frontiers in MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 06 August 2014
doi: 10.3389/fmicb.2014.00404

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

Lynnie S. Cua[1†] and Lisa Y. Stein[2]*

[1] Department of Environmental Sciences, University of California, Riverside, CA, USA
[2] Department of Biological Sciences, Faculty of Science, University of Alberta, Edmonton, AB, Canada

Edited by:
Martin G. Klotz, University of North Carolina at Charlotte, USA

Reviewed by:
Asa Frostegard, Norwegian University of Life Sciences, Norway
James Moir, University of York, UK

***Correspondence:**
Lisa Y. Stein, Department of Biological Sciences, Faculty of Science, University of Alberta, CW 405 Bio Sci Centre, Edmonton, AB, Canada
e-mail: lisa.stein@ualberta.ca

†Present address:
Lynnie S. Cua, County of San Diego, Department of Environmental Health, Vector Disease and Diagnostic Laboratory, San Diego, CA, USA

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_2^-) and produces nitrous oxide (N_2O), 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 $norZ_{ch}$ mutant of *S. wittichii* RW1 consumed NO_2^- and produced N_2O during stationary phase. The $norZ_{ch}$ mutant produced N_2O, although at significantly lower levels (c.a. 66–87%) relative to the WT. Rates of N_2O production were 2–3 times higher in cultures initiated at low relative to atmospheric O_2 per unit biomass, although rates of NO_2^- consumption were elevated in cultures initiated with atmospheric O_2 and 1 mM $NaNO_2$. Levels of mRNA encoding nitrite reductase (*nirK*), plasmid-encoded nitric oxide dioxygenase (hmp_p) and plasmid-encoded nitric oxide reductase ($norZ_p$) were significantly higher in the $norZ_{ch}$ mutant over a growth curve relative to WT. The presence of NO_2^- further increased levels of *nirK* and hmp_p mRNA in both the WT and $norZ_{ch}$ mutant; levels of $norZ_p$ mRNA compensated for the loss of $norZ_{ch}$ expression in the $norZ_{ch}$ mutant. Together, the results suggest that *S. wittichii* RW1 denitrifies NO_2^- to N_2O 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*.

Keywords: *Sphingomonas wittichii* RW1, nitrous oxide, nitrite reductase, nitric oxide reductase, nitric oxide dioxygenase, denitrification

INTRODUCTION

Denitrification is the sequential reduction of nitrate (NO_3^-) and nitrite (NO_2^-) to dinitrogen (N_2) via the gaseous intermediates, nitric oxide (NO) and nitrous oxide (N_2O) (Zumft, 1997). Respiratory denitrification is considered an anaerobic energy-generating metabolism; however, many bacteria can denitrify in the presence of O_2 starting with NO_3^- or NO_2^- and terminating with N_2O due to inhibition or absence of nitrous oxide reductase (Philippot et al., 2011; Stein, 2011; Chen and Strous, 2013). While some denitrifiers grow via hybrid respiration with O_2 and NO_3^- and/or NO_2^-, others respire N-oxides during late-log to stationary phase for detoxification and/or energy conservation, particularly under reduced O_2 (Takaya et al., 2003; Stein, 2011; Chen and Strous, 2013). The collective role of denitrifying bacteria in transformation and release of highly reactive N-oxides is of critical importance because of the effects these molecules have on environmental and human health, atmospheric chemistry, and global warming (Fields, 2004; Galloway et al., 2008).

