Title
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Permalink
https://escholarship.org/uc/item/3kz022cf

Journal
Applied and environmental microbiology, 82(1)

ISSN
0099-2240

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Publication Date
2016

DOI
10.1128/aem.02602-15

Peer reviewed
Determining Roles of Accessory Genes in Denitrification by Mutant Fitness Analyses

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Enzymes of the denitrification pathway play an important role in the global nitrogen cycle, including release of nitrous oxide, an ozone-depleting greenhouse gas. In addition, nitric oxide reductase, maturation factors, and proteins associated with nitric oxide detoxification are used by pathogens to combat nitric oxide release by host immune systems. While the core reductases that catalyze the conversion of nitrate to dinitrogen are well understood at a mechanistic level, there are many peripheral proteins required for denitrification whose basic function is unclear. A bar-coded transposon DNA library from Pseudomonas stutzeri strain RCH2 was grown under denitrifying conditions, using nitrate or nitrite as an electron acceptor, and also under molybdenum limitation conditions, with nitrate as the electron acceptor. Analysis of sequencing results from these growths yielded gene fitness data for 3,307 of the 4,265 protein-encoding genes present in strain RCH2. The insights presented here contribute to our understanding of how peripheral proteins contribute to a fully functioning denitrification pathway. We propose a new low-affinity molybdate transporter, OatABC, and show that differential regulation is observed for two MoaA homologs involved in molybdenum cofactor biosynthesis. We also propose that NnrS may function as a membrane-bound NO sensor. The dominant HemN paralog involved in heme biosynthesis is identified, and a CheR homolog is proposed to function in nitrate chemotaxis. In addition, new insights are provided into nitrite reductase redundancy, nitric oxide reductase maturation, nitrous oxide reductase maturation, and regulation.

Denitrification is the use of nitorgenous oxides as terminal electron acceptors for anaerobic respiration to produce nitrogen gas or, in some cases, nitrous oxide. It completes the nitrogen cycle by returning bioavailable nitrogen back to the atmosphere. Full denitrification from nitrate (NO$_3^-$) involves four consecutive reduction steps producing, in turn, nitrite (NO$_2^-$), nitric oxide (NO), nitrous oxide (N$_2$O), and dinitrogen (N$_2$). Each step is catalyzed by a complex metalloenzyme (Fig. 1), all of which have been characterized extensively (1–5). However, there are many accessory proteins that have been implicated in denitrification by genetic and gene cluster analyses but remain poorly characterized (6). These are the focus of this work, with Pseudomonas stutzeri strain RCH2 (7) as a model denitrifying system.

Strain RCH2 contains a respiratory nitrate reductase (NarGHI) that couples nitrate reduction and quinone oxidation with proton transport across the inner membrane (Fig. 2). Nar contains heme b, NarH and NarG contain iron-sulfur (Fe-S) clusters, and NarG contains molybdendum (Mo), which catalyzes nitrate reduction, coordinated in the bis-molybdopterin guanine dinucleotide (MGD) form of the molybdenum cofactor (Moco) (8). NarJ assists in assembly of the Nar complex, allows for insertion of the proximal Fe-S cluster, and delivers Moco to NarG (9). NarK is a nitrate importer with concomitant nitrite antiport or proton symport (10). NarXL forms a two-component sensor-regulator system that activates expression of nitrate reductase (11).

Nitrite reductase by strain RCH2 is carried out in the periplasm either by cytochrome-containing NirS or by NirK, which contains a type 1 copper (Cu) site for electron transfer and a catalytic type 2 Cu center (6). These two proteins are phylogenetically unrelated and have been reported to never occur in the same organism (12), although this was recently shown not to be the case (13). NirS contains the specialized heme $d_{1}$, and several accessory genes (nirDEFGHIJ) are involved in related cofactor maturation (6, 14). Several other genes, such as nirBCMT, encode cytochromes that have been proposed to be involved in electron transfer to NirS (6, 15, 16). Strain RCH2 also contains a CNOR-type nitric oxide reductase (NorBC) that harbors hemes b, b$_{2}$, and c, together with a nonheme iron site (Fig. 2). The main nir/nor regulator is an NO-responsive activator, DnrD (17). Several of the nitric oxide accessory proteins are located among nitrite reduction-related genes and have been given nir-based names (nirQOP). NirQ is proposed to work with NorD to generate active NorBC, although it is not known how the activation occurs (6). NnrS is a membrane-bound heme-Cu protein of unknown NO-related function (18, 19) that has not been characterized in gammaproteobacteria. Interestingly, NnrS and several other proteins connected with NO reduction, including NorBCD, are found in a variety of pathogens (see Fig. S1 in the supplemental material) (20, 21). NO is produced by inducible NO synthase (iNOS) in macrophages as part of the oxidative burst that is key to killing engulfed
pathogens; thus, NO reduction and detoxification are part of pathogen survival (22).

The nitrous oxide reductase (NosZ) of strain RCH2 is periplasmic and contains two Cu sites: binuclear CuA and tetranuclear Cu5 (Fig. 2). CuA is structurally and functionally similar to CuA of the cytochrome c oxidase of aerobic respiration, transferring electrons to active site Cu5, a Cu-S cluster, though the precise structure is still the subject of debate (5, 23). NosDFY and NosA transport sulfur and Cu, respectively, for assembly of Cu5, and NosL is a periplasmic Cu chaperone (24). NosR is a membrane-bound flavoprotein that is required for NosZ transcription, but the mechanism is not known (25).

In the present study, the roles of the various accessory proteins involved in denitrification were investigated in strain RCH2 by use of a recently developed randomly bar-coded transposon mutant (RB-TnSeq) library (26). This technique provides a way to quantitatively measure, by multiplexed short-read DNA sequencing, the fitness of hundreds of thousands of single-insertion transposon mutants simultaneously. Fitness is measured by the growth of an individual mutant relative to overall culture growth. Fitness values for mutants with insertions in the same gene are averaged to yield gene fitness values for nearly every gene in the genome. We used this approach to analyze genes that are peripherally involved in the process of denitrification, in addition to those that catalyze reduction of the nitrogenous oxides, to obtain a comprehensive picture of denitrifying growth in strain RCH2. We grew the RCH2 RB-TnSeq library anaerobically, with nitrate or nitrite as the terminal electron acceptor, and assayed the fitness of 3,307 nonessential coding genes (out of 4,265 in the genome) (26) by high-throughput sequencing of strain bar codes. In addition, the effects of limiting the availability of molybdenum (Mo), an essential component of nitrate reductase, were also investigated both by reducing the Mo concentration and by adding tungsten (W). Mo and W are found in the environment as the oxyanions molybdate (MoO₄²⁻) and tungstate (WO₄³⁻), respectively. While physically almost identical, they are different chemically, and WO₄³⁻ is a very effective antagonist of Mo uptake and metabolism. Mo limitation is of interest because soil and freshwater environments often contain Mo at very low concentrations that naturally limit denitrification and can lead to persistence of nitrate contamination (27, 28). Hence, we report here a comprehensive analysis of gene phenotypes related to denitrification in RCH2 by using genome-wide fitness profiling.

