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Nitrite oxidation kinetics of two *Nitrospira* strains: The quest for competition and ecological niche differentiation

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Nitrite oxidation is an aerobic process of the nitrogen cycle in natural ecosystems, and is performed by nitrite-oxidizing bacteria (NOB). Also, nitrite oxidation is a rate-limiting step of nitrogen removal in wastewater treatment plants (WWTPs). Although *Nitrospira* is known as dominant NOB in WWTPs, information on their physiological properties and kinetic parameters is limited. Here, we report the kinetic parameters and inhibition of nitrite oxidation by free ammonia in pure cultures of *Nitrospira* sp. strain ND1 and *Nitrospira japonica* strain NJ1, which were previously isolated from activated sludge in a WWTP. The maximum nitrite uptake rate ($V_{max\_NO_2}$) and the half-saturation constant for nitrite uptake ($K_{m\_NO_2}$) of strains ND1 and NJ1 were 45 ± 7 and 31 ± 5 (μmol NO_2^-/mg protein/h), and 6 ± 1 and 10 ± 2 (μM NO_2^-), respectively. The $V_{max\_NO_2}$ and $K_{m\_NO_2}$ of two strains indicated that they adapt to low-nitrite-concentration environments like activated sludge. The half-saturation constants for oxygen uptake ($K_{m\_O_2}$) of the two strains were 4.0 ± 2.5 and 2.6 ± 1.1 (μM O_2), respectively. The $K_{m\_O_2}$ values of the two strains were lower than those of other NOB, suggesting that *Nitrospira* in activated sludge could oxidize nitrite in the hypoxic environments often found in the interiors of biofilms and flocs. The inhibition thresholds of the two strains by free ammonia were 0.85 and 4.3 (mg-NH_3 l^{-1}), respectively. Comparing the physiological properties of the two strains, we suggest that tolerance for free ammonia determines competition and partitioning into ecological niches among *Nitrospira* populations.

[**Key words:** *Nitrospira*; Affinity for nitrite; Affinity for oxygen; Growth parameters; Free ammonia; Inhibitory constant; Activated sludge]

Nitrification, in which ammonia is transformed to nitrate via nitrite, is an aerobic step in the global nitrogen cycle. Nitrification consists of two stages, ammonia oxidation and nitrite oxidation, which are performed by ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA) and nitrite-oxidizing bacteria (NOB). Recently, it was reported that complete ammonia oxidation (COMAMMOX) was performed by a single *Nitrospira* bacterium, which was known previously as NOB that catalyzed only nitrite oxidation (1,2). Nitrification is also a key rate-limiting step in biological nitrogen removal from activated sludge in wastewater treatment plants (WWTPs), and contributes to prevention of eutrophication of coastal ecosystems (3). It is widely accepted that nitrification in activated sludge is stable in normal conditions, and that nitrite as an intermediate of nitrification is instantly converted into nitrate by close cooperation between AOB and NOB (4). However, nitrite occasionally accumulates when this cooperation collapses because of different growth rates of AOB and NOB (5). Nitrite accumulation may also be caused by inhibition of nitrite-oxidizing activity in NOB; temperature, pH, dissolved oxygen, light, free ammonia and free nitrous acid are known to inhibit nitrite oxidation (6). Thus, understanding of the microbial communities and physiological properties of NOB is required for stabilization of nitrite oxidation in activated sludge.

Over the last two decades and based on cultivation approaches, the genus *Nitrobacter* within the class *Alphaproteobacteria* has been considered as the dominant NOB in activated sludge. Since isolation and cultivation of *Nitrobacter* are relatively uncomplicated, the metabolism and biochemistry of NOB were investigated using pure cultures of *Nitrobacter* as a model bacterium (7–11). However, it was recently reported that previously unrecognized NOB belonging to genera outside the *Alphaproteobacteria* were found in activated sludge. *Candidatus* Nitrospira defluvii, belonging to the phylum *Nitrospirae*, was successfully enriched from activated sludge by cultivation at low nitrite concentration and selective repression with ampicillin, and appeared to prefer low-nitrite-concentration environments (12). *Candidatus* Nitrotoga arctica, belonging to the class *Betaproteobacteria*, was enriched from permafrost-affected soils in Siberia (13), and the genus *Nitrotoga* was recognized as cold-adapted NOB in activated sludge (14,15). *Nitrolancea hollandica*, belonging to the phylum *Chloroflexi*, was isolated from a nitrifying bioreactor with a high loading of ammonium bicarbonate and was characterized as a thermotolerant, neutrophilic NOB with high nitrite-tolerance (16,17). Thus, the huge phylogenetic diversity of

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nitrite oxidizers indicates that the insights obtained to date into the physiology of NOB are the tip of an iceberg.

