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Occurrence of Nitrate Reductase and Molybdopterin in *Xanthomonas maltophilia*

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Fifteen of 23 ATCC strains and 2 of 9 clinical isolates of *Xanthomonas maltophilia*, all of which grew aerobically on ammonia, but not nitrate, as a sole nitrogen source, reduced nitrate to nitrite. *X. maltophilia* failed to grow anaerobically on complex medium with or without nitrate, so it is considered an obligate aerobe. Nitrate-reducing strains contained reduced methyl viologen nitrate reductase (MVH-NR) with specific activities ranging from 49.2 to 192 U mg of protein^{-1}. Strain ATCC 17666 doubled its cell mass after 3 h of growth on nitrate broth under low aeration, possessed maximal MVH-NR activity, and converted the added nitrate to nitrite, which accumulated. Dissolved oxygen above 15% saturation greatly suppressed nitrite formation. All strains, except ATCC 14535, possessed between 0.25 and 5.05 pmol of molybdopterin mg of protein^{-1} as measured by the *Neurospora crassa nit-1* assay. The molybdopterin activity in the soluble fraction sedimented as a single symmetrical peak with an $s_{20,w}$ of 5.1. Studies identified MVH-NR in selected strains as a membrane-bound protein. The deoxycholate-solubilized MVH-NR sedimented as a single peak in sucrose density gradients with an $s_{20,w}$ of 8.8. The MVH-NR of *X. maltophilia* has the physical characteristics of a respiratory nitrate reductase and may enable cells to use nitrate as an electron sink under semiaerobic conditions.

Xanthomonas maltophilia, formerly *Pseudomonas maltophilia* (22, 36, 43), is a nonfermentative, oxidase-negative, aerobic rod that is widely distributed in soil and other natural environments (20, 29, 33), is present in the rhizosphere of certain plants (24, 25), and is an opportunistic human pathogen frequently encountered in clinical specimens (20, 21, 38). The broad antibiotic resistance of *X. maltophilia* (20) has been exploited to construct a selective medium for its isolation from soils and grasses (24). The ubiquitous distribution of this species, its importance in human disease, its purported ability to colonize small green roots and to stimulate wheat growth (25), and reports that some strains reduce nitrate led us to study the occurrence and physiology of nitrate reduction in *X. maltophilia*.

The four known physiological roles for bacterial nitrate reduction are assimilatory, dissimilatory (denitrification), respiratory, and as an auxiliary electron acceptor in fermentations. Assimilatory nitrate reductase (NR) initiates the assimilation of nitrate nitrogen via ammonia and is not affected by the presence of oxygen (12). In denitrification, the denitrifying NR reduces nitrate to nitrite, which is reduced subsequently to gaseous forms of nitrogen, such as nitrous oxide or dinitrogen or both (40). Nitrate respiration in *Escherichia coli* and other members of the family *Enterobacteriaceae* couples nitrate reduction to NADH oxidation while generating a proton motive force to generate ATP (42). The nitrite produced either accumulates or is reduced to nongaseous nitrogen compounds (5). Nitrate can also serve as an electron acceptor in facultative anaerobes such as *Staphylococcus aureus* (6) and in obligate anaerobes, such as *Clostridium* (15, 41), *Propionibacterium* (1), and *Mitsuokella* (46) spp., for generating ATP by either substrate-level reactions or electron transport phosphorylation.

Approximately half of the *X. maltophilia* strains studied previously (20, 21) produced nitrite from nitrate, even though they neither grew anaerobically on nitrate nor produced gas from nitrate. In the present study we surveyed the American Type Culture Collection (ATCC) strains of *X. maltophilia* and clinical isolates from Henry Ford Hospital (HFH) in Detroit, Mich., for the presence of NR and the ability to produce active molybdopterin (MPT) required for NR activity (9, 23, 31). Selected nitrate-reducing strains were then used to study the physiology of nitrate reduction.