Sphingomonas wittichii RW1 was isolated from the River Elbe (Germany) as a model organism for studying the bioremediation of dioxin-containing compounds (Wittich et al., 1992; Wilkes et al., 1996; Yabuuchi et al., 2001; Keum et al., 2008). 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*) 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 chromosomal (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 $norZ_p$ (Swit_5200) is the terminal member of a four-gene cluster; the first member of which encodes a nitric oxide dioxygenase (hmp_p; Swit_5203) with predicted function in NO oxidation to NO_3^- or NO reduction to N_2O depending on O_2 concentration (Bonamore and Boffi, 2008). Nitric oxide dioxygenases are present in both denitrifying and non-denitrifying microorganisms to combat nitrosative and oxidative

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_1434 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 spingomonad 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 N_2O 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_3^- or NO_2^- (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 NO_2^- to N_2O and thus can be classified as a denitrifying strain. The ability of *S. wittichii* RW1 to denitrify from NO_3^- 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 NO_3^- to NO_2^- (Yabuuchi et al., 2001). There is no identifiable sequence in the genome with similarity to nitrous oxide reductase; hence, this strain is predicted to denitrify only NO_2^- to N_2O. To provide support for *S. wittichii* RW1 as a denitrifier, objectives were to: (a) determine whether and when *S. wittichii* RW1 produces N_2O at the expense of NO_2^- (b) investigate the regulation of putative N-oxide metabolism genes in response to varying NO_2^-, 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 *norZ*$_{ch}$ MUTANT OF *S. WITTICHII* RW1

The region from bp 203 to 776 of the *norZ*$_{ch}$ gene was PCR-amplified from *S. wittichii* RW1 genomic DNA with primers 203F 5′ aactggaacaggccgatg 3′ and 776R 5′ cgatcgccttcatcttcg 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 *norZ*$_{ch}$ mutant strain was checked by PCR using additional primers: 45F 5′ agagacccaggaccacgac 3′, 854R 5′ tcaccgtcatggaatattgg 3′, pUCGM173F 5′ tgcctcgggcatccaagcagca 3′, pUCGM514R 5′ gagagcgccaacaaccgcttct 3′ and pUCGM519F 5′ cttacgttctgcccaggttt 3′. PCR products were purified and validated by Sanger sequencing. The *norZ*$_{ch}$ mutant strain was maintained on LB media with 50 μg/mL gentamycin.

GROWTH EXPERIMENTS

S. wittichii RW1 wildtype (WT) and *norZ*$_{ch}$ mutant cells from exponentially growing cultures were inoculated into LB media (500 μL into 100 mL) containing 0, 0.3, or 1 mM $NaNO_2$ into glass serum bottles (160 mL), which were then crimp-sealed with rubber septa and aluminum seals. Incubations of *norZ*$_{ch}$ mutant cells contained 50 μg/mL gentamycin. Triplicate incubations of each control condition included the same concentrations of $NaNO_2$ plus: (1) heat-inactivated cells, (2) no cells, or (3) live cells in bottles purged of O_2 by sparging the medium with N_2. All control incubations were treated identically to the

experimental incubations to determine whether chemical decomposition of NO_2^- contributed to NO_2^- loss or N_2O 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 RNAprotect™ 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 Skaletsky, 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- ^{32}P]-dCTP (3000 Ci mmol^{-1}; Perkin-Elmer 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 a 0.1× 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_2O were measured from the gas headspace of sample bottles by GC-TCD (Shimadzu, Kyoto Japan; Molesieve 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 [a]	Coding sequence ID	Enzyme commission number	F primer	R primer	Amplicon
Swit_1793	NO-forming nitrite reductase *(nirK)*	EC:1.7.2.1	ctgaccgcgaaggaagtatc	catggtcgacgatcacattg	742 bp
Swit_5203 (p)	Nitric oxide dioxygenase *(hmp)*	EC:1.14.12.17	tcgagcttgtccacattctg	attgtctccccaaaccatga	210 bp
Swit_R0031	16S rRNA	untranslated	gtacaaggcctgggaacgta	tttatcgcctgaggatgagc	1159 bp
*Swit_5200 (p)	Nitric oxide reductase *(norZ)*	EC:1.7.5.2	ccaacgccaatactcaacct	cagcatttctacggcatcaa	513 bp
*Swit_4614 (ch)	Nitric oxide reductase *(norZ)*	EC:1.7.5.2	gtggtgcccgagaaatagag	gccagagcttctacggtgtc	703 bp

[a] *Significant difference between atmospheric and reduced O_2 for wildtype (WT) cultures incubated with the same concentration of $NaNO_2$.*

(p), encoded on plasmid; (ch), encoded on chromosome.

