INTRODUCTION

Nitric oxide (NO), a diffusible free radical gas, is involved in many biological functions, including cell signaling, stress mediation, cytotoxicity, and defense (Wink et al., 1996). Because of its reactive nature, NO can directly interact with cellular components including cysteine thiolis, heme cofactors, iron–sulfur clusters, lipids, and DNA, as well as oxygen and other reactive oxygen and reactive nitrogen species (ROS/RNS) (Czapski & Goldstein, 1995; Jourd'heuil et al., 1997; Lancaster, 1994; Wink et al., 1996). The primary route for NO production in mammalian cells is through nitric oxide synthase (NOS; EC 1.14.13.39) (Knowles & Moncada, 1994). NOS catalyzes a two-step reaction to...
generate NO: In the first step, L-arginine is oxidized at its terminal (omega) guanidine nitrogen, resulting in the intermediate N(omega)-hydroxy-L-arginine (NOHA); in the second step, NOHA is oxidized releasing the terminal guanidine nitrogen as nitric oxide, with the generation of citrulline (Stuehr et al., 2004). Mammalian cells have three well-characterized isoforms of NOS (recently reviewed in Bogdan, 2015; Cinelli et al., 2019; Costa et al., 2016; Zhou & Zhu, 2009), two of which (neuronal NOS and endothelial NOS) participate in a wide variety of cell signaling processes, with the third isoform (inducible NOS; iNOS) an important component of immune cell oxidative burst.

Nitric oxide synthase homologs are also found in Gram-positive bacteria (Sudhamsu & Crane, 2009) such as \textit{Bacillus}, \textit{Staphylococcus}, \textit{Deinococcus}, and \textit{Streptomyces} (Bird et al., 2002; Johnson et al., 2008; Reece et al., 2009; Wang et al., 2007). These bacterial NOS (bNOS; EC 1.14.14.47) proteins are highly homologous to the oxygenase domain of mammalian NOS but lack the reductase domain that activates catalase (Gusarov & Nudler, 2005), while inactivation of nos as reductant (Gusarov et al., 2008). Previous studies suggested that bNOS can promiscuously associate with various reductases in the cell (Gusarov et al., 2008; Wang et al., 2007). In \textit{B. subtilis}, NOS-derived NO protects from oxidative stress caused by hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) by activating catalase (Gusarov & Nudler, 2005), while inactivation of nos in \textit{S. aureus} leads to increased sensitivity to H\textsubscript{2}O\textsubscript{2} despite an increase in catalase activity (Gusarov & Nudler, 2005; Mogen et al., 2017; Sapp et al., 2014). NOS-derived NO reduces antibiotic stress in \textit{B. subtilis} and \textit{S. aureus} (Gusarov et al., 2009; van Sorge et al., 2013), influences \textit{B. anthracis} and \textit{S. aureus} virulence (James et al., 2019; Popova et al., 2015; Sapp et al., 2014; van Sorge et al., 2013), modulates \textit{S. aureus} cell respiration (Chaudhari et al., 2017; Kinkel et al., 2016; Mogen et al., 2017), regulates growth and recovery after exposure to ultraviolet and ionizing radiation, and affects carotenoid formation in \textit{D. radiodurans} (Hansler et al., 2016; Patel et al., 2009).

A \textit{nos} homolog was previously identified in the \textit{Natronomonas pharaonis} genome (Gusarov et al., 2009; Sudhamsu & Crane, 2009), a haloalkaliphilic archaeon typically found in soda lakes (Gonzalez et al., 2010). Herein, we assess the prevalence of bNOS among archaeal genomes and characterize \textit{Nmn. pharaonis} \textit{nos} (npNOS) using a combination of bioinformatic, genetic, and biochemical approaches. Our results suggest that NOS-like homologs sharing high amino acid identity to bNOS are restricted primarily to haloalkaliphilic archaea. Furthermore, recombinant npNOS produces NO, as assessed by measuring nitrite production from NOHA. A \textit{Nmn. pharaonis} \textit{nos} mutant was also generated and characterized using new and/or optimized tools for probing the physiology of this organism.

