Characterization of denitrifying activity by the alphaproteobacterium, *Sphingomonas wittichii* RW1

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**INTRODUCTION**

Denitrification is the sequential reduction of nitrate (NO\textsubscript{3}{−}) and nitrite (NO\textsubscript{2}{−}) to dinitrogen (N\textsubscript{2}) via the gaseous intermediates, nitric oxide (NO) and nitrous oxide (N\textsubscript{2}O) (Zumft, 1997). Respiratory denitrification is considered an anaerobic energy-generating metabolism; however, many bacteria can denitrify in the presence of O\textsubscript{2} starting with NO\textsubscript{2}{−} or NO\textsubscript{−} and terminating with N\textsubscript{2}O due to inhibition or absence of nitrous oxide reductase (Hendriks et al., 2000; Cantera and Stein, 2007). The complete genome sequence of *S. wittichii* RW1 revealed the presence of a single circular chromosome and two megaplasmids (Miller et al., 2010). Although not known to denitrify, *S. wittichii* RW1 encodes in its genome a copper-containing nitrite reductase (nirK) and plasmid-encoded nitric oxide dioxygenase (hmp\textsubscript{p}; Swit_5200) with predicted function as the terminal member of a four-gene cluster with a NO-responsive NsrR regulator encoded upstream (Swit_1789-93). This gene cluster shares structural and sequence homology to ammonia- and nitrite-oxidizing bacteria in the *Nitrosomonas* and *Nitrobacter* genera, respectively (Cantera and Stein, 2007). *S. wittichii* RW1 also encodes a circular chromosome (Swit_4614) and plasmid copy (Swit_5200) of quinol-linked nitric oxide reductase (norZ). *NorZ* is often expressed in non-denitrifying pathogenic bacteria for NO detoxification (Hendriks et al., 2000), but can also act alongside the terminal oxidase in the aerobic respiratory chain for energy conservation (Chen and Strous, 2013). The plasmid-encoded *norZ\textsubscript{p}* (Swit_5200) is the terminal member of a four-gene cluster; the first member of which encodes a nitric oxide dioxygenase (hmp\textsubscript{p}; Swit_5203) with predicted function in NO oxidation to NO\textsubscript{2}{−} or NO reduction to N\textsubscript{2}O depending on O\textsubscript{2} concentration (Bonamore and Boffi, 2008). Nitric oxide dioxygenases are present in both denitrifying and non-denitrifying microorganisms to combat nitrosative and oxidative stress, which is critical for bacterial survival in environments with high NO concentrations (Müller et al., 2010).

*Sphingomonas wittichii* RW1 has no reported denitrifying activity yet encodes nitrite and nitric oxide reductases. The aims of this study were to determine conditions under which *S. wittichii* RW1 consumes nitrite (NO\textsubscript{2}{−}) and produces nitrous oxide (N\textsubscript{2}O), examine expression of putative genes for N-oxide metabolism, and determine the functionality of chromosomal (ch) and plasmid (p) encoded quinol-dependent nitric oxide reductases (*NorZ*). Batch cultures of wildtype (WT) and a norZ\textsubscript{ch} mutant of *S. wittichii* RW1 consumed NO\textsubscript{2}{−} and produced N\textsubscript{2}O during stationary phase. The norZ\textsubscript{ch} mutant produced N\textsubscript{2}O, although at significantly lower levels (c.a. 66–87%) relative to the WT. Rates of N\textsubscript{2}O production were 2–3 times higher in cultures initiated at low relative to atmospheric O\textsubscript{2} per unit biomass, although rates of NO\textsubscript{2}{−} consumption were elevated in cultures initiated with atmospheric O\textsubscript{2} and 1 mM NaNO\textsubscript{2}. Levels of mRNA encoding nitrite reductase (nirK), plasmid-encoded nitric oxide dioxygenase (hmp\textsubscript{p}) and plasmid-encoded nitric oxide reductase (norZ\textsubscript{p}) were significantly higher in the norZ\textsubscript{ch} mutant over a growth curve relative to WT. The presence of NO\textsubscript{2}{−} further increased levels of *nirK* and *hmp\textsubscript{p} mRNA* in both the WT and norZ\textsubscript{ch} mutant; levels of *norZ\textsubscript{p} mRNA* compensated for the loss of *norZ\textsubscript{ch}* expression in the norZ\textsubscript{ch} mutant. Together, the results suggest that *S. wittichii* RW1 denitrifies NO\textsubscript{2}{−} to N\textsubscript{2}O and expresses gene products predicted to detoxify N-oxides. So far, only *S. wittichii* strains within four closely related taxa have been observed to encode both *nirK* and *norZ* genes, indicating a species-specific lateral gene transfer that may be relevant to the niche preference of *S. wittichii*.
stresses (Bonamore and Boffi, 2008; Forrester and Foster, 2012). Although nitric oxide dioxygenases are usually conserved members of the NO-controlled NsrR transcriptional regulon in bacteria (Rodionov et al., 2005), the plasmid-encoded gene cluster in S. wittichii RW1 that includes both NorZ and nitric oxide dioxygenase is preceded by a CDS for the NO-responsive NnrR transcriptional regulator (Swit_5204). Aside from Swit_5203, S. wittichii RW1 encodes three other putative hmp genes, the plasmid-encoded Swit_5299 and the chromosomal Swit_4344 and 3173. A comparison of 51 genome-sequenced sphingomonad strains (encompassing the Sphingomonas, Sphingobium, Novosphingobium, and Sphingopyxis genera) by BLAST searches through the Integrated Microbial Genomes database (http://img.jgi.doe.gov) revealed that only the two strains of S. wittichii (RW1 and DP58) encode the complete nirK gene cluster, whereas eight sphingomonad genomes encode either one or two copies of norZ and 17 genomes encode one or more hmp genes whose translated sequences share >60% protein identity to Swit_5203. Hence, the potential for sphingomonad bacteria to transform nitrogen oxides appears to be fairly restricted.