MATERIALS AND METHODS

Organism and growth conditions. The ATCC collection of *X. maltophilia* strains was kindly provided by Robert Gherna, American Type Culture Collection, Bethesda, Md. The clinical isolates were collected at Henry Ford Hospital during October and November of 1987. All strains were maintained on nutrient agar slants and/or stored at −20°C in 40% (wt vol^{-1}) glycerol. Cultures were grown in nutrient broth (Difco Laboratories, Detroit, Mich.), in nitrate broth (nutrient broth plus 0.1% [wt vol^{-1}] $NaNO_3$); in complex nitrate broth (tryptic soy broth [Difco] containing 0.1% [wt vol^{-1}] yeast extract [Difco], 1.0% glycerol, and 0.5% $NaNO_3$); or in a defined medium composed of Vogel's (44) salts (wt vol^{-1}: 0.2% $MgSO_4 \cdot 7H_2O$, 0.2% citric acid, 1.0% K_2HPO_4, and 0.232% NaH_2PO_4), pantothenic acid and nicotinic acid at 2.5 μg/ml, methionine, valine, and leucine at 20 μg/ml, and 0.1% (wt vol^{-1}) $NaNO_3$, 0.1% (wt/vol^{-1}) NH_4Cl, or 0.1% (wt vol^{-1}) of both. Inocula were grown in the defined NH_4 medium and then diluted 1/30 in sterile water, and a drop was transferred to 5 ml of defined medium.

Anaerobic growth in tubes of nitrate broth sealed with paraffin wax-paraffin oil (1:3, wt wt^{-1}) and nutrient agar plugs was the test for denitrification. The studies on dissolved oxygen (DO_2) were performed in a New Brunswick BioFlo III fermentor equipped with a polarographic oxygen sensor calibrated to zero with argon and to 100% dissolved

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oxygen with compressed air at a flow of 1 ft^3 (2.83×10^{-2} m^3)/min.

Extracts were prepared from cells grown under low aeration in 200 ml of nitrate broth in 500-ml Erlenmeyer flasks for 24 h at 30°C in a New Brunswick G76 water bath shaker at 134 rpm. Cultures were harvested and then washed by centrifugation three times in phosphate buffer (0.02 M NaH_2PO_4/Na_2HPO_4, 1 mM EDTA, pH 7.2) to remove nitrite. Washed cells were resuspended in phosphate buffer (1 g [wet weight] 3 ml^{-1}) and stored frozen at −20°C. Thawed cell suspensions were ruptured by passage through a French pressure cell at 20,000 lb/in^2. The supernatant from the 20-min 20,000 × *g* centrifugation was designated the crude extract.

Neurospora crassa nit-1 was grown on Fries medium as described previously (37). Nitrate-induced mycelia were harvested, washed, and dispersed in pH 7.2 buffer composed of 0.1 M Na_2HPO_4/NaH_2PO_4, 1% (wt vol^{-1}) NaCl, and 5×10^{-3} M EDTA (1 g of mycelia 3 ml^{-1}), using an IKA UT-dispersing tool (Tekmar Co., Cincinnati, Ohio). This mycelial suspension was ground in a cold TenBroeck tissue homogenizer, and the cell debris was removed by centrifugation at 20,000 × *g* for 20 min. The decanted supernatant solution was frozen at −20°C until used in the MPT assay.

Assay for reduced MVH-NR, MPT, and protein. Reduced methyl viologen nitrate reductase (MVH-NR) was measured at 23°C as described before (14). One unit of NADPH-nitrate reductase (NR) or MVH-NR equals 1 nmol of nitrite formed per min at room temperature. MPT was assayed by the reconstitution of NADPH-NR in extracts of *N. crassa nit-1*. One unit of complementing activity equals 1 U of NADPH-NR formed during 10 min at 23°C (37). Control assays determined nitrite and NADPH-NR in the bacterial extract during each assay. Nitrite was measured with the diazo procedure (14). Nitrate was measured with the same assay after being reduced to nitrite with a few crystals of zinc dust. The control experiments also revealed the presence of excess aponitrate reductase in *nit-1* extracts with acid-treated xanthine oxidase as a source of MPT (26). The measured concentration of MPT in crude extracts correlated linearly with protein concentration. Moles of MPT were calculated by using the value of 26 μmol of NO_2^- min^{-1} ng-atom of Mo^{-1} in reconstituted NADPH-NR (16). Protein was determined with the Bradford protein assay, using the Bio-Rad dye and bovine serum albumin standard (4).