**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 Skaletsky, 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).

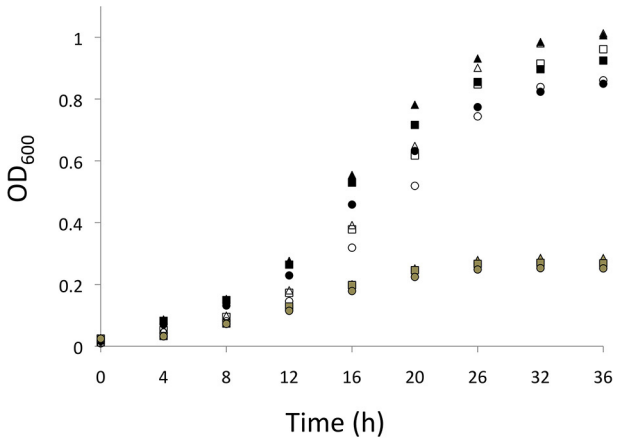

FIGURE 1 | Growth (OD_{600}) measured over time (h) for *S. wittichii* RW1 WT cultures initiated at atmospheric O_2 (black symbols) or reduced O_2 (gray symbols), and for *norB*$_{ch}$ mutant *S. wittichii* RW1 cultures initiated at atmospheric O_2 (white symbols). Triangles = 0 mM added $NaNO_2$, squares = 0.3 mM added $NaNO_2$, and circles = 1 mM added $NaNO_2$. Points on the growth curves represent averaged values from 5 independent experiments for each incubation condition.

RESULTS

EFFECT OF O_2 AND NO_2^- ON GROWTH OF WT AND *norZ*$_{ch}$ MUTANT STRAINS OF *S. WITTICHII* RW1

S. wittichii RW1 is an aerobic heterotrophic bacterium; hence, the doubling time (calculated from 12 to 20 h growth) and final yields of non-mutagenized cells were significantly faster and higher, respectively, for cultures initiated under atmospheric compared to reduced (ca. 3%) O_2 levels (**Figure 1** and **Table 2**). Doubling times of the *norZ*$_{ch}$ mutant were significantly shorter than those of the WT during exponential growth; thus, even though the *norZ*$_{ch}$ 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 $NaNO_2$ to cultures initiated at atmospheric O_2 only significantly increased the doubling time of WT cultures, but significantly reduced the final yields of both WT and *norZ*$_{ch}$ mutant cultures (**Table 2**).

Table 2 | Growth of WT and *norZ*$_{ch}$ mutant strains of *S. wittichii* RW1 at variable $NaNO_2$ and O_2 concentrations.

Variable in growth condition	Doubling time (h)		Yield (OD600 nm)	
	WT	*norZ*$_{ch}$	WT	*norZ*$_{ch}$
Atmospheric O_2, no $NaNO_2$	5.3[abc]	4.4[c]	0.98[ab]	0.98[b]
Reduced O_2, no $NaNO_2$	8.2[a]	N.D.	0.25[a]	N.D.
Atmospheric O_2, 0.3 mM $NaNO_2$	5.6[abc]	4.4[c]	0.90[ab]	0.91[b]
Reduced O_2, 0.3 mM $NaNO_2$	8.5[a]	N.D.	0.25[a]	N.D.
Atmospheric O_2, 1.0 mM $NaNO_2$	5.5[abc]	4.4[c]	0.82[ab]	0.84[b]
Reduced O_2, 1.0 mM $NaNO_2$	8.4[a]	N.D.	0.22[ab]	N.D.