Previous studies in Neisseria and Synechococcus demonstrated that disruption of norZ expression resulted in increased NO sensitivity, diminished NO consumption and N2O production, and decreased growth under anoxia (Householder et al., 2000; Busch et al., 2002). Interestingly,Ralstonia eutropha H16 also possesses two independent quinol-linked nitric oxide reductases. Deletion of either gene in R. eutropha H16 resulted in no phenotypic change under aerobic or anaerobic growth at the expense of NO or NO2 (Cramm et al., 1997). Therefore, in the present study we tested the hypothesis that the norZ genes in S. wittichii RW1 are similarly isofunctional.

The overarching hypothesis of the present study is that S. wittichii RW1 reduces NO2 to N2O and thus can be classified as a denitrifying strain. The ability of S. wittichii RW1 to denitrify from NO2 was not investigated as the genome of S. wittichii RW1 encodes only the alpha subunit of assimilatory nitrate reductase (Swit_1709) and no features of dissimilatory nitrate reductases. Furthermore, this strain tested negative for reduction of NO2 to NO (Yabuchi et al., 2001). There is no identifiable sequence in the genome with similarity to nitrous oxide reductase; hence, this strain is predicted to denitriify only NO2 to N2O. To provide support for S. wittichii RW1 as a denitrifier, objectives were to: (a) determine whether and when S. wittichii RW1 produces N2O at the expense of NO2; (b) investigate the regulation of putative N-oxide metabolism genes in response to varying NO2, and (c) determine whether the chromosomal- and plasmid-encoded norZ genes in S. wittichii RW1 are isofunctional.

**MATERIALS AND METHODS**

**CULTURE MAINTENANCE**

*Sphingomonas wittichii* RW1 was provided as a gift from Dr. Rolf Halden. Cultures were grown in 5 mL Luria-Bertani Broth (LB) in sterilized 15 mL capped-polystyrene tubes in a rotary shaker (180 r.p.m.) at 28°C. Cultures were periodically streaked and grown on LB agar plates for single colony isolation to maintain culture purity.