Sucrose density gradients. Sucrose gradients were prepared with 15.5 and 33% (wt vol^{-1}) sucrose solutions in 0.02 M phosphate (Na_2HPO_4/NaH_2PO_4) buffer, pH 7.2. The gradients were centrifuged at 110,000 × *g* in a Beckman SW65 rotor at 4°C for 18 h. Estimation of the sedimentation coefficient was made by the Martin and Ames method (34), with yeast alcohol dehydrogenase as the marker protein.

RESULTS

Metabolism of nitrate. Of the strains examined, 15 of the 23 ATCC strains and 2 of the 9 HFH clinical isolates reduced nitrate to nitrite during semiaerobic growth (Table 1). Nitrite accumulated in these cultures and was not metabolized further. The nitrite-negative cultures did not reduce nitrate, which remained in the cultures after growth had ceased. Tests for anaerobic growth on nutrient broth-nitrate medium were negative for all test strains. Since some bacteria require a rich medium for denitrification, we tested the nitrate-reducing strains on tryptic soy broth supplemented with yeast extract, glycerol, and nitrate. They all grew aerobically on this complex nitrate medium, but none grew under anaerobic conditions.

All test strains grew on defined media containing ammonium ions as the nitrogen source and on ammonium ions and nitrate, but none were able to grow on nitrate or nitrite as the sole nitrogen source. Nitrite (0.01%, wt vol^{-1}) did not inhibit growth since each strain grew within 48 h of inoculation in nutrient broth containing 0.01% (wt vol^{-1}) nitrite. This nitrite remained after 8 days of incubation. Unlike the nitrite reductase of *E. coli*, which is inhibited by high but not low (0.01%, wt vol) nitrite (11), *X. maltophilia* does not metabolize nitrite even at low concentrations.

The production of nitrite by 53% of the strains tested is consistent with previous reports of nitrate reduction in this species (20, 21). The lower incidence of nitrate reduction in the clinical isolates (2 of 9) appears related to sample size, since 5 of an additional 12 *X. maltophilia* strains we isolated at HFH in 1988 reduced nitrate. That nitrite accumulated in nitrate-reducing cultures and was not metabolized by the other strains indicates that *X. maltophilia* does not produce a nitrite reductase.

Occurrence of MVH-NR in *X. maltophilia*. All nitrate-reducing cultures exhibited an MVH-NR with specific activities between 49.2 and 191 U mg of $protein^{-1}$ (Table 1), similar to the NR activities in crude extracts of *Pseudomonas aeruginosa* (8) and *Paracoccus denitrificans* (7). Strain ATCC 17666 was selected for studies on the effects of nitrate on growth and induction of MVH-NR (Fig. 1). The pattern of MVH-NR formation and nitrite accumulation observed resembled that reported for *Pseudomonas aeruginosa* (45), except in the latter the nitrite produced was metabolized after it reached a concentration of 3 to 8 μmol ml^{-1}. In our experiments, cells converted the added nitrate (11.8 μmol ml^{-1}) completely to nitrite within 150 min. Unexpectedly, more nitrite appeared than reduction of the nitrate added to the medium would provide. This surpassing of stoichiometry could be caused by the production of nitrite by heterotrophic nitrification (39), a possibility not yet tested. Zero-time cells displayed a low level of NR (26.6 U mg of $protein^{-1}$), which increased dramatically during the first 60 min and reached 225 U mg of $protein^{-1}$ after 3 h, activity comparable to that of cells of the same strain grown on nitrate broth for 24 h. Under semi-aerobic growth conditions (Fig. 1), cell mass doubled during the 3-h experiment. The accumulated nitrite did not measurably inhibit the final growth yield, which equaled that of cultures grown in the absence of nitrate.