Doubling times were calculated during exponential growth from 12 to 20 h. Final cell yields were reported at 32 and 20 h growth when initiated at atmospheric (ca. 22%) and reduced (ca. 3%) O_2 levels, respectively. Values represent averages from 5 separate experiments. Statistically significant differences between groups were determined by Student's t-test at $p < 0.05$ and are designated as follows:

[a] Significant difference between atmospheric and reduced O_2 for wildtype (WT) cultures only.

[b] Significant difference between WT or norZ$_{ch}$ mutant cultures incubated with $NaNO_2$ relative to unamended controls.

[c] Significant difference between WT and norZ$_{ch}$ mutant cultures grown under identical conditions. N.D., not determined.

CONSUMPTION OF NO_2^- AND PRODUCTION OF N_2O

Cultures of WT and *norZ*$_{ch}$ mutant *S. wittichii* RW1 were incubated in the presence of $NaNO_2$ to assess whether expression of *norZ*$_{ch}$ was required for aerobic denitrifying activity. Amounts of remaining NO_2^-, remaining O_2, and headspace N_2O levels were compared over stationary phase (**Table 3**). NO_2^- was consumed nearly to completion in both WT and *norZ*$_{ch}$ mutant cultures by 96 h incubation. Neither WT nor *norZ*$_{ch}$ mutant cultures consumed O_2 to complete anoxia and headspace O_2 levels remained largely stable following 72 h incubation, even with continuous shaking at 180 rpm. N_2O was measurable in the gas headspace starting after 48 h of incubation and continued to accumulate proportionally with the amount of added $NaNO_2$ (**Table 3**). The *norZ*$_{ch}$ mutant cultures produced significantly less N_2O than the WT cultures (66–87% of WT levels) at both NO_2^- concentrations. Nitrate production was not observed, which would be an expected aerobic activity of Hmp. NH_4^+ concentrations also did not vary between treatment groups, which would be expected if *S. wittichii* RW1 reduced NO_2^- directly to NH_4^+ and allowed its accumulation prior to assimilation (data not shown). N_2 was not measured. Control incubations containing heat-inactivated cells, no cells, or live cells inoculated into bottles sparged of O_2 with N_2 gas showed no consumption of NO_2^- and no production of N_2O.

We next tested whether lower oxygen had an effect on the rates of NO_2^- or O_2 consumption or N_2O production in non-mutated *S. wittichii* RW1. To address this question, *S. wittichii* RW1 cultures were inoculated with 0, 0.3, or 1 mM $NaNO_2$ at either atmospheric or reduced (ca. 3%) O_2 levels. Cultures initiated at atmospheric O_2 and 1 mM $NaNO_2$ consumed O_2 and NO_2^- significantly faster than cultures initiated at reduced O_2 and 1 mM $NaNO_2$, yet the rate of N_2O production was 2–3 times faster for cultures initiated at reduced relative to atmospheric O_2 levels (**Table 4**). The N_2O-N measured in the gas headspace of the cultures was orders of magnitude lower than the amount of NO_2^- consumed per unit biomass (i.e., nmol N_2O produced from μmol NO_2^- consumed). Even though N_2O is highly soluble, the vast difference between NO_2^- consumption and N_2O production implies conversion of NO_2^- into a product other than N_2O; however, NO_3^- was undetectable and NH_4^+ levels did not vary in any culture at any time point (data not shown).

Table 3 | Consumption of nitrite and oxygen and production of nitrous oxide by wild-type and $norZ_{ch}$ mutant cultures of *S. wittichii* RW1 initiated at atmospheric oxygen headspace and with 0.3 or 1 mM $NaNO_2$.