## 2 | MATERIALS AND METHODS

### 2.1 | Strains and culture conditions

A complete list of strains and plasmids used in this study is found in Table A1 in Appendix 1. \textit{Escherichia coli} strains DH5\textalpha and JM110 were used for plasmid construction and/or maintenance, whereas strain BL21(DE3) was used for all protein expression studies. Antibiotics (ampicillin and kanamycin) for \textit{E. coli} plasmid maintenance were used at 50 µg/ml final concentration. \textit{E. coli} strains were grown on lysogeny broth (LB) agar plates from glycerol stocks (25% v/v) and incubated at 37°C for 16–24 h. A single colony was inoculated into 3 ml of LB broth in a sterile 15-ml aerated culture tube and grown as specified below for npNOS or saNOS expression. These starter cultures were each used to inoculate 1-L LB medium containing 0.5% (w/v) glucose.

\textit{Natronomonas pharaonis} DSM 2160 and isogenic nos::gyrB mutant (created in this study, see below) were used for all other experiments. NVM+medium was used for transformation and was prepared as described in (Haider, 2009) and Methods in Appendix 1. DSM 205, a casamino acid-based complex medium for growth of \textit{Natronobacterium} described by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (https://www.dsmz.de/collection/catalogue/microorganisms/culture-technology/list-of-media-for-microorganisms), was also used for indicated experiments and was prepared with modifications as described in Methods in Appendix 1 (referred to as “modified DSM 205”). \textit{Nmn. pharaonis} was grown at 40°C and 250 rpm in a 1:5 volume-to-flask ratio (aerobically) unless otherwise stated. The recipe for the acetate minimal medium from (Gonzalez et al., 2010) was used for Bioscreen growth experiments. The nos::gyrB mutant was cultured with novobiocin (3-5 µg/ml final concentration, depending on the experiment). For each experiment, \textit{Nmn. pharaonis} glycerol stocks (20% v/v) were struck on NVM+ or modified DSM 205 agar plates and incubated at 42°C for 5–10 days in plastic bags to minimize evaporation of plates. Starter cultures were inoculated by placing a loop-full of colonies into 10 ml of appropriate broth in a sterile 50 ml conical screw-top tube with the cap loosened and grown at 40°C and 250 rpm for 48–72 h. All experiments were performed using 10 ml starter cultures as the inoculum source for final media volumes adjusted as stated below for each experiment. Unless otherwise noted below, cultures were incubated at 40°C and 250 rpm.

### 2.2 | pET expression vector construction

All primers used in this study are listed in Table A2 in Appendix 1. The \textit{N. pharaonis} \textit{nos} gene was PCR amplified from genomic DNA with primer pair Npnos_NdeI_F/Npnos_NotI_R, and the \textit{S. aureus} \textit{nos} gene was amplified from strain UAMS-1 (Gillaspie et al., 1995) genomic DNA with primer pair Sanos_NdeI_F/Sanos_XhoI_R, using the Phusion High-Fidelity DNA Polymerase Kit. Each PCR product was ligated into pCR-Blunt (Thermo Fisher Scientific), transformed into \textit{E. coli} DH5\textalpha, and sequenced. The \textit{Nmn.} and \textit{S. aureus} \textit{nos} inserts were then cut from pCR-Blunt with Ndel and NotI (\textit{Nmn. nos}) or Ndel and Xhol (\textit{S. aureus nos}), ligated into pET24b, and transformed into \textit{E. coli} DH5\textalpha. Plasmids containing inserts were sequenced before transformation into \textit{E. coli} BL21(DE3) for protein purification.
2.3 | Protein purification

Escherichia coli BL21(DE3) containing pET24bnpNOS and pET24bsaNOS were each grown in LB supplemented with 5% (w/v) glucose in 1 L batches at 37°C until an OD_{600} of 0.6 was reached; then, 100 µg/ml of γ-aminobutyric acid was added. To induce the expression of npNOS, the pET24bnpNOS cultures were first incubated at 37°C shaking at 250 rpm to an OD_{600} of 0.6, induced with 0.5 mM IPTG, and then shifted to an 18°C incubator and grown overnight with shaking at 250 rpm. To induce the expression of saNOS, the pET24bsaNOS cultures were incubated at 37°C shaking at 250 rpm, induced with 0.5 mM IPTG at an OD_{600} of 0.6, and grown for 3–5 h at 37°C shaking at 250 rpm. After growth, cells were harvested and lysed via FRENCH Press method (Thermo Electron Co.).

