antibodies Specific for Dissimilatoric Copper Nitrite Reductase

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## ABSTRACT

Several hybridoma cell lines from mice were established, producing monoclonal antibodies (MAbs) directed against the dissimilatoric copper nitrite reductase (dNIR) to detect actual denitrifying bacteria at the single cell level under nondestructive conditions in the environment. The mice were immunized with native or recombinant enzyme gained from two different bacteria, *Ochrobactrum anthropi* and *Alcaligenes faecalis*. The antibodies obtained could be divided into two groups according to their different specificities for dNIRs of different bacteria: One group of MAbs had a broad specificity for dissimilatoric copper nitrite reductases from bacteria of different phylogenetic taxa; the other group gave only a clear signal with the corresponding immunogen. None of the raised MAbs showed a cross reactivity with the dissimilatoric heme nitrite reductase. One MAb from each group (MAb dNIR1a and MAb dNIR29) has been selected for further investigation. Data of enzyme-linked immunosorbent assay (ELISA), Western blotting, and immunofluorescence-microscopy are presented and compared with phylogenetic data. Furthermore, results of Western blotting experiments with cells, grown without nitrate under aerobic conditions, and cells cultivated with nitrate under anaerobiosis, are shown.

## INTRODUCTION

DENITRIFICATION is one of the key processes in the nitrogen cycle, as nitrite is reduced to gaseous products. During this process, nitrate is reduced stepwise via nitrite, NO and N<sub>2</sub>O to N<sub>2</sub>. The genes for denitrification are usually expressed at low levels of oxygen or anoxic conditions in the presence of an N-oxide.<sup>(1)</sup> However, there are also some organisms known that denitrify at oxic conditions.<sup>(2-4)</sup> The key enzyme of this process is nitrite reductase (dNIR). It reduces nitrite to N<sub>2</sub>O, which is one of the most important greenhouse gases and responsible for the destruction of the ozone-layer.<sup>(5)</sup> Denitrifiers are spread over different bacterial taxa. Denitrification enzymes have even been found in mitochondria of fungi.<sup>(6)</sup> Thus, phylogenetic analysis of a microbial community cannot yield information. However, knowledge of the expression-level of nitrite reductase in a (natural) sample would lead to a better understanding of the denitrification process and its regulation by environmental conditions.

Antibodies are an excellent tool for the detection of the expressed enzyme *in situ* and for the separation of the relevant organism for further phylogenetical investigations. Thus, they should be helpful in determining the conditions that control the release of N<sub>2</sub>O, for example, in agricultural soils or in wastewater treatment plants and finally help to improve the management of these complex systems.

Two main groups of nitrite reductases among *Bacteria* and *Archaea* are known: (1) one type with heme c and heme d<sub>1</sub> as a prosthetical group (cd<sub>1</sub>-dNIR) and (2) proteins with a copper-site at the active center (copper-dNIR). The latter seems to be more common among different bacterial taxa<sup>(7)</sup> and the structure of the enzyme appears to be more conserved as compared to the heme cd<sub>1</sub>-dNIR.<sup>(8)</sup>

In this paper, we characterize two groups of new monoclonal antibodies (MAbs) against the copper dNIR and describe their applicability for expression studies of the enzyme *ex situ* and *in situ*. For a first direct preparation of the antigen, the copper dNIR *Ochrobactrum anthropi* DSM 14396, a Gram-nega-