Time (h)	NO_2^- remaining (mM)				%O_2 remaining in the headspace				N_2O produced (nmolOD^{-1})			
	WT		*norZ*$_{ch}$		WT		*norZ*$_{ch}$		WT		*norZ*$_{ch}$	
	0.3 mM	1.0 mM	0.3 mM	1.0 mM	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	0.24^{ab}	0.78^{a}	0.27^{ab}	0.79^{a}	7.24^{ab}	7.62^{a}	7.82^{b}	7.55	2.66^{ab}	3.19^{ab}	1.76^{ab}	2.40^{ab}
72	0.01^{a}	0.24^{ab}	0.04^{a}	0.33^{ab}	4.50^{b}	4.65	4.71^{b}	4.63	10.30^{ab}	34.68^{ab}	8.80^{ab}	24.99^{ab}
96	0.00	0.01	0.00	0.02	4.19^{b}	4.12	4.42^{b}	4.45	17.69^{ab}	63.62^{ab}	14.79^{ab}	49.31^{ab}
120	0.00	0.00	0.00	0.00	4.31	3.89	4.48	4.33	19.28^{ab}	73.06^{ab}	16.83^{ab}	59.91^{ab}

Values represent averages from 5 experiments initiated on different cultures and on different days. N_2O 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:

[a] Significant difference between 0.3 and 1 mM $NaNO_2$ treatment groups of wildtype (WT) or $norZ_{ch}$ mutant cultures of S. wittichii RW1 at each time point.

[b] Significant difference between WT and $norZ_{ch}$ mutant S. wittichii RW1 cultures incubated with 0.3 mM $NaNO_2$ or 1 mM $NaNO_2$.

Table 4 | Maximum rates of nitrite and oxygen consumption and nitrous oxide production by stationary phase *S. wittichii* RW1 wildtype cultures grown at atmospheric (ca. 22%) or reduced (ca. 3%) O_2 headspace.

$NaNO_2$ (mM) added to growth medium	Rate of O_2 consumption (% headspaceOD^{-1} h^{-1})		Rate of NO_2^- consumption (μmolOD^{-1} h^{-1})		Rate of N_2O production (nmolOD^{-1} h^{-1})	
	Atmos. O_2	Red. O_2	Atmos. O_2	Red. O_2	Atmos. O_2	Red. O_2
0	0.03	0.02	0	0	0	0
0.3	0.03	0.02	0.9	0.9	5.2*	16.1*
1.0	0.04*	0.02*	2.2*	1.5*	13.9*	29.8*

Values represent slopes of averaged measurements from 5 experiments initiated on different cultures and on different days (y-axis) over time (48–72 h; x-axis). The "" indicates a significant difference between incubations initiated at atmospheric or reduced (3%) O_2 headspace as determined by Student's t-test ($p < 0.05$). Calculated rates of were normalized to OD units of the cultures due to the difference in maximum biomass between cultures (**Table 2**).*

EXPRESSION LEVELS OF PUTATIVE AEROBIC DENITRIFICATION GENES

Levels of specific mRNAs encoding *nirK*, hmp_p, $norZ_{ch}$, and $norZ_p$, as normalized to levels of 16S rRNA, were compared between WT and $norZ_{ch}$ 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 NO_2^- and production of N_2O is measurably active. Expression of $norZ_p$ substituted for $norZ_{ch}$ in the $norZ_{ch}$ 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_p transcript were significantly higher in the $norZ_{ch}$ mutant than in the WT strain at nearly all time points. Whereas *nirK* and hmp_p transcript levels increased between 24 and 66 h in WT cells, both transcript levels remained relatively high in the $norZ_{ch}$ mutant over the full time course (**Figure 2**).

Finally, the effect of NO_2^- on transcript levels was examined in $norZ_{ch}$ mutant and WT cultures, and for non-mutated cultures initiated under atmospheric and reduced O_2 levels. Each hybridization signal was normalized to that for 16S rRNA, after which the ratio of hybridization intensity between $NaNO_2$-treated and untreated sample was calculated for every culture and each transcript pool. There was no significant effect of NO_2^- treatment on any transcript level for any culture until late log phase (i.e., 24 h for WT and $norZ_{ch}$ mutant cultures and 20 h for WT cultures initiated at reduced O_2). At least a two-fold increase between $NaNO_2$-treated and untreated cells was considered a significant effect; thus, *nirK* and hmp_p were positively responsive to $NaNO_2$ in both WT and $norZ_{ch}$ mutant cultures (**Table 5**). For both *norZ* genes, only transcription levels of $norZ_p$ in the $norZ_{ch}$ mutant were responsive to 1 mM $NaNO_2$ treatment.

