



# Denitrifying metabolism of the methylotrophic marine bacterium *Methylophaga nitratreducenticrescens* strain JAM1

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## ABSTRACT

**Background.** *Methylophaga nitratreducenticrescens* 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 ( $\text{NO}_3^-$ ) to nitrite ( $\text{NO}_2^-$ ). These physiological traits are attributed to gene clusters that encode two dissimilatory nitrate reductases (Nar). Strain JAM1 also contains gene clusters encoding two nitric oxide (NO) reductases and one nitrous oxide ( $\text{N}_2\text{O}$ ) reductase, suggesting that NO and  $\text{N}_2\text{O}$  can be reduced by strain JAM1. Here we characterized further the denitrifying activities of *M. nitratreducenticrescens* JAM1.

**Methods.** Series of oxic and anoxic cultures of strain JAM1 were performed with  $\text{N}_2\text{O}$ ,  $\text{NO}_3^-$  or sodium nitroprusside, and growth and  $\text{N}_2\text{O}$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{N}_2$  concentrations were measured. Ammonium ( $\text{NH}_4^+$ )-free cultures were also tested to assess the dynamics of  $\text{N}_2\text{O}$ ,  $\text{NO}_3^-$  and  $\text{NO}_2^-$ . Isotopic labeling of  $\text{N}_2\text{O}$  was performed in  $^{15}\text{NH}_4^+$ -amended cultures. Cultures with the JAM1 $\Delta narG1narG2$  double mutant were performed to assess the involvement of the Nar systems on  $\text{N}_2\text{O}$  production. Finally, RT-qPCR was used to measure the gene expression levels of the denitrification genes cytochrome *bc*-type nitric oxide reductase (*cnorB1* and *cnorB2*) and nitrous oxide reductase (*nosZ*), and also *nnrS* and *norR* that encode NO-sensitive regulators.

**Results.** Strain JAM1 can reduce NO to  $\text{N}_2\text{O}$  and  $\text{N}_2\text{O}$  to  $\text{N}_2$  and can sustain growth under anoxic conditions by reducing  $\text{N}_2\text{O}$  as the sole electron acceptor. Although strain JAM1 lacks a gene encoding a dissimilatory  $\text{NO}_2^-$  reductase,  $\text{NO}_3^-$ -amended cultures produce  $\text{N}_2\text{O}$ , representing up to 6% of the N-input.  $\text{NO}_2^-$  was shown to be the key intermediate of this production process. Upregulation in the expression of *cnorB1*, *cnorB2*, *nnrS* and *norR* during the growth and the  $\text{N}_2\text{O}$  accumulation phases suggests NO production in strain JAM1 cultures.

**Discussion.** By showing that all the three denitrification reductases are active, this demonstrates that *M. nitratreducenticrescens* 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 an incomplete pathway could be related to the specific role of strain JAM1 in the denitrifying biofilm of the denitrification reactor from which it originates. The production of  $\text{N}_2\text{O}$  in strain JAM1 did not involve Nar, contrary to what was demonstrated in *Escherichia coli*. *M. nitratreducenticrescens* JAM1 is the only reported *Methylophaga* species that has the

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capacity to grow under anoxic conditions by using  $\text{NO}_3^-$  and  $\text{N}_2\text{O}$  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.

**Subjects** Environmental Sciences, Microbiology, Molecular Biology, Biogeochemistry

**Keywords** Denitrification, Marine bacterium, *Methylophaga*, Nitrous oxide, Nitrate

## INTRODUCTION

The complete denitrification pathway describes the successive reduction of nitrate ( $\text{NO}_3^-$ ) to nitrite ( $\text{NO}_2^-$ ), nitric oxide (NO), nitrous oxide ( $\text{N}_2\text{O}$ ), and nitrogen ( $\text{N}_2$ ) (Van Spanning, Delgado & Richardson, 2005). This process is used by bacteria for respiration in environments with low oxygen concentrations and with  $\text{NO}_3^-$  as an electron acceptor. The process is driven by metalloenzymes  $\text{NO}_3^-$  reductase,  $\text{NO}_2^-$  reductase, NO reductase, and  $\text{N}_2\text{O}$  reductase (Einsle & Kroneck, 2004). As a facultative trait, denitrification occurs frequently across environments and is performed by bacteria of diverse origins (Zumft, 1997). 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 (Zumft, 1997), the four steps of reduction from  $\text{NO}_3^-$  to  $\text{N}_2$  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  $\text{NO}_2^-$  and NO are deleterious molecules (Kaspar, 1982; Poole, 2005; Schreiber et al., 2012; Simon & Klotz, 2013).

*Methylophaga nitratireducentescens* JAM1 is a marine methylotrophic gammaproteobacterium 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 (Auclair et al., 2010; Villeneuve et al., 2013). This biofilm is composed of at least 15 bacterial species and of numerous protozoans (Labbé et al., 2003; Laurin et al., 2008), among which *Methylophaga* spp. and *Hyphomicrobium* spp. compose more than 50% of the biofilm (Labbé et al., 2007). Along with the denitrifying bacterium *Hyphomicrobium nitrativorans* NL23, *M. nitratireducentescens* JAM1 was shown to be the representative of the *Methylophaga* population in the biofilm (Auclair et al., 2010).

*M. nitratireducentescens* JAM1 is considered as a nitrate respirer as it can grow under anoxic conditions through the reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ , which accumulates in the culture medium (Auclair et al., 2010). 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. nitratireducentescens* JAM1, which we showed that both contribute to  $\text{NO}_3^-$  reduction during strain JAM1 growth (Mauffrey, Martineau & Villemur, 2015). Anaerobic growth by strain JAM1 is a unique among *Methylophaga*

spp. that were described as strictly aerobic bacteria (Boden, 2012). 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* and *cnor2*) and one putative dissimilatory N<sub>2</sub>O reductase, but lacks gene encoding a dissimilatory copper- (NirK) or cytochrome *cd1*-type (NirS) NO<sub>2</sub><sup>-</sup> reductase. These gene clusters have been shown to be transcribed. However, the capacity of *M. nitratireducenticrescens* JAM1 to consume NO and N<sub>2</sub>O has not been fully determined. In addition to these gene clusters, genes involved in the NO response such as *nnrS* and *norR* are present (Mauffrey, Martineau & Villemur, 2015) suggesting tight regulation of denitrification genes such as *cnorB* and *nosZ*. Finally, the genome has a gene cluster encoding assimilatory nitrate and NADH-dependent nitrite reductases.

In this study, we assessed further the denitrification activities of strain JAM1 in pure cultures by demonstrating the consumption of NO and N<sub>2</sub>O by strain JAM1 in cultures amended with N<sub>2</sub>O or sodium nitroprusside as NO provider. Through our investigation, we found that strain JAM1 cultured with NO<sub>3</sub><sup>-</sup> generates a small amount of N<sub>2</sub>O. We assessed whether nitrate and the nitrate reductases, nitrite and ammonium are directly involved in this N<sub>2</sub>O production, and found that NO<sub>2</sub><sup>-</sup> is a key intermediate of this production process. Finally, we showed that the N<sub>2</sub>O accumulation/consumption cycle in NO<sub>3</sub><sup>-</sup>-amended cultures affects the expression of denitrification genes *cnorB* (*cnorB1* and *cnorB2*) and *nosZ*, and also *nnrS* and *norR*, which encode NO-sensitive regulators. These results suggest that NO is also generated in NO<sub>3</sub><sup>-</sup>-amended cultures.