TABLE 1. LIST OF MICROORGANISMS

<i>Organism</i>	<i>Origin</i>
<i>Agrobacterium tumefaciens</i>	DSM 30205
<i>Alcaligenes denitrificans</i>	DSM 30026
<i>Alcaligenes faecalis</i> S6	Dr. M. Nishiyama, University of Tokyo
<i>Alcaligenes faecalis</i>	DSM 30030
<i>Alcaligenes</i> sp.	DSM 30128
<i>Bacillus azotoformans</i>	DSM 1046
<i>Bradyrhizobium japonicum</i>	DSM 30131
<i>Corynebacterium</i> sp.	DSM 20150
<i>Escherichia coli</i> JM 105	Amersham Pharmacia Biotech
<i>Gluconacetobacter diazotrophicus</i>	DSM 5601
<i>Hyphomicrobium zavarzinii</i>	DSM 1566
<i>Ochrobactrum anthropi</i>	LMG 2136
<i>Ochrobactrum anthropi</i>	LMG 3333
<i>Ochrobactrum anthropi</i>	LMG 5440
<i>Ochrobactrum anthropi</i>	DSM 14396
<i>Ochrobactrum grignonense</i>	LMG 18955
<i>Ochrobactrum tritici</i>	LMG 18957
<i>Pseudomonas aeruginosa</i>	DSM 10
<i>Pseudomonas alcaligenes</i>	DSM 50342
<i>Pseudomonas denitrificans</i> sp. den.	DSM 1650
<i>Pseudomonas fluorescens</i>	DSM 50090

tive  $\alpha$ -proteobacterium, which is known as one of the dominant members of the soil microflora, was used. In a second approach, the copper dNIR of *Alcaligenes faecalis* S6 that has the same genotype, as copper dNIR from *Ochrobactrum anthropi*, was subcloned and expressed in *E. coli*.

## MATERIALS AND METHODS

### *Bacterial strains and cultivation*

Most organisms were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and the Laboratorium voor Microbiologie (LMG, Gent, Belgium), as listed in Table 1. *Alcaligenes faecalis* S6 was a kind gift from Dr. M. Nishiyama, University of Tokyo.<sup>(9)</sup> The bacteria were grown, as described in Table 2.

For the induction of the denitrification enzymes, 2 g/L nitrate was added. Denitrification was confirmed by the detec-

tion of N<sub>2</sub>O using a gas-chromatograph according to Lotfield et al.<sup>(10)</sup>

### *Preparation of copper-nitrite reductase*

*Purification of the native enzyme from Ochrobactrum anthropi.* A cell-free crude extract was prepared from 40 g of *Ochrobactrum anthropi* cells (DSM 14396; wet weight). After removing heat-sensitive proteins (10 min, 70°C, centrifugation) the proteins in the supernatant were separated by ion-exchange with DEAE-cellulose, followed by molecular-sieving with Sephacel S100. Finally, proteins were separated in native polyacrylamide gel (PAGE) and the copper-dNIR was detected by activity-staining.<sup>(11)</sup> The active band was excised and the copper dNIR was electroeluted. The purity was confirmed by sodium dodecyl sulfate (SDS)-PAGE.

*Production and purification of recombinant dNIR from Alcaligenes faecalis S6.* Full-length nitrite reductase cDNA from

TABLE 2. CULTURE MEDIA FOR MICROORGANISMS

<i>Microorganism</i>	<i>Medium</i>
<i>Ochrobactrum anthropi</i>	Meat peptone 5.0 g
	Meat extract 3.0 g
	Yeast-extract 10.0 g
	Glucose 16.0 g
	Ad 1000 mL <sub>demin.</sub> pH 7.0
<i>Hyphomicrobium zavarzinii</i>	Medium 162 (DSMZ)
<i>Bradyrhizobium japonicum</i>	Medium 98 (DSMZ)
<i>Bacillus</i> sp.	Medium 257 (DSMZ)
All other bacteria	Luria-Bertani-medium

*Alcaligenes faecalis* S6 was subcloned into the bacterial expression vector pQE 13 (Qiagen, Hilden, Germany) and the plasmid was introduced into *E. coli* JM 105. The enzyme was expressed as a fusion protein with N-terminal-6x His-tag under the control of a phage T5 Promotor/2 lac-Operator sequence and purified by affinity-chromatography with Ni-NTA (Qiagen) under denaturing conditions. The purity was confirmed by SDS-PAGE.