DISCUSSION

SPHINGOMONAS WITTICHII RW1 DENITRIFIES NO_2^- TO N_2O

Rapid consumption of NO_2^- by *S. wittichii* RW1 occurred only once the cells reached stationary phase (**Table 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 O_2, an increased rate of NO_2^- conversion to N_2O (**Table 4**) relative to cultures initiated at atmospheric O_2 implies that O_2 limitation must be reached for denitrifying activity to commence as would be commonly expected (Zumft, 1997). It is interesting that a faster rate of NO_2^- consumption occurred for cultures initiated at atmospheric than at reduced O_2in the presence of 1 mM $NaNO_2$ as this implies an additional process from denitrification for NO_2^- loss. Although a substantial quantity of NO_2^- consumed by *S. wittichii* RW1 was converted to N_2O, there was a considerable pool of transformed NO_2^- that could not be accounted for in NH_4^+

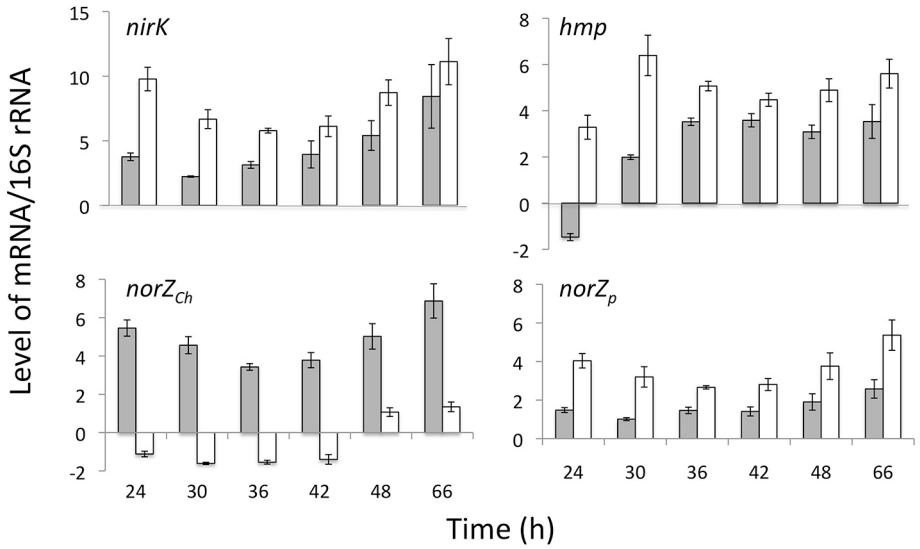

FIGURE 2 | Levels of mRNA of select genes in *S. wittichii* RW1 WT (gray bars) and $norZ_{ch}$ mutant (white bars) in batch cultures over time. Error bars represent standard error for 5 replicate experiments performed with separate cultures on different days.

Table 5 | Effect of nitrite and O_2 on levels of specific transcripts of *S. wittichii* RW1 from mid-log and early stationary phase cultures.

mRNA	WT (atmospheric O_2)				WT (reduced O_2)				$norZ_{ch}$ (atmospheric O_2)			
	0.3 mM		1.0 mM		0.3 mM		1.0 mM		0.3 mM		1.0 mM	
	24 h	36 h	24 h	36 h	20 h	24 h	20 h	24 h	24 h	36 h	24 h	36 h
nirK	1.68[a]	1.56[a]	**3.98[a]**	**2.17[ab]**	0.89[a]	0.96	**2.00[a]**	1.38	1.81[a]	**2.28[a]**	**4.53[a]**	1.56[ab]
hmp	1.23	**2.20**	1.24[b]	**2.52**	1.26[a]	1.47[a]	**3.89[a]**	**2.65[a]**	1.18[a]	**2.28**	**2.00[ab]**	**2.79**
$norZ_{ch}$	1.37[b]	1.51[ab]	1.42[b]	1.00[ab]	0.80	0.93	1.15	1.14	*Not expressed in this strain*			
$norZ_p$	0.88[b]	0.88[b]	0.89[b]	0.91[b]	1.09	1.31	1.63	1.69	1.39[ab]	1.70[b]	**2.21[ab]**	1.58[b]