**CONSTRUCTION OF norZch MUTANT OF S. WITTICHIU RW1**

The region from bp 203 to 776 of the norZch gene was PCR-amplified from S. wittichii RW1 genomic DNA with primers 203F 5′ aactggaacaggccgatg 3′ and 776R 5′ cgatgctgcctactctcg 3′ to make use of an internal BclI restriction site [ Primer3 Input 0.4.0 software (Rozen and Skaletsky, 2000)]. The amplification product was purified and ligated to the pGEM®-T Vector according to manufacturers’ instructions (Promega Corp., Madison, WI). The ligation mixture was transformed into dam−/dcm− competent *E. coli* cells (New England BioLabs Inc., Ipswich, MA) and transformants were selected via blue-white screening on LB agar plates containing 0.5 mM IPTG, 80 μg/mL X-Gal, and 100 μg/mL ampicillin. Plasmids from positive transformants were purified using Wizard® Plus SV Minipreps DNA Purification System kit (Promega Corp., Madison, WI) and digested with the BclI restriction enzyme (New England BioLabs Inc., Ipswich MA). The digest was run on a 0.8% agarose gel and linearized vector was gel-purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega Corp., Madison, WI).

A gentamycin-resistance cassette (871 bp) was digested from the pUCGM vector (gift from N. Hommes) using the BamHI restriction enzyme (New England BioLabs Inc., Ipswich MA). The digest was gel-purified from a 0.8% agarose gel and ligated to the previously BclI-digested pGEM-T-norZ vector. The ligation mixture was transformed into competent *E. coli* JM109 cells. Transformed cells were plated onto LB agar containing 100 μg/mL ampicillin and 10 μg/mL gentamycin. Positive transformants were verified by PCR and Sanger sequencing using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City USA). Plasmids containing the correct inserts were purified as described above and electroporated into *S. wittichii* RW1 cells using an *E. coli* Pulser™ Transformation Apparatus (BioRad Laboratories, Hercules, CA). Competent *S. wittichii* RW1 cells were prepared by harvesting in exponential phase, washing three times with 20 mL ice-cold and nuclease-free water, washing twice with 2 mL ice-cold 10% glycerol, and resuspended in 10% glycerol to a final volume of 100 μL. Electroporated cells were plated onto LB agar containing 10 μg/mL gentamycin. The norZch mutant strain was checked by PCR using additional primers: 45F 5′ agagacccagcagcagc 3′, 854R 5′ taccgctgtgatattgg 3′, pUCGM173F 5′ tgtcctgccatccagacga 3′, pUCGM514R 5′ gagagcccaacagctct 3′ and pUCGM519F 5′ cattgcctgcagcttt 3′. PCR products were purified and validated by Sanger sequencing. The norZch mutant strain was maintained on LB media with 50 μg/mL gentamycin.

**GROWTH EXPERIMENTS**

*S. wittichii* RW1 wildtype (WT) and norZch mutant cells from exponentially growing cultures were inoculated into LB media (500 μL into 100 mL) containing 0, 0.3, or 1 mM NaNO2 into glass serum bottles (160 mL), which were then crimp-sealed with rubber septa and aluminum seals. Incubations of norZch mutant cells contained 50 μg/mL gentamycin. Triplicate incubations of each control condition included the same concentrations of NaNO2 plus: (1) heat-inactivated cells, (2) no cells, or (3) live cells in bottles purged of O2 by sparging the medium with N2. All control incubations were treated identically to the
experimental incubations to determine whether chemical decomposition of NO$_2^-$ contributed to NO$_3^-$ loss or N$_2$O accumulation. Gas headspace (60 mL) was either left unchanged (atmospheric O$_2$) or, for WT cells, sparged with N$_2$ and injected with pure O$_2$ prior to inoculation (ca. 3% O$_2$ in gas headspace as validated by gas chromatography; GC-TCD, Shimadzu, Kyoto, Japan; Molecular Sieve 6A column, Alltech, Deerfield IL). Experimental and control bottles were incubated in a rotary shaker (180 r.p.m.) at 28°C. Starting at $t = 0$ h, 2 mL samples were extracted every 4 h using a sterile 1 mL needle and syringe. Growth was determined by measuring OD 600 nm using a Spectronic 20 Genesys spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA). Cells were immediately treated with 500 μL RNNaprotect™ Bacteria Reagent (Qiagen, Valencia, CA), and kept at −80°C. Experiments consisted of five independent trials performed on different days for both strains and under every condition.

NUCLEIC ACID EXTRACTION

Genomic DNA was isolated using the Wizard® SV Genomic DNA Purification System kit (Promega Corp., Madison, WI). Total RNA was extracted using the Aurum™ Total RNA Mini kit (Bio-Rad Laboratories, Hercules, CA). Nucleic acid concentration was determined using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). DNA and RNA samples were kept at −20 and −80°C, respectively.