*Immunization and establishment of the hybridoma cell lines.* Female BALB/c mice, 7–10 weeks old, were immunized on Day 1 by subcutaneous injection with 100  $\mu$ g nitrite reductase emulsified in Freund's complete adjuvant. On Days 15 and 22, the mice were boosted with 100  $\mu$ g nitrite reductase suspended in Freund's incomplete adjuvant. Blood samples were taken before the first immunization and 5 days after the first boost to check the immunoreaction. Four days after the last injection, the mice were splenectomized and the spleen cells were fused with X63AG8.563 myeloma cells according to Köhler and Milstein.<sup>(12)</sup> Ten days after cell fusion, culture supernatants were screened for antibodies against nitrite reductase. Positive clones were cultivated and recloned twice by the method of limiting dilution. For antibody-production, the cell lines were scaled up in 100-mL cell-culture-flasks containing about 50 mL of hybridoma cultures. After 5 days, the cells were separated by centrifugation, the supernatant was filtered (0.2  $\mu$ m, Millipore, Eschborn, Germany) and stored at +4°C.

#### *Determination of immunoglobulin (Ig)-subclass*

The immunosubclasses were determined by the Mouse Iso-typing Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's specifications.

#### *Enzyme-linked Immunosorbent Assay (ELISA)*

Screening of hybridoma supernatant for antibody production was performed by ELISA in 96-well microtiter-plates for luminometric detection (white-colored FluoroNunc™-microtiter-plates with Maxisorp™-surface; Nunc, Denmark) or transparent Microlon 96-K microtiterplates (Greiner Labortechnik GmbH, Frickenhausen, Germany). The plates were coated with nitrite-reductase (1  $\mu$ g/well, 200 mM carbonat-buffer, pH 9.6) overnight at 4°C. Unspecific binding capacities were blocked by bovine serum albumine (BSA)-buffer (3% BSA (w/v); phosphate-buffered saline [PBS]), at 37°C for 60 min. Hybridoma supernatant was added and incubated at 37°C for 60 min. Horseradish peroxidase (HRP) conjugated goat anti-mouse (Amersham Pharmacia Biotech, Freiburg, Germany) was diluted 1:200 in PBS/0.5% BSA (w/v) and incubated at 37°C for 45 min. The detection was performed either by 2,2'-Azino-di-[3-ethylbenzthiazolin]sulfonic acid (ABTS) and a microtiter-plate reader at 450 nm or a chemoluminescence-substrate (BM Chemoluminescence ELISA Substrat/POD; Roche Diagnostics GmbH, Mannheim) according to the manufacturer's recommendations and a chemoluminometer (Dynatech ML 1000, Dynatech; Denkendorf, Germany), respectively.

#### *SDS gel electrophoresis and Western blotting*

Proteins were separated in a 10% acrylamide-matrix, and stained with Coomassie Brilliant Blue or silver or transferred

to a nitrocellulose membrane (Hybond™ECL™, Amersham-Pharmacia Biotech, Freiburg, Germany) under semi-dry conditions (Biometra-Fast Blot, Biometra, Göttingen, Germany) following the manufacturer's instructions. Immunodetection was carried out as follows: after 60 min blocking at room temperature or overnight at 4°C with 5% (w/v) skim milk in PBS, the membrane was rinsed twice with washing solution (0.5% skim milk (w/v); 0.1% Tween 80) and incubated 3 times in washing solution for 10 min. After incubation with hybridoma supernatant (diluted in washing solution) for 90 min at room temperature the membrane was washed again and incubated with HRP conjugated goat anti-mouse (Amersham Pharmacia Biotech) diluted 1:3000 in washing solution at room-temperature for 60 min. After another washing step, the membrane was incubated with streptavidine-peroxidase-conjugate (1:3000 diluted with washing solution) for 60 min at room temperature. After a final washing step, bound antibodies were detected by an enhanced chemoluminescence (ECL) system RPN 2109 (Amersham-Pharmacia Biotech) following the instructions of the distributor. The results were documented on x-ray film (X Ray 90, CEA, Stängnäs, Sweden).

