Denitrifying metabolism of the methylotrophic marine bacterium *Methylophaga nitratireducenticrescens* strain JAM1

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**Abstract**

*Methylophaga nitratireducenticrescens* strain JAM1 is a methylotrophic, marine bacterium that was isolated from a denitrification reactor treating a closed-circuit seawater aquarium. It can sustain growth under anoxic conditions by reducing nitrate (NO\(_3^–\)) to nitrite (NO\(_2^–\)), which accumulates in the medium. These physiological traits are attributed to gene clusters that encode two dissimilatory nitrate reductases (NarGHJI). *M. nitratireducenticrescens* strain JAM1 also contains gene clusters encoding two putative nitric oxide reductase (NO) reductases and one putative nitrous oxide (N\(_2O\)) reductase, suggesting that NO and N\(_2O\) can be reduced by strain JAM1. In this study, we show that strain JAM1 can reduce NO to N\(_2O\) and N\(_2O\) to N\(_2\) and can sustain growth under anoxic conditions by reducing N\(_2O\) as the sole electron acceptor. Although strain JAM1 lacks a gene encoding a dissimilatory copper-(NirK) or cytochrome cd1-type (NirS) NO\(_2^-\) reductase, NO\(_3^-\)-amended strain JAM1 cultures produce N\(_2O\), representing up to 6% of the N-input. NO\(_2^-\) was shown to be the key intermediate of this production process. In NO\(_3^-\)-amended cultures, we analyzed denitrification genes in succession of net N\(_2O\)-production and -consumption phases at the gene expression level. These phases were found to correlate with changes in the expression levels of the NO reductase gene *cnorB1* and *nnrS*, which indicated NO production in the cultures.

**Importance**

By showing that all the three denitrification reductases are active, this demonstrates that *Methylophaga nitratireducenticrescens* JAM1 is one of many bacteria species that maintain genes associated primarily with denitrification, but not necessarily related to the maintenance of the entire pathway. The reason to maintain such incomplete pathway could be related to the specific role of strain JAM1 in the denitrifying biofilm of a denitrification reactor from which it originates. The small production of N\(_2O\) via NO in strain JAM1 did not involve Nar contrary to what was demonstrated in *Escherichia coli*. *M. nitratireducenticrescens* JAM1 is the only reported *Methylophaga* species that has the capacity to grow under anoxic conditions by using NO\(_3^-\) and N\(_2O\) as sole electron acceptors for its growth. It is also one of a few marine methylotrophs that is studied at the physiological and genetic levels in relation to its capacity to perform denitrifying activities.
Introduction

The complete denitrification pathway describes the successive reduction of nitrate (NO$_3^-$) to nitrite (NO$_2^-$), nitric oxide (NO), nitrous oxide (N$_2$O), and nitrogen (N$_2$) (1). This process is used by bacteria for respiration in environments with low oxygen concentrations and with NO$_3^-$ as an electron acceptor. The process is driven by metalloenzymes NO$_3^-$ reductase, NO$_2^-$ reductase, NO reductase, and N$_2$O reductase (2). As a facultative trait, denitrification occurs frequently across environments and is performed by bacteria of diverse origins (3). However, numerous bacterial strains have been isolated with incomplete denitrification pathway, meaning that at least one reductase-encoding gene cluster is missing. As proposed by Zumft (3), the four steps of reduction from NO$_3^-$ to N$_2$ could be seen as a modular assemblage of four partly independent respiratory processes that respond to combinations of different external and internal signals. This could explain the vast diversity of bacteria with incomplete denitrification pathway that can sustain growth with one of the four nitrogen oxides as electron acceptor. Another purpose of the incomplete pathway is related to detoxification, as nitrite and NO are deleterious molecules (4-7).

*Methylophaga nitratireducenticrescens* JAM1 is a marine methylotrophic gammaproteobac-
terium that was isolated from a naturally occurring multispecies biofilm that has developed in a methanol-fed, fluidized denitrification system that treated recirculating water of the marine aquarium in the Montreal Biodome (8, 9). This biofilm is composed of at least 15 bacterial species and of numerous protozoans (10, 11), among which *Methylophaga* spp. and *Hyphomicrobium* spp. compose more than 70% of the biofilm (12). Along with the denitrifying bacterium *Hyphomicrobium nitrativorans* NL23, *M. nitratireducenticrescens* JAM1 was shown to be the representative of the *Methylophaga* population in the biofilm (8).

*M. nitratireducenticrescens* JAM1 is considered as a nitrate respirer as it can grow under anoxic conditions through the reduction of NO$_3^-$ to NO$_2^-$, which accumulates in the culture medium (8). This trait is correlated with the presence of two gene clusters encoding dissimilatory nitrate reductases (narGHJI, referred as Nar1 and Nar2) in the genome of *M. nitratireducenticrescens* JAM1, which we showed that both contribute to NO$_3^-$ reduction during strain JAM1 growth (13). Anaerobic growth by strain JAM1 is a unique among *Methylophaga* spp. that were described as strictly aerobic bacteria (14). Genome annotation revealed that strain JAM1 seems to maintain an incomplete denitrification pathway with the presence of gene clusters encoding two putative cytochrome bc-type complex NO reductase (cNor) (cnor1: norQDBCRE and cnor2: norCBQD) and one putative dissimilatory N$_2$O reductase (N$_2$OR) (nosRZDFYYL), but lacks gene encoding a dissimilatory copper- (NirK) or cytochrome cd1-type (NirS) NO$_2^-$ reductase. These gene clusters have been shown to be transcribed (13). These data suggest that *M. nitratireducenticrescens* JAM1 has other respiratory capacities by performing NO and N$_2$O reduction.

