Purification of Two Nitrate Reductases from \textit{Xanthomonas maltophilia} Grown in Aerobic Cultures

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\textit{Xanthomonas maltophilia} ATCC 17666 is an obligate aerobe that accumulates nitrite when grown on nitrate. Spectra of membranes from nitrate-grown cells exhibited \textit{b}-type cytochrome peaks and $A_{515-430}$ indicative of \textit{d}-type cytochrome but no absorption peaks corresponding to \textit{c}-type cytochromes. The nitrate reductase (NR) activity was located in the membrane fraction. Triton X-100-extracted reduced methyl viologen-NRs were purified on DE-52, hydroxylapatite, and Sephacryl S-300 columns to specific activities of 52 to 67 $\mu$mol of nitrite formed per min per mg of protein. The cytochrome-containing NRs, separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis into a 135-kDa $\alpha$-subunit, a 64-kDa $\beta$-subunit, and a 23-kDa $\gamma$-subunit with relative band intensities indicative of a 1:1:1 $\alpha/\beta/\gamma$ subunit ratio and a $M_r$ of 222,000. The electronic spectrum of dithionite-reduced purified NR displayed peaks at 425, 528, and 558 nm, indicative of the presence of a cytochrome $b$, an interpretation consistent with the pyridine hemochrome spectrum formed. The cytochrome $b$ of the NR was reduced under anaerobic conditions by menadiol and oxidized by nitrate with the production of nitrite. This NR contained 0.96 M, 12.5 n mole iron, and 1 hem per 222 kDa: molybdopterin was detected with the Neurospora crassa nit-i assay. A smaller reduced methyl viologen-NR (169 kDa), present in various concentrations in the Triton X-100 preparations, lacked a cytochrome spectrum and did not oxidize menadiol. The characteristics of the NRs and the absence of \textit{c}-type cytochromes provide insights into why \textit{X. maltophilia} accumulates nitrite.

Nitrified nitrogen is of considerable biological and economic importance in agriculture and aquatic ecosystems where it serves as a nitrogen source for plants, fungi, and algae. When not assimilated, animal waste- and fertilizer-derived nitrate in groundwater is a major pollutant (2) and/or is reduced to nitrite and gaseous nitrogen oxides by bacteria (49). The economics of waste management and agricultural loss are compounded by the concerns for the participation of nitrite in the formation of nitrosamines and nitrous oxide in the depletion of stratospheric ozone (33, 38).

Nitrate reductase (NR) catalyzes the first reaction in a variety of metabolic pathways whose physiological significance may depend on or be used for the reduced products (s). Plants, fungi, and some bacteria assimilate nitrate nitrogen after reducing it to ammonia. The microaerophilic to anaerobic process of bacterial denitration generates NO, $N_2O$, and/or $N_2$ as gaseous products of nitrate (through nitrite) reduction, whereas environmental ammonia is the product of dissimilatory nitrate reduction (14). Bacterial denitrification has long been considered an anaerobic process (49, 56), in part because oxygen represses NR in many bacteria (56, 59) or prevents nitrate transport to the active enzyme site (27).

Detailed experiments have shown, however, that the effects of oxygen on the cellular enzymes of denitrification vary greatly (16, 40, 42, 44, 58). For example, the obligate aerobes \textit{Aquaspirillum magnetotacticum} and \textit{Nitrosomonas europaea}, which will not grow without $O_2$, denitrify under microaerophilic conditions (57), and \textit{Thiosphaera pantotropha} respires on nitrate at oxygen concentrations of up to 90% of air saturation (51). Moreover, oxygen differentially affects the enzymes of denitrifiers, with NR least affected by oxygen in \textit{Pseudomonas stutzeri} (40) and present at all concentrations of oxygen tested in \textit{Achromobacter cycloclastes} (16). Nitrite is often the product of aerobic nitrate reduction among aerobes (5, 63), and many facultative anaerobes accumulate nitrite when grown anaerobically on nitrate (54).