#### *Immunofluorescence-staining and epifluorescence microscopy*

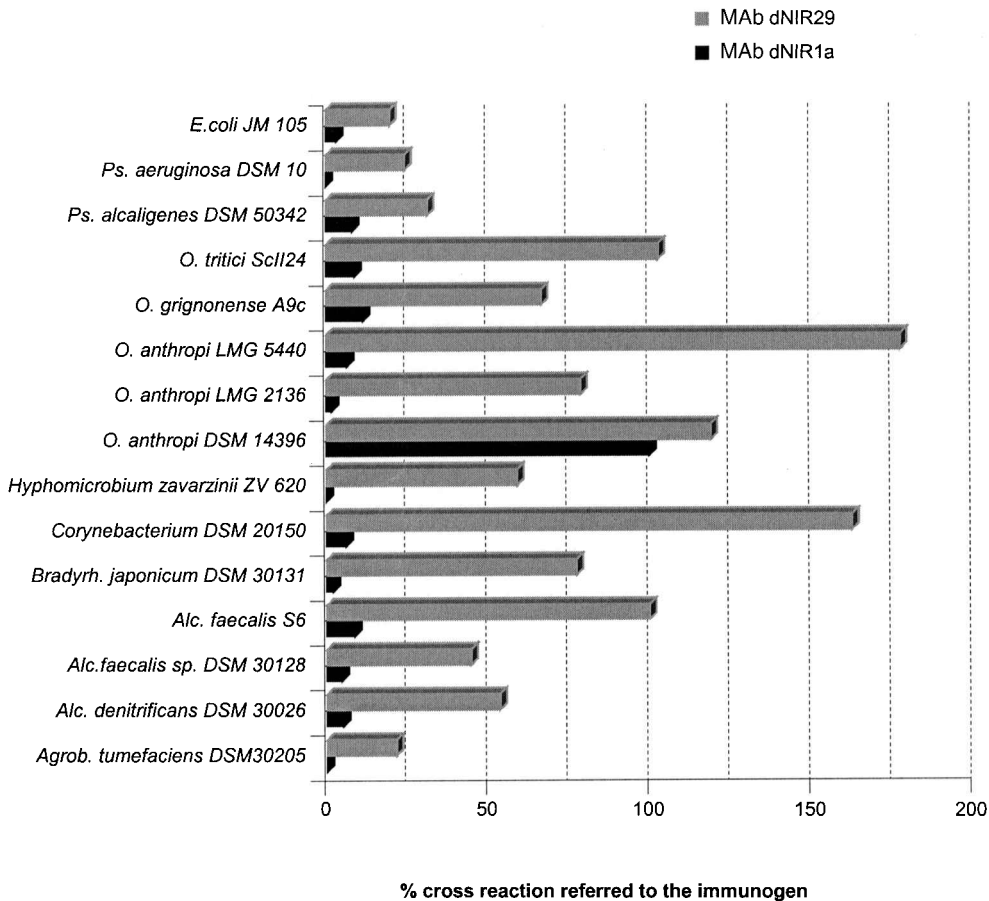
The cells were fixed in PFA, as described by Amann et al.<sup>(13)</sup> The fixed cells were permeabilized by digestion with lysozyme (662 U/ $\mu$ L PBS; 15 min; room temperature). The reaction was stopped by two washes in PBS. Some droplets of the cell suspension were transferred to a glass slide and dried by room temperature. After blocking of unspecific binding sites by BSA (3% in PBS; 60 min); the cells were covered with the anti-dNIR-antibody (in a suitable dilution in washing solution; 90 min). Unbound antibody was removed by two incubation steps in washing solution (0.5% BSA; 0.5% Tween 80; PBS; 5 min). The cells were incubated with a fluorescence-labelled secondary-antibody (anti-mouse-FLUOS-Fab-Fragment Roche Diagnostics GmbH; Mannheim; diluted 1:20 in washing-solution; 60 min), washed as described above, rinsed with A<sub>demin</sub> and air-dried. All incubation steps were conducted in a wet-chamber at room temperature. Controls were incubated in washing solution only instead of MAb dNIR.

The slides were mounted in the fluorescence enhancer Citifluor AF1 (Citifluor Ltd., London) and examined by an epifluorescence microscope (Axioplan, Carl Zeiss, Jena) using an oil immersion objective (Plan-Neofluor 100  $\times$  1.3); and a 50-W mercury lamp. The staining was visualized by the band pass filters 359–371 nm for DAPI; 450–490 nm for FLUOS and 540–552 nm for Cy3 and excitation longpass filters of 397, 520, and 590 nm, respectively. Photographs were taken by using a MC 100 camera (Carl Zeiss, Germany) and a EPS 800/1600-film (Kodak, Rochester, NY).