In this study, we aimed to assess the denitrification capacities of *M. nitratireducenticrescens* JAM1. Our results show that strain JAM1 can reduce NO to N$_2$O and then to N$_2$. It can use N$_2$O as a source of energy for its growth under anoxic conditions. Through our investigation, we found that strain JAM1 cultured with NO$_3^-$ under anoxic and oxic conditions generates a small amount of N$_2$O, despite the absence of gene encoding NirK or NirS. NO$_2^-$ was found to be a key intermediate of this production process. By using the JAM1ΔnarG1-narG2 double mutant, we showed that the two Nar were not involved in N$_2$O production via NO. We analyzed at the gene expression level the succession of N$_2$O production and consumption with the denitrification genes cnorB (cnorB1 and cnorB2) and nosZ, and also mnrS that encodes a NO-sensitive regulator. We found that gene expression level of cnorB1 and mnrS increased during the N$_2$O production phase, which suggest the presence of NO.
Results

*M. nitratireducenticrescens* JAM1 grows on N₂O under anoxic conditions

Strain JAM1 was cultured under anoxic conditions with either NO₃⁻ in the medium or with N₂O injected in the headspace as the sole electron acceptor. Both types of culture received the same electron equivalent of NO₃⁻ or N₂O (1.3 mmole vial⁻¹ or 18.2 and 36.4 mg-N vial⁻¹, respectively) according to:

\[
\text{NO}_3^- + 2e^- + 2H^+ \rightarrow \text{NO}_2^- + H_2O \quad [1]
\]

\[
\text{N}_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \quad [2]
\]

![Figure 1: Methylophaga nitratireducenticrescens JAM1 growth with N₂O or NO₃⁻ as an electron acceptor](image1)

Strain JAM1 was cultured with 36.4 mg-N vial⁻¹ N₂O (A) or 18.2 mg-N vial⁻¹ NO₃⁻ (B) under anoxic conditions. N₂O, NO₃⁻ and NO₂⁻ concentrations and growth were measured over different time intervals. Control (A): N₂O injected in non-inoculated vials. To minimize oxygen contamination, sampling was performed using a glove bag inflated with nitrogen gas. Data represent mean values ± standard deviation (SD; n=3).

In N₂O-amended cultures, N₂O decrease was apparent from the start and consumption continued for 48 hours (Fig. 1A). The N₂O decrease paralleled strain JAM1 growth with almost complete N₂O consumption. The NO₃⁻-amended cultures showed complete NO₃⁻ consumption and equivalent nitrite accumulation after 24 h (Fig. 1B). However, slower growth than that recorded for the N₂O cultures was observed. Such growth kinetics could be related to the toxicity of nitrite that accumulated in the medium. Both types of culture reached equivalent biomass concentration (t test on the last 4-time points, P >0.05).

*M. nitratireducenticrescens* JAM1 consumes N₂O under oxic conditions

In a previous study, we demonstrated that strain JAM1 can consume NO₃⁻ under oxic growth conditions with equivalent accumulation of NO₂⁻ (13). Culturing strain JAM1 under oxic conditions with N₂O (3.5 mg-N vial⁻¹) also showed a complete N₂O consumption within 24 h (Fig. 2). In presence of O₂, the strain JAM1 oxic cultures reached higher (4-5-times) biomass concentration than the anoxic cultures. N₂O-amended cultures yielded equivalent biomass (O.D. ~1.2) than NO₃⁻-amended oxic cultures (20 mg-N vial⁻¹) (Fig. 2).

![Figure 2: N₂O consumption by Methylophaga nitratireducenticrescens JAM1 under oxic conditions](image2)

Strain JAM1 was cultured with 3.5 mg-N vial⁻¹ N₂O or 22 mg-N vial⁻¹ NO₃⁻ under oxic conditions. N₂O and growth were measured over different time intervals. Data represent mean values ± SD (n=3).
During the first assays to test the capacity of strain JAM1 to reduce N₂O, cultures were performed with N₂O (3.5 mg-N vial⁻¹) but with the addition of NO₃⁻ (20 mg-N vial⁻¹) to make sure that growth would occur. Although N₂O was completely consumed within 24 h, a net production of N₂O was observed after 48 h (data not shown). The production of N₂O by strain JAM1 is puzzling, as its genome does not contain NirS or NirK.

Figure 3. N₂O production by Methylophaga nitratireducenticrescens JAM1

Strain JAM1 was cultured under anoxic (A) or oxic (B) conditions with NO₃⁻ (22 mg-N vial⁻¹). NO₃⁻, NO₂⁻, N₂O and N₂ (panel A only) concentrations were measured over different time intervals. Data represent mean values ± SD (n=3).

To further investigate this observation, strain JAM1 was cultured under anoxic conditions with NO₃⁻, and NO₃⁻, NO₂⁻, N₂O and N₂ were measured (Fig. 3A). Complete NO₃⁻ reduction (19.3 ± 0.3 mg-N vial⁻¹) was performed within 55 h. The nitrite level reached 17.5 ± 0.2 mg-N vial⁻¹ over this period and decreased slowly to 15.9 ± 0.5 mg-N vial⁻¹. N₂O production initiated when NO₃⁻ was nearly reduced and reached 0.70 ± 0.21 mg-N vial⁻¹ after 55 h of incubation (Fig. 3A). N₂O was completely reduced after 127 h. In parallel, for cultures in which the headspace was flushed with argon, N₂ production was also measured. The corresponding results show an increase of N₂ in the headspace (Fig. 3A) by 1.14 ± 0.54 mg-N vial⁻¹ after 127 h, which represent 6.0 ± 2.9% of the N input.