Expression of npNOS and saNOS was confirmed in the cytosolic and pellet fractions of the cell lysate, using SDS-PAGE and immunoblots with an anti-C-terminal His-tag primary mouse antibody (Invitrogen). Proteins were purified via nickel affinity chromatography and eluted at 100 mM imidazole. The purified fractions were buffer exchanged to remove imidazole by centrifuging samples (9000 g for 10 min) on 30-kDa centrifugal filters (Microcon) and exchanging the buffer 3 times with the following reaction buffers: npNOS buffer (50 mM HEPES, 3 M NaCl, pH 8) and saNOS buffer (50 mM HEPES, 500 mM NaCl, pH 8).

2.4 | Hydrogen peroxide (H_2O_2)-assisted NOS oxidation of N(omega)-hydroxy-L-arginine (NOHA)

Nitric oxide synthase activity of the recombinant proteins was validated through H_2O_2-assisted NOS oxidation of NOHA assay as first described in (Pufahl et al., 1995) and adapted from (Bird et al., 2002). One milliliter reactions were set up with 50 µl or 25 µl of NOS (either npNOS or saNOS), 10 units of superoxide dismutase, and 0.5 mM DTT. To begin the reaction, 50 µl of 10 mM NOHA and 3.4 µl of 0.3 mg/ml of pronase E and incubated in a 42°C water bath for 48 h. At this point, 3 ml of cells was centrifuged at 10,000 g, resuspended in 150 µl of buffered spheroplasting solution—glycerol. 15 µl of EDTA pH 8.0 was carefully added to the cells, and the mixture was incubated for 10 min at room temperature. Linearized pUC19nos::gyrB DNA (cut with Scal digestion enzyme) was added afterward, and cells were incubated as described above for 5 min.

Thereafter, 150 µl of PEG solution (60% (v/v) PEG 600 at 65°C and 40% (v/v) unbuffered spheroplasting solution) was carefully added to the cells. One milliliter of NVM+medium was added, and cells were subsequently centrifuged for 3 min. All supernatant was removed, and cells were resuspended in 1 ml of medium and recovered at 37°C shaking at 250 rpm for 2 days. Cells were plated on medium containing 5 µg/ml of novobiocin and incubated at 42°C. Once colonies appeared on plates, they were placed in culture tubes containing 4 ml of NVM+medium and 5 µg/ml of novobiocin and grown at 40°C, 250 rpm. Cells were re-cultured and diluted 1/10 in fresh medium containing novobiocin (5 µg/ml) every 3 days for a total of 15 passages. At passages 10 and 15, cells were plated on novobiocin (5 µg/ml) and individual colonies were screened for complete gene disruption (primers NpNOS_promoter_F, gyrB_check_R, and NpNOS_check_R) with primers flanking the cloning region. Several mutants lacking wild-type nos alleles were found at passage 15 and were stocked in glycerol, and one consistent isolate (A2) was used for further experiments.

2.5 | Creation of nos::gyrB mutant

The Nmn. pharaonis nos gene was amplified from genomic DNA with primers NpNOS_EcoRI_F, and NpNOS_HindIII_R, digested with EcoRI and HindIII and ligated into pUC19 cut with the same enzymes (New England Biolabs) to generate pUC19-npunos. The novobiocin antibiotic resistance gene gyrB from plasmid pJAM809 (Humbard et al., 2009) was excised by Blpl and Nsil digestion, followed by Klenow treatment (New England Biolabs) to generate blunt ends. The Nmn. nos gene contains a naturally occurring Nael site located 560-bp downstream of the ATG start codon. Since this restriction site is not present in the pUC19 sequence, digestion of pUC19-npunos with Nael was performed to linearize the plasmid, followed by dephosphorylation with Antarctic Phosphatase and Klenow treatment to generate blunt ends. This linearized DNA was then ligated to blunt-ended gyrB and transformed into E. coli DH5α to generate pUC19nos::gyrB. The orientation of the gyrB insertion in this plasmid was determined via Sanger sequencing. Plasmid pUC19nos::gyrB was then transformed into E. coli JM110, and plasmid was isolated in quantities of 20–30 µg for transformation into Nmn. pharaonis.