The effect of oxygen on the formation of nitrite was studied in cultures growing in a New Brunswick BioFlo III fermentor. The medium, strain, and initial cell concentration were the same as the experiment reported in Fig. 1. Less than 2% of the nitrate appeared as nitrite after 3 h of growth at a DO_2 of 50% saturation. Significant quantities of nitrite (e.g., representing >15% of the nitrate) accumulated only after the DO_2 fell to <15% saturation. These results are consistent with the effects of oxygen on the formation of NR and the reduction of nitrate in *Pseudomonas stutzeri* (30) and other denitrifiers (7, 42).

Membrane-bound nature of MVH-NR. More than 80% of the MVH-NR activity was present in the membrane fraction separated from crude extracts of *X. maltophilia* ATCC 13637, prepared in 10 mM $MgSO_4$ to stabilize membranes (2, 8), by centrifugation at 90,000 × *g* for 2 h. The specific activity of the pelleted MVH-NR increased threefold over that of the crude extract (Table 2). The NR solubilized from the membranes by deoxycholate used MVH, but not

TABLE 1. NR activity and MPT in *X. maltophilia*

Strain	Nitrite (nitrate[a]) in medium	U mg of protein^{-1}		MPT (pmol mg of protein^{-1})
		MVH-NR	*N. crassa nit-1* complementation	
ATCC				
12714	− (+)	0	0.98	0.25
13270	+	56.7	16.5	4.25
13636	− (+)	0	10.9	2.80
13637	+	136.0	15.1	3.89
13843	− (+)	0	2.3	0.59
14535	− (+)	0	0	0
15099	− (+)	0	4.72	1.22
17445	+	53.7	5.56	1.43
17666	+	192.0	19.6	5.05
17671	+	92.3	6.44	1.66
17672	− (+)	0	11.6	2.99
17673	+	129.0	11.2	2.89
17674	+	117.0	5.93	1.53
17675	+	90.3	5.67	1.46
17576	+	90.1	2.25	0.58
17677	− (+)	0	1.80	0.46
17678	+	81.8	8.69	2.24
17679	+	49.2	12.5	3.22
17806	+	79.9	4.23	1.09
17807	+	149.6	6.13	1.58
17808	+	135.6	5.36	1.38
19374	− (+)	0	2.73	0.70
21879	+	65.8	6.06	1.56
HFH				
1	− (+)	0	2.90	0.74
2	− (+)	0	2.48	0.64
3	− (+)	0	3.36	0.87
4	− (+)	0	4.78	1.23
5	− (+)	0	3.60	0.93
6	+	118.3	14.50	3.74
7	− (+)	0	4.40	1.13
8	+	155	12.30	3.17
9	− (+)	0	5.10	1.31

[a] Measured after growth following reduction to nitrite with zinc dust.

NADPH or NADH, as electron donor and sedimented as a single peak in sucrose gradients with an $s_{20,w}$ of 8.8.

Presence of MPT. Many bacteria contain MPT (10, 23, 27, 28), as an essential component of most known molybdoenzymes (19), including bacterial NRs (9, 41, 46). When bound to a molybdoenzyme, MPT remains nonreactive in the *nit-1* assay until released by high ionic strength or acid treatment (16, 26). A freely accessible, dialyzable form of MPT active in the *nit-1* assay without prior treatment also exists in bacterial extracts (28) and this form of MPT was measured. Crude extracts of all strains of *X. maltophilia* tested possessed detectable MPT activity, except ATCC 14535 (Table 1). Since no NAD(P)H-NR activity was detected in either the bacterial or the *nit-1* extracts, endogenous NR activity did not interfere with the assay.