DOT-BLOT HYBRIDIZATION

Gene-specific primers were designed from CDS's of selected genes from the S. wittichii RW1 genome sequence (Genbank accession: CP000699 to CP000701) using Primer3 Input 0.4.0 software (Rozen and Skaltsky, 2000) (Table 1). PCR reactions included standard reagents for Taq polymerase and genomic DNA as template in 25 μL reactions (Sambrook and Russell, 2001). Thermal cycler (iCycler, BioRad, Hercules, CA) amplification conditions were: 95°C for 5 min, 30 cycles of 95°C for 40 s, 55°C for 40 s and 72°C for 50 s, with an additional extension cycle of 72°C for 7 min. PCR products were checked by agarose gel (1%) to verify single products of appropriate size. Amplification products were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega Corp., Madison, WI). Amplification products were labeled using the Prime-a-Gene labeling system (Promega Corp., Madison, WI) with [α- 32P]-dCTP (3000 Ci mmol$^{-1}$; PerkinElmer Inc., Waltham, MA) and random hexamers. The dynamic range of detection for each probe was tested using a concentration series of specific mRNA from 0.1 to 3 μg from control incubations (0 mM NaNO$_2$). The $r^2$ values for the slope of hybridization intensity/μg mRNA was from 0.94 to 1.0 for all probes.

Two μg total RNA from each sample was blotted onto a Zeta-Probe® GT nylon membrane (Bio-Rad Laboratories, Hercules, CA) using a Minifold® microsample filtration manifold (Dot-Blot System, Schleicher & Schuell, Keene, NH) following the Zeta-Probe® protocol. Membranes were allowed to dry overnight and UV-crosslinked (FB-UVXL-1000, Fisher Scientific, Pittsburgh, PA). Prehybridization, hybridization, and washing of Zeta-Probe® nylon membranes were done according to manufacturer’s instructions at 30°C. To allow re-probing, membranes were stripped of radioactivity by washing twice in 1.0x SSC/0.5% SDS solution at 95−100°C for 20 min. All blots were hybridized to gene-specific probes to normalize hybridization signals to the 16S rRNA pool. Hybridization intensity was analyzed using a Typhoon Phosphorimager and Imagequant software (Amersham, Piscataway, New Jersey).

DATA ANALYSIS

Background and signal from non-specific binding was subtracted, after which the relative hybridization intensity of specific probes was normalized by dividing gene-specific signal by signal from 16S rRNA probe hybridizations. The fold difference in levels of mRNA for each gene and time point was determined by dividing hybridization intensities from dot blots of RNA extracted from NO$_2^-$ amended by those from unamended cultures. Student’s t-test ($p < 0.05$) was performed to determine significant differences between treatments.

ANALYTICAL MEASUREMENTS

Nitrite and ammonium were measured colorimetrically using standard methods (Clesceri et al., 1998). Nitrate was measured using a Standard Range Lab Nitrate Test kit (NECi, Lake Linden, Michigan). O$_2$ and N$_2$O were measured from the gas headspace of sample bottles by GC-TCD (Shimadzu, Kyoto Japan; Molecise 5A and Hayesep Q columns, Alltech, Deerfield IL). Concentrations were determined by comparing to standard curves generated for each reagent and gas within the limits of detection.

Table 1: Primers used to generate probes for RNA dot-blot hybridizations.

| Locus Tag | Coding sequence ID | Enzyme commission number | F primer | R primer | Amplicon |
|-----------|--------------------|--------------------------|----------|----------|----------|
| Swit_1793 | NO-forming nitrite reductase (nirK) | EC:1.7.2.1 | ctgaccgcgaaggaagatgc | catgctgcatagcatagc | 742 bp |
| Swit_5203 (p) | Nitric oxide dioxygenase (hmp) | EC:1.14.12.17 | tcaagtcgtcctcactcg | attgctcctcctcactc | 210 bp |
| Swit_R0031 | 16S rRNA | untranslated | gtaaagcctgagggcatga | tttacgcgtcaggtgac | 1159 bp |
| Swit_5200 (p) | Nitric oxide reductase (norZ) | EC:1.7.5.2 | ccaacgccaatactcactc | cgacagtctcaggtgac | 513 bp |
| Swit_4614 (ch) | Nitric oxide reductase (norZ) | EC:1.7.5.2 | gtttgcccccagagaattgag | gccagacgtctcaggtgac | 703 bp |

*Significant difference between atmospheric and reduced O$_2$ for wildtype (WT) cultures incubated with the same concentration of NaNO$_2$.