## MATERIALS AND METHODS

### Bacterial growth conditions

*M. nitratireducenticrescens* JAM1 and the JAM1  $\Delta narG1 narG2$  double mutant were cultured in the American Type Culture Collection (ATCC, Manassas, VA, USA) *Methylophaga* medium 1403 (Villeneuve et al., 2013; Mauffrey, Martineau & Villemur, 2015). When required, NO<sub>3</sub><sup>-</sup> (NaNO<sub>3</sub>) or NO<sub>2</sub><sup>-</sup> (NaNO<sub>2</sub>) (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  $\mu$ L methanol (final concentration 0.3% [vol/vol]; 74.3 mM), 800  $\mu$ L solution T (per 100 mL: 0.7 g KH<sub>2</sub>PO<sub>4</sub>, 10 g NH<sub>4</sub>Cl, 10 g Bis-Tris, 0.3 g ferric ammonium citrate (pH 8)), 400  $\mu$ L Wolf's mineral solution (pH 8) (ATCC), and 40  $\mu$ L vitamin B<sub>12</sub> (stock solution 0.1 mg/mL). The Wolf mineral solution is composed of (per liter) 0.5 g EDTA, 3.0 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g MnSO<sub>4</sub>·H<sub>2</sub>O, 1.0 g NaCl, 0.1 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, 0.1 g CaCl<sub>2</sub> (anhydrous), 0.1 g ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.010 g CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.010 g AlK(SO<sub>4</sub>)<sub>2</sub> (anhydrous), 0.010 g H<sub>3</sub>BO<sub>3</sub>, 0.010 g Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.001 g Na<sub>2</sub>SeO<sub>3</sub> (anhydrous), 0.010 g Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O, and 0.020 g NiCl<sub>2</sub>·6H<sub>2</sub>O. The final concentration of ammonium (NH<sub>4</sub><sup>+</sup>) in the *Methylophaga* 1403 medium was measured as 21 mg-N vial<sup>-1</sup> (20.9 mg-N vial<sup>-1</sup> from NH<sub>4</sub>Cl and 0.1 mg-N vial<sup>-1</sup> from ferric ammonium citrate). The amount of NO<sub>3</sub><sup>-</sup> carried by the Wolf mineral solution (0.0038 mg-N vial<sup>-1</sup>)

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,  $N_2O$  (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  $N_2O$  studies to observe  $N_2O$  production in cells (*Klemetsson et al., 1977*). 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  $\geq 99.0\%$ , Sigma-Aldrich, St. Louis, MO, USA) as the NO source. To avoid SNP toxicity, strain JAM1 was first cultured in *Methylophaga* 1403 medium under oxic conditions without  $NO_3^-$  for 24 h. The cells were then centrifuged (8,000 g 5 min) and dispersed into fresh medium supplemented with 2 mM, 5 mM, or no SNP. Culture medium with 5 mM SNP and no biomass was also used as a control. Cells were incubated under oxic conditions at 30 °C in the dark, and  $N_2O$  production was monitored. To investigate the potential role of  $NH_4^+$  in  $N_2O$  production,  $NH_4Cl$ -free cultures were employed under oxic and anoxic conditions using solution T containing no  $NH_4Cl$ . 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 ( $T0\ h = 20.4 \pm 0.3\%$ ;  $T100\ h = 19.7 \pm 0.9\%$ ).

### **$^{15}N$ -labeling of $N_2O$**

Strain JAM1 cultures were made with 22 mg-N vial<sup>-1</sup>  $Na^{15}NO_3$  (Sigma-Aldrich) in  $NH_4Cl$ -free medium or with 22 mg-N vial<sup>-1</sup>  $Na^{14}NO_3$  and 20.7 mg-N vial<sup>-1</sup>  $^{15}NH_4Cl$  (Sigma-Aldrich). Both cultures were used under anoxic conditions, and 10% (vol/vol) acetylene was added to allow  $N_2O$  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.  $N_2O$ -isotope measurements were performed at the Environmental Isotope Laboratory (Earth & Environmental Sciences; University of Waterloo, ON, Canada) via Trace Gas-GVI IsoPrime-Isotope Ratio Mass Spectrometry (TG-IRMS).  $^{45}[N_2O]/^{44}[N_2O]$  and  $^{46}[N_2O]/^{44}[N_2O]$  ratios were calculated according to the peak intensity measured for  $^{46}[N_2O]$ ,  $^{45}[N_2O]$  and  $^{44}[N_2O]$ . The  $^{15}N/^{14}N$  isotopic ratio was derived from the previous results from Eq. (1).

$$R_s = \sum(^{15}N\ \text{vial}^{-1}) / \sum(^{14}N\ \text{vial}^{-1}) = [^{15}N45 + 2(^{15}N46)] / [2(^{14}N44) + ^{14}N45] \quad (1)$$

where  $R_s$  is the sample isotopic ratio. Calculated from the  $^{45}[N_2O]/^{44}[N_2O]$  and  $^{46}[N_2O]/^{44}[N_2O]$  isotopic ratios,  $^{14}N45$  is the quantity of  $^{14}N$  in  $^{45}[N_2O]$ ,  $^{15}N45$  is

the quantity of  $^{15}\text{N}$  in  $^{45}[\text{N}_2\text{O}]$ ,  $^{14}\text{N}44$  is the quantity of  $^{14}\text{N}$  in  $^{44}[\text{N}_2\text{O}]$  and  $^{15}\text{N}46$  is the quantity of  $^{15}\text{N}$  in  $^{46}[\text{N}_2\text{O}]$ . We considered the isotope fractionation by denitrification enzymes as negligible in our calculations (delta values ranging from  $-10\text{‰}$  to  $-40\text{‰}$ ) (Snider, Schiff & Spoelstra, 2009).

### Measurements of nitrogenous compounds

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

$\text{N}_2\text{O}$  and  $\text{N}_2$  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  $\text{N}_2\text{O}$  was assessed before each set of measurements via the repeated analysis of certified  $\text{N}_2\text{O}$  standard gas with standard deviations  $<5\%$ .  $\text{N}_2\text{O}$  standards (500 ppmv and 250 ppmv) were created based on dilutions from the 10,000 ppmv  $\text{N}_2\text{O}$  stock standard. The 10,000 ppmv stock standard was obtained by injecting 1% pure  $\text{N}_2\text{O}$  (Praxair) into a 720 mL gastight bottle. The detection limit of the  $\text{N}_2\text{O}$  was set to  $<10$  ppbv, corresponding to the 0.3 nmol/vial composition of our bioassays. No significant  $\text{N}_2\text{O}$  production patterns were observed through our blank experiments involving sterile media and empty glass bottles. The total quantity of  $\text{N}_2\text{O}$  in the culture bottle (aqueous phase and headspace) ( $X_{\text{N}_2\text{O}}$  in  $\mu\text{mole vial}^{-1}$ ) was calculated according to Eq. (2).

$$X_{\text{N}_2\text{O}} = [K_{\text{cpH}30\text{sw}} * A_{\text{N}_2\text{O}} * P * V_1]_{\text{aq}} + [A_{\text{N}_2\text{O}} * V_g / V_n]_{\text{gaz}} \quad (2)$$

where  $A_{\text{N}_2\text{O}}$ : the  $\text{N}_2\text{O}$  mixing ratio measured in the headspace ( $\mu\text{mole}_{\text{N}_2\text{O}} \text{mole}^{-1}$ );  $P$ : 1 atm;  $V_1$  and  $V_g$ : volume of the aqueous (0.04 or 0.06 L  $\text{vial}^{-1}$ ) and gaseous phases (0.68 or 0.66 L  $\text{vial}^{-1}$ ), respectively; and  $V_n$ : molar volume (RT (gas constant):  $0.08206 \text{ L atm K}^{-1} \text{ mol}^{-1} * 303 \text{ K} = 24.864 \text{ L mol}^{-1}$ ).  $K_{\text{H}30\text{sw}}$  is the corrected Henry's constant for seawater at  $30^\circ\text{C}$  ( $0.01809 \text{ mol L}^{-1} \text{ atm}^{-1}$ ) according to Weiss and Price (1980).  $X_{\text{N}_2\text{O}}$  was then converted (Eq. (3)) into mg-N  $\text{vial}^{-1}$  for an easier calculation of mass balances using the other nitrogenous compounds:

$$X_{\text{N-N}_2\text{O}} = X_{\text{N}_2\text{O}} * [2\text{N}/\text{N}_2\text{O}] * [0.014 \text{ mg-N } \mu\text{mole}^{-1}]. \quad (3)$$

The reproducibility of the  $\text{N}_2$  was assessed before each set of measurements via a repeated analysis of  $\text{N}_2$  (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  $\text{N}_2$  in the culture bottles was only considered for the headspace, as the quantity of dissolved  $\text{N}_2$  in the aqueous phase was considered to be negligible in our experimental design based on Henry's constant ( $0.0005 \text{ mol L}^{-1} \text{ atm}^{-1}$ ) and was thus calculated according to Eq. (4).

$$X_{\text{N-N}_2}(\text{mg-N } \text{vial}^{-1}) = [A_{\text{N}_2} * V_g / V_n]_{\text{gaz}} * [2\text{N}/\text{N}_2] * [0.014 \text{ mg-N } \mu\text{mole}^{-1}]. \quad (4)$$