To estimate the concentration of active MPT per mg of bacterial protein, we compared our assay conditions with the 4°C 24-h assay procedure of Hawkes and Bray (16) for the quantitative measurement of MPT in purified molybdoenzymes. Simultaneous assays using bacterial extracts and our preparations of *nit-1* were performed at 23 and 4°C for up to 24 h, with and without Na_2MoO_4 (Table 3). Assays of sufficiently numerous dilutions of the bacterial extract assured the presence of excess *nit-1*. Addition of molybdate to the bacterial extracts slightly inhibited the MPT assay (Table 3) in contrast to the stimulation of the MPT assay by molybdate when purified molybdoenzymes served as the source of MPT. Molybdate stabilizes the MPT dissociated from molybdoenzymes with acids, detergents, or salts (16, 19), but the dissociable MPT present in bacterial extracts needs no stabilizing (27).

The quantity of MPT in the assay of bacterial extracts maximized after 24-h incubation at 4°C, revealing 6.7 times more NADPH-NR than our standard assay (10 min at 23°C). The MPT concentrations calculated from the 10-min 23°C assay were multiplied by 6.7 to approximate the picomoles of MPT per milligram of protein in the bacterial extract (Table 3).

MPT concentrations in nitrate-reducing strains ranged from 0.58 to 5.05 pmol mg of protein^{-1}, with an average of 2.40 pmol mg of protein^{-1}. In strains lacking NR, the MPT concentration ranged from 0 to 2.99 pmol mg of protein^{-1}, with an average of 1.06 pmol mg of protein^{-1}. If this assay measures the molybdopterin active in bacterial NR formation, then NR formation is not prevented by lack of MPT in any strain except ATCC 14535.

The dissociable form of MPT behaves as a large molecule in bacterial extracts despite the molecular weight estimate of 760 for bacterial MPT (32). Presumably MPT is bound to a "carrier" protein within cells and in crude extracts. MPT from *X. maltophilia* ATCC 13637 likewise behaved as a large molecule by sedimenting as a single symmetrical peak with

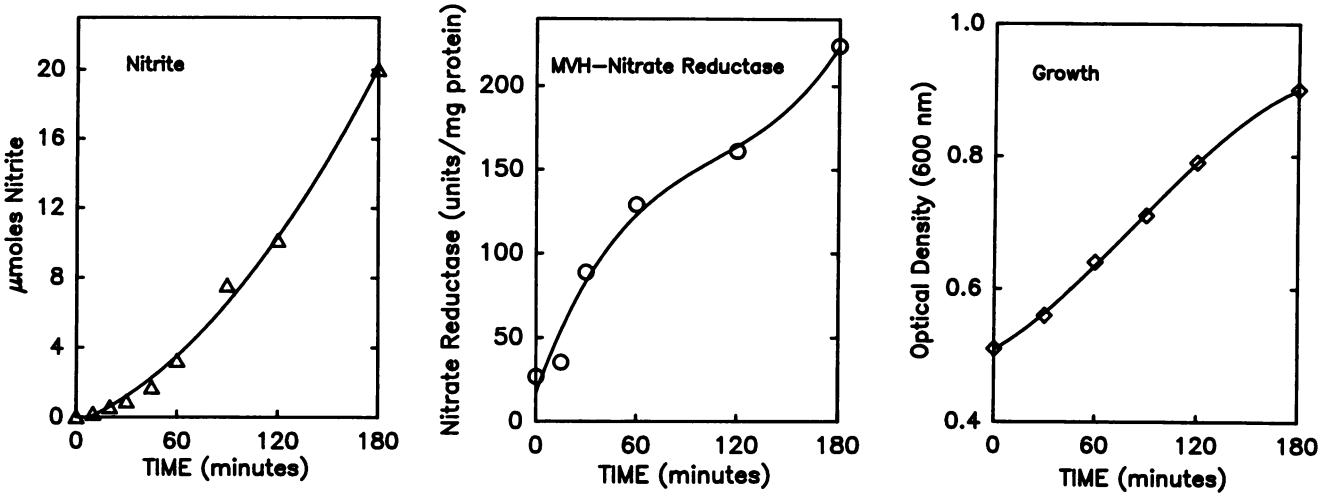

FIG. 1. Induction of NR activity in *X. maltophilia* ATCC 17666. Aerobic nutrient broth cultures were diluted in nitrate broth (11.8 mM nitrate) to an optical density at 600 nm of 0.500 and shaken in a 30°C water bath as described in Materials and Methods. Samples, for nitrite and optical density (600 nm) of cell suspensions, were taken from the 3-h culture. Cells from six flasks harvested at timed intervals were used to prepare crude extracts for determining the specific activity of MVH-NR. Nitrite is expressed as micromoles per milliliter of culture.

an $s_{20,w}$ of 5.1 in sucrose gradients. This bound form of MPT readily dissociates from its carrier since it was active in the *nit-1* assay without acid or salt treatment.