Under oxic conditions, NO₃⁻ reduction (17.4 ± 2.1 mg-N vial⁻¹) was complete after 24 h with equivalent NO₂⁻ accumulation (17.1 ± 1.3 mg-N vial⁻¹). N₂O accumulation started after complete nitrate reduction (Fig. 3B) and increased to reach 0.31 ± 0.32 mg-N vial⁻¹ after 96 h of incubation (1.7% of N input). Unlike trends observed for the anoxic cultures, no N₂O consumption was observed in the oxic cultures.

Influence of ammonium on NO₃⁻ and NO₂⁻ consumption, and N₂O production and consumption by M. nitratireducenticrescens JAM1

The original 1403 medium recommended by the ATCC for culturing Methylophaga spp. contains 20.9 mg-N vial⁻¹ NH₄Cl and 0.1 mg-N vial⁻¹ ferric ammonium citrate (see Material and Methods). Therefore, NO₃⁻ transformation in NH₄⁺ should not be necessary in Methylophaga metabolism for nitrogen assimilation in biomass (Fig. S1). For the next set of experiments, we aimed to determine the effect of the absence of NH₄⁺ on the dynamics of NO₃⁻ and NO₂⁻ consumption, and N₂O production and consumption. We hypothesized that that forcing strain JAM1 to reroute some NO₃⁻ for N assimilation would affect denitrification and thus growth rates. Strain JAM1 was cultured with ca. 20 mg-N vial⁻¹ NO₃⁻ under anoxic or oxic conditions in NH₄Cl-free medium (Fig. 4A and B). Growth pattern observed under anoxic conditions was similar between the regular and NH₄Cl-free cultures, as also the growth pattern under oxic conditions between the regular
and NH₄Cl-free cultures.

![Graph showing nitrate (NO₃⁻), nitrite (NO₂⁻), and N₂O concentrations over time](image)

Figure 4: Influence of ammonium on NO₃⁻ and NO₂⁻ consumption, and N₂O production and consumption by Methylophaga nitratireducenticrescens JAM1.

Strain JAM1 was cultured under anoxic (A) or oxic (B) conditions with NO₃⁻ (22 mg-N vial⁻¹) in NH₄Cl-free 1403 medium. NO₃⁻, NO₂⁻ and N₂O concentrations were measured over different time intervals. The results are derived from triplicate cultures. In panel A, asterisks denote the sampling times used for RNA extraction (see Figure 6). Data represent mean values ± SD (n=3).

Under anoxic NH₄Cl-free conditions, full nitrate reduction (19.1 ± 0.6 mg-N vial⁻¹) occurred within 48 h (Fig. 4A). The N₂O production and consumption profile found was similar to that observed in regular cultures (Fig. 3A), though lower N₂O concentrations were detected during the accumulating phase. The nitrite level reached 18.5 ± 0.8 mg-N vial⁻¹ after 24 h and then slowly decreased to 12.8 ± 0.5 mg-N vial⁻¹ after 96 h. Nitrogen assimilation by the biomass and the production of N₂O and its reduction to N₂ could account for the difference in nitrogen mass balance (32.7 ± 2.5%).

Unlike the cultures in regular medium (Fig. 3B), NO₃⁻ (21.3 ± 1.0 mg-N vial⁻¹) was not completely reduced under oxic NH₄Cl-free conditions, and it stopped after 24 h at 2.9 ± 2.7 mg-N vial⁻¹ (Fig. 4A). In conjunction with NO₃⁻ reduction, NO₂⁻ levels stopped accumulating at 13.0 ± 2.6 mg-N vial⁻¹ after 24 h. N₂O was observed after 48 h of incubation (Fig. 4A), after which it slowly accumulated and reached a concentration of 0.043 ± 0.048 mg-N vial⁻¹. This level is 7 times lower than that of the regular culture medium (Fig. 3B). Nitrogen assimilation by the biomass under oxic conditions could account for the difference in nitrogen mass balance (25.5 ± 4.3%) found between the initial concentration of NO₃⁻ and residual concentrations of NO₃⁻, NO₂⁻ and N₂O (13.9%, 60.4% and 0.20% of the N input, respectively). In addition, N₂O production and consumption could have reached an equilibrium and loss of nitrogen would occur by N₂ production.

To assess whether N₂O could have been generated through NH₄⁺, strain JAM1 was cultured under anoxic conditions with 22 mg-N vial⁻¹ NO₃⁻, 20.7 mg-N vial⁻¹¹⁵NH₄⁺, and acetylene to prevent the reduction of N₂O to N₂. Unfortunately, strain JAM1 cannot be cultured without NO₃⁻ under anoxic conditions, and growth is inhibited in NO₂⁻-amended cultures (8). If NH₄⁺ is involved in N₂O production, high proportion of labelled N₂O is expected. If NH₄⁺ is not involved in N₂O production, we expected the production of labeled N₂O to be derived from ¹⁵NO₃⁻ naturally present in NaNO₃ at a natural ¹⁵N/¹⁴N isotopic ratio of 0.0036765. In the ¹⁵NH₄⁺-amended cultures, the ¹⁵N/¹⁴N ratios measured were 0.008 and 0.0165, respectively, with an ¹⁵N/¹⁴N isotopic ratio of 0.020418. As a control, strain JAM1 cultured under anoxic conditions with ¹⁵NO₃⁻ in NH₄Cl-free medium with acetylene showed, as was expected, all N₂O recovered in ¹⁴N₁⁶O. Because low ¹⁵N/¹⁴N isotopic ratio were found in the ¹⁵NH₄⁺-amended cultures, our results suggest that N₂O do not proceed through NH₄⁺.
NO reduction by *M. nitratireducenticrescens* JAM1