*Swit_4614 and Swit_5200 share 54% amino acid sequence identity based on BLAST.

Primers were designed using Primer 3 Input 0.4.0 software (Rozen and Skaltsky, 2000) against the full CDS’s from the complete genome sequence of Sphingomonas wittichii RW1, which includes a single circular chromosome and two megaplasmids (Genbank accession: CP000699–CP000701).
RESULTS

EFFECT OF O2 AND NO2 ON GROWTH OF WT AND norZch MUTANT STRAINS OF S. WITTICHII RW1

S. wittichii RW1 is an aerobic heterotrophic bacterium; hence, the doubling time (calculated from 12 to 20h growth) and final yields of non-mutagenized cells were significantly faster and higher, respectively, for cultures initiated under atmospheric compared to reduced (ca. 3%) O2 levels (Figure 1 and Table 2). Doubling times of the norZch mutant were significantly shorter than those of the WT during exponential growth; thus, even though the norZch mutant exhibited a longer lag phase, the cell density of the cultures were equivalent in stationary phase (Figure 1 and Table 2). The addition of NaN2O to cultures initiated at atmospheric O2 only significantly increased the doubling time of WT cultures, but significantly reduced the final yields of both WT and norZch mutant cultures (Table 2).

CONSUMPTION OF NO2 AND PRODUCTION OF N2O

Cultures of WT and norZch mutant S. wittichii RW1 were incubated in the presence of NaN2O to assess whether expression of norZch was required for aerobic denitrifying activity. Amounts of remaining NO2−, remaining O2, and headspace N2O levels were compared over stationary phase (Table 3). NO2− was consumed nearly to completion in both WT and norZch mutant cultures by 96 h incubation. Neither WT nor norZch mutant cultures consumed O2 to complete anoxia and headspace O2 levels remained largely stable following 72 h incubation, even with continuous shaking at 180 rpm. N2O was measurable in the gas headspace starting after 48 h of incubation and continued to accumulate proportionally with the amount of added NaN2O (Table 3). The norZch mutant cultures produced significantly less N2O than the WT cultures (66–87% of WT levels) at both NO2− concentrations.

Nitrate production was not observed, which would be an expected aerobic activity of Hmp. NH4+ concentrations also did not vary between treatment groups, which would be expected if S. wittichii RW1 reduced NO2 directly to NH3 and allowed its accumulation prior to assimilation (data not shown). N2 was not measured. Control incubations containing heat-inactivated cells, no cells, or live cells inoculated into bottles sparged of O2 with N2 gas showed no consumption of NO2 and no production of N2O.

We next tested whether lower oxygen had an effect on the rates of NO2− or O2 consumption or N2O production in non-mutated S. wittichii RW1. To address this question, S. wittichii RW1 cultures were inoculated with 0, 0.3, or 1 mM NaN2O at either atmospheric or reduced (ca. 3%) O2 levels. Cultures initiated at atmospheric O2 and 1 mM NaN2O consumed O2 and NO2− significantly faster than cultures initiated at reduced O2 and 1 mM NaN2O, yet the rate of N2O production was 2–3 times faster for cultures initiated at reduced relative to atmospheric O2 levels (Table 4). The N2O-N measured in the gas headspace of the cultures was orders of magnitude lower than the amount of NO2− consumed per unit biomass (i.e., nmol N2O produced from μmol NO2− consumed). Even though N2O is highly soluble, the vast difference between NO2− consumption and N2O production implies conversion of NO2− into a product other than N2O; however, NO3− was undetectable and NH4+ levels did not vary in any culture at any time point (data not shown).