## RNA extraction

Anoxic cultures of strain JAM1 were created in an  $\text{NH}_4\text{Cl}$ -free 1403 medium supplemented with  $22 \text{ mg-N vial}^{-1} \text{ NO}_3^-$ . Cells were harvested at specific times, and RNA was immediately extracted using the PureLink RNA mini kit (Ambion Thermo 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  $1 \mu\text{g}$  of RNA and quantified by spectrophotometry. 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  $25 \text{ ng}$  of cDNA and  $300 \text{ nM}$  of primers (Table 1). Genes tested included *cnorB1*, *cnorB2*, *nnrS*, *nosZ*, *norR* and *nr*, and the reference genes *dnaG*, *rpoD* and *rpoB* (Mauffrey, Martineau & Villemur, 2015) and the PCR began with an initial denaturation step of  $10 \text{ min}$  at  $95 \text{ }^\circ\text{C}$  followed by  $40$  cycles of  $10 \text{ s}$  at  $95 \text{ }^\circ\text{C}$ ,  $15 \text{ s}$  at  $60 \text{ }^\circ\text{C}$ , and  $20 \text{ s}$  at  $72 \text{ }^\circ\text{C}$ . To confirm the purity of the amplified products, a melting curve analysis was performed by increasing the temperature from  $65 \text{ }^\circ\text{C}$  to  $95 \text{ }^\circ\text{C}$  at increments of  $1 \text{ }^\circ\text{C}$  per step with a pause of  $5 \text{ s}$  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. The gene expression levels of the targeted genes were standardized with the three reference genes. The RNA extraction and qPCR were performed with three to four independent biological replicates. The significance of differential expression levels was tested for each phase against the pre-culture phase using One-way ANOVA tests with Tukey *post hoc* tests.

## RESULTS

### *M. nitratireducens* JAM1 grows on $\text{N}_2\text{O}$ under anoxic conditions

Strain JAM1 was cultured under anoxic conditions with either  $\text{NO}_3^-$  in the medium or with  $\text{N}_2\text{O}$  injected in the headspace as the sole electron acceptor. Both types of culture received the same electron equivalent of  $\text{NO}_3^-$  or  $\text{N}_2\text{O}$  ( $1.3 \text{ mmole vial}^{-1}$  or  $18.2$  and  $36.4 \text{ mg-N vial}^{-1}$ , respectively) according to:



**Table 1** Primers used for RT-qPCR.

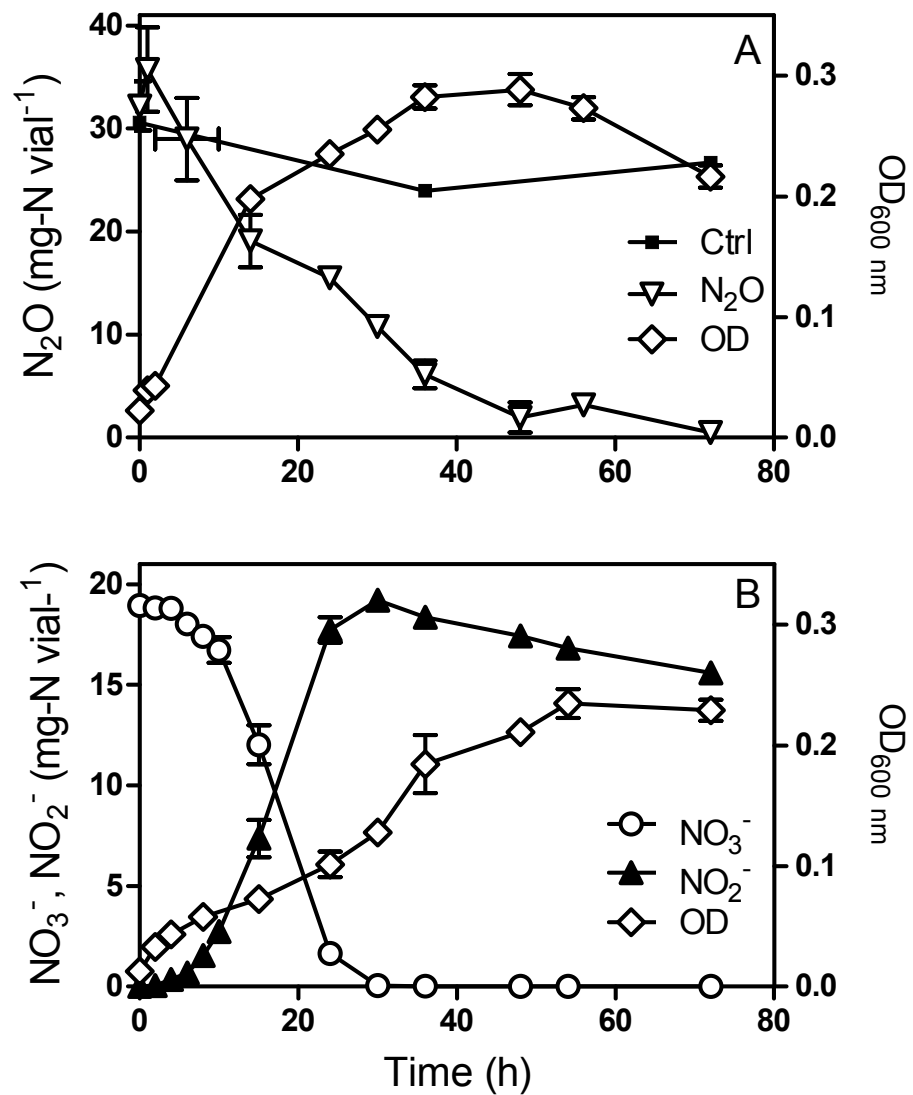
Primers	Target gene	Locus tag*	Sequence (5'–3')
cytochrome <i>bc</i> -type complex NO reductases			
cnorB1-510f	<i>cnorB1</i>	Q7A_0433	CCTGATCGGTTTGGCTCTC
cnorB1-635r			CCCATGATCAATTCCCAGAC
cnorB2-334f	<i>cnorB2</i>	Q7A_0487	GGCAACAAGCTATTGGAGCA
cnorB2-449r			GTGGTGGTAAAGCGACCAGA
N <sub>2</sub> O reductase			
nosZ-826f	<i>nosZ</i>	Q7A_0459	GAGCGTGACTGGGTAGTCGT
nosZ-952r			GTGTCAACTCGCTCCCTTTG
NO-sensitive regulators			
nnrs-749f	<i>nnrS</i>	Q7A_1801	TGTTCCGCATTTACGCAATA
nnrs-848r			TAACCGATGTGCAAAGACCA
norR-265f	<i>norR</i>	Q7A_0435	CGGTTTGCTGCAGATAGTGA
norR-386r			CCCCAGGGCCTGTTATTAT
Assimilatory nitrate reductase			
nr-1350f	<i>nr</i>	Q7A_2619	ATTCGGTACAGTCGGTTTGC
nr-1474r			TGTCTGGATTATTGCCACCA
Reference genes			
dnaG-774f	<i>dnaG</i>	Q7A_342	CATCCTGATCGTGGAAGGTT
dnaG-894r			GCTGCGAATCAACTGACGTA
rpoB (3861F)	<i>rpoB</i>	Q7A_2329	TGAGATGGAGGTTTGGGCAC
rpoB (4006R)			GCATACCTGCATCCATCCGA
rpoD (10F)	<i>rpoD</i>	Q7A_343	CAGCAATCACGCGTTAAAGA
rpoD(153R)			ACCCAGGTCGCTGAACATAC

**Notes.**\*from GenBank accession number [CP003390.3](https://www.ncbi.nlm.nih.gov/nuclot/CP003390.3).

In N<sub>2</sub>O-amended cultures, N<sub>2</sub>O decrease was apparent from the start and consumption continued for 48 h (Fig. 1A). The N<sub>2</sub>O decrease paralleled strain JAM1 growth with almost complete N<sub>2</sub>O consumption. The NO<sub>3</sub><sup>-</sup>-amended cultures showed complete NO<sub>3</sub><sup>-</sup> consumption and equivalent NO<sub>2</sub><sup>-</sup> accumulation after 24 h (Fig. 1B). However, slower growth than that recorded for the N<sub>2</sub>O cultures was observed. Such growth kinetics could be related to the toxicity of NO<sub>2</sub><sup>-</sup> 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 N<sub>2</sub>O under oxic conditions**

In a previous study, [Mauffrey, Martineau & Villemur \(2015\)](#) demonstrated that strain JAM1 can consume NO<sub>3</sub><sup>-</sup> under oxic growth conditions with equivalent accumulation of NO<sub>2</sub><sup>-</sup>. We tested if this was also the case with N<sub>2</sub>O. Culturing strain JAM1 under oxic conditions with N<sub>2</sub>O (3.5 mg-N vial<sup>-1</sup>) showed a complete N<sub>2</sub>O consumption within 24 h (Fig. 2). Growth patterns illustrated in Fig. 2 were similar between oxic cultures amended with or without either N<sub>2</sub>O or NO<sub>3</sub><sup>-</sup>. In the presence of O<sub>2</sub>, cultures reached higher (4–5 times) biomass concentration than the anoxic cultures.