Current research emphasis on denitrification and nitrate respiration has overshadowed the enzymology of nitrate reduction by aerobic bacteria, which, except for \textit{T. pantotropha} (4, 51), is poorly characterized. The classic aerobic systems for nitrate reduction are the assimilatory NRs of fungi, algae, and plants, which are synthesized when oxygen, an energy source, and nitrate are present in the absence of an usable reduced-nitrogen source (24). Assimilatory NRs are large, soluble enzymes that oxidize reduced pyridine nucleotides as substrates. Although genetic evidence for \textit{Pseudomonas aeruginosa} supports the existence of distinct assimilatory and dissimilatory NRs (32), no "assimilatory" NR from bacteria has been purified and characterized.

In contrast, the NRs from gram-positive (37, 61) and gram-negative (3, 6, 9) denitrifying bacteria and from the facultative anaerobes that accumulate nitrite or reduce nitrite to ammonia (12, 21, 60) are large membrane-bound molybdo-iron-sulfur proteins (28, 55). The NR complex consists of three subunits ($\alpha$, $\beta$, and $\gamma$)—the small $\gamma$ subunit is a cytochrome $b$ (11, 20, 37). Quinols or menaquinols function as the substrate for NRs containing cytochrome $b$ (17, 20, 37, 47), but not in heme-lacking NRs of the $\delta$-subunit structure. The menaquinol or quinol nitrate:oxidoreductases probably function in anaerobic membranes to couple proton pumping to ATP and nitrite formation (46), which explains the biochemistry of anaerobic respiration on nitrate.

To better understand aerobic nitrate reduction in bacteria, we have studied the enzymology of NR in \textit{Xanthomonas}.
maltophilia (63). This oxidase-negative, aerobic rod occurs in soil and other natural environments (39) and in the rhizosphere of certain plants (36) and is often encountered in human clinical specimens (29, 30). It stoichiometrically converts nitrate to nitrite while growing aerobically and is unable to respire anaerobically on nitrate or to metabolize nitrite (63). Our results show that in aerobic culture, X. maltophilia cells make two nitrate reductases, one of which contains cytochrome b and oxidizes menaquinol.

MATERIALS AND METHODS

Organism and growth conditions. X. maltophilia ATCC 17666, which produces high NR activity (63), was maintained in stock culture at −20°C in 40% (wt/vol) glycerol. Cells were grown in enriched nitrate broth containing (per liter of distilled water) Bacto Tryptone (10 g), Bacto Yeast Extract (5 g), dextrose (5 g), NaNO₃ (4 g), NaCl (5 g), and KH₂PO₄ (2.5 g). Cultures grown in a 10-liter New Brunswick fermentor at 28°C, stirred at 400 rpm with compressed air entering at 4 liters min⁻¹, yielded 160 g (wet weight) of cells in 24 h when inoculated with a 200-ml culture. The cells were collected by continuous-flow centrifugation, washed in phosphate buffer (50 mM KH₂PO₄/K₂HPO₄, 10 mM MgSO₄, pH 7.6), suspended in 2 volumes of identical buffer, and then frozen at −20°C until used.

Preparation of membranes. Thawed cell suspensions supplemented with 1 mM phenylmethylsulfonyl fluoride to inhibit proteolysis were passed through a French pressure cell at 20,000 lb/in². This and all subsequent procedures were carried out at 4°C. A few milligrams of DNase I was added to decrease viscosity before treatment with neutralized streptomycin sulfate (0.5 mg/ml of protein). After stirring for 20 min, the mixture was centrifuged at 13,200 × g for 30 min. The pellet was discarded, and the supernatant solution was centrifuged at 144,000 × g for 90 min to sediment the membranes. The red-brown pellet, called the membrane fraction, was suspended in 10 mM KH₂PO₄/K₂HPO₄ (pH 7.6) with 0.1 mM phenylmethylsulfonyl fluoride and used as the source of NR.