Molecular approaches have revealed that *Nitrospira*, not *Nitrobacter*, are the dominant NOB in ubiquitous activated sludge (15,18–21). Since *Nitrospira* are known as notoriously recalcitrant bacteria and the availability of pure strains is limited, detailed physiological characteristics of *Nitrospira* are still uncertain. Affinities for substrates (nitrite and oxygen) and inhibition by environmental factors (pH, temperature and free ammonia) of enriched *Nitrospira* cells in nitrifying bioreactors have been investigated using a microsensor system (20,22,23). Based on the physiological characteristics determined from enrichment samples, it was revealed that the affinity for nitrite and maximum nitrite uptake rates were major factors in the ecological niche differentiation between *Nitrobacter* and *Nitrospira* (20). It was suggested that their differentiation could be explained by *K* and *r* strategies: *Nitrobacter* (*r* strategists) have relatively low affinity for nitrite and a high maximum nitrite uptake rate, whereas *Nitrospira* (*K* strategists) have relatively high affinity for nitrite and a low maximum nitrite uptake rate (20,24). Moreover, it was recently reported that competition and partitioning between ecological niches among phylogenetically different populations of *Nitrospira* in WWTPs were caused by different physiological properties such as affinities for substrates, formate utilization and relationships with AOB (25–27). However, these insights were obtained from mixed cultures containing other species, not from pure *Nitrospira* cultures.

The kinetic and growth parameters of NOB were determined using pure cultures of *Nitrobacter* and *Nitrospira*, and major factors determining ecological niche differentiation among NOB communities were also demonstrated (28). However, the nitrite affinities and maximum uptake rates determined by a few strains are not enough to explain competition and partitioning into ecological niches among *Nitrospira* communities in activated sludge, and more information is required from the available pure cultures. Here, using two pure *Nitrospira* cultures of *Nitrospira* sp. strain ND1 and *Nitrospira japonica* strain NJ1, we investigated kinetics and growth parameters, and evaluated inhibition by free ammonia, which is well known as an inhibitor of nitrite oxidation (29). In previous studies (30–32), we selectively enriched phylogenetically distinct *Nitrospira* lineages (I and II) using a continuous feeding bioreactor, and successfully isolated strains ND1 and NJ1 using a cell sorting system. Although both strains were selectively enriched by controlling nitrite concentration in the continuous feeding bioreactor, it was not clear whether nitrite concentration or other factors determined their competition and ecological niche partitioning in activated sludge. Thus, different properties of the two strains as representative strains within two *Nitrospira* lineages, were expected to help elucidate the competition and ecological niche partitioning between two *Nitrospira* lineages found in activated sludge.

MATERIALS AND METHODS

***Nitrospira* strains and culture conditions** In this study, we investigated pure cultures of *Nitrospira* sp. strain ND1 and *N. japonica* strain NJ1, which were previously isolated from activated sludge in a wastewater treatment plant by our research group (30,31). The two *Nitrospira* strains were cultured in mineral medium containing nitrite in the same manner as in previous reports (30,31). The medium consisted of $NaNO_2$ (49.3 mg l^{-1}), KH_2PO_4 (38.2 mg l^{-1}), $MgSO_4{\cdot}7H_2O$ (61.1 mg l^{-1}), $CaCl_2{\cdot}2H_2O$ (10.0 mg l^{-1}), $FeSO_4{\cdot}7H_2O$ (5.0 mg l^{-1}), $NaHCO_3$ (200.0 mg l^{-1}), $MnSO_4{\cdot}5H_2O$ (54.2 μg l^{-1}), H_3BO_3 (49.4 μg l^{-1}), $ZnSO_4{\cdot}7H_2O$ (43.1 μg l^{-1}), $Na_2Mo_4O_4$ (27.6 μg l^{-1}) and $CuSO_4{\cdot}5H_2O$ (25.0 μg l^{-1}). Other culture conditions were darkness, pH 7.8–8.0, 29°C, and shaking at 100 rpm.

Determination of kinetic parameters for nitrite oxidation To investigate kinetic parameters of nitrite oxidation, nitrite affinity and the maximum nitrite uptake rate, nitrite concentration was measured over time. Fig. S1 shows a flow chart of the sampling method. As in a previous report (28), we used pure cultures in early stationary phase, which is between 12 and 48 h after nitrite consumption. Since the cell number in *Nitrospira* cultures is low, 40-ml aliquots of the pure cultures were enriched using a membrane filter with a pore size of 0.22 μm (Merck Milipore, Billerrica, MA, USA), and the cells were resuspended in 4 ml mineral medium. The initial nitrite concentration of the samples was adjusted to 3 mg-N l^{-1} (214 μM NO_2^-). Incubation conditions were: shaking at 150 rpm, 25°C, pH 8.0. Every 5–20 min during incubations, 50 μl aliquots of incubated samples were collected and treated at 95°C for 10 min to inactivate the nitrite-oxidizing activity. Occasionally, we checked the remaining nitrite concentration in the samples using Griess reagent (33). The initial interval of sampling was 20 min; once the remaining nitrite concentration in the incubated samples was below approximately 1.5 mg-N l^{-1}, the sampling interval was changed to 5 min. When the remaining nitrite concentration in the incubated samples reached 0 mg-N l^{-1}, the nitrite concentrations of all the samples collected were measured using Griess reagent and a microplate spectrophotometer (PowerScan HT, BioTek, Winooski, VT, USA). The nitrite uptake rate of pure *Nitrospira* cultures was calculated based on the measured nitrite concentrations. Subsequently, the nitrite affinities and the maximum nitrite uptake rates of *Nitrospira* cultures were calculated by fitting the data to a Michaelis–Menten kinetic equation (Eq. 1):