To verify NO reduction by strain JAM1, N$_2$O generation was monitored in cultures without NO$_3^-$ and supplemented with sodium nitroprusside hypochloride (SNP) used as an NO donor (Fig. 5). Because N$_2$O is quickly reduced under anoxic conditions but accumulates under oxic conditions, these assays were performed under oxic conditions. N$_2$O started to accumulate in both 2 mM and 5 mM SNP-supplemented media after 24 h of incubation, reaching 7.9 ± 0.5 µg-N vial$^{-1}$ and 14.5 ± 0.4 µg-N vial$^{-1}$, respectively, after 168 h. No N$_2$O production was observed in strain JAM1 cultures without SNP or in the controls with non-inoculated culture medium supplemented with SNP or inoculated with autoclaved biomass.

**Figure 5: Reduction of NO to N$_2$O by *Methylphaga nitratireducenticrescens* JAM1**

![Graph showing N$_2$O concentration over time](image)

Strain JAM1 was cultured under oxic conditions without NO$_3^-$ and with 2 mM (square), with 5 mM (triangle), or with no (circle) sodium nitroprusside. N$_2$O concentrations were measured over different time intervals. Controls with 5 mM SNP in non-inoculated culture medium (reverse triangle) and in culture medium inoculated with autoclaved biomass (diamond) were also performed. Data represent mean values ± SD (n=3).

Role of Nar systems in NO/N$_2$O production

In the absence of NirK or NirS, N$_2$O could have been generated via NO by the Nar system (see discussion). We used the JAM1ΔnarG1narG2 double mutant, which lacks functional Nar-type nitrate reductases and which cannot grow under anoxic conditions (13). Strain JAM1 and the JAM1ΔnarG1narG2 were cultured with 16.8 mg-N vial$^{-1}$ NO$_3^-$ under oxic conditions. The growth of strain JAM1 and the mutant was similar (13). After 96 h of incubation, strain JAM1 completely reduced NO$_3^-$ to NO$_2^-$ and produced 0.14 mg-N vial$^{-1}$ of N$_2$O (Table 1). As was expected, NO$_3^-$ was not reduced, and NO$_2^-$ was not produced by JAM1ΔnarG1narG2. Contrary to the wild type strain, the mutant did not produce N$_2$O.

**Table 1: Production of N$_2$O by strain JAM1 and the JAM1ΔnarG1narG2 double mutant.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Conditions</th>
<th>Nitrate</th>
<th>Nitrite</th>
<th>N$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAM1</td>
<td>A</td>
<td>0.17(0.06)</td>
<td>16.6(0.7)</td>
<td>0.14(0.01)</td>
</tr>
<tr>
<td>JAM1ΔnarG1G2</td>
<td>A</td>
<td>17.1(0.1)</td>
<td>0.22(0.22)</td>
<td>0.004(0.002)</td>
</tr>
<tr>
<td>JAM1</td>
<td>B</td>
<td>0</td>
<td>4.25(0.09)</td>
<td>0.11(0.03)</td>
</tr>
<tr>
<td>JAM1ΔnarG1G2</td>
<td>B</td>
<td>0</td>
<td>4.87(0.39)</td>
<td>0.18(0.02)</td>
</tr>
</tbody>
</table>

Concentrations of nitrate, nitrite and nitrous oxide (mg-N vial$^{-1}$) were measured after 96 h (OD$_{600nm}$ ~ 1.2) of incubation in strain JAM1 and JAM1ΔnarG1narG2 cultured under oxic conditions with (A) 16.8 mg-N vial$^{-1}$ NO$_3^-$ added at T0h or (B) 4.7 mg-N vial$^{-1}$ NO$_2^-$ added at T24h. The results are derived from triplicate cultures. Data represent mean values (SD) (n=3).

The influence of NO$_2^-$ was also tested. As the toxicity of NO$_2^-$ has been attested from 0.36 mM (0.2 mg-N vial$^{-1}$) (8), strain JAM1 and the mutant were cultured without NO$_3^-$ under oxic conditions to allow for biomass growth. After 24 h, 4.7 mg-N vial$^{-1}$ NO$_2^-$ was added to the cultures, which were incubated for another 72 h. Strain JAM1 and the mutant produced 0.11 mg-N vial$^{-1}$ and 0.18 mg-N vial$^{-1}$ of N$_2$O, respectively, reflecting N$_2$O concentrations produced by strain JAM1 under oxic conditions with NO$_3^-$ (Table 1). Our results show that NO$_2^-$ and not NO$_3^-$ is directly involved in N$_2$O production, and the Nar systems are not involved in N$_2$O production via NO.

**Indirect detection of NO production by *M. nitratireducenticrescens* JAM1**

We assessed whether variations in the expression levels of denitrification genes correlate with the N$_2$O production and consumption cycles of strain JAM1 cultures. Strain JAM1 was cultured in NH$_4$Cl-free medium with 22 mg-N vial$^{-1}$ NO$_3^-$ under...
anoxic conditions. RNA was extracted from cells harvested over four different phases (Fig. 4): 1) at T0 for the pre-cultures, 2) during the growth phase with nitrate reduction and no N₂O production, 3) during the N₂O-production phase, and 4) during the N₂O-consumption phase. The relative transcript levels of cnorB1, cnorB2 and nosZ, which encode the catalytic subunits of the corresponding NO and N₂O reductases, and nnrS, were measured by RT-qPCR. nnrS encodes a NO-sensitive regulator and was used as an indicator of the presence of NO in the cultures. cnorB1 and nnrS expression patterns observed were similar, with the highest expression levels observed during the N₂O-production phase (Fig. 6), which suggests the presence of NO. The cnorB2 expression level remained stable except during the initial phase, when it was significantly lower. With the exception of that of the pre-cultures, the cnorB2 transcript level was always lower than those detected for cnorB1. No significant changes in nosZ expression levels were observed over the four phases.