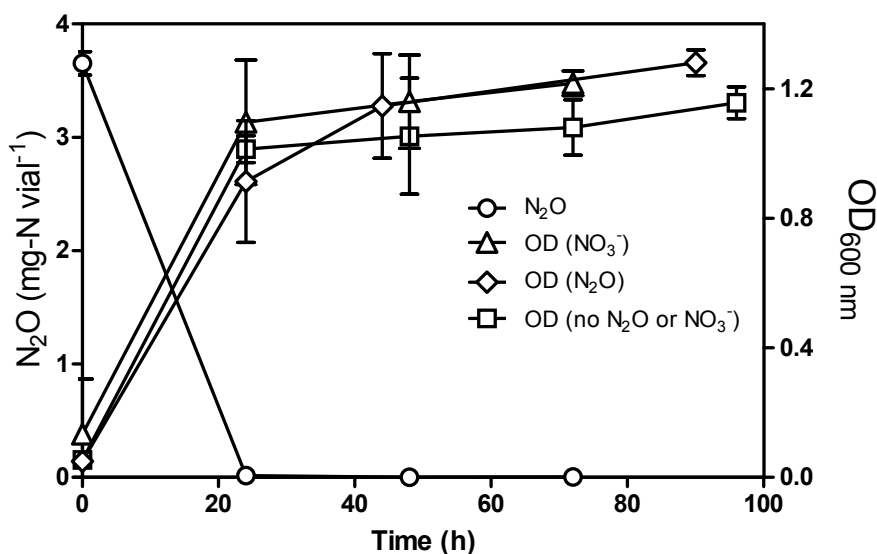
**Figure 1** *Methylophaga nitratireducentescens* JAM1 growth with N<sub>2</sub>O or NO<sub>3</sub><sup>-</sup> as an electron acceptor. Strain JAM1 was cultured with 36.4 mg-N vial<sup>-1</sup> N<sub>2</sub>O (A) or 18.2 mg-N vial<sup>-1</sup> NO<sub>3</sub><sup>-</sup> (B) under anoxic conditions. N<sub>2</sub>O, NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> concentrations and growth were measured over different time intervals. Control (A): N<sub>2</sub>O 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).

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### N<sub>2</sub>O production in NO<sub>3</sub><sup>-</sup>-amended cultures

During the first assays to test the capacity of strain JAM1 to reduce N<sub>2</sub>O under anoxic conditions, cultures were performed with N<sub>2</sub>O (3.5 mg-N vial<sup>-1</sup>) but with the addition of NO<sub>3</sub><sup>-</sup> (20 mg-N vial<sup>-1</sup>) to make sure that growth would occur. Although N<sub>2</sub>O was completely consumed within 24 h, a net production of N<sub>2</sub>O was observed after 48 h. To further investigate this observation, strain JAM1 was cultured under anoxic conditions with NO<sub>3</sub><sup>-</sup>, and NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup> and N<sub>2</sub>O were measured (Fig. 3A). Complete NO<sub>3</sub><sup>-</sup> reduction



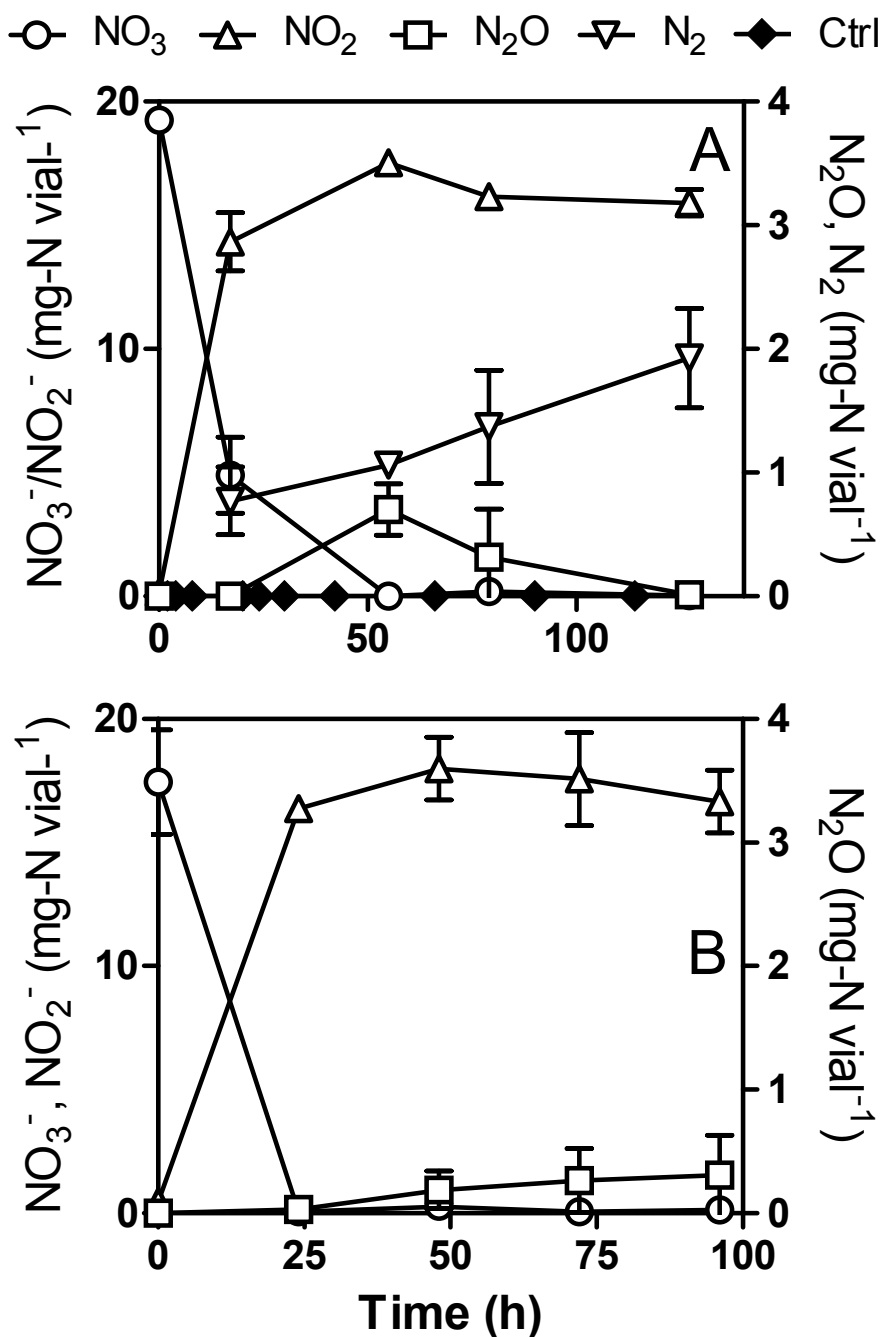


**Figure 2** N<sub>2</sub>O consumption by *Methylophaga nitritireducens* JAM1 under oxic conditions. Strain JAM1 was cultured with 3.5 mg-N vial<sup>-1</sup> N<sub>2</sub>O, with 22 mg-N vial<sup>-1</sup> NO<sub>3</sub><sup>-</sup> or without N<sub>2</sub>O and NO<sub>3</sub><sup>-</sup>, and under oxic conditions. N<sub>2</sub>O concentration was measured in N<sub>2</sub>O-amended cultures only. Growth were measured over different time intervals. Data represent mean values ± SD ( $n = 3$ ).