Purification of NR. Membranes were extracted overnight by stirring with 2% Triton X-100 added as a filtered 10% solution. Debris was removed by centrifugation at 144,000 × g for 90 min, and the supernatant solution was diluted with an equal volume of 10 mM KH₂PO₄/K₂HPO₄ (pH 7.6) before loading on a DE-52 column (4.5 by 24 cm) equilibrated in 10 mM Tris-HCl buffer (pH 7.6) containing 1% Triton X-100. A dark-brown band was clearly visible on the top of the column. After a wash with 2 column volumes of the equilibration buffer, the column was eluted with a 0 to 400 mM NaCl gradient (1,000 ml total) in the equilibration buffer with the peak NR yield eluted at 190 mM NaCl. The pooled NR fractions were loaded on a hydroxylapatite-HTP column equilibrated with 3 mM KH₂PO₄/K₂HPO₄ buffer (pH 7.6) containing 0.1% Triton X-100. The column was subjected to a 3 to 200 mM gradient of KH₂PO₄/K₂HPO₄ (pH 7.6) containing 0.1% Triton X-100. Reduced methyl viologen (MVH)-NR was associated with a red band (NRb) that eluted between 35 and 41 mM KH₂PO₄/K₂HPO₄, whereas the remaining MVH-NR eluted as a brown band (NRg) between 86 and 102 mM KH₂PO₄/K₂HPO₄. The two NR peaks were pooled and concentrated with an Amicon YP-10 membrane to small samples (2 to 3 ml), each of which was loaded on a Sephacryl S-300 column (3 by 90 cm) equilibrated in 50 mM KH₂PO₄/K₂HPO₄ buffer containing 0.1% Triton X-100 and eluted at a flow rate of 5 and 7 ml h⁻¹. Eluates containing the peak NR activity were pooled and concentrated before being stored at −20°C in 20% sucrose.

Subunit structure and molecular weight analysis. Molecular weights of the two NRs were estimated (62) by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (43) with the following proteins obtained from Bio-Rad serving as standards: myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase a (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Gradient gels of 7.5 to 18% acrylamide were used for subunit separation, whereas 7.5% acrylamide gels were used to determine the molecular mass of the α-subunit as previously described (37).

Optical spectra and metal analysis. Spectra were obtained for samples assayed at room temperature with a Shimadzu UV-2100 double-beam spectrophotometer. Heme content was determined from spectra of the alkaline pyridine hemochrome prepared in 0.075 M NaOH and 25% pyridine (23). The optical density at 554 to 570 nm obtained after reduction with a few crystals of dithionite was used to calculate heme b content by using the millimolar extinction coefficient of 34.4 (53). Flavin was assayed spectrophotometrically after extracting the sample with 8% trichloroacetic acid (52). Metal content was determined by plasma emission spectroscopy with a Mark II Jarrel-Ash model 965 ICAP. Separate calibration curves were run for Fe and Mo in Triton X-100.

Enzyme and protein assays. MVH-NR was assayed at room temperature in 50 mM KH₂PO₄/K₂HPO₄ buffer, pH 7.2, by using the diazo procedure for measuring nitrite (24). One unit of nitrate reductase activity equals 1 μmol of nitrite formed per min. The preparation of menadion and its oxidation in the presence of nitrate was followed at 266 nm under anaerobic conditions in 50 mM Tris-HCl, pH 8.2, containing menadion (333 μM), sodium nitrate, and enzyme as previously described (37). Glucose oxidase (2 U), catalase (6 U), and glucose (20 mM) were used to scavenge oxygen and ensure anaerobiosis (10). Molybdenopterin (MPT) was assayed, and its concentration was calculated as described elsewhere (25). Protein was determined by the Bradford method (7) by employing the Bio-Rad reagent with bovine serum albumin as the standard.

Materials. Hydroxyapatite Bio-Gel-HTP and acrylamide were obtained from Bio-Rad Laboratories, Richmond, Calif.; Sephacryl S-300 was from Pharmacia, Uppsala, Sweden; 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfo- nate (CHAPS) and 3-[3-cholamidopropyl]dimethylammonio]-2-hydroxypropane-1-sulfonate (CHAPSO) were from Calbiochem, San Diego, Calif.; DE-52 was from Whatman Ltd., Kent, United Kingdom; and buttermilk xanthine oxidase, phenylmethylsulfonyl fluoride, menadione, catalase, and glucose oxidase were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Membrane cytochromes. Membranes isolated from cells grown aerobically on nutrient broth with and without nitrate contained MVH-NR at specific activities of 0.1 and 0.15, respectively. Spectra of dithionite-reduced membranes revealed absorbance peaks at 428, 527, 560, and 629 nm indicative of b-type cytochromes and a cytochrome d. Ascorbate had no effect on the absorption spectrum of the isolated membranes. Peaks at 427, 532, and 560 nm appeared after NADH addition under anaerobic conditions. The A₄₅₀ decreased significantly in the presence of nitrate.
TABLE 1. Purification of NR from aerobically grown X. maltophilia cells