![Figure 6: Relative transcript levels of cnorB1, cnorB2, nnrS and nosZ.](image)

Strain JAM1 was cultured under anoxic conditions in NH₄Cl-free 1403 medium with 22 mg-N vial⁻¹ NO₃⁻. Growth patterns were similar to those shown in Figure 1B under the same conditions with regular 1403 medium. Samples were drawn from the pre-culture and during the growth phase (no N₂O production), N₂O-production phase, and N₂O-consumption phase (see Figure 4D), from which total RNA was extracted. Gene expression levels of cnorB1, cnorB2, nnrS and nosZ were measured by RT-qPCR and were reported as the gene copy number per copy of dnaG (reference gene). Student's t-tests were performed for each phase to draw comparisons to the pre-culture phase. *: 0.05<P<0.01; **: 0.01<P<0.001. The results are derived from triplicate cultures from different inoculums. Data represent mean values ± SD (n=3).

Discussion

Our results show that *M. nitratireducens* JAM1 can consume NO and N₂O via the mechanism of reduction of NO to N₂O and then to N₂ as predicted by the genome sequence (Fig. S1) (9, 13). The N₂O-amended cultures yielded equivalent biomass results to those of the NO₃⁻-amended cultures as predicted by the respiratory electron transport chains of the denitrification pathway (15). Therefore, in addition of reducing nitrate, strain JAM1 has another respiratory capacity under anoxic conditions by reducing N₂O for its growth. As observed with nitrate reduction, NO and N₂O reduction can occur under oxic conditions, reinforcing the lack of a functional oxygen regulation response in strain JAM1 (see Discussion in 13).

N₂O production was observed in NO₃⁻-amended cultures either under oxic or anoxic conditions when NO₂⁻ was accumulating. This production represented up to 6% of N-input in the anoxic cultures, and NO₂⁻ was shown to be the key element of this production process. Because, we showed that the NO reductase activities were carried out in strain JAM1 cultures, the N₂O could originate from NO production despite the absence of gene encoding NirS or NirK. Intermediate NO creates problems as this molecule is highly toxic to microorganisms, inducing nitrosative stress in cells (5). Reducing NO is a key step in denitrification and is closely regulated by various sensors and regulators. NnrS is involved in cell defense against nitrosative stress and is positively regulated by the presence of NO (16-18). Therefore, nnrS expression reflects NO concentrations in a medium and was used as a marker of NO presence. The higher expressions of nnrS found during N₂O production strongly suggest that NO is produced during this phase. This correlates with higher expressions of cnorB1, which can also be regulated by NO-sensitive regulators such as NnrS or NorR (19). Moreover, the increase in cnorB1 expression found can be directly linked to observed N₂O production levels. During the
N₂O-consumption phase, only cnorB1 expression decreased and could have changed the balance between N₂O production and consumption. As nosZ expression is mainly reduced by the presence of O₂, the stable expression of this gene under constant anoxic conditions was expected (19). Interestingly, cnorB2 expression was not affected by the presence of NO, unlike cnorB1. This suggests a putatively different regulation mechanism for this gene like those observed for narG1 and narG2 in a previous study (13). Finally, the succession of different phases of N₂O production/consumption correlates with the presence of NO through an increased expression of nnrS, which strongly suggests that NO is an intermediate in N₂O production in strain JAM1.

Other nitrate respiring bacteria that lack NirK or NirS have been shown to be N₂O producers (20-22). For instance, Bacillus vireti contains three denitrification reductases (Nar, qCu₅Nor, N₂OR) and lacks, like M. nitratireducenticrescens JAM1, gene encoding NirK or NirS (23). This bacterium also produces NO and N₂O in anaerobic, NO₃⁻-amended TSB cultures during NO₂⁻ accumulation. NO was shown to originate from chemical decomposition of NO₂⁻ (6) and from an unknown biotic reaction. In our study, the abiotic control of the Methylophaga 1403 medium amended with NO₃⁻ and NO₂⁻ did not show N₂O production. Furthermore, no N₂O was detected in this medium inoculated with autoclaved biomass (Fig. 5). These results rule out NO/N₂O production by chemical decomposition of NO₂⁻ in strain JAM1 cultures. The possible biotic source of NO in absence of NirS or NirK has been studied in Escherichia coli (see review by Vine and Cole (24)). There are supporting evidence that NO is generated in E. coli as a side product during nitrite reduction (i) by the cytoplasmic, NADH-dependent nitrite reductase (NirBD), (ii) by the nitrite reductase NrfAB, and (iii) by NarGHI. Vine et al. (25) showed, with mutants defective in these reductases, that NarGHI is the major enzyme responsible of NO production. However, a small production of NO was still occurring in narG mutant, suggesting the involvement of another molybdoprotein. In M. nitratireducenticrescens JAM1, the double-knockout mutant JAM1ΔnarG1narG2, which lacks the two dissimilatory NO₃⁻ reductases, was still able to produce N₂O under oxic conditions at the same level of the wild type when NO₂⁻ was added to the cultures. These results suggest the two Nar systems are not involved in NO production. The genome of strain JAM1 did not reveal gene encoding NrfAB, but contain a gene cluster encoding a cytoplasmic, NADH-dependent nitrite reductase (CP003390.3; Q7A_2620 and Q7A_2621), which may be the source of NO (Fig. S1).