MATERIALS AND METHODS

Growth screens. Pseudomonas stutzeri RCH2 was cultured in 100-well plates in a Bioscreen C apparatus enclosed in a Plas-Labs La Petite anaerobic glove box under an argon atmosphere with <5% hydrogen for catalytic removal of oxygen. RCH2 grew on a defined medium, passing electrons from lactate to either nitrate or nitrite. The medium contained 4.7 mM ammonium chloride, 1.3 mM potassium chloride, 2 mM magnesium sulfate, 0.1 mM calcium chloride, 0.3 mM sodium chloride, 29.7 mM sodium bicarbonate, 5 mM sodium dihydrogen phosphate, 20 mM sodium lactate, and either 20 mM sodium nitrate or 20 mM sodium nitrite. Vitamins and minerals were added as described by Widdel and Bak (29), except for molybdenum and tungsten, which were varied in 10-fold increments from 1 nM to 1 mM. Various molybdenum and tungsten conditions were tested to find molybdenum-limited conditions that reproducibly reached about 50% of the maximum (molybdenum replete) optical density (OD) in 8 to 12 h. Yeast extract and/or casein (0.5 g/liter [each]) was added to some conditions to test the effects on observed growth defects.

Mutant library growth. The P. stutzeri RCH2 RB-TnSeq library, containing 166,448 single-transposon insertions with known, sequence-identifiable genome locations, was grown under the same conditions as those under which it was constructed (aerobic, rich medium) (26). Anaerobic experimental growths were inoculated from these (starting OD of 0.02) into sealed Hungate tubes under an argon atmosphere and shaken at 160 rpm on an orbital shaker. Nitrate versus nitrite growths (5 ml) used the same medium composition that was used for growth screens, with both casein (0.5 g/liter) and yeast extract (0.5 g/liter) added to avoid eliminating insertion mutants from the population due to nutrient auxotrophies. Mo-limited and associated Mo-replete control growths (10 ml) used the same medium without casein and with different amounts of Mo and W, based on growth screens. In order to achieve consistent Mo limitation, a small amount of Mo was added with a higher concentration of W to act as
an antagonist and induce Mo limitation. The Mo and W concentration for nitrate, nitrite, and Mo limitation control media was 0.8 μM (each). The two Mo limitation conditions had Mo concentrations of 9 nM and 700 nM and W concentrations of 500 nM and 1 mM, respectively. Triplicate growth experiments were carried out for all conditions.

**DNA isolation, PCR, sequencing, and sequence analysis.** Sample processing, DNA sequencing, and analysis of sequence data were conducted as described previously (26). DNA from nitrate versus nitrite samples and the corresponding time-zero reference samples was processed using both of the PCR techniques described previously (26). PCR products were sequenced using an Illumina MiSeq instrument, along with samples from three other conditions not discussed here. Strain fitness values were calculated for each individual transposon insertion strain. Gene fitness values for each replicate are reported in Table S1 in the supplemental material. Quality control and normalization of data were conducted using an Agilent 7500c inductively coupled plasma mass spectrometer (ICP-MS). Sample processing, instrument settings, and analysis of mass spectrometry data have been described previously (30).

**Bioinformatics.** Protein sequence alignments were done using CLC Genome Workbench 6 (Qiagen). Phylogenetic trees were calculated using the neighbor-joining algorithm and the Jukes-Cantor protein distance measure with 100 bootstrap replicates. Tree visualization and analysis were done using iTOL (31).

### RESULTS AND DISCUSSION

**Analysis of RB-TnSeq fitness data.** The RCH2 mutant library was grown anaerobically with lactate (20 mM) as the carbon source and electron donor and with nitrate or nitrite (20 mM) as the electron acceptor. Comparison of nitrite- and nitrate-based growth discriminates between genes that are specifically required for nitrate reduction and those that are more essential for subsequent steps of denitrification. In the present study, gene fitness ($w$) reflects the relative growth rate (see Materials and Methods), such that an increase in relative abundance of mutants in a gene gives positive fitness for that gene and a decrease in relative abundance gives negative fitness (26). Thus, in the following data, fitness under nitrate growth conditions ($w_{\text{nitrate}}$) subtracted from fitness under nitrite growth conditions ($w_{\text{nitrite}}$) is the key metric ($w_{\text{nitrate}}-w_{\text{nitrite}}$). As an example, in Fig. 3, $narG$ is shown in red, with a $w_{\text{nitrate}}$ value of −1.3 and a $w_{\text{nitrite}}$ value of +0.2, showing that mutants in $narG$ grow poorly with nitrate as an electron acceptor, but much better (above average) with nitrite, and the positive $w_{\text{nitrate}}-w_{\text{nitrite}}$ value indicates better growth with nitrate than with nitrite.

**Role of Nar proteins in nitrate reduction.** Mutants in genes encoding the nitrate reductase structural subunits ($narGHI$), the molybdenum cofactor chaperone ($narJ$), nitrate/nitrite transporters ($narCK$), the nitrate sensor ($narX$), and certain Moco biosyn-
thesis proteins (mobA, moaBCE, and moeAB) exhibited strong, nitrate-specific fitness defects (\( w_{\text{nitrate}}-\text{nitrate} \) values of >1) (Fig. 3; see Table S3 in the supplemental material). Most of these (mobA, moaBCE, moeAB, and narHIJ) had lower fitness values (with a \( w_{\text{nitrate}} \) value as low as −2.2 for mobA) than that of narG (−1.3), which encodes the Mo-containing subunit of nitrate reductase (Fig. 3). That is, paradoxically, the nitrate reductase catalytic site is not the most critical component for nitrate reduction on a genome-wide basis. While the cause of this is not clear for NarHIJ, for the Moco biosynthetic genes it is likely because another Moco-based nitrate-reducing enzyme, either Nap (periplasmic) or Nas (cytoplasmic), replaces Nar to a limited extent in the absence of a proper functioning Nar active site, as previously proposed for the closely related organism Pseudomonas aeruginosa (Fig. 4) (32).