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( $19.3 \pm 0.3$  mg-N vial<sup>-1</sup>) was performed within 55 h. The NO<sub>2</sub><sup>-</sup> level reached  $17.5 \pm 0.2$  mg-N vial<sup>-1</sup> over this period and decreased slowly to  $15.9 \pm 0.5$  mg-N vial<sup>-1</sup>. N<sub>2</sub>O production initiated when NO<sub>3</sub><sup>-</sup> was nearly reduced and reached  $0.70 \pm 0.21$  mg-N vial<sup>-1</sup> after 55 h of incubation (Fig. 3A). N<sub>2</sub>O was completely reduced after 127 h. In parallel, for cultures in which the headspace was flushed with argon, N<sub>2</sub> production was also measured. The corresponding results show an increase of N<sub>2</sub> in the headspace (Fig. 3A) by  $1.14 \pm 0.54$  mg-N vial<sup>-1</sup> after 127 h, which represent  $6.0 \pm 2.9\%$  of the N input. As the *Methylophaga* 1403 medium contains ferrous chloride ( $216 \mu\text{mole vial}^{-1}$ ), N<sub>2</sub>O production could originate from the abiotic reaction between NO<sub>2</sub><sup>-</sup> that accumulated in the cultures and the ferrous ion (Klueglein et al., 2014). An abiotic control was performed with  $18.2$  mg-N vial<sup>-1</sup> NO<sub>2</sub><sup>-</sup>. N<sub>2</sub>O was detected in the abiotic control after 20 h and reached  $0.00172 \pm 0.00012$  mg-N vial<sup>-1</sup> after 114 h, which is 407 times lower than the N<sub>2</sub>O concentration measured in the anoxic cultures. This results showed that the abiotic reaction generated negligible amount of N<sub>2</sub>O.

Under oxic conditions, NO<sub>3</sub><sup>-</sup> reduction ( $17.4 \pm 2.1$  mg-N vial<sup>-1</sup>) was complete after 24 h with equivalent NO<sub>2</sub><sup>-</sup> accumulation ( $17.1 \pm 1.3$  mg-N vial<sup>-1</sup>). N<sub>2</sub>O production started after complete NO<sub>3</sub><sup>-</sup> reduction (Fig. 3B) and increased to reach  $0.31 \pm 0.32$  mg-N vial<sup>-1</sup> after 96 h of incubation (1.7% of N input). Unlike trends observed for the anoxic cultures, N<sub>2</sub>O concentration did not decrease in the oxic cultures. N<sub>2</sub>O production and consumption could have reached an equilibrium and loss of nitrogen would occur by N<sub>2</sub> production.



**Figure 3**  $\text{N}_2\text{O}$  production by *Methylophaga nitratreducentescens* JAM1. Strain JAM1 was cultured under anoxic (A) or oxic (B) conditions with  $\text{NO}_3^-$  (22 mg-N vial $^{-1}$ ).  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$  concentrations were measured over different time intervals.  $\text{N}_2$  concentration was measured in anoxic cultures that were flushed with argon. Ctrl: Abiotic control with 18.2 mg-N vial $^{-1}$   $\text{NO}_2^-$ . Data represent mean values  $\pm$  SD ( $n=3$ ).

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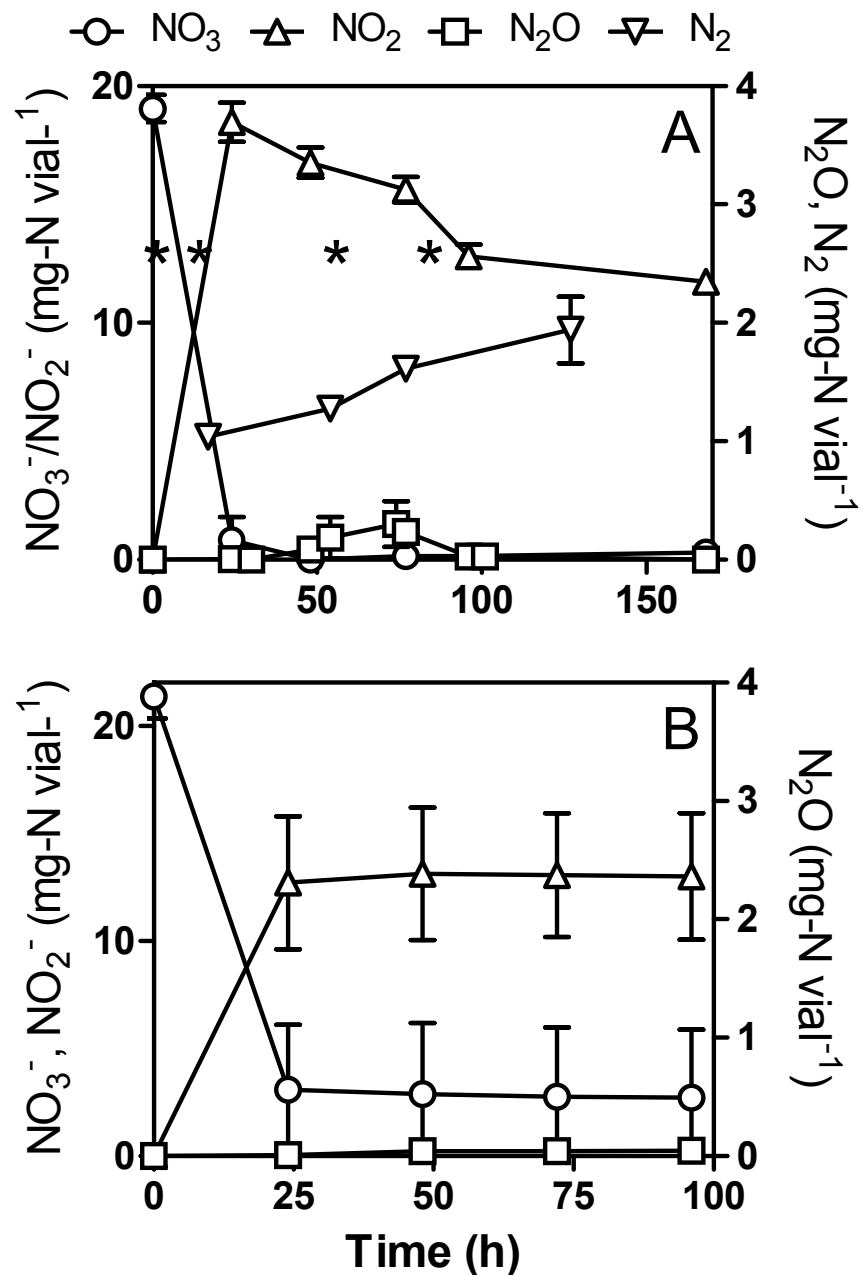
### $\text{NO}_3^-$ , $\text{NO}_2^-$ and $\text{N}_2\text{O}$ dynamics in $\text{NH}_4\text{Cl}$ -free cultures

The original 1403 medium recommended by the ATCC for culturing *Methylophaga* spp. contains 20.9 mg-N vial<sup>-1</sup>  $\text{NH}_4\text{Cl}$  and 0.1 mg-N vial<sup>-1</sup> ferric ammonium citrate (see ‘Material and Methods’). Based on the deduced nitrogen metabolic pathways from strain JAM1 genome (Fig. S1), N-assimilation into the biomass should proceed directly from  $\text{NH}_4^+$  and minimal  $\text{NO}_3^-$  reduction to  $\text{NH}_4^+$  would be occurring. For the next set of experiments, we aimed to determine the effect of removing  $\text{NH}_4\text{Cl}$ , which provides most of the  $\text{NH}_4^+$  (99.5%), on the dynamics of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$ . We hypothesized that forcing strain JAM1 to reroute some  $\text{NO}_3^-$  for N assimilation would affect denitrification and thus growth rates. Strain JAM1 was cultured with ca. 20 mg-N vial<sup>-1</sup>  $\text{NO}_3^-$  under anoxic or oxic conditions in  $\text{NH}_4\text{Cl}$ -free medium (Fig. 4). Growth pattern observed under anoxic conditions was similar between the regular and  $\text{NH}_4\text{Cl}$ -free cultures, as also the growth pattern under oxic conditions between the regular and  $\text{NH}_4\text{Cl}$ -free cultures.