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Vol (ml)</th>
<th>Total U</th>
<th>Total protein (mg)</th>
<th>Sp act</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>460</td>
<td>1,853</td>
<td>3,358</td>
<td>0.55</td>
<td>100</td>
</tr>
<tr>
<td>High-speed pellet</td>
<td>125</td>
<td>932</td>
<td>1,006</td>
<td>0.93</td>
<td>50</td>
</tr>
<tr>
<td>Triton X-100 high-speed supernatant</td>
<td>136</td>
<td>756</td>
<td>385</td>
<td>1.91</td>
<td>39.7</td>
</tr>
<tr>
<td>DE-52 pool</td>
<td>103</td>
<td>441</td>
<td>60.2</td>
<td>7.35</td>
<td>24</td>
</tr>
<tr>
<td>Hydroxylapatite pool</td>
<td>24</td>
<td>436</td>
<td>14.1</td>
<td>30.9</td>
<td>23.5</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>20</td>
<td>377.2</td>
<td>7.2</td>
<td>52.4</td>
<td>20.3</td>
</tr>
</tbody>
</table>

Purification of MVH-NR. The presence of 10 mM MgSO₄ in the crude extract facilitated sedimentation of NR with the membrane fraction (3, 9). Triton X-100 (2%) treatment of the membrane fraction released the most NR activity, with 2% deoxycholate, 2% CHAPS, and 2% CHAPSO releasing 85, 30, and 28% as much activity, respectively. Heating for 30 min at 60°C released 60% of the NR activity solubilized by Triton X-100 with a high specific activity (5.83), in part because only 10% of the membrane protein was released. As recovery of b cytochromes is not observed routinely with heat- or deoxycholate-solubilized NRs (1, 6, 9), we chose to use Triton X-100.

The cytochrome-containing NR was purified >90-fold through DE-52, Bio-Gel-HP, and Sephacryl S-300 columns to a specific activity of 52 with 20% recovery (Table 1). Two distinct MVH-NRs separated during the Bio-Gel-HP step. The cytochrome b containing NR₁ (75% of MVH-NR activity) eluted first from the HTP column between 35 and 41 mM phosphate, whereas a second MVH-NR (NR₁) eluted at between 86 to 102 mM phosphate (Fig. 1). The relative amounts of the two NRs varied greatly, with NR₁ being the prominent form in three of the four preparations. Upon concentration, both NRs were brown; however, NR₁ exhibited no cytochrome spectrum. Each NR was further purified on Sephacryl S-300 columns.

Enzyme purity and subunit structure. Purified NR₁ is a heteromultimeric protein that separates into three major protein-staining bands on SDS-PAGE (Fig. 2). Minor bands observed below the middle (β) band may represent degradation products or a processing of the β-subunit as observed for the Escherichia coli NR (18, 47) and the Pseudomonas sp. NRs (6, 31). These latter bands were more pronounced after enzyme storage. The three major bands shown in Fig. 2 accounted for more than 95% of the Coomassie blue-staining protein. Moreover, the MVH-NR specific activity of the X. maltophilia NR₁ was greater than that reported for NR from Bacillus licheniformis (61), Bacillus halodenitrificans (37), P. stutzeri (6), and P. aeruginosa (9) and approaches that of cytochrome-lacking respiratory NR isolated from E. coli and Paracoccus denitrificans (1, 17, 47).