Moco does not appear to have any critical function under these conditions, other than nitrate reduction, since fitness defects for the Moco biosynthetic genes have \( w_{\text{nitrate}} \) values near zero.

**Mo limitation RB-TnSeq fitness data.** Two growth media were used to study Mo limitation. One contained 9 nM MoO\(_4^{2-}\) with 500 nM WO\(_4^{2-}\) (limited because Mo is scarce; fitness is denoted \( w_{9\text{nM MoO}_4^{2-}} \)). The other contained 0.7 \( \mu \text{M MoO}_4^{2-}\) and 1.0 mM WO\(_4^{2-}\) (Mo limited because W is inhibitory; fitness is denoted \( w_{1.0\text{mM WO}_4^{2-}} \)). Growth with 0.8 \( \mu \text{M MoO}_4^{2-}\) and 0.8 \( \mu \text{M WO}_4^{2-}\) was used as a Mo-replete control (fitness is denoted \( w_{\text{replete}} \)). Casein was omitted from these media because it contains Mo as a contaminant. Mo limitation fitness data are presented as \( w_{5\text{nM MoO}_4^{2-}} \) or \( w_{700\text{nM MoO}_4^{2-}} \) to distinguish Mo limitation-influenced fitness from effects due simply to denitrifying growth. Since RB-TnSeq gives fitness values for most genes nonessential to the growth condition used during library construction (aerobic, rich medium) (26), our data gave insight into many pathways involved in denitrification. Figure 4 shows the genes discussed and how they relate to denitrification. A discussion of cofactor-related genes follows the discussion of the enzymes in which the cofactors are found.

**Role of molybdate uptake in denitrification.** The modABC genes encode the most common transporter for molybdenum in bacteria (33), and as might be expected, their \( w_{9\text{nM MoO}_4^{2-}} \) values were negative (−3.2, −3.1, and −1.6, respectively) (see Table S4 in the supplemental material). Mutants in only 12 genes exhibited significant phenotypic differences between the two Mo limitation conditions (\( w_{700\text{nM MoO}_4^{2-} - 9\text{nM MoO}_4^{2-}} > 1 \)) (Table 1). Surprisingly, the modABC genes had the highest \( w_{700\text{nM MoO}_4^{2-} - 9\text{nM MoO}_4^{2-}} \) values (3.4, 3.3, and 2.0, respectively) (Table 1), suggesting that although growth was Mo limited, Mo uptake through ModABC was not essential under tungsten-inhibited conditions, in contrast to low-Mo conditions. Mo at micromolar to millimolar concentrations, however, can enter the cell via sulfate transporters or low-specificity anion transporters (LSATs) as well as via ModABC (34). LSATs have long been suspected to exist, but the encoding genes have yet to be identified, with the exception of a low-specificity permease that transports MoO\(_4^{2-}\), SO\(_4^{2-}\), VO\(_4^{3-}\), and WO\(_4^{2-}\) in Rhodobacter capsulatus (34, 35).

Our RB-TnSeq data for RCH2 now provide evidence for additional LSATs. Genes encoding two members of another ABC-type transporter (Psest_2542-4), here called oat (oxanion transporter), exhibited \( w_{700\text{nM MoO}_4^{2-} - 9\text{nM MoO}_4^{2-}} \) values of about −2 (oatAC) (Fig. 5; Table 1). The transmembrane subunit-encoding gene, oatB, had relatively few insertion strains and sequencing reads, so a gene fitness value is not reported; however, the fitness for these few strains (data not shown) appeared to be consistent with the results for oatA and oatC. The oatBCA genes are annotated to encode proteins transporting nitrate, sulfonate, and/or bicarbonate (GenBank accession no. AGA87064 to AGA87066). Negative \( w_{700\text{nM MoO}_4^{2-} - 9\text{nM MoO}_4^{2-}} \) values for oatABC combined with positive \( w_{700\text{nM MoO}_4^{2-} - 9\text{nM MoO}_4^{2-}} \) values for modABC suggest that under conditions of highly inhibitory WO\(_4^{2-}\), the role of ModABC in MoO\(_4^{2-}\) uptake diminishes, and the lower-affinity OatABC system becomes the dominant path for Mo uptake. OatABC does not appear to transport nitrate, as the genes encoding the known nitrate transporters NarCK have fitness values dissimilar to those of oatABC. Curiously, the gene preceding oatBCA in the genome is a homolog of oatA (here called oatA’), suggesting that OatA and OatA’ share the same BC components. STRING analysis of oatBC...
offers further support for this idea, associating members of four other ABC transporter operons with \textit{oatBC} with a high confidence (0.9) \(H11022\). Confidence scores indicate a much closer association for \textit{oatA} (0.99) than for \textit{oatA}' (0.87). Component sharing could also explain why \textit{oatB} had few mutants, which can be caused by low aerobic fitness, though this is not necessarily the case. The ModABC system is found in 90% of sequenced Moco-producing bacteria. Three percent use more tungsten-specific systems (WtpABC and TupABC); however, 7% have no recognized Mo uptake system \(H33\). Thus, we propose that LSATs, such as OatABC, are the primary Mo uptake system in such species. The high degree of similarity among ABC transporters can make bioinformatic classification of proteins into subfamilies quite challenging, especially for LSATs that are multifunctional and are not evolutionarily driven toward a particular (single) optimization. RB-TnSeq provides a powerful tool for identifying the most functionally important transporters both under conditions where there is a clear hypothesis (as in this study) and under conditions where the answer is less clear (many or no candidate transporters annotated).