The Nmn. pharaonis transformation protocol was adapted from (Haider, 2009). Nmn. pharaonis cultures were grown at 37°C 250 rpm in 10 ml of modified DSM 205 containing 70 µg/ml of bacitracin in a 50 ml conical screw-cap tube to an OD_{600} of 0.4–0.6 (about 3 days). Then, cells were centrifuged at 3900 g and resuspended with a half volume of buffered spheroplasting solution + glycerol containing 0.3 mg/ml of pronase E and incubated in a 42°C water bath for 48 h. At this point, 3 ml of cells was centrifuged at 10,000 g, resuspended in 150 µl of buffered spheroplasting solution—glycerol. 15 µl of EDTA pH 8.0 was carefully added to the cells, and the mixture was incubated for 10 min at room temperature. Linearized pUC19nos::gyrB DNA (cut with Scal digestion enzyme) was added afterward, and cells were incubated as described above for 5 min. Thereafter, 150 µl of PEG solution (60% (v/v) PEG 600 at 65°C and 40% (v/v) unbuffered spheroplasting solution) was carefully added to the cells. One milliliter of NVM+medium was added, and cells were subsequently centrifuged for 3 min. All supernatant was removed, and cells were resuspended in 1 ml of medium and recovered at 37°C shaking at 250 rpm for 2 days. Cells were plated on medium containing 5 µg/ml of novobiocin and incubated at 42°C. Once colonies appeared on plates, they were placed in culture tubes containing 4 ml of NVM+medium and 5 µg/ml of novobiocin and grown at 40°C, 250 rpm. Cells were re-cultured and diluted 1/10 in fresh medium containing novobiocin (5 µg/ml) every 3 days for a total of 15 passages. At passages 10 and 15, cells were plated on novobiocin (5 µg/ml) and individual colonies were screened for complete gene disruption (primers NpNOS_promoter_F, gyrB_check_R, and NpNOS_check_R) with primers flanking the cloning region. Several mutants lacking wild-type nos alleles were found at passage 15 and were stocked in glycerol, and one consistent isolate (A2) was used for further experiments.

2.6 | Growth curves

Wild-type and nos::gyrB mutant strains were streaked on modified DSM 205 medium (supplemented with 5 µg/ml novobiocin, as
necessary) and grown at 42°C for 5–6 days. A loop-full of colonies were used to inoculate a 50 ml conical tube containing 10 ml of media, and cultures were grown at 40°C and 250 rpm for about 2 days (to mid/late exponential growth phase). To measure initial OD600 for inoculation, 500 µl of each culture was centrifuged at 14,000 g for 5 min to remove excess S-layer in the supernatant, and pellets were resuspended in a fresh sterile medium. This was found to give a more accurate measure of viable cells. Unless otherwise stated, cultures were inoculated to an OD600 = 0.01 with each appropriate strain and grown in 50 ml of media in a 250 ml sterile foil top Erlenmeyer flask (1:5 volume-to-flask ratio, 40°C, and 250 rpm). The OD600, CFU/ml of DSM 205 medium was used to control for background.

Cultures were grown in modified DSM 205 medium as described above. One milliliter samples were taken at 0, 12, 24, 36, 48, 72, 96, 120, and 144 h of growth to determine extracellular acetate concentrations. Acetate was measured using an Acetic Acid Kit (R-Biopharm) that links acetyl-CoA and oxaloacetate to NAD+ reduction by citrate synthase and L-malate dehydrogenase. Procedures for measuring acetate were conducted according to the methods outlined in the R-Biopharm kit instruction manual, with an adjustment of the reaction volume to 283 µl. Assays were performed in 96-well culture plates (Costar 3596), and the absorbance at 340 nm was measured in a Cytation 3 Imaging Microplate Reader (BioTek). Each supernatant had n = 3 technical replicates and 2 biological replicates per strain per time point and modified DSM 205 medium was used to control for background.

2.7 Quantification of extracellular acetate

Cultures were grown in modified DSM 205 medium as described above. One milliliter samples were taken at 0, 12, 24, 36, 48, 72, 96, 120, and 144 h of growth to determine extracellular acetate concentrations. Acetate was measured using an Acetic Acid Kit (R-Biopharm) that links acetyl-CoA and oxaloacetate to NAD+ reduction by citrate synthase and L-malate dehydrogenase. Procedures for measuring acetate were conducted according to the methods outlined in the R-Biopharm kit instruction manual, with an adjustment of the reaction volume to 283 µl. Assays were performed in 96-well culture plates (Costar 3596), and the absorbance at 340 nm was measured in a Cytation 3 Imaging Microplate Reader (BioTek). Each supernatant had n = 3 technical replicates and 2 biological replicates per strain per time point and modified DSM 205 medium was used to control for background.