Table 2 | Growth of WT and norZch, mutant strains of S. wittichii RW1 at variable NaN2O and O2 concentrations.

| Variable in growth condition | Doubling time (h) | Yield (OD600 nm) |
|-----------------------------|------------------|-----------------|
|                            | WT               | norZch          | WT               | norZch          |
| Atmospheric O2, no NaN2O    | 5.3abc           | 4.4c            | 0.98ab           | 0.98b           |
| Reduced O2, no NaN2O        | 8.2a             | N.D.            | 0.25a            | N.D.            |
| Atmospheric O2, 0.3 mM NaN2O| 5.6abc           | 4.4c            | 0.90ab           | 0.91b           |
| Reduced O2, 0.3 mM NaN2O    | 8.5a             | N.D.            | 0.25a            | N.D.            |
| Atmospheric O2, 1.0 mM NaN2O| 5.5abc           | 4.4c            | 0.82ab           | 0.84b           |
| Reduced O2, 1.0 mM NaN2O    | 8.4a             | N.D.            | 0.22ab           | N.D.            |

Doubling times were calculated during exponential growth from 12 to 20h. Final cell yields were reported at 32 and 20h growth when initiated at atmospheric (ca. 22%) and reduced (ca. 3%) O2 levels, respectively. Values represent averages from 5 independent experiments for each incubation condition.

aSignificant difference between atmospheric and reduced O2 for wildtype (WT) cultures only.
bSignificant difference between WT or norZch mutant cultures incubated with NaN2O relative to unamended controls.
cSignificant difference between WT and norZch mutant cultures grown under identical conditions. N.D., not determined.
Table 3 | Consumption of nitrite and oxygen and production of nitrous oxide by wild-type and norZch mutant cultures of S. wittichii RW1 initiated at atmospheric oxygen headspace and with 0.3 or 1 mM NaNO2.

| Time (h) | NO2− remaining (mM) | %O2 remaining in the headspace | N2O produced (nmolOD−1) |
|---------|----------------------|-------------------------------|-------------------------|
|         | WT       | norZch | WT       | norZch | WT       | norZch | WT       | norZch |
| 0.3 mM  | 1.0 mM   |        | 0.3 mM  | 1.0 mM | 0.3 mM  | 1.0 mM | 0.3 mM  | 1.0 mM |
| 48      | 0.24ab   | 0.78a  | 0.27ab  | 0.79a  | 7.24ab  | 7.62a  | 7.82ab  | 7.55a  |
| 72      | 0.01*    | 0.24ab | 0.04a   | 0.33ab | 4.50b   | 4.65a  | 4.71b   | 4.63a  |
| 96      | 0.00     | 0.00   | 0.00    | 0.02   | 4.19p   | 4.12a  | 4.42b   | 4.45a  |
| 120     | 0.00     | 0.00   | 0.00    | 0.00   | 4.31    | 3.89a  | 4.48    | 4.33a  |

Values represent averages from 5 experiments initiated on different cultures and on different days. N2O amounts were normalized to OD of each culture at each time point. Statistically significant differences between treatments were determined by Student’s t-test at p < 0.05 and are designated as follows:

*Significant difference between 0.3 and 1 mM NaNO2 treatment groups of wildtype (WT) or norZch mutant cultures of S. wittichii RW1 at each time point.

EXPRESSIVE LEVELS OF PUTATIVE AEROBIC DENITRIFICATION GENES

Levels of specific mRNAs encoding nirK, hmp, norZch, and norZp, as normalized to levels of 16S rRNA, were compared between WT and norZch mutant S. wittichii RW1 from mid-log and into stationary phase (24–66 h). This period of time covers the interval over which consumption of NO2− and production of N2O is measurably active. Expression of norZp substituted for norZch in the norZch mutant strain and the levels of respective norZ transcript remained relatively high in both cell lines over time (Figure 2). Levels of nirK and hmp, transcript levels increased between 24 and 66 h in WT cells, both transcript levels remained relatively high in the norZch mutant over the full time course (Figure 2).

Finally, the effect of NO2− on transcript levels was examined in norZch mutant and WT cultures, and for non-mutated cultures initiated under atmospheric and reduced O2 levels. Each hybridization signal was normalized to that for 16S rRNA, after which the ratio of hybridization intensity between NaNO2-treated and untreated sample was calculated for every culture and each transcript pool. There was no significant effect of NO2− treatment on any transcript level for any culture until late log phase (i.e., 24 h for WT and norZch mutant cultures and 20 h for WT cultures initiated at reduced O2). At least a two-fold increase between NaNO2-treated and untreated cells was considered a significant effect; thus, nirK and hmp were positively responsive to NaNO2 in both WT and norZch mutant cultures (Table 5). For both norZ genes, only transcription levels of norZp in the norZch mutant were responsive to 1 mM NaNO2 treatment.