Our modABC/oatBCA results are consistent with the action of \textit{modE}, which represses modABC transcription in the MoO\(_4\)\(^2-\) bound state, but modE is absent from the RCH2 genome. Hence, either there is a novel regulator present in RCH2 or OatBCA has a higher selectivity for MoO\(_4\)\(^2-\) than for WO\(_4\)\(^2-\), resulting in the observed phenotypes. The absence of ModE-type regulators is somewhat common: 29% of Moco-utilizing bacteria have a full-

### TABLE 1 Fitness differences between Mo limitation conditions for mutants with \(W^{700\, nM\, Mo-9\, nM\, Mo}\) of \(>1^a\)

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Gene annotation</th>
<th>Fitness value</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psest_2543</td>
<td>Nitrate/sulfonate/bicarbonate transport ATPase</td>
<td>-0.1</td>
<td>\textit{oatC}\</td>
</tr>
<tr>
<td>Psest_0520</td>
<td>Hypothetical protein</td>
<td>0.1</td>
<td>\textit{oatA}\</td>
</tr>
<tr>
<td>Psest_2544</td>
<td>Nitrate/sulfonate/bicarbonate transport, periplasmic</td>
<td>0.1</td>
<td>\textit{moaE}\</td>
</tr>
<tr>
<td>Psest_3168</td>
<td>Moco step 2 (sulfur insertion)</td>
<td>-1.5</td>
<td>\textit{nirQ1}\</td>
</tr>
<tr>
<td>Psest_0814</td>
<td>AAA ATPase (Nor activation)</td>
<td>-1.8</td>
<td>\textit{polA}\</td>
</tr>
<tr>
<td>Psest_4219</td>
<td>DNA polymerase I</td>
<td>-0.6</td>
<td>\textit{norD1}\</td>
</tr>
<tr>
<td>Psest_0828</td>
<td>Nitric oxide reductase activation protein</td>
<td>-1.8</td>
<td>\textit{modC}\</td>
</tr>
<tr>
<td>Psest_3170</td>
<td>Moco biosynthesis step 1 (GTP cyclization)</td>
<td>-1.5</td>
<td>\textit{modB}\</td>
</tr>
<tr>
<td>Psest_3001</td>
<td>Mo transport, ATPase</td>
<td>-0.8</td>
<td>\textit{modA}\</td>
</tr>
<tr>
<td>Psest_3000</td>
<td>Mo transport, permease</td>
<td>-0.5</td>
<td>\textit{modC}\</td>
</tr>
<tr>
<td>Psest_2999</td>
<td>Mo transport, periplasmic Mo binding</td>
<td>-0.6</td>
<td>\textit{modB}\</td>
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</tbody>
</table>

\(a\) \textit{nirD1} was included for comparison. Genes are sorted by \(W^{700\, nM\, Mo-9\, nM\, Mo}\) values, from lowest to highest.

![FIG 5 Molybdenum cofactor biosynthesis](image_url)

Roles of Denitrification Genes Based on Mutant Fitness

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TABLE 2 Paralogs distinguished by RB-TnSeq data

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Gene function/annotation</th>
<th>$w_{\text{nitr}}$</th>
<th>$w_{\text{anut}}$</th>
<th>$w_{\text{nutr}}$</th>
<th>Name</th>
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</thead>
<tbody>
<tr>
<td>Psest_0814</td>
<td>MoxR-like AAA ATPase (Nor activation)</td>
<td>-1.8</td>
<td>-2.4</td>
<td>-0.6</td>
<td>nirQ1</td>
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<tr>
<td>Psest_2290</td>
<td>MoxR-like AAA ATPase (Nor activation)</td>
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<td>-0.2</td>
<td>0.0</td>
<td>nirQ2</td>
</tr>
<tr>
<td>Psest_0815</td>
<td>Cytochrome cd$_2$, nitrite reductase</td>
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<td>-2.2</td>
<td>-2.2</td>
<td>nir5</td>
</tr>
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<td>Psest_2294</td>
<td>Cu-containing nitrite reductase (NiRK)</td>
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<td>0.0</td>
<td>0.0</td>
<td>nirK</td>
</tr>
<tr>
<td>Psest_0826</td>
<td>Cytochrome c subunit of NO reductase</td>
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<td>-3.5</td>
<td>-1.7</td>
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<td>Psest_2292</td>
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<td>-0.1</td>
<td>norC2</td>
</tr>
<tr>
<td>Psest_0827</td>
<td>Cytochrome b subunit of NO reductase</td>
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<td>-3.1</td>
<td>-1.4</td>
<td>norB1</td>
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<tr>
<td>Psest_2291</td>
<td>Cytochrome b subunit of NO reductase</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>norB2</td>
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<tr>
<td>Psest_0828</td>
<td>Nitric oxide reductase activation protein</td>
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<td>-3.3</td>
<td>-1.4</td>
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<td>Psest_2289</td>
<td>Nitric oxide reductase activation protein</td>
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<td>0.0</td>
<td>norD2</td>
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<td>Psest_2511</td>
<td>Moco BS step 1 (GTP rearrangement)</td>
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<td>-0.1</td>
<td>moaA1</td>
</tr>
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<td>Psest_3481</td>
<td>Moco BS step 1 (GTP rearrangement)</td>
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<td>0.1</td>
<td>0.9</td>
<td>moaA2</td>
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<tr>
<td>Psest_0799</td>
<td>NnrS (heme-copper protein; response to NO)</td>
<td>-2.0</td>
<td>-2.2</td>
<td>-0.2</td>
<td>nnnS1</td>
</tr>
<tr>
<td>Psest_3754</td>
<td>NnrS (heme-copper protein; response to NO)</td>
<td>-0.1</td>
<td>-0.2</td>
<td>-0.2</td>
<td>nnnS2</td>
</tr>
<tr>
<td>Psest_4279</td>
<td>NnrS (heme-copper protein; response to NO)</td>
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<td>0.1</td>
<td>0.0</td>
<td>nnnS3</td>
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<tr>
<td>Psest_0276</td>
<td>Oxygen-independent coproporphyrinogen III oxidase</td>
<td>-0.3</td>
<td>0.0</td>
<td>0.3</td>
<td>hemN1</td>
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<tr>
<td>Psest_2234</td>
<td>Oxygen-independent coproporphyrinogen III oxidase</td>
<td>-3.2</td>
<td>-3.0</td>
<td>0.2</td>
<td>hemN2</td>
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<tr>
<td>Psest_2404</td>
<td>Coproporphyrinogen III oxidase, Fe-S oxidoreductase</td>
<td>-0.2</td>
<td>0.1</td>
<td>0.3</td>
<td>hemN3</td>
</tr>
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<td>Psest_2537</td>
<td>Oxygen-independent coproporphyrinogen III oxidase</td>
<td>-0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>hemN4</td>
</tr>
</tbody>
</table>

*Genes shown in bold have a more pronounced mutant fitness defect. Shaded genes are found in the main nir/nor/nos genome region. Underlined genes are part of the nirK operon.