2.8 Pigment quantification via methanol extraction

*Natronomonas pharaonis* wild-type and nos mutant strains were grown in modified DSM 205 (complex medium) at 40°C and 250 rpm for 120 h. At this point, 4.5 ml of each culture was centrifuged at 14,000 g for 5 min and then washed in 1 ml in Halo-HBSS (filter-sterilized Hank’s balanced salt solution (Hanks & Wallace, 1949) without phenol red), supplemented with 3.5 M NaCl, and then adjusted to pH 8.5 with 6 N NaOH. Cells were resuspended in 1 ml of Halo-HBSS again and 100 µl was removed and added to 900 µl of sterile Halo-HBSS to determine OD600 of cells for standardization. Cells were then centrifuged and resuspended in 650 µl of methanol. Cells were vortexed for at least 30 s and heated at 55°C for 5–10 min until cell debris became colorless. At this point, cells were centrifuged at 14,300 g for 3 min, and 600 µl of pigmented containing methanol was added to 400 µl of fresh methanol and the absorbance at both 490 and 525 nm was measured using a GENESYS 10 Bio Spectrophotometer (Thermo Scientific). Wild-type OD600 was adjusted to 1.0, and all other sample ODs were adjusted to a relative OD ratio based on wildtype. The absorption for 490 nm and 525 nm was each normalized to the sample's respective relative OD ratio.

2.9 DAF-FM diacetate staining

This assay was performed following protocols described in (Lewis et al., 2016) with slight modifications. Cultures were grown in modified DSM 205 medium, and at 24 h growth, a 5 ml aliquot was harvested by centrifugation at 3901 g for 10 min. Cells were resuspended in 1 ml Halo-HBSS with 10 µM DAF-FM diacetate and incubated statically at 37°C for 60 min. Cells were then pelleted (12,000 g for 5 min) and washed once in fresh Halo-HBSS. Cells were finally resuspended in 700–900 µl of Halo-HBSS to adjust for cell density differences, triplicate aliquots (200 µl) of stained cell suspensions were transferred to black optically clear-bottom 96-well plates (Costar 3904), and both OD600 and fluorescence (relative fluorescent units, RFU) were measured as described in (Lewis et al., 2016) using a Biotek Synergy HT fluorescent plate reader.

2.10 CTC staining

Respiration was measured using 5 mM 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) reduction, adapted from (Lewis et al., 2015; Mogen et al., 2017). Briefly, cultures were grown in modified DSM 205 medium at 40°C and 250 rpm as described above. At 24 h of growth, a culture volume equivalent to OD600 = 1.0 from each strain was harvested by centrifugation at 3901 g for 10 min. Cells were washed once with 1 ml of Halo-HBSS and resuspended in 650 µl of Halo-HBSS with 5 mM CTC. Triplicate aliquots (200 µl) of stained cell suspensions were transferred to Costar 3904 plates. The plate was shaken 5 s before each read, and then, RFU (EX: 485 ± 20 nm, EM: 645 ± 40 nm) and OD600 of each were measured at 10-min intervals at 37°C for 60 min using a Biotek Synergy HT Plate Reader. RFU collected at 30 min (the time point at which optimal fluorescence was measured, presented in Figure 4) was then normalized to the first (t = 0) OD600 reading of each well.
Oxygen consumption was measured using a 4-Channel Free Radical Analyzer (TBR-4100, World Precision Instruments) and Clark-type electrode (ISO-Oxy-2, World Precision Instruments) with methods adapted from (Mogen et al., 2017). Exponential-phase cultures (24-h) growing aerobically in 100 ml (1:5 volume-to-flask ratio) of modified DSM 205 medium were harvested by centrifugation (3901 g, 22°C for 5 min), supernatant media was discarded, and pellets were resuspended in 700 μl of Halo-HBSS. The O₂ assay was conducted in a 20-ml disposable scintillation vial with 13 ml of Halo-HBSS buffer amended with 4 mM potassium acetate. The solution was stirred at 350 rpm and prewarmed to 40°C. O₂ consumption was measured for 25 min after the addition of 500 μl of the resuspended culture, and 2 ml of mineral oil overlay added to the vial to prevent oxygen equilibration from the atmosphere. The rate of O₂ loss for the nos mutant was determined using the slope O₂ decrease after 10 minutes incubation and normalized to wildtype. O₂ consumption rate was calculated by O₂ decrease after 10 minutes incubation normalized to OD₆₀₀ of resuspended culture (remaining 200 μl used).