DISCUSSION

Sphingomonas wittichii RW1 denitrifies NO2− to N2O

Rapid consumption of NO2− by S. wittichii RW1 occurred only once the cells reached stationary phase (Figure 3), suggesting that S. wittichii RW1 performs this process for detoxification or maintenance metabolism rather than for generating proton motive force for cellular growth. During growth under reduced O2, an increased rate of NO2− conversion to N2O (Table 4) relative to cultures initiated at atmospheric O2 implies that O2 limitation must be reached for denitrifying activity to commence as would be commonly expected (Zumft, 1997). It is interesting that a faster rate of NO2− consumption occurred for cultures initiated at atmospheric than at reduced O2 in the presence of 1 mM NaNO2 as this implies an additional process from denitrification for NO2− loss. Although a substantial quantity of NO2− consumed by S. wittichii RW1 was converted to N2O, there was a considerable pool of transformed NO2− that could not be accounted for in NH4+.
or NO$_3^\text{-}$ pools. There is no homolog for nitrous oxide reductase (nosZ) in the genome sequence of S. wittichii RW1; hence, denitrification to N$_2$ is unlikely. Sphingomonads are also not known to produce N-storage polymers, but S. wittichii RW1 does encode an assimilatory nitrite reductase (nirBD; Swit_1707-8). Thus, the fate of the remaining NO$_2^\text{-}$N remains unknown.

**GENES FOR NITROGEN OXIDE TRANSFORMATIONS ARE EXPRESSED IN S. WITTICHII RW1, AND THE norZ GENES ARE ISOFUNCTIONAL**

Levels of nirK and hmp$_p$ and either norZ$_{ch}$ (WT) or norZ$_p$ (norZ$_{ch}$ mutant) transcripts remained relatively high through stationary phase of S. wittichii RW1 (Figure 2), supporting the stationary phase onset of denitrifying activity (Table 3). The absence of norZ$_{ch}$ expression in S. wittichii RW1 had the effects of increasing the exponential growth rate and preventing slowed growth upon exposure to NO$_2^\text{-}$ (Figure 1 and Table 1). This phenotype may be in part due to increased expression of genes for handling nitrosative stress, that is nirK, hmp$_p$, and norZ$_p$, in the norZ$_{ch}$ mutant compared to the WT (Figure 2). The increase in transcript pools corresponded to a decrease in the amount of NO$_2^\text{-}$ converted to N$_2$O (Table 3), further suggesting that the norZ$_{ch}$ mutant cells were not as susceptible to nitrosative stress as the WT. While other unexamined genetic factors were likely at play in mediating these phenotypes of the norZ$_{ch}$ mutant, the present data clearly show that the loss of norZ$_{ch}$ expression was compensated for by expression of norZ$_p$; hence, the norZ genes of S. wittichii RW1 are isofunctional. As with the WT cells, expression of both nirK and hmp$_p$ genes were positively affected by exposure to NO$_2^\text{-}$ in the norZ$_{ch}$ mutant (Table 5). This increased expression was potentially a function of nirK and hmp$_p$ genes being regulated by NsrR (Swit_1789) and NnrR (Swit_5204) NO responsive regulators, respectively. In addition, the increased level of norZ$_p$ transcript in the norZ$_{ch}$ mutant upon exposure to 1 mM NaNO$_2$ (Table 5), suggests a conditional co-regulation...
of hmp-orf1-orf2-norZ genes when norZp expression is required.

CONCLUSIONS

Results from this study confirm the ability of S. wittichii RW1 to reduce NO_3^- to N_2O and also to transform excess NO_2^{-} via another mechanism. This metabolic capability may be restricted to the Spingomonas wittichii species of the spingomonads based on the limited coinocurrence of nirK and norZ genes in their genomes. This denitrification module was likely acquired by S. wittichii strains by lateral gene transfer as a function of ecological niche and need for N-oxide detoxification. As meta-omic studies often rely on correlating functional genes to 16S rRNA phylotypes, this study sheds light on the complication of relatively rare LGT events that can confer biogeochemically important functions to individual species of broadly distributed bacterial families.

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