## RESULTS

#### *Cross-reactivity of the MAbs*

After immunization with the denatured recombinant protein, 40 hybridoma cell lines were established producing specific antibodies (MAbs dNIR 1–40) against copper-nitrite reductase.



**FIG. 1.** Cross-reactivity of the antibodies MAb dNIR1a and mAb dNIR29 with different copper-dNIRs. The wells of a microtiter plate were covered with crude-extracts of different bacteria ( $1 \mu\text{g protein}_{\text{total}}$ ). A chemoluminescence-ELISA with the anti-dNIR-antibodies and a secondary anti-mouse peroxidase-conjugate was performed. The values are expressed as a percent of the value of the immunogen (*Alcaligenes faecalis* S6 for MAb dNIR29 or *Ochrobactrum anthropi* DSM 14396 for MAb dNIR1a).

Ten of the MABs were tested by Western blotting for cross-reactivity with nitrite reductases from bacteria of different phylogenetic taxa. Most of the MABs recognized the dNIRs from *Ochrobactrum* spp., whereas the enzyme of *Bacillus azotofornans* could not be detected by any of the antibodies. No cross-reaction occurred with the cd<sub>1</sub>-dNIR of *Ps. aeruginosa* and also no cross-reaction could be observed with the crude-extract of *E. coli*. These results could be confirmed by chemoluminescence-ELISA. MAb dNIR29 was selected for further characterization from taxonomically different typical soil bacteria, because of its broad spectrum for isoenzymes. Figure 1 shows positive reactions by chemoluminescence-ELISA with bacteria including organisms from the  $\alpha$ - and  $\beta$ -subgroups of the Proteobacteria, as well as Gram-positives of the low-GC-group. No cross-reaction with the crude extracts of *E. coli* or the cd<sub>1</sub>-dNIR of *Ps. aeruginosa* occurred. Comparing the cross-reactivity profile of MAb dNIR29 with the similarities of partial DNA sequences from different nitrite-reductases (Fig. 2), we conclude that this antibody has the potential to recognize dNIRs of a wide variety of taxa, even those with low DNA sequence similarity.

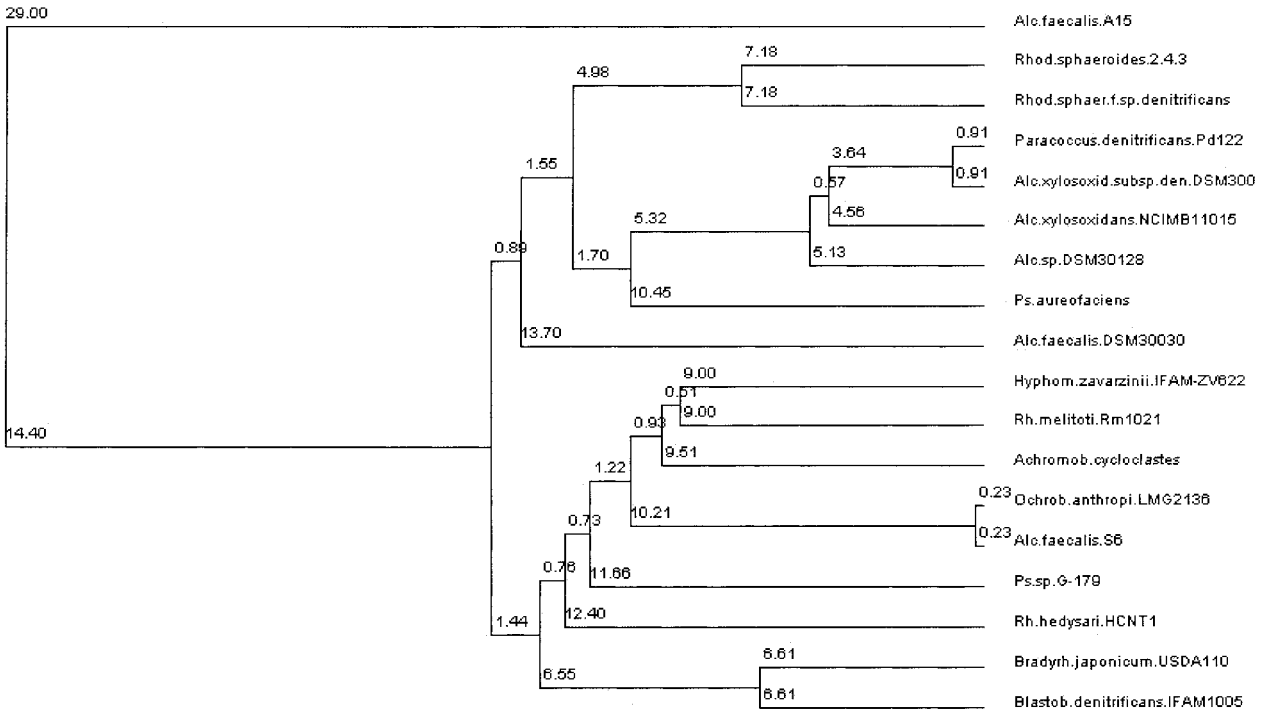
For further investigations about the specificity of the MAB,