$$V_{NO_2} = (V_{max\_NO_2} \cdot [S]) / (K_{m\_NO_2} + [S]) \quad (1)$$

Here, V_{NO_2} is the nitrite uptake rate (μmol NO_2^-/mg protein/h), $V_{max\_NO_2}$ is the maximum nitrite uptake rate (μmol NO_2^-/mg protein/h), $K_{m\_NO_2}$ is the half-saturation constant for nitrite uptake (μM NO_2^-), and S is the nitrite concentration (μM NO_2^-). The data fitting was performed using non-linear least square method with solver in Excel. In these experiments, we did not use purified nitrite-oxidizing enzymes (nitrite oxidoreductases) from *Nitrospira*. Since the kinetic parameters were determined in short-term (2–3 h) experiments, we can ignore growth of the *Nitrospira* cells and regard them as enzymes. Thus, we prefer to use the terms V_{max} and K_m.

Oxygen affinity of pure *Nitrospira* cultures To investigate the oxygen affinity of strains ND1 and NJ1, the dissolved oxygen concentration was measured over time. The strains were harvested in early stationary phase. After they were concentrated using a membrane filter with a pore size of 0.22 μm (Merck Milipore), the initial nitrite concentration and pH were adjusted to approximately 21 mg-N l^{-1} (1.5 mM NO_2^-) and 8.0, respectively. Each suspension was transferred into a 2-ml glass chamber, followed by commencement of an oxygen consumption test at 29°C with complete mixing by a magnetic stirrer. The oxygen consumption of each strain was monitored using a microrespiration system (Unisense AS, Denmark). After measurements, the dissolved oxygen concentration data were subjected to smoothing to remove noise using Sigma plot 13.0 software (Systat Software GmbH, Erkrath, Germany). The oxygen affinity and the maximum oxygen uptake rate of strains ND1 and NJ1 were calculated by fitting the data to the following a Michaelis–Menten kinetic equation (Eq. 2):

$$V_{O_2} = (V_{max\_O_2} \cdot [S]) / (K_{m\_O_2} + [S]) \quad (2)$$

Here, V_{O_2} is the oxygen uptake rate (μmol O_2/h), $V_{max\_O_2}$ is the maximum oxygen uptake rate (μmol O_2/h), $K_{m\_O_2}$ is the half-saturation constant for oxygen (μM O_2), and S is the dissolved oxygen concentration (μM O_2). The data fitting was performed using non-linear least square method with solver in Excel.

Determination of growth parameters Subcultured pure *Nitrospira* cultures (50 ml) were added to an autoclaved 5-l glass bottle containing 950-ml mineral medium. The initial nitrite concentration was adjusted to 1 mM, and other culture conditions were 29°C, pH 8.0, shaking at 100 rpm and darkness. Sampling was performed once a day during several days of incubation. During sampling, since both strains ND1 and NJ1 formed large flocs, 20 ml aliquots were taken from incubated samples and sonicated at 25% amplitude for 20 s to disrupt the flocs using an ultrasonic processor (Vibra-cell VCX500, Sonics & Materials, Inc., CT, USA). After sonication treatment, 1 ml aliquots were taken from the sonicated samples for chemical analysis and DNA extraction, and were stored at −20°C. After the incubation finished, total DNA was extracted from 1 ml aliquots of each sampling point using a DNA extraction kit (NucleoSpin Tissue, Takara Bio, Otsu, Japan), and stored at −20°C. The cell growth yield of *Nitrospira* pure cultures was calculated based on cells produced per millimole of nitrite oxidized.

Measurement of nitrite concentration Nitrite concentration in samples taken from nitrite-oxidizing activity experiments was measured using ion chromatography (IC 2010, Tosoh, Tokyo, Japan). Before measuring nitrite concentrations, samples were sterilized using a membrane filter with a pore size of 0.22 μm (Advantec, Tokyo, Japan).

Extraction and quantification of protein The incubated samples in each experiment were concentrated by centrifuge (15,000 rpm, 15 min), and lysed by incubation with 0.15 M NaOH at 90°C for 30 min following a published protocol (28). Protein quantification was performed using the Qubit protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Calculation of *Nitrospira* cell numbers To calculate cell numbers of the two *Nitrospira* strains, qPCR was performed targeting the *nxrB* gene in the DNA extracted from each sample. The *nxrB* gene is known as a functional and phylogenetic marker gene for *Nitrospira*. Based on genomic information for both strains (unpublished data), the genome of strain ND1 contains one copy of the *nxrB* gene, and the strain NJ1 genome contains three copies of the *nxrB* gene. Thus, the cell numbers of strains

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