Molecular mass estimates from SDS-PAGE for the α, β, and γ bands were 135, 64, and 23 kDa, respectively (Fig. 2). Distribution of the stained protein by densitometer scans revealed 66% associated with the α band, 26% with β, and 8% with γ, which is consistent with a 1:1:1 ratio of these subunits—assuming they stain equally with Coomassie blue. The presumed Mr of 222,000 for the X. maltophilia enzyme was essentially identical to that of NR from B. halodenitrificans (37). However, the X. maltophilia NR₁ exhibited a smaller α-subunit and a larger β-subunit than the B. halodenitrificans NR.

Spectral and metal analysis of purified NR. Electronic spectra of NR as prepared (native) exhibited a broad absorption between 400 and 450 nm with a major peak at 409 nm (Fig. 3). Absorption maxima at 425, 528, and 558 nm observed upon reduction with dithionite are characteristic of b-type cytochromes. The absorption spectrum of the pyridine hemochrome extracted from purified NR was characteristic of a protoporphyrin IX, thus identifying the heme of NR as b type. Because flavin was not detected in trichloroacetic acid extracts of the purified enzyme, we attribute the high absorption between 400 and 450 nm to iron-sulfur clusters.

Molybdenum, heme iron, and total iron were determined on two samples of purified NR indicating 0.96 molybdenum, 1 heme iron, and 12.5 nonheme iron per mol of NR, assuming a Mr of 222,000. Standards containing Triton X-100 equivalent to its concentration in the enzyme samples were used in both the molybdenum and iron analyses. These data are consistent with a molybdenum/heme/nonheme iron ratio.

FIG. 1. Separation of MVH-NR on a hydroxylapatite column by using a linear gradient of 3 to 200 mM KH₂PO₄/K₂HPO₄ (pH 7.6). The ratio of NR₁ to NR₁ varied among preparations.

FIG. 2. SDS-PAGE of purified NR₁ from X. maltophilia run on 7.5 to 18% acrylamide gel showing the α-, β-, and γ-subunits and a densitometry scan of the gel revealing the relative concentrations of each band.

FIG. 3. Absorption spectra of NR₁ and NR₁ and the ratios of iron to molybdenum, heme to nonheme iron, and molybdenum to heme iron.
of 1:1:12, suggesting that for each molybdenum there is one heme iron and at least three iron-sulfur clusters.

Two preparations of purified NR\textsubscript{I} contributed MPT to the reconstitution of NADPH-NR in the Neurospora crassa nit-i assay (25). Although restoration of NADPH-NR was proportional to the amount of denatured NR added to the nit-i extract, less than 1% of the expected MPT in the bacterial NR was converted to NADPH-NR (0.138 pmol of MPT per 35 pmol of bacterial NR). The same procedure was more efficient in transferring MPT from xanthine oxidase to NADPH-NR (2.08 pmol of MPT per 45 pmol of xanthine oxidase). The recent discovery that, unlike MPT from eukaryotes, MPT in CO dehydrogenase contains a cysteine residue (35, 41) and MPT in dimethyl sulfoxide reductase contains a guanosine residue (34) suggests bactopterins need to be processed before participating in the nit-i reactivation. A greater understanding of the reconstitution process may lead to improved techniques for measuring bacterial MPTs with the nit-i assay.

**Characteristics of NR\textsubscript{II}**. Purified NR\textsubscript{II} eluted from the HTP column at 86 to 102 mM KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{2}HPO\textsubscript{4} (Fig. 1) well separated from NR\textsubscript{I}. Indicative of its smaller size, NR\textsubscript{II} eluted 75 ml after NR\textsubscript{I} on the Sephacryl-300 column. On SDS-PAGE, NR\textsubscript{II} separated into major bands at 125 and 44 kDa and a minor 70-kDa band. The major bands are characteristic of an \(\alpha\beta\) subunit structure observed in other bacterial NR preparations (3, 6, 17, 31). Assuming that the weak 70-kDa band is derived from the \(\alpha\)-subunit by proteolysis, as demonstrated for NRs from pseudomonads (6, 31), we estimated the \(M_\text{r}\) of NR\textsubscript{II} to be 169,000. Spectra of concentrated preparations from the HTP and Sephacryl columns contained no indication of cytochromes in NR\textsubscript{II}. Purified preparations of NR\textsubscript{II} had specific activities for MVH-NR of between 30 and 67. Metal analysis of two NR\textsubscript{II} samples indicated 6 mol of iron and 0.2 mol of molybdenum per mol of enzyme.