The significance of maintaining an incomplete pathway by M. nitratireducenticrescens JAM1 is unclear and may depend upon the original habitat and environment, here the denitrifying biofilm. While M. nitratireducenticrescens JAM1 serves as an important actor among the microbial community of the marine biofilm in performing optimal denitrifying activities (10, 26), it was thought to participate uniquely in the reduction of NO₃⁻ to NO₂⁻. It was previously proposed that NO₂⁻ reduction to N₂ is carried out by Hyphomicrobium nitrativorans NL23, the second most represented bacterium in the biofilm (12, 27). Its capacity to reduce NO and N₂O and to grow on N₂O suggests that M. nitratireducenticrescens JAM1 may participate in the reduction of NO and N₂O during denitrification in the biofilm. Although our culture assays were performed with high levels of NO₃⁻ (37 mM), which is rarely exceeds a value of 0.7 mM in natural environments (28), similar levels can be reached in closed-circuit systems like the seawater aquarium tank located in the Montreal Biodome, where NO₃⁻ levels reached up to 14 mM (29). Rissanen et al. (30) observed also the combination of Methylophaga spp. and Hyphomicrobium spp. in the fluidized-bed type denitrification reactors treating the recirculating seawater of the public fish aquarium SEA LIFE at Helsinki, Finland. Although, this study provided no indication of the denitrification pathway in these Methylophaga and Hyphomicrobium, it reinforces the importance of the natural combination of these two genera in marine denitrification.
environment.

**Conclusions**

*M. nitratireducenticrescens* JAM1 is one of few isolated marine methylotrophic bacterial strains to exhibit anaerobic respiratory capacities by reducing NO$_3^−$ to NO$_2^−$ and, as reported here, by reducing N$_2$O to N$_2$. It can also generate N$_2$O via NO by an unknown biotic system. Very few marine denitrifying bacteria have been isolated from recirculating marine systems (31-34). No previous studies have generated genetic information related gene arrangement or expression on these bacteria. Based on substantial data accumulated on the genome, gene arrangement and gene expression of denitrification and on methylotrophy, *M. nitratireducenticrescens* JAM1 can serve as a model for studying such activities in marine environments. Finally, our results enable a better understanding of the ecophysiological role of *M. nitratireducenticrescens* JAM1 in the original biofilm developed in the denitrification reactor of a closed-circuit marine aquarium.

**Materials and Methods**

**Bacterial growth conditions**

*M. nitratireducenticrescens* JAM1 and the JAM1ΔnarG1narG2 double mutant were cultured in the American Type Culture Collection (ATCC, Manassas, VA, USA) *Methylophaga* medium 1403 (9, 13). When required, NO$_3^−$ (NaNO$_3$) or NO$_2^−$ (NaNO$_2$) (Fisher Scientific Canada, Ottawa, ON, Canada) were added to the medium. Medium (40 or 60 mL) was dispensed into 720-mL bottles (680- or 660-mL head space) that were sealed with caps equipped with septum and which were then autoclaved. After autoclaving, the following filter-sterilized solutions were added to the bottles (40 mL volume): 120 µL methanol (final concentration 0.3% [vol/vol]; 74.3 mM), 800 µL solution T [per 100 mL: 0.7 g KH$_2$PO$_4$, 10 g NH$_4$Cl, 10 g Bis-Tris, 0.3 g ferric ammonium citrate (pH 8)], 400 µL Wolf’s mineral solution (pH 8) (ATCC), and 40 µL vitamin B$_{12}$ (stock solution 0.1 mg/mL). The Wolf mineral solution is composed of (per liter) 0.5 g EDTA, 3.0 g MgSO$_4$.7H$_2$O, 0.5 g MnSO$_4$.H$_2$O, 1.0 g NaCl, 0.1 g FeSO$_4$.7H$_2$O, 0.1 g Co(NO$_3$)$_2$.6H$_2$O, 0.1 g CaCl$_2$ (anhydrous), 0.1 g ZnSO$_4$.7H$_2$O, 0.010 g CuSO$_4$.5H$_2$O, 0.010 g AlK(SO$_4$)$_2$ (anhydrous), 0.010 g H$_2$BO$_3$, 0.010 g Na$_2$MoO$_4$.2H$_2$O, 0.001 g Na$_2$SeO$_3$ (anhydrous), 0.010 g Na$_2$WO$_4$.2H$_2$O, and 0.020 g NiCl$_2$.6H$_2$O. The final concentration of ammonium (NH$_4^+$) in the *Methylophaga* 1403 medium was measured as 21 mg-N vial$^{-1}$ (20.9 mg-N vial$^{-1}$ from NH$_4$Cl and 0.1 mg-N vial$^{-1}$ from ferric ammonium citrate). The amount of NO$_3^−$ carried by the Wolf mineral solution (0.0038 mg-N vial$^{-1}$) was deemed negligible. For the anoxic cultures, bottles were flushed with nitrogen gas (N$_2$, purity >99.9%; Praxair, Mississauga, ON, Canada) or argon (purity 99.9%, Praxair) for 20 min prior to autoclaving. When necessary, N$_2$O (purity 99.9%, Praxair) and acetylene (10% [vol/vol] of headspace; Praxair) were injected into the headspace before autoclaving. Acetylene is an inhibitor of nitrous oxide reductase and has been extensively used in N$_2$O studies to observe N$_2$O production in cells (35). Inoculums were made from fresh culture cultivated under oxic conditions without NO$_3^−$ to reach an optical density (OD$_{600}$) of 0.025. Culture bottles were incubated at 30°C in the dark. For oxic cultures, bottles were shaken at 150 rpm.