Under anoxic  $\text{NH}_4\text{Cl}$ -free conditions, full  $\text{NO}_3^-$  reduction ( $19.1 \pm 0.6$  mg-N vial<sup>-1</sup>) occurred within 48 h (Fig. 4A). The  $\text{N}_2\text{O}$  profile found was similar to that observed in regular cultures (Fig. 3A), though lower  $\text{N}_2\text{O}$  concentrations were detected during the accumulating phase. The  $\text{NO}_2^-$  level reached  $18.5 \pm 0.8$  mg-N vial<sup>-1</sup> after 24 h and then slowly decreased to  $12.8 \pm 0.5$  mg-N vial<sup>-1</sup> after 96 h. Cultures flushed with argon showed an increase of  $\text{N}_2$  in the headspace (Fig. 4A) by  $0.90 \pm 0.28$  mg-N vial<sup>-1</sup> after 127 h, which is similar to  $\text{N}_2$  production in the regular culture medium. Nitrogen assimilation by the biomass could account for the difference in nitrogen mass balance (28.3%).

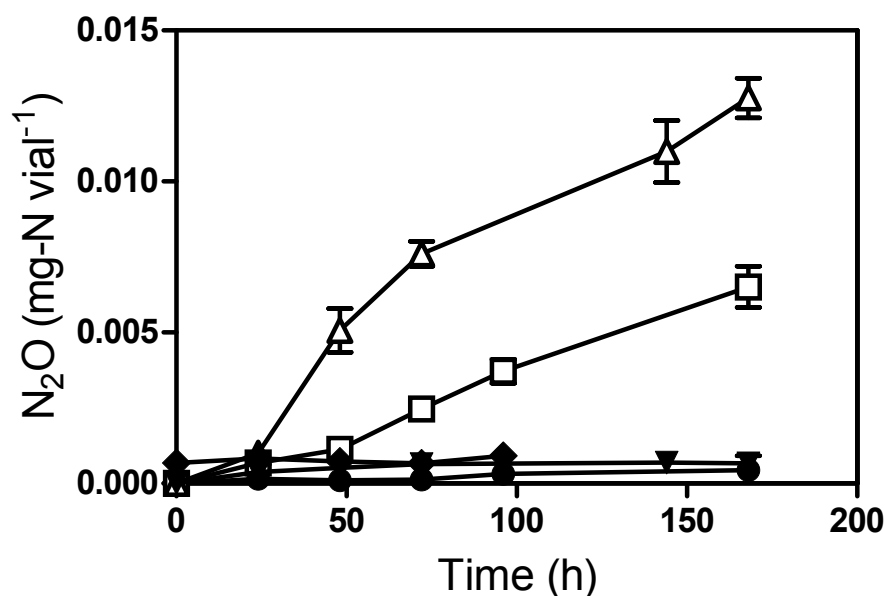
Unlike the cultures in regular medium (Fig. 3B),  $\text{NO}_3^-$  ( $21.3 \pm 1.0$  mg-N vial<sup>-1</sup>) was not completely reduced under oxic  $\text{NH}_4\text{Cl}$ -free conditions, and it stopped after 24 h at  $2.9 \pm 2.7$  mg-N vial<sup>-1</sup> (Fig. 4B). In conjunction with  $\text{NO}_3^-$  reduction,  $\text{NO}_2^-$  levels stopped accumulating at  $13.0 \pm 2.6$  mg-N vial<sup>-1</sup> after 24 h.  $\text{N}_2\text{O}$  was observed after 48 h of incubation (Fig. 4B), after which it slowly accumulated and reached a concentration of  $0.043 \pm 0.048$  mg-N vial<sup>-1</sup>. This level is seven times lower than that of the regular culture medium (Fig. 3B).

To assess whether  $\text{N}_2\text{O}$  could have been generated through  $\text{NH}_4^+$ , strain JAM1 was cultured under anoxic conditions with 22 mg-N vial<sup>-1</sup>  $\text{NO}_3^-$ , 20.7 mg-N vial<sup>-1</sup>  $^{15}\text{NH}_4^+$ , and acetylene to prevent the reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$ . If  $\text{NH}_4^+$  is involved in  $\text{N}_2\text{O}$  production, high proportion of labelled  $\text{N}_2\text{O}$  is expected. If  $\text{NH}_4^+$  is not involved in  $\text{N}_2\text{O}$  production, we expected the production of labeled  $\text{N}_2\text{O}$  to be derived from  $^{15}\text{NO}_3^-$  naturally present in  $\text{NaNO}_3^-$  at a natural  $^{15}\text{N}/^{14}\text{N}$  isotopic ratio of 0.0036765. In the  $^{15}\text{NH}_4^+$ -amended cultures, the  $^{45}[\text{N}_2\text{O}]/^{44}[\text{N}_2\text{O}]$  and  $^{46}[\text{N}_2\text{O}]/^{44}[\text{N}_2\text{O}]$  ratios measured were 0.008 and 0.0165, respectively, with an  $^{15}\text{N}/^{14}\text{N}$  isotopic ratio of 0.020418. As a control, strain JAM1 cultured under anoxic conditions with  $^{15}\text{NO}_3^-$  in  $\text{NH}_4\text{Cl}$ -free medium with acetylene showed, as was expected, all  $\text{N}_2\text{O}$  recovered in  $^{46}[\text{N}_2\text{O}]$ . Because low  $^{15}\text{N}/^{14}\text{N}$  isotopic ratio were found in the  $^{15}\text{NH}_4^+$ -amended cultures, our results suggest that  $\text{N}_2\text{O}$  do not proceed through  $\text{NH}_4^+$ .



**Figure 4** NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup> and N<sub>2</sub>O dynamics by *Methylophaga nitratireducentiscrescens* JAM1 in NH<sub>4</sub>Cl-free cultures. Strain JAM1 was cultured under anoxic (A) or oxic (B) conditions with NO<sub>3</sub><sup>-</sup> (22 mg-N vial<sup>-1</sup>) in NH<sub>4</sub>Cl-free 1403 medium. NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup> and N<sub>2</sub>O concentrations were measured over different time intervals. N<sub>2</sub> concentration was measured in anoxic cultures that were flushed with argon. The results are derived from triplicate cultures. In (A) asterisks denote the sampling times used for RNA extraction (see Fig. 6). Data represent mean values ± SD (*n* = 3).

Full-size DOI: 10.7717/peerj.4098/fig-4



**Figure 5** Reduction of NO to N<sub>2</sub>O by *Methylophaga nitratireducens* JAM1. Strain JAM1 was cultured under oxic conditions without NO<sub>3</sub><sup>-</sup> and with 2 mM (square), with 5 mM (triangle), or with no (circle) sodium nitroprusside (SNP). N<sub>2</sub>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 ( $n = 3$ ).

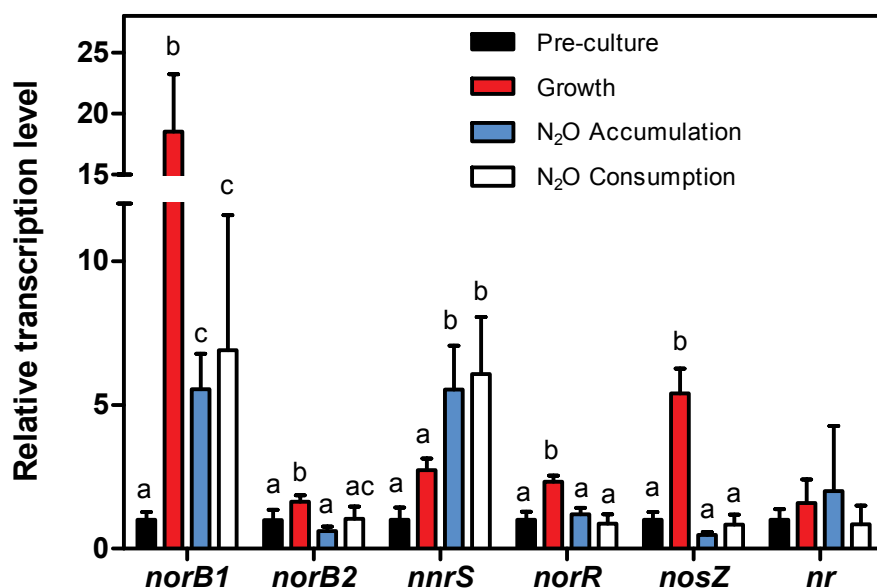
Full-size DOI: [10.7717/peerj.4098/fig-5](https://doi.org/10.7717/peerj.4098/fig-5)

### NO reduction by *M. nitratireducens* JAM1

To verify NO reduction by strain JAM1, N<sub>2</sub>O generation was monitored in cultures without NO<sub>3</sub><sup>-</sup> and supplemented with sodium nitroprusside hypochloride (SNP) used as an NO donor (Moore et al., 2004). Because N<sub>2</sub>O is quickly reduced under anoxic conditions but accumulates under oxic conditions, these assays were performed under oxic conditions (Fig. 5). N<sub>2</sub>O started to accumulate in both 2 mM and 5 mM SNP-supplemented media after 24 h of incubation, reaching  $7.9 \pm 0.5 \mu\text{g-N vial}^{-1}$  and  $14.5 \pm 0.4 \mu\text{g-N vial}^{-1}$ , respectively, after 168 h. No N<sub>2</sub>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.