**Substrates for NR**. Under anaerobic conditions, the cytochrome \(b\) in purified NR\textsubscript{I} was reduced by menadiol and oxidized by nitrate (Fig. 4). The rate of menadiol oxidation followed at 266 nm was proportional to NR concentration, and the end product formed was nitrite. NR\textsubscript{II} did not oxidize menadiol, and neither of the NRs used NADPH or NADH (200 \(\mu\)M) as a substrate for nitrate reduction in the presence or absence of flavin adenine dinucleotide (10 \(\mu\)M). Characteristics of the two X. maltophilia NRs are summarized in Table 2.

**DISCUSSION**

About half of the known strains of X. maltophilia rapidly reduce nitrate to nitrite (63). Despite numerous attempts to

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**FIG. 3.** Spectrum of purified NR\textsubscript{I} (0.5 mg of protein ml\textsuperscript{-1}) as prepared (-----) and reduced with a few crystals of dithionite (-----).

**FIG. 4.** Spectrum of purified NR\textsubscript{I} (72 \(\mu\)g of protein) in 2.0 ml of 50 mM KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{2}HPO\textsubscript{4} buffer, pH 7.6, as prepared (-----), 1 min after adding 100 nmol of menadiol (-----), and 1 min after adding 3 \(\mu\)mol of NaNO\textsubscript{3} (-----).
TABLE 2. Characteristics of X. maltophilia NRs

<table>
<thead>
<tr>
<th>NR</th>
<th>Sp act</th>
<th>Absorption spectrum (nm)</th>
<th>Home/iron/molybdenum ratio</th>
<th>M&lt;sub&gt;r&lt;/sub&gt;</th>
<th>Subunit structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR&lt;sub&gt;I&lt;/sub&gt;</td>
<td>52.4</td>
<td>425, 528, 558</td>
<td>1:12:1</td>
<td>222,000</td>
<td>αβγ</td>
</tr>
<tr>
<td>NR&lt;sub&gt;II&lt;/sub&gt;</td>
<td>60.0</td>
<td>No peaks</td>
<td>0:60:2</td>
<td>169,000</td>
<td>αβ</td>
</tr>
</tbody>
</table>

The inability of X. maltophilia to metabolize nitrate may be related to the absence of c-type cytochromes in this oxidase-negative bacterium. c-type cytochromes are essential to key reactions in denitriﬁcation and the pathway for nitrite reduction to ammonia. The cd<sup>−</sup> nitrite reductase of several Pseudomonas spp. (15, 28), the E. coli and Desulfovibrio desulfuricans cytochrome c<sub>553</sub> nitrite reductase (13, 45), and the bc complex of the nitric oxide reductase in P. stutzeri recently characterized by Heiss et al. (26) all involve a c-type cytochrome. As shown by the elegant experiments of Braun and Zumft (8), the Nor phenotype in P. stutzeri (mutants defective in nitric oxide reductase) is conditionally lethal for growth on nitrate or nitrite, conditions permitting NO to accumulate. However, the Nir<sup>−</sup> Nor<sup>−</sup> double mutant, lacking both nitrite reductase and nitric oxide reductase, was viable when grown anaerobically solely at the expense of nitrate respiration. The absence of c-type cytochromes in X. maltophilia may be the key biochemical factor limiting its ability to metabolize nitrite.

In some ways, nitrate reduction in X. maltophilia is similar to that in E. coli K-12 strains that lack the cytochrome c<sub>553</sub> nitrite reductase and to Rhodobacter capsulatus, which lacks nitrite reductase (22). However, unlike E. coli and R. capsulatus, X. maltophilia is an obligate aerobe and does not respire anaerobically on nitrate (63). The insensitivity of NR formation to oxygen in X. maltophilia resembles aerobic denitriﬁcation seen in T. pantotropha. Although the physiological beneﬁt of reducing nitrite to nitrate in X. maltophilia is unknown, nitrate-reducing strains may function in the cycling of nitrogen in aerobic environments, generating nitrate that can be oxidized by nitrifying bacteria (5).

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