The capacity for strain JAM1 to reduce NO was tested with sodium nitroprusside (sodium nitroprusside hypochloride ([SNP]; purity ≥ 99.0%, Sigma-Aldrich, St. Louis, MO, USA) as the NO source. Strain JAM1 was cultured in *Methylophaga* 1403 medium under oxic conditions without NO$_3^−$ to reach an optical density (OD$_{600}$) of 0.025. Culture bottles were incubated at 30°C in the dark. For oxic cultures, bottles were shaken at 150 rpm.

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production, NH$_4$Cl-free cultures were employed under oxic and anoxic conditions using solution T containing no NH$_4$Cl. Prior to inoculation, cells from start-up cultures were centrifuged and rinsed three times with saline solution to remove any residual traces of NH$_4$.

Bacterial growth was monitored by spectrophotometry (OD$_{600}$). Bacterial flocs were dispersed with a Potter-Elvehjem homogenizer prior to measurement. Oxygen concentrations in the headspace were monitored in cultures under oxic conditions by gas chromatography using a temperature conductivity detector (7890B series GC Custom, SP1 option 7890-0504/0537; Agilent Technologies, Mississauga, ON, Canada). Although vials were capped in the oxic cultures, O$_2$ concentrations in the headspace (680 ml) did not significantly decrease (T0 h = 20.4 ± 0.3%; T100 h = 19.7 ± 0.9%).

$^{15}$N-labeling of N$_2$O

Strain JAM1 cultures were made with 22 mg-N vial$^{-1}$ Na$^{15}$NO$_3$ (Sigma-Aldrich) in NH$_4$Cl-free medium or with 22 mg-N vial$^{-1}$ Na$^{14}$NO$_3$ and 20.7 mg-N vial$^{-1}$ $^{15}$NH$_4$Cl (Sigma-Aldrich). Both cultures were used under anoxic conditions, and 10% (vol/vol) acetylene was added to allow N$_2$O to accumulate. Cultures were made in triplicate. After 14 days of incubation, the headspace of each replicate was pooled, and 100 mL of the gaseous phase was sampled in Tedlar bags. N$_2$O-isotope measurements were performed at the Environmental Isotope Laboratory (Earth & Environmental Sciences; University of Waterloo, ON, Canada) via Trace Gas-VGI IsoPrime-Isotope Ratio Mass Spectrometry (TG-IRMS). $^{45}$[N$_2$O]/$^{44}$[N$_2$O] and $^{46}$[N$_2$O]/$^{44}$[N$_2$O] ratios were calculated according to the peak intensity measured for $^{46}$[N$_2$O], $^{45}$[N$_2$O] and $^{44}$[N$_2$O]. The $^{15}$N/$^{14}$N isotopic ratio was derived from the previous results according to Eq. 3.

$$Rs = \frac{\Sigma(15N \text{ vial}^{-1})}{\Sigma(14N \text{ vial}^{-1})} = \frac{[^{15}N45 + 2(15N46)]}{[2(14N44) + ^{14}N45]} \quad [3]$$

where Rs is the sample isotopic ratio. Calculated from the $^{45}$[N$_2$O]/$^{44}$[N$_2$O] and $^{46}$[N$_2$O]/$^{44}$[N$_2$O] isotopic ratios, $^{14}$N45 is the quantity of $^{14}$N in $^{45}$[N$_2$O], $^{15}$N45 is the quantity of $^{15}$N in $^{45}$[N$_2$O], $^{14}$N44 is the quantity of $^{14}$N in $^{44}$[N$_2$O] and $^{15}$N46 is the quantity of $^{15}$N in $^{46}$[N$_2$O]. We considered the isotope fractionation by denitrification enzymes as negligible in our calculations (delta values ranging from -10 ‰ to -40 ‰) (36).

Measurements of nitrogenous compounds

NO$_3^-$ and NO$_2^-$ concentrations were determined by ion chromatography using the 850 Professional IC (Metrohm, Herisau, Switzerland) with a Metrosep A Supp 5 analytical column (250 mm x 4.0 mm).

N$_2$O and N$_2$ concentrations were determined by gas chromatography. Headspace samples (10 mL) were collected using a Pressure Lok gastight glass syringe (VICI Precision Sampling Inc., Baton Rouge, LA, USA) and were injected through the injection port of a gas chromatograph equipped with a thermal conductivity detector and electron-capture detector (7890B series GC Custom, SP1 option 7890-0504/0537; Agilent Technologies). The reproducibility of the N$_2$O was assessed before each set of measurements was conducted via the repeated analysis of certified N$_2$O standard gas with standard deviations <5%. N$_2$O standards (500 ppmv and 250 ppmv) were created based on dilutions from the 10,000 ppmv N$_2$O stock standard. The 10,000 ppmv stock standard was obtained by injecting 1% pure N$_2$O (Praxair) into a 720 mL gastight bottle. The detection limit of the N$_2$O was set to <10 ppbv, corresponding to the 0.3 nmol/vial composition of our bioassays. No significant N$_2$O production patterns were observed through our blank experiments involving sterile media and empty glass bottles. The total quantity of N$_2$O in the culture bottle (aqueous phase and headspace) (X$_{N2O}$ in µmole vial$^{-1}$) was calculated according to Eq. 4.

$$X_{N2O} = \frac{[K_{cpH30sw} * A_{N2O} * P * V_{i}]_{aq} + [A_{N2O} * V_{g}/ V_{n}]_{gaz}}{[A_{N2O} * V_{g}/ V_{n}]}_{gaz}$$