*BS, biosynthesis.

length moeD gene, and 34% do not have even partial homologs (33). No clear MoO$_4^{2-}$–binding candidates were apparent from a search for proteins related to MoE, WtpA, or TupA (37).

Role of MoaA in Moco biosynthesis. MoaA is a radical SAM enzyme that catalyzes the complex rearrangement of GTP, together with MoaC, to generate cyclic pyranopterin monophosphate (cPMP), a reaction that requires a high level of catalytic control and specificity (38). There has been debate concerning what intermediate is passed from MoaA to MoaC, including recent evidence for 3′,8′-CH$_2$GTP (66); however, the identity of this intermediate does not affect the conclusions presented here, and it is clear that MoaA is the first enzyme in the pathway. RCH2 contains two moaA homologs (Fig. 4), Psest_2511 and Psest_3481, referred to here as moaA1 and moaA2. moaA2 exhibits a lower $w_{\text{nitr}}$ value (~0.8) than that of moaA1 (0.0) (Table 2). Thus, it appears that RCH2 relies predominantly on MoaA2. However, the average $w_{\text{nitr}}$ value among other genes in the biosynthesis pathway is much lower (~1.8) (see Table S3 in the supplemental material) than that for moaA2, indicating that MoaA1 can substitute somewhat. Under Mo limitation conditions, moaA2 fitness was less negative ($w_{9 \text{nm} \text{Mo}}$ value of ~0.3) than the $w_{\text{repl}}$ value of ~0.8. Surprisingly, moaA1 showed the opposite effect, with a $w_{9 \text{nm} \text{Mo}}$ value of ~0.7 and a $w_{\text{repl}}$ value of ~0.1 (Fig. 5; see Table S4). In addition, moaA1 had strong negative fitness when RCH2 was grown aerobically with nitrate as the sole nitrogen source (K. M. Wetmore, M. N. Price, and A. Deutschbauer, unpublished data). Hence, depending on the conditions, either MoaA1 or MoaA2 is primarily used. The strong fitness difference observed under aerobic conditions suggests a possible difference in oxygen sensitivity for MoaA1 and MoaA2; however, further investigation is required to discover the basis for their differential use.

Roles of Nir and Nor paralogs in nitrite and nitric oxide reduction. Genes involved in the reduction of nitrite have negative $w_{\text{nitr}}$–$w_{\text{nutr}}$ values (Fig. 3; see Table S3 in the supplemental material). These include genes encoding the cytochrome cd$_2$-containing nitrite reductase (nirS), the maturation factors for NirS (specialized heme biosynthesis genes nirCDEFJL), the diheme cytochrome $c_{552}$ (nirB) connected with nitrite reduction, and the activator (dnrD) for nitrite reductase and also for nitric oxide reductase. Values of $w_{\text{nutr}}$ near zero indicate that strain RCH2 obtains sufficient energy for robust growth from nitrate reduction alone, and in contrast to the growth of individual mutants, nitrite accumulation does not occur in a mutant library, because most of the population is competent for nitrite reduction. Furthermore, $w_{9 \text{nm} \text{Mo} \text{--reple}}$ values of ~1 indicate that Mo limits growth by reducing the rate of nitrate reduction such that it becomes rate limiting to respiration. Hence, reduction of nitrite (and nitric oxide and nitrous oxide) is needed to increase the rate of overall electron transfer in order to support maximal growth.

In addition to nirS, RCH2 also contains a nirK homolog, which codes for the copper-containing nitrite reductase. Under the growth conditions tested, however, nirK mutants had no phenotype (Table 2). Although it has been claimed that the presence of one of these nitrite reductase homologs in a given organism precludes the presence of the other (12, 39, 40), this is obviously not true (13). In fact, P. stutzeri strains BAL361, ATCC 14405, CCUG 29243, DSM 10701, F13, RCH2, and T13, as well as members of 37 other species, representing five bacterial phyla, one archaeal phylum (Euryarchaeota), and one green alga (Chlamydomonas), also contain genes encoding proteins of both types, according to InterPro (see Table S5 in the supplemental material). Outside Pseudomonas stutzeri, it is remarkable how infrequently and widely distributed such species are. The presence of two copies each of norBCD and nirQ lends credence to the hypothesis that both nitrite reductases are functional in strain RCH2 (Fig. 4). One copy is in the genome region that contains nirS and other nir genes (nirQ1 is adjacent to nirS on the opposite strand), while the other copy is found in the nirK operon (the gene order is norD2, nirQ2, norB2C2, and nirK). The Nir and Nor systems are known to work in tight cooperation to avoid the accumulation of toxic amounts of NO, and nirK has its own copy of the nor system that is likely
coexpressed. NirK was not required under the conditions tested, however, as mutations in the nirK cassette had no fitness effect, while single mutants of norB1C1D1 or nirQ1 all had strong negative \( w_{\text{nitrate}} \) values (Table 2).

Unlike nitrite reductase-related genes, however, nitrite oxide reductase-related genes, namely, norB1C1D1, nirQ1, and nmrS1, had \( w_{\text{nitrate}} \) and \( w_{\text{nitrite}} \) values of \(< -1\) (Table 2). The NorBC proteins are nitrite oxide reductase subunits, while NorD and NirQ are maturation factors required for NorBC activity (NrnS is discussed below). The negative fitness values of norB1C1D1 and nirQ1 mutants are likely due to the accumulation of toxic NO in the absence of these genes. As with nir genes, norB1C1D1 had \( w_{\text{gut}} \) Mo-replete values of \(< -1\) (Table S4 in the supplemental material); however, norD1 and nirQ1 had less negative values (\(-0.8\) and \(-0.5\), respectively). Phenotypes for norD1 and nirQ1 were further distinguished using Mo limitation growth conditions. For nirQ1, the \( W_{\text{700 nM Mo-9 nM Mo}} \) Value was \(-1.6\), compared to \(-0.2\) for norD1 (Table 1). While this in itself does not clarify the specific functions of NirQ and NorD, it does indicate that NirQ and NorD may not be completely interdependent and identifies a growth condition that is able to phenotypically distinguish between them, which may facilitate elucidation of their mechanism of action.