2.12 | UV stress assay

*Natronomonas pharaonis* wild-type and nos mutant strains were grown in modified DSM 205 medium at 40°C and 250 rpm for 48 h in 50-ml conical screw-cap tubes, followed by sub-culturing to an OD₆₀₀ = 0.02 into 50-ml media in 250-ml sterile foil top Erlenmeyer flasks. Cultures were then incubated for an additional 48 hours, followed by centrifugation at 4000 g at 20°C for 10 min. Cells were resuspended in Halo-HBSS to an OD₆₀₀ = 0.4–0.5, and the volume of each culture was split into two square plastic Petri dishes. One plate was exposed to 750 mJ/cm² UV in HL-2000 HybriLinker Hybridization Oven/ UV Crosslinker (UVP), and the other plate was the untreated control. After UV exposure, cultures were transferred to 125 ml sterile foiled Erlenmeyer flasks and diluted 1:2 with modified DSM 205 medium. Flasks were incubated and recovered under light at 40°C and 250 rpm, and CFU/ml via serial dilution was measured at t = 0, 1, 3, 6, and 24 h. Data are expressed as percent survival between the untreated and UV treated samples for each strain at each time point.

2.13 | Statistical analysis

All quantitative data were analyzed for statistical significance using SigmaPlot software version 14, build 14.0.3.192 (Systat Software Inc.). Data were tested for normality and equal variance before choosing the appropriate parametric or non-parametric test, respectively.

### RESULTS AND DISCUSSION

#### 3.1 | *Nmn. pharaonis* NP_1908A encodes a predicted bacterial NOS-like protein

The *S. aureus* MRSA252 nos open reading frame (GenPept accession| CAG40992) was used to tBLASTn search the NCBI RefSeq genome database, restricting the search to Archaea (TAXID 2157). This analysis yielded 15 hits (Table A3 in Appendix 1) with extensive query coverage (94%–98%), and a high degree of amino acid (AA) identity to *S. aureus* NOS (saNOS; 45%–51%). All identified NOS-containing genomes belonged to the Halobacteria class. Of these, 8 belonged to the Natrialbaceae, 5 to the Halobacteriales, and 2 to the Haloferacales. Interestingly, proteins with high AA identity to saNOS were not identified in methanogen or Crenarchaeota genomes in this analysis. Clustal-Omega AA alignment (Sievers et al., 2011) of a subset of these archaeal NOS homologs to saNOS and *B. subtilis* NOS (bsNOS) indicated high conservation of most active site, heme-binding and dimerization residues (Figure A1 in Appendix 1). At *B. subtilis* isoleucine 218, *Nmn. pharaonis* and most other Halobacteria NOS contain a valine, which is conserved in eukaryotic NOS proteins (Pant et al., 2002; Wang et al., 2004). This switch has been previously characterized in the context of *B. subtilis* NOS (bsNOS) and mammalian iNOS: When bsNOS was mutated I218V, the transition state of the heme was directly affected, while the mutation of iNOS V346I had a decrease in the dissociation rates of NO (Wang et al., 2004; Weisslocker-Schaetzel et al., 2017). Although the occupation of isoleucine versus valine at this conserved domain appears to play a role in NOS enzyme kinetics, this residue is not conserved among all potential nos homologs from archaeal genomes: *Nbt. gregoryi* contains the bacterial-like isoleucine, while most other archaeal NOS homologs (including *Nmn. pharaonis*) contain valine at this AA position (Figure A1 in Appendix 1).