Values represent the ratio of hybridization intensity between nitrite-treated and untreated cells, previously normalized to 16S rRNA levels, and averaged from 5 experiments initiated on separate cultures on separate days. Bold values indicate a two-fold or greater increase in transcript level between $NaNO_2$-treated and untreated cells. Statistically significant differences between treatments were determined by Student's t-test at $p < 0.05$ and are designated as follows:

[a] Significant difference between 0.3 and 1 mM $NaNO_2$ treatment groups of wild-type (WT) or $norZ_{ch}$ mutant cultures of S. wittichii RW1 at the same time point.

[b] Significant difference between WT and $norZ_{ch}$ mutant cultures initiated at atmospheric O_2, incubated with 0.3 or 1 mM $NaNO_2$.

or NO_3^- 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^--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^- (**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^- converted to N_2O (**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^- 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 $norZ_p$ expression is required.

CONCLUSIONS

Results from this study confirm the ability of *S. wittichii* RW1 to reduce NO_2^- 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 sphingomonads based on the limited co-occurrence 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.

ACKNOWLEDGMENTS

This work was supported by the Kearny Foundation for Soil Science and the Environmental Toxicology Program at the University of California, Riverside.

REFERENCES

Bonamore, A., and Boffi, A. (2008). Flavohemoglobin: structure and reactivity. *IUBMB Life* 60, 19–28. doi: 10.1002/iub.9

Busch, A., Friedrich, B., and Cramm, R. (2002). Characterization of the *norB* gene, encoding nitric oxide reductase, in the nondenitrifying cyanobacterium *Synechocystis* sp strain PCC6803. *Appl. Environ. Microbiol.* 68, 668–672. doi: 10.1128/AEM.68.2.668-672.2002

Cantera, J. J. L., and Stein, L. Y. (2007). Molecular diversity of nitrite reductase (*nirK*) genes in nitrifying bacteria. *Environ. Microbiol.* 9, 765–776. doi: 10.1111/j.1462-2920.2006.01198.x

Chen, J. W., and Strous, M. (2013). Denitrification and aerobic respiration, hybrid electron transport chains and co-evolution. *Biochim. Biophys. Acta Bioenerg.* 1827, 136–144. doi: 10.1016/j.bbabio.2012.10.002

Clesceri, L. S., Greenberg, A. E., and Eaton, A. D. (1998). *Standard Methods for the Examination of Water and Wastewater.* Washington, DC: American Public Health Assoc.

Cramm, R., Siddiqui, R. A., and Friedrich, B. (1997). Two isofunctional nitric oxide reductases in *Alcaligenes eutrophus* H16. *J. Bacteriol.* 179, 6769–6777.

Fields, S. (2004). Global nitrogen - Cycling out of control. *Environ. Health Perspect.* 112, A556–A563. doi: 10.1289/ehp.112-a556

Forrester, M. T., and Foster, M. W. (2012). Protection from nitrosative stress: a central role for microbial flavohemoglobin. *Free Rad. Biol. Med.* 52, 1620–1633. doi: 10.1016/j.freeradbiomed.2012.01.028

Galloway, J. N., Townsend, A. R., Erisman, J. W., Bekunda, M., Cai, Z., Freney, J. R., et al. (2008). Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science* 320, 889–892. doi: 10.1126/science.1136674

Hendriks, J., Oubrie, A., Castresana, J., Urbani, A., Gemeinhardt, S., and Saraste, M. (2000). Nitric oxide reductases in bacteria. *Biochim. Biophys. Acta Bioenerg.* 1459, 266–273. doi: 10.1016/S0005-2728(00)00161-4