NirQ and NorD are reported to share a phenotype in P. stutzeri and likely work together to generate active nitric oxide reductase (NorBC), although it is not known how the activation occurs (6). NirQ encodes a member of the MoxR AAA protein family, which is widespread in both bacteria and archaea and whose members act cooperatively with von Willebrand type A (VWA) domain-containing proteins (such as NorD) as ATP-driven molecular chaperones (41). NirQ and NorD are therefore proposed to function as molecular chaperones in NorBC maturation, involving heme insertion, multisubunit complex assembly, and/or membrane insertion. It has been suggested that their function does not involve cofactors, because organisms containing norZ, encoding a longer-chain, single-subunit, quinol-oxidizing nitric oxide reductase, do not use nirQ/norD (17). However, it is now known that heme c interacts with both NorB and NorC subunits, and heme c is not found with norZ (4). Furthermore, it has been reported that nirQ deletion in P. stutzeri and Paracoccus denitrificans results in elevated production of NorBC and NrnS but that they have a greatly reduced heme content (42, 43). Hence, it seems reasonable that NirQ and NorD are involved in heme c insertion. This conclusion has important implications for the study of certain pathogens as well as denitrifiers (see above) (44). In fact, norD has been identified as a virulence factor in Brucella suis (20). Further characterization of norD and nirQ deletion mutants in RCH2 in terms of their response to Mo limitation and NorBC abundance and cofactor content should reveal more details of their maturation roles.

**Role of NrnS in NO metabolism.** While norBCD and nirQ are all known to be required for Nor activity, nmrS has not been shown to be required for denitrification or for the activity of any of the four reductases. However, the RB-TnSeq data reveal that NrnS is needed for efficient denitrification in RCH2. This organism contains three NrnS homologs, encoded by nmrS1 to -3 (Table 2). While the presence of two or three divergent nmrS paralogs is quite common (see Fig. S1 in the supplemental material), the reason for this is unknown. RCH2 nmrS1 (Psest_0799) is a monocistronic gene located within the cluster of nir, nor, and nos genes. In our experiments, inactivation of nmrS1 caused severe fitness defects (\( w_{\text{nitrate}} = -2.0; w_{\text{nitrite}} = -2.2\)), while nmrS2 or nmrS3 mutations had no effect (Table 2). However, in contrast to norB1C1D1 and nirQ1, nmrS1 unexpectedly had a positive \( w_{\text{gut}} \) Mo-replete value (\( w_{\text{gut}} = 1.4; w_{\text{nGut t}} = -0.4\)) (see Table S4). Consequently, it does not appear that NrnS contributes to NO reduction. This is consistent with work in Vibrio cholerae where an nmrS mutant showed hypersensitivity to NO under anaerobic conditions or in a \( \Delta \text{hmpA} \) (NO dioxygenase) background but showed no decrease in the NO degradation rate. There was also an increase in the formation of DNIC (dinitrosyl iron complex), the product of NO reacting with Fe-S clusters, indicating a connection with NO toxicity without direct removal of NO (19).

The connection between NrnS family proteins and NO seems quite strong from bioinformatic analyses. For example, of the 1,774 sequenced genomes that contain an nmrS gene in the InterPro family (IPR010266), 56% also contain a gene encoding a NO-forming nitrite reductase (a member of the Nir family [IPR001287] or containing a NrnS domain [IPR003143], with the same domain architecture as RCH2 NrnS). There are also many pathogens represented (see Fig. S1 in the supplemental material), and if all members of the genera Vibrio, Bordetella, and Neisseria are included, over 75% of nmrS-containing organisms are predicted to encounter NO either as a respiratory intermediate or as the result of a host attack. Furthermore, STRING analysis of RCH2 NrnS1 most strongly connects it with NorB and NorC by cooccurrence (data not shown).

Moreover, the Mo limitation data suggest that NrnS may be involved in NO sensing. A lack of Mo decreases the flux of nitrogenous oxides through denitrification (see above). While increasing the need for Nir and Nor (see above), this would also be expected to decrease steady-state NO concentrations during denitrification. Increased fitness with decreased NO is consistent with NrnS responding to NO toxicity, either via signaling/regulation or by repairing NO damage. The latter seems unlikely given that NrnS is membrane bound (18). A repair protein would be expected to be cytoplasmic so it can interact efficiently with damaged metal sites or redox molecules. However, NrnS is likely to interact directly with NO, as it contains heme and copper cofactors (18), and hemes are known for their exquisite sensitivity in binding NO (22). In addition, NO partitions to membranes (45). Hence, we suggest that NrnS may sense NO and signal to cytoplasmic transcription factors or Fe-S cluster repair proteins that result in the NO-protective effect previously observed (19).

Possible partner proteins for NO-activated NrnS include YggX and CyaY, which are both involved in the repair or protection of iron-sulfur proteins in response to nitrosative as well as oxidative damage (46). In support of this, the fitness values for yggX (Psest_0144) and cyaY (Psest_3762) match those for nmrS1. For example, yggX has \( w_{\text{nitrat}} \) \( w_{\text{nitrite}} \) and \( w_{\text{gut t}} \) Mo-replete values of \(-1.6, -2.4\), and \( 0.6\), respectively, and for cyaY, the values are \(-0.9, -1.6, \) and 0.4, respectively. This compares with respective values for nmrS1 of \(-2.0, -2.2\), and 1.0 (Table 2; see Table S4 in the supplemental material). Previous work showed that a yggX cyaY double deletion mutant has a more severe phenotype than that of either of the individual mutants (46), so if NrnS-based NO sensing leads to activation of both proteins, the nmrS mutant could share this more severe phenotype. A sensing role for NrnS also explains how an nmrS mutant in the photosynthetic denitrifier Rhodobacter sphaeroides has a disparate phenotype affecting nitrate and nitrite chemotaxes (18). Both nitrate and nitrite are reduced to NO, and thus the presence of all three electron acceptors
could be detected and responded to by NnrS. The membrane localization of NnrS would allow for detection of NO on one side of the cell versus another, preserving directional information that could be translated into chemotactic behavior. It should be noted that RCH2 has two known NO-responsive regulators: DnrD, recently shown to use heme to bind NO (47), and NorR. Of the two, DnrD plays a more prevalent role in denitrification, as shown by its much more negative $w_{nitrite}$ value ($-2.68$) than that for NorR ($-0.81$). DnrD is predicted to activate $mnrS$ expression (48). In contrast to NnrS, both DnrD and NorR are cytoplastic proteins, and while this facilitates direct gene regulation, the intracellular NO concentration would correlate less with extracellular NO for chemotactic signaling. In addition, the biological effects of NO are known to depend on its concentration (58, 22, 49, 50), which is one reason for the presence of multiple NO sensors in many bacteria (48, 51), enabling different responses at different NO concentrations. A low NO concentration might signify a nearby source of electron acceptor and induce chemotaxis toward that source, whereas a high NO concentration would be toxic and require a very different response. In accord with the multiple NO sensors in RCH2, there is evidence for a multilevel response. NO is required for transcription of $mnrSTB$ and $norBC$ via activation by DnrD, but transcription is prevented if the concentration reaches 500 nM, through an unknown mechanism (49).