### Role of Nar systems in NO/N<sub>2</sub>O production

In the absence of NirK or NirS, N<sub>2</sub>O could have been generated via NO by the Nar system (see ‘Discussion’). We used the JAM1  $\Delta narG1 narG2$  double mutant, which lacks functional Nar-type nitrate reductases and which cannot grow under anoxic conditions (Mauffrey, Martineau & Villemur, 2015). Strain JAM1 and the JAM1  $\Delta narG1 narG2$  were cultured with  $16.8 \text{ mg-N vial}^{-1}$  NO<sub>3</sub><sup>-</sup> under oxic conditions. The growth of strain JAM1 and the mutant was similar (Mauffrey, Martineau & Villemur, 2015). After 96 h of incubation, strain JAM1 completely reduced NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup> and produced  $0.14 \text{ mg-N vial}^{-1}$  of N<sub>2</sub>O (Table 2). As



**Figure 6** Relative transcript levels of *cnorB1*, *cnorB2*, *nnrS*, *nosZ*, *norR* and the assimilatory nitrate reductase (*nr*). Strain JAM1 was cultured under anoxic conditions in  $\text{NH}_4\text{Cl}$ -free 1403 medium with  $22 \text{ mg-N vial}^{-1} \text{NO}_3^-$ . Growth patterns were similar to those shown in Fig. 1B under the same conditions with regular 1403 medium. Samples were drawn from the pre-cultures (2–3 day old oxic cultures with no  $\text{NO}_3^-$ ) and during the growth phase ( $\text{NO}_3^-$  reduction),  $\text{N}_2\text{O}$  accumulation phase, and  $\text{N}_2\text{O}$  consumption phase (see Fig. 4A), from which total RNA was extracted for RT-qPCR assays. Changes in the levels of *cnorB1*, *cnorB2*, *nnrS*, *nosZ*, *norR* and *nr* transcripts were calculated relative to their expression during the pre-culture phase (set to one, black column). One-way ANOVA tests with Tukey *post hoc* tests were performed within each phase. Columns represented by different letters are significantly different ( $P < 0.05$ ). Data represent mean values  $\pm$  SD.

Full-size DOI: 10.7717/peerj.4098/fig-6

**Table 2** Production of  $\text{N}_2\text{O}$  by strain JAM1 and the JAM1  $\Delta narG1narG2$  double mutant. Concentrations of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$  were measured after 96 h ( $\text{OD}_{600\text{nm}} \sim 1.2$ ) of incubation in strain JAM1 and JAM1  $\Delta narG1narG2$  cultured under oxic conditions with (A)  $16.8 \text{ mg-N vial}^{-1} \text{NO}_3^-$  added at T0h or (B)  $4.7 \text{ mg-N vial}^{-1} \text{NO}_2^-$  added at T24h. The results are derived from triplicate cultures. Data represent mean values (SD) ( $n = 3$ ).

Strain	Conditions	$\text{NO}_3^-$ (mg-N vial <sup>-1</sup> )	$\text{NO}_2^-$ (mg-N vial <sup>-1</sup> )	$\text{N}_2\text{O}$ (mg-N vial <sup>-1</sup> )
JAM1	A	$0.17 \pm 0.06$	$16.6 \pm 0.7$	$0.14 \pm 0.01$
JAM1 $\Delta narG1narG2$	A	$17.1 \pm 0.1$	$0.22 \pm 0.22$	$0.004 \pm 0.002$
JAM1	B	0	$4.25 \pm 0.09$	$0.11 \pm 0.03$
JAM1 $\Delta narG1narG2$	B	0	$4.87 \pm 0.39$	$0.18 \pm 0.02$

was expected,  $\text{NO}_3^-$  was not reduced, and  $\text{NO}_2^-$  was not produced by JAM1  $\Delta narG1narG2$ . Contrary to the wild type strain, the mutant did not produce  $\text{N}_2\text{O}$ .

The influence of  $\text{NO}_2^-$  was also tested. As the toxicity of  $\text{NO}_2^-$  has been attested from  $0.36 \text{ mM}$  ( $0.2 \text{ mg-N vial}^{-1}$ ) (Auclair *et al.*, 2010), strain JAM1 and the mutant were cultured without  $\text{NO}_3^-$  under oxic conditions to allow for biomass growth. After 24 h,  $4.7 \text{ mg-N vial}^{-1} \text{NO}_2^-$  was added to the cultures and was incubated for another 72 h. Strain JAM1 and the mutant produced  $0.11 \text{ mg-N vial}^{-1}$  and  $0.18 \text{ mg-N vial}^{-1}$  of  $\text{N}_2\text{O}$ , respectively,

reflecting N<sub>2</sub>O concentrations produced by strain JAM1 under oxic conditions with NO<sub>3</sub><sup>-</sup> (Table 2). Our results show that NO<sub>2</sub><sup>-</sup> and not NO<sub>3</sub><sup>-</sup> is directly involved in N<sub>2</sub>O production, and the Nar systems are not involved in N<sub>2</sub>O production via NO.

### Relative expression levels of denitrification genes in *M. nitratireducentis* JAM1

We assessed whether variations in the expression levels of denitrification genes correlate with the N<sub>2</sub>O accumulation and consumption cycles of strain JAM1 cultures. Strain JAM1 was cultured in NH<sub>4</sub>Cl-free medium with 22 mg-N vial<sup>-1</sup> NO<sub>3</sub><sup>-</sup> under anoxic conditions. RNA was extracted from cells harvested over four different phases (Fig. 4): (1) at T0 for the pre-cultures (oxic cultures with no NO<sub>3</sub><sup>-</sup>), (2) during the growth phase with NO<sub>3</sub><sup>-</sup> reduction and no N<sub>2</sub>O accumulation, (3) during the N<sub>2</sub>O accumulation phase, and (4) during the N<sub>2</sub>O consumption phase. The transcript levels of *cnorB1*, *cnorB2* and *nosZ*, which encode the catalytic subunits of the corresponding NO and N<sub>2</sub>O reductases, and *nnrS* and *norR*, were measured by RT-qPCR. *nnrS* and *norR* encode NO-sensitive regulators and were used as an indicator of the presence of NO in the cultures. Because the assimilatory nitrate reductase is involved in the re-routing of NO<sub>3</sub><sup>-</sup> to the biomass, RT-qPCR assays were also performed on the gene encoding this reductase (named here *nr*). The expression levels were calculated relative to the transcript levels measured during the preculture phase (set to one) (Fig. 6).

The relative *cnorB1* transcript levels showed an 18.5-fold increase during the growth phase. *cnorB1* expression was still upregulated during the N<sub>2</sub>O accumulation and consumption phases (5.5 and 6.9-fold increases, respectively). The relative *cnorB2* transcript levels had a 1.6-fold increase during the growth phase. These levels returned nearly to the same levels of those in the preculture phase. Significant increases (5.5- and 6.0-fold) of the relative expression levels of *nnrS* were observed in the N<sub>2</sub>O accumulation and consumption phases. *norR* was upregulated (2.3-fold increase) during the growth phase. The *nosZ* expression levels had a 5.4-fold increase during the growth phase relative to the preculture phase, and decreased to the preculture levels during the N<sub>2</sub>O accumulation phase. No significant difference was observed in the relative transcript levels of the *nr* gene between all phases.