Householder, T. C., Fozo, E. M., Cardinale, J. A., and Clark, V. L. (2000). Gonococcal nitric oxide reductase is encoded by a single gene, *norB*, which is required for anaerobic growth and is induced by nitric oxide. *Infect. Immunol.* 68, 5241–5246. doi: 10.1128/IAI.68.9.5241-5246.2000

Keum, Y. S., Lee, Y. J., and Kim, J. H. (2008). Metabolism of nitrodiphenyl ether herbicides by dioxin-degrading bacterium *Sphingomonas wittichii* RW1. *J. Agricult. Food Chem.* 56, 9146–9151. doi: 10.1021/jf801362k

Miller, T. R., Delcher, A. L., Salzberg, S. L., Saunders, E., Detter, J. C., and Halden, R. U. (2010). Genome sequence of the dioxin-mineralizing bacterium *Sphingomonas wittichii* RW1. *J. Bacteriol.* 192, 6101–6102. doi: 10.1128/JB.01030-10

Philippot, L., Andert, J., Jones, C. M., Bru, D., and Hallin, S. (2011). Importance of denitrifiers lacking the genes encoding the nitrous oxide reductase for N_2O emissions from soil. *Global Change Biol.* 17, 1497–1504. doi: 10.1111/j.1365-2486.2010.02334.x

Rodionov, D. A., Dubchak, I. L., Arkin, A. P., Alm, E. J., and Gelfand, M. S. (2005). Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks. *PLoS Comp. Biol.* 1:e55. doi: 10.1371/journal.pcbi.0010055

Rozen, S., and Skaletsky, H. J. (2000). "Primer3 on the WWW for general users and for biologist programmers," in *Bioinformatics Methods and Protocols: Methods in Molecular Biology,* eds S. Krawetz and S. Misener (Totowa, NJ: Humana Press), 365–386.

Sambrook, J., and Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual.* Cold Springs Harbor, NY: Cold Springs Harbor Laboratory Press.

Stein, L. Y. (2011). "Heterotrophic nitrification and nitrifier denitrification," in *Nitrification,* eds B. B. Ward, D. J. Arp, and M. G. Klotz (Washington, DC: ASM Press), 95–114.

Takaya, N., Kuwazaki, S., Adachi, Y., Suzuki, S., Kikuchi, T., Nakamura, H., et al. (2003). Hybrid respiration in the denitrifying mitochondria of *Fusarium oxysporum*. *J. Biochem.* 133, 461–465. doi: 10.1093/jb/mvg060

Wilkes, H., Wittich, R. M., Timmis, K. N., Fortnagel, P., and Francke, W. (1996). Degradation of chlorinated dibenzofurans and dibenzo-p-dioxins by Sphingomonas sp. strain RW1. *Appl. Environ. Microbiol.* 62, 367–371.

Wittich, R. M., Wilkes, H., Sinnwell, V., Francke, W., and Fortnagel, P. (1992). Metabolism of dibenzo-para-dioxin by *Sphingomonas* sp. strain RW1 O. *Appl. Environ. Microbiol.* 58, 1005–1010.

Yabuuchi, E., Yamamoto, H., Terakubo, S., Okamura, N., Naka, T., Fujiwara, N., et al. (2001). Proposal of *Sphingomonas wittichii* sp. nov. for strain RW1T, known as a dibenzo-p-dioxin metabolizer. *Int. J. Syst. Evol. Microbiol.* 51, 281–292.

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* 61, 533–616.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 06 May 2014; accepted: 17 July 2014; published online: 06 August 2014.
Citation: Cua LS and Stein LY (2014) Characterization of denitrifying activity by the alphaproteobacterium, Sphingomonas wittichii RW1. Front. Microbiol. ***5****:404. doi: 10.3389/fmicb.2014.00404*
This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Microbiology.