All wild-type bacterial strains we have tested to date, with the exception of three folic acid-requiring strains, possess an active form of MPT. The absence of MPT in ATCC 14535 was therefore of interest, and we attempted to reverse this deficiency by growing cells in the presence of possible MPT precursors. Addition of pterin or neopterin (1 μg ml^{-1}) to the growth medium of ATCC 14535 failed to alter results of subsequent MPT assays. To determine whether this strain produced an inactive NR capable of activation by an exogenous source of MPT, extracts of nitrate-grown ATCC 14535 were mixed with bacterial extracts known to contain MPT, or acid-treated xanthine oxidase as sources of MPT, and incubated as described for the in vitro restoration reaction. We detected no MVH-NR in any of these mixtures, suggesting that ATCC 14535 generates neither the aponitrate reductase nor MPT.

DISCUSSION

As found in previous growth studies (20, 21) of nitrate reduction in *X. maltophilia*, we showed that approximately half the strains tested reduce nitrate to nitrite, which accumulates. Nitrate reduction in *X. maltophilia*, however, fails to fulfill one of the classic physiological functions of bacterial NRs. The absence of a nitrite reductase means that nitrate nitrogen can neither be assimilated nor reduced to a gaseous product(s) characteristic of denitrification, and *Xanthomonas* appears to be an obligate aerobe incapable of fermentation with or without nitrate added to simple or complex media.

Although no current observation links the NR of *X. maltophilia* to conservation of energy, many characteristics of this enzyme resemble the respiratory NRs of gram-negative bacteria. Nitrate-induced synthesis of MVH-NR activity by *X. maltophilia* occurred only at low oxygen concentrations, conditions required for the synthesis of respiratory NRs in other gram-negative bacteria. For example, formation of NR in *Pseudomonas stutzeri* requires nitrate or nitrite during growth and maximizes at 5 mg of O_2 per liter, being repressed by higher concentrations (30). Extensive studies of the influence of oxygen on NR formation in *E. coli* indicate that oxygen affects the coordinated control of respiratory operons through the *fnr* locus (42).

TABLE 2. Membrane-associated NR in cell extracts of *X. maltophilia* ATCC 13637

Source	Total units	Total protein (mg)	Sp act (U mg of protein^{-1})	% of total
Crude extract	2,134	56.6	37.7	100
High-speed[a] supernatant	393	49.3	7.1	19
High-speed[a] pellet	1,641	15.4	106.6	81

[a] Centrifugation at 90,000 × *g* for 2 h. Recovery was 95%.

TABLE 3. Characteristics of the MPT assay[a]

Na_2MoO_4	Time	U of NADPH-NR formed ml of bacterial extract^{-1}	% NR at 24 h
Minus	10 min	539	15
	1 h	1,107	30
	2 h	1,476	41
	3 h	1,445	39
	4 h	2,460	68
	24 h	3,619	100
Plus	10 min	579	19
	1 h	1,037	34
	2 h	1,674	55
	3 h	1,389	46
	4 h	2,175	72
	24 h	3,039	100

[a] Crude extracts of *X. maltophilia* were diluted in 0.02 M Na_2HPO_4/NaH_2PO_4–1 mM EDTA buffer, pH 7.2, with and without 10 mM Na_2MoO_4 before mixing with the *nit-1* extract. These mixtures were incubated at 4°C until brought to 25°C and assayed for NADPH-NR at the times indicated. Comparison of the 24-h results (Minus) with those of our standard assay (10 min at 23°C) yielded the multiplier of 6.7 we used to estimate the moles of MPT in the bacterial extracts.