cells of *Alcaligenes faecalis* S6 were grown anaerobically in the presence of 2 mg/mL nitrate in the medium to induce the denitrification process. As a negative control, cells were cultivated aerobically without nitrate. The crude extracts of the two cultures were investigated by Western blot with MAb dNIR29. No cross-reaction with proteins from the noninduced sample could be detected. In the induced sample, the antibody specifically recognized a protein band at 39 kDa, the expected molecular weight for copper-dNIR. No unspecific binding could be observed (Fig. 3).

After immunization with the native enzyme from *Ochrobactrum anthropi*, only one stable MAB producing cell line could be established (MAb dNIR1a). The antibody reacts with the nitrite-reductase of *O. anthropi* DSM 14396 only, which has been used for immunization (Fig. 1).

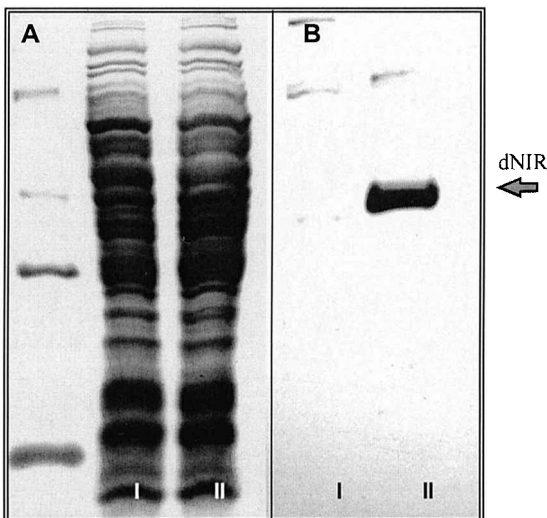
#### Detection limit and immunoglobulin classes

Both antibodies were of the IgG-type (IgG<sub>1</sub> or IgG<sub>3</sub> subtype). The light-chains could be classified as  $\lambda$ -chain-typus for MAb dNIR1a and  $\kappa$ -chain-typus for MAb dNIR29 (data not shown).



**FIG. 2.** Average distance tree calculated from conserved regions of the *nirK*-gene. The tree was calculated from dNIR-sequences of the EMBL nucleotide sequence database with the program ClustalW.

MAb dNIR1a showed a detection limit of 2 ng pure enzyme in ELISA, whereas it was possible to determine 5 ng dNIR with MAb dNIR29 (Fig. 4).



**FIG. 3.** Polyacrylamide gel-electrophoresis and western blot analysis of a crude extract from *A. faecalis* S6 with MAb dNIR29. (A) Shows the SDS-PAGE after silver-staining. (B) Shows an immunoblot with anti-dNIRMAb (detection by anti-mouse peroxidase-conjugate and chemoluminescence). *Alcaligenes faecalis* S6 was cultivated aerobically without nitrate (I) or anaerobically plus 2 mg/mL nitrate in the medium (II). Crude extracts of the 2 cultures were prepared and separated by SDS-PAGE.