[4]
where $A_{N_2O}$: the $N_2O$ mixing ratio measured in the headspace (ppmv/10^6; no unit); P: 1 atm; $V_1$ and $V_g$: volume of the aqueous (0.04 or 0.06 L vial⁻¹) and gaseous phases (0.68 or 0.66 L vial⁻¹), respectively; and $V_a$: molar volume [RT (gas constant): 0.08206 L atm K⁻¹ mol⁻¹ * 303K = 24.864 L mol⁻¹]. $K_{H30sw}$ is the corrected Henry’s constant for seawater at 30°C (0.01809 mol L⁻¹ atm⁻¹) according to Weiss and Price (1980). $X_{N_2O}$ was then converted (eq.5) in an mg-N vial⁻¹ for an easier calculation of mass balances using the other nitrogenous compounds:

$$X_{N,N_2O} = X_{N_2O} [2N/N_2O] [0.014 \text{ mg-N \ \mu mole}^{-1}] \quad [5]$$

The reproducibility of the $N_2$ was assessed before each set of measurements was made by a repeated analysis of $N_2$ (purity >99.99%, Praxair) diluted in a 720 mL gastight bottle (0 and 500 ppmv) flushed with argon (purity >99.99%, Praxair). The total quantity of $N_2$ in the culture bottles was only considered for the headspace, as the quantity of dissolved $N_2$ in the aqueous phase was considered to be negligible in our experimental design based on Henry’s constant (0.0005 mol L⁻¹ atm⁻¹) and was thus calculated according to Eq. 6.

$$X_{N,N_2} (\text{mg-N vial}^{-1}) = \frac{A_{N_2} \cdot V_g}{V_a} [2N/N_2] \cdot [0.014 \text{ mg-N \ \mu mole}^{-1}] \quad [6]$$

RNA extraction

Anoxic cultures of strain JAM1 were created in an NH₄Cl-free 1403 medium supplemented with 22 mg-N vial⁻¹ NO₃⁻. Cells were harvested at specific times, and RNA was immediately extracted using the PureLink RNA mini kit (Ambion Therm Fisher Scientific, Burlington, ON, Canada). RNA extracts were treated twice with TurboDNase (Ambion), and RNA quality was verified by agarose gel electrophoresis. The absence of remaining DNA was checked via the end-point polymerase chain reaction (PCR) amplification of the 16S rRNA gene using RNA extracts as the template.

Gene expression

cDNAs samples were generated from the RNA using hexameric primers and the Reverse Transcription System developed by Promega (Madison, WI, USA) with 1 µg of RNA. Real-time quantitative PCR (qPCR) assays were performed using the Faststart SYBR Green Master (Roche Diagnostics, Laval, QC, Canada) according to the manufacturer’s instructions. All reactions were performed in a Rotor-Gene 6000 real-time PCR thermocycler (Qiagen Inc. Toronto, ON, Canada), and each reaction contained 25 ng of cDNA and 300 nM of primers (Table 2). Genes tested included $cnorB_1$, $cnorB_2$, nosZ and $nnrS$. PCR began with an initial denaturation step of 10 min at 95°C followed by 40 cycles of 10 s at 95°C, 15 s at 60°C, and 20 s at 72°C. To confirm the purity of the amplified products, a melting curve was performed by increasing the temperature from 65°C to 95°C at increments of 1°C per step with a pause of 5 s included between each step. All genes for each sample and standard were tested in a single run. The amplification efficiency level was tested for each set of primer pairs by qPCR using a dilution of strain JAM1 genomic DNA as the template. The amplification efficiencies for all primer pairs varied between 0.9 and 1.1. The copy number of each gene was calculated according to standard curves using dilutions of strain JAM1 genomic DNA. To normalize the gene expression of the different growth phases, results were expressed as copy numbers per dnaG copy numbers for each sample. In accordance with previous studies (13), dnaG generated the least variability of the reference genes tested (dnaG, rpoD and rpoB) in strain JAM1. dnaG encodes for a DNA primase and is present in one copy in strain JAM1 genomes. RNA extraction and qPCR were performed with three independent biological replicates. The significance of differential expression levels was tested for each phase against the pre-culture phase via Student’s $t$-test.
Table 2: Primers used for RT-qPCR

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<th>Primers</th>
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<tr>
<td>dnaG-894r</td>
<td></td>
<td>GCTGCGAATGCAACTGACGTA</td>
</tr>
</tbody>
</table>

Acknowledgments

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References


Figure S1: Schematic of the denitrification pathway and the ammonium pathways in *Methylophaga nitratireducens* JAM1

Legend:
- **Green**: Denitrification pathway
- **Blue**: NH₄⁺ and NO₃⁻ assimilatory pathways
- **Red**: Respiratory pathways
- **Yellow**: C1 assimilatory pathway

Functions are based on the annotations of strain JAM1 genome (CP003390.3). Numbers are locus tag of the corresponding gene preceding by Q7A_.

TCA: tricarboxylic acid cycle; GS: glutamine synthase; GlnA: Glutamine synthetase; GOGAT: Glutamate dehydrogenase; trp: transporter; deh: dehydrogenase; MDH: methanol dehydrogenase; Cyt: cytochrome; Q: quinolone; Fae: formaldehyde-activating enzyme; HPS: 3-hexulose-6-phosphate synthase; NO₃⁻ red and NO₂⁻ red: assimilatory NADH-dependent nitrate and nitrite reductases, NarK1: nitrate/H+ symporter; NarK2: Nitrate/nitrite antiporter; NarK12f: fused NarK1-NarK2 transporter; NarXL: two-component nitrate/nitrite sensor system.

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