Role of HemN in denitrification. Denitrification uses heme in three of the four core enzymes (Fig. 4) as well as in electron transfer cytochromes, and HemN catalyzes the oxygen-independent oxidation of coproporphyrinogen III to protoporphyrinogen IX as part of heme biosynthesis (Fig. 4). RCH2 contains four $hemN$ homologs, Psest_0276, Psest_2234, Psest_2404, and Psest_2537, referred to here as hemN1 to -4. Our data show a phenotype for hemN2 alone, with $w_{nitrate}$ and $w_{nitrite}$ values of $-3.2$ and $-3.0$, respectively (Table 2). However, hemN4 has previously been reported to encode the functional HemN protein (52), based on genomic proximity to and activation by the O$_2$-responsive regulator $frrA$/$FnrA$ (Psest_2538), although this was prior to the availability of the $P. stutzeri$ genome sequence (53). We propose that HemN2, not HemN4, is the primary HemN protein in heme biosynthesis. Many other microorganisms contain multiple hemN paralogs, as found in InterPro families IPR004558 and IPR004559, but a function other than the oxidation of coproporphyrinogen III has yet to be identified (37).

Role of NosL in nitrous oxide reduction. As with nitrite reduction, the genes known to be involved with nitrous oxide reduction exhibited consistently negative $w_{nitrite-nitrate}$ values. Hence, genes encoding nitrous oxide reductase (nosZ), the maturation factors (nosDFLY) for NosZ, and a membrane-bound Fe-S flavoprotein (nosR) exhibited similar phenotypes ($w_{nitrite-nitrate}$ value of $-1.4 \pm 0.2$ and $w_{nitrite}$ value of $-1.6 \pm 0.2$) (see Table S3 in the supplemental material). Thus, it is clear that at 20 mM nitrate, RCH2 grows well using nitrate alone as the terminal electron acceptor, whereas growth on nitrite requires the reduction of nitrite, nitric oxide, and (to a lesser extent) nitrous oxide. It was previously shown that heterologous expression of nosL was not required to produce active NosZ (unlike nosDFY) in $Pseudomonas putida$ (24). Our results demonstrate, however, that at least in RCH2, NosL is required for full nitrite-dependent growth of RCH2, presumably in its proposed role as a copper chaperone given the large negative $w_{nitrate}$ value ($-1.7$).

Role of the global regulator HsbA in denitrification. RB-TnSeq analysis revealed positive $w_{nitrite-nitrate}$ values for three regulatory/signaling genes associated with cell motility/biofilm formation, two of which have no known connection to nitrate reduction. One of these, Psest_1724 ($hsbA$) ($w_{nitrate} = -1.6$ [see Table S3 in the supplemental material]), had the largest $w_{TnSeq-Mb-require}$ value (2.5) (see Table S4) of any gene and is annotated as an anti-sigma factor antagonist. In $P. aeruginosa$, HsbA binds the anti-sigma factor FlgM, thereby freeing sigma factor 28, FliA, to facilitate transcription of flagellar and other motility-related genes (54, 55). RCH2 has two $fla$ homologs, Psest_1740 ($fla$A1) and Psest_4160 ($fla$A2), with 82% and 38% sequence identity, respectively. Curiously, $fla$A1 insertion mutants had mildly positive fitness ($w_{nitrate}$ and $w_{nitrite}$ values of 0.2 and 0.3, respectively), while $fla$A2 had little effect ($w_{nitrate}$ and $w_{nitrite}$ of <0.09). An anti-sigma factor antagonist should have a fitness with the same sign as that of the associated sigma factor, as it has the net effect of activating the sigma factor. These results imply that HsbA has another major regulatory effect on denitrification. Indeed, it was reported that deletion of $hsbA$ and site-directed mutagenesis to simulate constitutive activation of HsbA shared a hyperswarming phenotype (54). This is likely due to other regulatory effects that are not yet understood. HsbA is activated in a regulatory phospho-relay resulting in dephosphorylation by HsbR, and it has been suggested that HsbR may itself be an anti-sigma factor (making HsbA an antagonist to two anti-sigma factors), though the target sigma factor could not be determined (54). However, cofitness analysis (56, 57) of the entire RB-TnSeq data set from strain RCH2 (26) revealed that $hsbR$ (Psest_1725) mutants had strong cofitness (0.82) with the gene that encodes the RpoE sigma factor (Psest_3100), suggesting a functional connection between the proteins. Thus, we propose that HsbR acts as an anti-sigma factor to RpoE, a sigma factor known to be involved in promoting transcription of genes required for proper maintenance and repair of periplasmic and outer membrane components (58). The only gene with a higher cofitness with $hsbR$ (0.85) was a gene encoding a histidine kinase (Psest_0538), LadhS, which is known to promote biofilm formation and to decrease cytotoxicity in $P. aeruginosa$ (59). It appears that in $P. stutzeri$ these two systems may be connected and that LadhS participates in the same phospho-relay that activates HsbA.

$hsbA$ comprises a single anti-sigma factor antagonist domain (IPR002645) that is found throughout biology. For example, InterPro identifies 18,008 single-domain members, including 6 in strain RCH2. A phylogenetic tree of the 960 single-domain members of the $Pseudomonadaceae$ (215 species) shows six well-differeniated clades (see Fig. S2 in the supplemental material). A total of 203 of the 215 species contain a member of the specific HsbA clade, demonstrating conservation throughout this family of organisms. In addition, using the gene synteny tool SyntTax (60), it was found that the $hsbA$-$hsbR$-$hptB$ operon that encodes this regulatory phospho-relay is conserved in 77 of 78 members of the $Pseudomonadaceae$ that contain an $hsbA$ homolog (there are 81 $Pseudomonadaceae$ species in the SyntTax database).