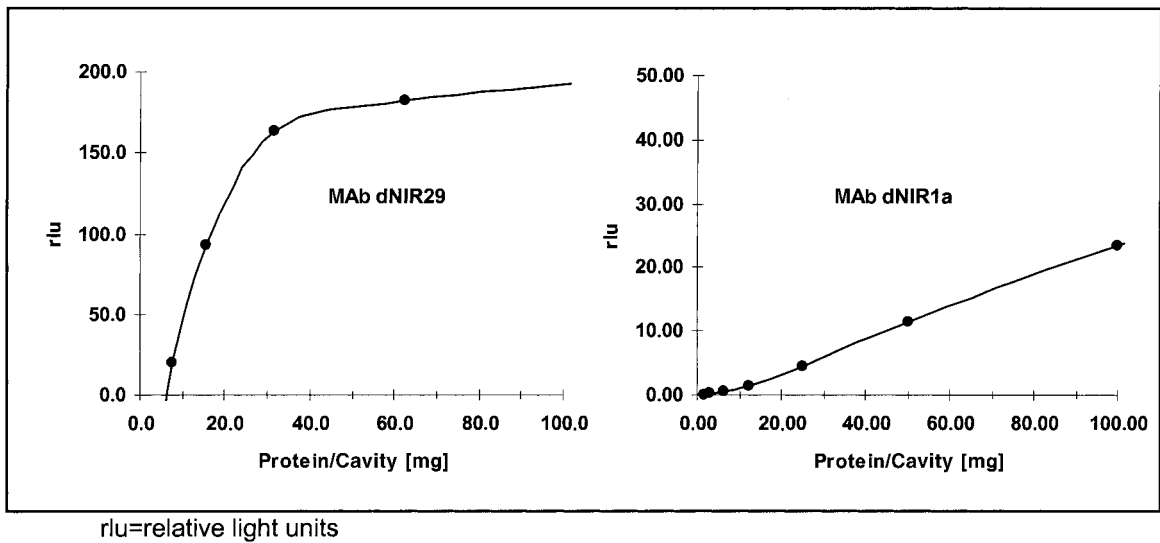
#### Immunofluorescence labeling

A procedure was established to detect dNIR-induced bacteria at the single-cell level, by immunofluorescence labeling. Fixing of the cells was carried out with paraformaldehyde (PFA) or ethanol. Whereas the PFA-fixed cells could be stored for several weeks with only slight losses of signal intensity, the signal-intensity of the ethanol-fixed cells was brighter immediately after fixing but declined to the detection limit during 2–4 weeks of storage. The developed protocol resulted in a clear labeling with no background of nonspecific cross-reaction. The immunofluorescence showed a ring-like structure reflecting the location of nitrite reductase in the periplasm of the bacteria (Fig. 5). No signal could be observed when labeling *Ps. aeruginosa* cells, which contain  $cd_1$ -dNIR, or by incubation with washing solution instead of the anti-dNIR antibody (data not shown).

## DISCUSSION

In this paper, we describe the generation and characterization of two types of MABs against the dissimilatory copper nitrite reductase (copper-dNIR), one of the key enzymes of the denitrification process, to determine its expression as a parameter for denitrification activity of bacteria.

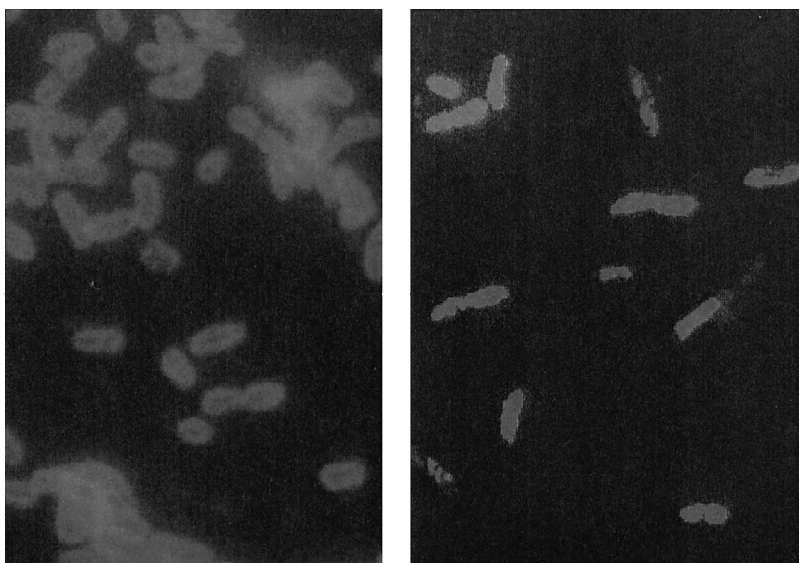
Two very distinct anti-dNIR antibodies were established: one that has a very broad specificity for copper dNIRs from bacte-