High oxygen concentrations also prevent nitrate uptake in a variety of nitrate-reducing bacteria (18). Such control mechanisms may also operate in nitrate-reducing *Xanthomonas* strains.

The specific activity of MVH-NR in crude extracts of *X. maltophilia* approximate those reported for other gram-negative bacteria (7, 8), and NR from selected strains of *X. maltophilia* occurred as a membrane-bound enzyme. Its physical characteristics, membrane location, induction by nitrate, and repression by oxygen are properties consistent with those of counterpart enzymes in facultative gram-negative (42) and denitrifying (2, 5, 8) bacteria, but different from the 88-kDa, soluble, periplasmic NR of *Rhodobacter capsulatus* (35).

Denitrifying bacteria typically reduce nitrate to nitrite, which accumulates before nitrite reductase is formed (45), presumably induced by nitrite (42). *X. maltophilia* did not metabolize nitrite, even when grown in the presence of 0.01% (wt vol^{-1}) nitrite to dispel concern that high nitrite represses nitrite reductase formation as in *E. coli* (11). The inability to reduce nitrite is not unique among nitrate reducers. *S. aureus* grows anaerobically on glycerol, a nonfermentable substrate, when nitrate is present. Under these conditions, a NR is formed that reduces nitrate to nitrite, which accumulates (6).

Both *X. maltophilia* and *S. aureus* are oxidase-negative organisms with no *c*-type cytochromes, although both possess *b*-type cytochromes. This may explain in part the inability of these nitrate-reducing organisms to grow as denitrifiers. Growth by denitrification involves a *c*-type cytochrome as a component of the dissimilatory cd_1 nitrite reductase in roughly half of the oxidase-positive denitrifiers (13). Cytochrome *c*, recently demonstrated by Heiss et al. (17) to be a component of the nitric oxide reductase, may also be required. Another group of denitrifiers use a copper-containing nitrite reductase to reduce nitrite to nitric oxide (13), but such an enzyme has yet to be demonstrated in an oxidase-negative bacterium. The involvement of *c*-type cytochromes in known nitrite and/or nitric oxide reductases could limit the ability of oxidase-negative organisms to reduce nitrite and/or to remove the more toxic nitric oxide produced during denitrification. (Some oxidase-negative bacteria, such as *E. coli*, possess a siroheme nitrite reductase for reducing nitrite to ammonia during nitrate respiration [12], which is part of the assimilatory pathway not considered above.)

The almost universal occurrence of MPT in bacteria suggests the importance of this pterin to molybdoenzymes and to general metabolism. The biosynthesis of MPT in *E. coli* is an involved process requiring at least seven genes located at various positions on the chromosome (42). The absence of MPT in one strain of *X. maltophilia* is therefore unusual. All other strains possess an active form of MPT that we presume is suitable for the synthesis of bacterial NR.

More than one MPT, however, may be involved in molybdoenzymes. Kruger and Meyer (32) proposed that bacteria contain bactopterin (molecular weight, 760), which is as effective as the MPT (molecular weight, 360) from xanthine oxidase and sulfite oxidase in restoring activity to the aponitrate reductase of *N. crassa nit-1*. If bacteria produce more than one MPT, only one of which is active in bacterial nitrate reduction, strains unable to reduce nitrate may lack the pterin for bacterial NR. The assays needed to resolve this question have not been developed, and the isolation and chemical characterization of the active form(s) of MPT have yet to be accomplished.

In natural environments, *X. maltophilia* could benefit from using nitrate as an alternative electron acceptor for oxidizing excess NADH and NADPH when growing under semiaerobic conditions. Such a system would function as an electron sink for the bacterium and result in the release of nitrite into the environment. This process would also generate substrate for the nitrite-oxidizing bacteria in soil, which derive a significant portion of the nitrite they oxidize from nitrate reduction, as opposed to ammonia oxidation (3).

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