Role of the chemotaxis protein CheR in denitrification. Another motility-associated gene with a denitrifying phenotype is the $cheR$ homolog (Psest_0393), which has a $w_{nitrite-nitrate}$ value of 1.2 (see Table S3 in the supplemental material). CheR proteins are class I methyltransferases that catalyze methylation of the signaling domains of certain chemoreceptors as part of a chemosensory cascade. They are annotated “methylase of chemotaxis methyl-
accepting protein,” after the function of the sole ortholog in *Escherichia coli* and *Salmonella* (61). RCH2 contains five homologs, and Psest_0393 is a close homolog of cheR3 of *P. putida* (71% sequence identity). Phenotypes for the two other cheR genes from *P. putida* were recently reported (biofilm deficiency for cheR1 and a chemotaxis defect for cheR2), but none could be identified for cheR3 (61). Our data suggest that Psest_0393 (and possibly CheR3) is involved in a nitrate-specific signal cascade, which could facilitate nitrate-responsive cell motility, such as nitrate chemotaxis, as well as other adaptive responses. This is compatible with the physiology of *P. putida*, while because it is not a denitrifier, it can use nitrate as a nitrogen source, using an assimilatory nitrate reductase, and thus is likely to have nitrate-responsive genes (62). Indeed, in a phylogenetic tree of the 5,861 CheR family (IPR00780) proteins with this domain architecture (see Fig. S3), Psest_0393 and CheR3 (but not CheR1 or CheR2) are found in a clade containing 205 *Pseudomonas* strains and several betaproteobacteria, all of which contain nitrate transporter genes. These organisms are predominantly aerobic, and several lack the gene encoding the NarX nitrate sensor. Interestingly, *E. coli* and *Salmonella* homologs cluster at the opposite end of the tree (see Fig. S3).

How Psest_0393 helps to regulate denitrification in RCH2, however, is not clear. Our data indicate that it plays a very important role, since it has a $w_{\text{nitrate}}$ value of $-0.8$ similar to that of NarX ($-0.9$) (see Table S3, the well-characterized nitrate sensor that activates the NarL transcription factor.

**Role of the hypothetical protein Psest_1511 in denitrification.** Psest_1511 also exhibited a strongly positive $w_{\text{nitrate}}$--nitrate value (1.3) (see Table S3 in the supplemental material), indicating a role in nitrate utilization. This is in accord with a previous report that mutation of the homolog in *P. aeruginosa* (PA1006) resulted in the loss of two Moco-requiring enzyme activities (nitrate reductase and xanthine oxidase) and a decreased intracellular concentration of Mo, indicating that PA1006 has a specific role in Mo homeostasis (63). Our Mo limitation results, however, suggest that in RCH2, Psest_1511 does not eliminate Moco production, because the value for $w_{\text{mM Mo} - \text{mM rep}}$ was only $-0.5$, compared to an average of $-2.5 \pm 0.3$ for Moco biosynthesis genes (excluding moaA). Previous work showed that during aerobic growth in the presence of nitrate, PA1006 mutation changed the expression of over 550 genes at least 2-fold and inhibited biofilm formation (64). Our findings support a more general regulatory role, possibly including Mo-independent effects on nitrate reduction. Psest_1511 and PA1006 are small proteins (<85 residues) containing a single, poorly characterized TusA-like domain (IPR001455). There are 7,568 single-domain (IPR001455) proteins found across a wide variety of bacterial and archeal species. Clearly, more remains to be discovered concerning this broad regulatory factor and how it functions in different organisms. Taken together, the denitrifying phenotypes of hsbA, Psest_0393, and Psest_1511 indicate common regulatory factors, and thus coordination, between denitrification, biofilm formation, and possibly nitrate chemotaxis, such that denitrifying growth is dependent on these regulatory factors in strain RCH2 and possibly other *Pseudomonas* species.

**Summary.** The use of genome-wide fitness analysis of transposon insertion mutants under denitrifying growth conditions for *P. stutzeri* strain RCH2 has given many new insights into our understanding of signaling, regulation, cofactor biosynthesis, and the maturation of the holoenzymes involved in denitrification (Fig. 4). Two homologs involved in Moco biosynthesis for nitrate reductase, MoaA1 and MoaA2, were both shown to be active under denitrifying conditions and are differentially regulated under aerobic, anaerobic, and Mo-limited conditions. In addition, a new transporter complex, OatBCA (for ABC-type oxanion transporter), encoded by Psest_2542 to Psest_2544, was identified that is able to transport molybdate at a concentration of 700 nM, although it likely does not exclusively transport molybdate. For nitrite reduction, we demonstrated that strain RCH2 uses the hemeccontaining NirS protein rather than the copper-containing NirK protein under the conditions tested, although it appears that both forms are likely functional. In heme biosynthesis, of the four HemN homologs encoded in the RCH2 genome, HemN2, not the previously proposed HemN4 (52), is the primary heme biosynthesis version. One of three mnrS paralogs in RCH2, mnrS1, is suggested to encode a membrane-bound NO sensor that participates in regulation of NO detoxification. Our data also indicate that in contrast to previous reports (24, 65), NosL, the proposed Cu chaperone for the nitrous oxide reductase (NosZ), is required for proper function of this enzyme in RCH2. Finally, the scope of regulatory influence held by several proteins was further defined, revealing regulatory connections between cell motility and denitrification. In particular, we propose that HsbR acts as an anti-sigma factor to the RpoE sigma factor and that HsbA may act as an antagonist to two anti-sigma factors, its established partner FlgM and HsBR. We propose that Psest_0393 participates in a nitrate-related signal cascade that involves nitrate chemotaxis, and our data show that a regulatory protein, Psest_1511, does not strongly perturb Mo homeostasis in RCH2, as was shown for *P. aeruginosa* (63), and more likely plays a general regulatory role in denitrification. In summary, we report evidence to further the understanding of the complex network of accessory proteins that support this important biochemical pathway.

**ACKNOWLEDGMENTS** We thank Pavel S. Novichkov and Alexey E. Kazakov for advice and discussion of the manuscript.

**FUNDING INFORMATION** This material by ENIGMA (Ecosystems and Networks Integrated with Genes and Molecular Assemblies) [http://enigma.lbl.gov/], a scientific focus area program at Lawrence Berkeley National Laboratory, is based upon work supported by the U.S. Department of Energy, Office of Science, Office of Biological & Environmental Research, under contract number DE-AC02-05CH11231. All authors are associated with this contract. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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Roles of Denitrification Genes Based on Mutant Fitness