**FIG. 4.** Detection limit of the MAb dNIR1a and dNIR29 for copper-dNIR in ELISA using a POD-coupled secondary antibody and chemoluminescence substrate.

ria of different taxa and a second, strain-specific one, that exclusively recognizes the enzyme of one particular strain, used for immunization of mice (Fig. 1). No cross-reaction with the heme type of the dissimilatoric nitrite reductase (cd<sub>1</sub>-dNIR-type) was found. According to Braker et al.,<sup>(14)</sup> the DNA sequences for the nitrite reductases from *Ochrobactrum anthropi* and *Alcaligenes faecalis* are nearly identical in the conserved regions of the enzyme. Thus, the strain-specific antibody (MAb dNIR1a) obviously reacts with less conserved regions of the protein. Because of its missing cross-reaction with dNIRs from other bacteria, this antibody is only useful for a specific monitoring purpose of the expressions level of the dNIR of strain *O. anthropi* DSM 14396 in different environments.

The broad cross-reaction profile of MAb dNIR29 for phylogenetically different bacteria in contrast, indicates that it reacts with more conserved regions of nitrite reductase, for example at the C-terminus<sup>(14,15)</sup> or at the highly conserved copper binding sites<sup>(16)</sup> of the enzyme. The antibody recognizes nitrite reductases of organisms from the  $\alpha$ - and  $\beta$ -subclass of the Proteobacteria as well as the enzyme of *Corynebacterium*, which belongs to the low-GC group of the Gram-positive bacteria. The clustertree based on partial DNA sequences of nitrite reductases does not follow the phylogenetic clustertree (based on 16S rDNA sequences). It seems that phylogenetic different bacteria possess similar nitrite reductases. Thus, only the comparison of the cross-reaction profile of the MAb with the sequence simi-



**FIG. 5.** Immunofluorescence-labeling of *O. anthropi* and *A. faecalis* with the antibodies MAb dNIR1a and MAb dNIR29. Left = *O. anthropi* labeled with MAb-dNIR1a and anti-mouse-fluorescein; Right = *A. faecalis* (magnification: 100 $\times$ ) labeled with MAb dNir29 and anti-mouse-fluorescein.

larity is meaningful. It can be seen that MAb dNIR29 recognizes dNIRs with rather low DNA nirK sequence similarity too.

The antibodies show a detection limit for nitrite reductase of about 2 or 5 ng (Fig. 3). This is in the same range as reported for the serum cd<sub>1</sub>-NIR from Ward et al.,<sup>(17)</sup> who were able to determine the enzyme in crude extracts of denitrifying cultures of *Ps. stutzeri* down to 2.5 ng or  $2 \times 10^5$  cells by Western blotting.

Due to the inducible manner of nitrite reductase and the fast-changing temporal and spatial conditions in a natural sample, like in soil, the expression level in a bacterial community will not be homogenous. Thus, for a detailed understanding of the denitrification process, it is necessary not only to determine the overall number of nitrite reductase molecules, but to investigate microsites conducive for the introduction of denitrification enzymes. Only by analyzing with spatial resolution is it possible to assign the inducing conditions to the expression level of the enzyme. The *in situ* immunostaining protocol for the nitrite reductase on a single cell level under nondestructing conditions will be one possible way to reach this goal.

Another application of the antibodies of the MAb DNIR29 type on natural samples could be the sorting of bacteria that have expressed copper nitrite reductase after immunostaining by flow cytometry followed by a further characterization of these organisms by molecular techniques.

Furthermore, the MAbs could be used for detailed biochemical studies of the periplasm membrane, for example, using immunogold labeling techniques (Fig. 5).

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