## DISCUSSION

Our results show that *M. nitratireducentis* JAM1 can consume NO and N<sub>2</sub>O via the mechanism of reduction of NO to N<sub>2</sub>O and then to N<sub>2</sub> as predicted by the genome sequence (Fig. S1) (Villeneuve et al., 2013; Mauffrey, Martineau & Villemur, 2015). The N<sub>2</sub>O-amended cultures yielded equivalent biomass results to those of the NO<sub>3</sub><sup>-</sup>-amended cultures as predicted by the respiratory electron transport chains of the denitrification pathway (Simon, 2011). Therefore, in addition of reducing NO<sub>3</sub><sup>-</sup>, strain JAM1 has another respiratory capacity under anoxic conditions by reducing N<sub>2</sub>O for its growth.

Although denitrification is generally an anaerobic process, there are cases where it occurs under oxic conditions (Otani, Hasegawa & Hanaki, 2004). As observed with NO<sub>3</sub><sup>-</sup> reduction, NO and N<sub>2</sub>O reduction can occur under oxic conditions, reinforcing the

lack of a functional oxygen regulation response in strain JAM1 (Mauffrey, Martineau & Villemur, 2015). However, there is little benefit to this consumption, as  $\text{N}_2\text{O}$  or  $\text{NO}_3^-$  amended cultures have a similar growth pattern than the cultures with only oxygen as terminal electron acceptor. The methylamine-utilizing bacterium *Methylothermobacter mobilis* strain JLW8 also showed denitrifying activities under oxic conditions (Kalyuzhnaya et al., 2009). This freshwater bacterium has an incomplete denitrification pathway with gene clusters encoding a periplasmic NapA-type nitrate reductase, NirK and cNor. Although no growth were recorded in methylamine-amended culture supplemented with  $\text{NO}_3^-$  under oxic conditions, growth occurred in methanol-amended cultures with reduction of  $\text{NO}_3^-$  to  $\text{N}_2\text{O}$ . Contrary to *M. nitratireducenticrescens* JAM1, *Methylothermobacter mobilis* JLW8 cannot grow under anoxic conditions with  $\text{NO}_3^-$  (Kalyuzhnaya et al., 2006; Mustakhimov et al., 2013). Denitrification enzymes were showed to be active under anoxic conditions but oxygen is required for strain JLW8 growth (Kalyuzhnaya et al., 2009).

$\text{N}_2\text{O}$  production was observed in  $\text{NO}_3^-$ -amended cultures either under oxic or anoxic conditions when  $\text{NO}_2^-$  was accumulating. This production represented up to 6% of N-input in the anoxic cultures, and  $\text{NO}_2^-$  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  $\text{N}_2\text{O}$  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 (Poole, 2005). Reducing NO is a key step in denitrification and is closely regulated by various sensors and regulators. NnrS and NorR are involved in cell defense against nitrosative stress and are positively regulated by the presence of NO (Stern et al., 2013; Stern et al., 2012; Bartnikas et al., 2002). Therefore, the expression of *nnrS* and *norR* reflects NO concentrations in a medium and was used as a marker of NO presence. The upregulation of the expression of *norR* during the growth phase strongly suggest that NO is produced during this phase. This correlates with higher expressions of both *cnorB*, and *nosZ* during the growth phase, which can be regulated by NO-sensitive regulators such as NorR (Spiro, 2012). *nnrS* is upregulated during the  $\text{N}_2\text{O}$  accumulation and consumption phases, which suggests that NO is still generated during these phases. This upregulation can be linked to the decrease of the relative transcript levels of both *cnorB*, and of *nosZ* and *norR*, but also to the highest level of  $\text{NO}_2^-$  concentrations in the culture medium. It is therefore possible that NO is not reduced quickly enough in the cells by cNorB and strain JAM1 must rely on another mechanism, such as NnrS, to protect itself from NO toxicity. Stern et al. (2013) suggest that NnrS does not remove NO directly, but protects cells against the formation of iron-NO complexes, which are inhibitory to iron-sulfur cluster proteins. Moreover, Vaccaro et al. (2016) proposed that NnrS senses NO and signals to cytoplasmic transcription factors or Fe-S cluster repair proteins.

Other  $\text{NO}_3^-$  respiring bacteria that lack NirK or NirS have been shown to be  $\text{N}_2\text{O}$  producers (Bleakley & Tiedje, 1982; Smith & Zimmerman, 1981; Sun, Vos & Heylen, 2016). For instance, *Bacillus vireti* contains three denitrification reductases (Nar, qCu<sub>A</sub>Nor,  $\text{N}_2\text{OR}$ ) and lacks, like *M. nitratireducenticrescens* JAM1, gene encoding NirK or NirS (Mania et al., 2014). This bacterium also produces NO and  $\text{N}_2\text{O}$  in anaerobic,  $\text{NO}_3^-$ -amended TSB cultures during  $\text{NO}_2^-$  accumulation. NO was shown to originate from chemical



decomposition of  $\text{NO}_2^-$  (Schreiber *et al.*, 2012) and from an unknown biotic reaction. In our study, the abiotic control of the *Methylophaga* 1403 medium amended with  $\text{NO}_3^-$  did not show significant  $\text{N}_2\text{O}$  production. Also, the abiotic reaction between the ferrous ion in the medium and  $\text{NO}_2^-$  generated negligible amount of  $\text{N}_2\text{O}$ . Furthermore, no  $\text{N}_2\text{O}$  was detected in this medium inoculated with autoclaved biomass (Fig. 5). These results rule out abiotic reactions as the source of the total amount of  $\text{N}_2\text{O}$  produced 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 & Cole, 2011). There are supporting evidence that NO is generated in *E. coli* as a side product during  $\text{NO}_2^-$  reduction (i) by the cytoplasmic, NADH-dependent nitrite reductase (NirBD), (ii) by the nitrite reductase NrfAB, and (iii) by NarGHI. Vine, Purewal & Cole (2011) 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. nitratireducentescens* JAM1, the double-knockout mutant JAM1 $\Delta$ *narG1narG2*, which lacks the two dissimilatory  $\text{NO}_3^-$  reductases, was still able to produce  $\text{N}_2\text{O}$  under oxic conditions at the same level of the wild type when  $\text{NO}_2^-$  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). In the latter case, this could be verified by generating a knockout mutant of this gene.

The significance of maintaining an incomplete pathway by *M. nitratireducentescens* JAM1 is unclear and may depend upon the original habitat and environment, here the denitrifying biofilm. While *M. nitratireducentescens* JAM1 serves as an important actor among the microbial community of the marine biofilm in performing optimal denitrifying activities (Labbé *et al.*, 2003; Labbé, Parent & Villemur, 2003), it was thought to participate uniquely in the reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ . It was previously proposed that  $\text{NO}_2^-$  reduction to  $\text{N}_2$  is carried out by *Hyphomicrobium nitrativorans* NL23, the second most represented bacterium in the biofilm (Labbé *et al.*, 2007; Auclair, Parent & Villemur, 2012). Its capacity to reduce NO and  $\text{N}_2\text{O}$  and to grow on  $\text{N}_2\text{O}$  suggests that *M. nitratireducentescens* JAM1 may participate in the reduction of NO and  $\text{N}_2\text{O}$  during denitrification in the biofilm. Although our culture assays were performed with high levels of  $\text{NO}_3^-$  (37 mM), which rarely exceeds a value of 0.7 mM in natural environments (Yeats, 1990), similar levels can be reached in closed-circuit systems like the seawater aquarium tank located in the Montreal Biodome, where  $\text{NO}_3^-$  levels reached up to 14 mM (Parent & Morin, 2000). Rissanen *et al.* (2016) also observed 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  $\text{NO}_3^-$  to  $\text{NO}_2^-$  and, as reported here, by reducing  $\text{N}_2\text{O}$  to  $\text{N}_2$ . It can also generate  $\text{N}_2\text{O}$  via  $\text{NO}$  by an unknown biotic system. Very few marine denitrifying bacteria have been isolated from recirculating marine systems (Borges *et al.*, 2008; Foessel, Drake & Schramm, 2011; Zheng *et al.*, 2012; Zheng *et al.*, 2011). 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.

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The authors declare there are no competing interests.

### Author Contributions

- Florian Mauffrey conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Alexandra Cucaita performed the experiments, analyzed the data.
- Philippe Constant conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Richard Villemur conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

## Data Availability

The following information was supplied regarding data availability:

The raw data was provided as a [Supplemental File](#).

## Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.4098#supplemental-information>.

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