#### 3.2 | Demonstration of in vitro recombinant npNOS activity

To verify its gene function, npNOS (RefSeq locus tag NP_RS04750, original locus tag NP_1908A) and saNOS (RefSeq locus tag SAR_RS10475, original locus tag SAR2007) as a control were both heterologously expressed and purified from *E. coli*. NO production by each purified recombinant protein was measured using a H₂O₂-assisted NOHA oxidation assay (Pufahl et al., 1995), as previously described for saNOS and other NOS enzymes (Bird et al., 2002; Montgomery et al., 2010). In this assay, the NOS enzyme catalyzes the oxidation of N(omega)-hydroxy-L-arginine (NOHA) to NO, with H₂O₂ providing electrons instead of a redox partner. NO production by NOS is coupled to nitrite formation, that is, subsequently quantified using the Griess reagent. The saNOS and npNOS proteins produced 72.07 ± 26 and 77.35 ± 19 μmol nitrite min⁻¹ mg⁻¹, respectively.
These results confirm that nNOS catalyzes NO production in an in vitro assay at a comparable catalytic rate to the well-characterized sNOS (Bird et al., 2002), which suggests its physiological function may also be similar.

3.3 Generation of a Nmn. pharaonis nos mutant

To further investigate the physiological role of the NP_1908A gene (henceforth referred to as nos; Figure 1A), we generated a Nmn. pharaonis nos mutant through gene disruption, in which the Nmn. nos gene sequence interrupted with a novobiocin resistance gene (nos::gyrB) was cloned into a suicide plasmid and subsequently introduced into Nmn. pharaonis using a modified transformation protocol (Figure 1B) (Derntl et al., 2015). A representative double-crossover clone which demonstrated a mixture of wild-type and nos::gyrB alleles by PCR was then sub-cultured for ≥15 passages, yielding several colonies that completely lost the nos wild-type allele. Mutant A2 (nos::gyrB; Figure 1C) was chosen for further experiments.

Several attempts were made to complement the nos::gyrB mutant with cloning vector pRO5 developed for Natrilaiba magadii (Mayrhofer-Iro et al., 2013) modified to replace novobiocin selection with a mevinolin resistance cassette and containing the Nmn. nos gene and upstream promoter region. However, transformation efforts were not successful in either the wild-type or mutant strains, highlighting the complexity of transforming haloalkaliphilic archaea. In the absence of a genetic counterselection system, the Nmn. nos::gyrB mutant had to be sub-passaged 15 times to completely lose the nos wild-type allele after the original integration event of the nos::gyrB allele into its genome. This sub-passaging step was necessary since Nmn. pharaonis, like many other halophilic archaea, is polyploid (Breuert et al., 2006). This, combined with our inability to perform genetic complementation of the nos::gyrB mutant, prompted our re-sequencing of the genomes of both wild-type and nos::gyrB mutant, which were analyzed for single nucleotide polymorphisms (SNPs) (Table A4 in Appendix 1), comparing each to the Nmn. pharaonis DSM 2160 reference genome (NC_007426.1). This analysis revealed that both the wild-type and nos::gyrB mutant shared eleven identical SNPs in coding regions that were distinct from the published genome sequence (Table A4 in Appendix 1). Additionally, the nos::gyrB mutant contained three SNPs that were not detected in the re-sequenced wild-type genome (Table A4 in Appendix 1): a P16S substitution in a predicted restriction endonuclease (NP_RS00005), a stop codon at position 23 in a predicted CheY response regulator (NP_RS05195), and a frameshift mutation at A227 in NP_RS12645 (NP_5136A), which bears 60-82% amino acid identity to proteins annotated as cation acetate symporters in several halophilic archaea. The observed accumulation of genomic

![Figure 1](image-url)
SNPs in the Nmn. nos::gyrB mutant highlights the need for the development of future tools for Nmn. genetic manipulation that are similar in strategy to the pyrE(F)-positive selection/counterselection systems for mutant generation in Haloferax species (Bitan-Banin et al., 2003; Liu et al., 2011), or possibly CRISPR-based gene silencing as recently described for Hfx. volcanii (Stachler & Marchfelder, 2016).

3.4 Growth and physiological differences observed in the nos::gyrB mutant

When grown aerobically in shaking flasks using modified DSM 205 (a complex casamino acid-based medium), a slight increase in mid-late exponential-phase growth was observed in the nos::gyrB mutant (Figure 2A,B). Additionally, a significant reduction in viable cells (as assessed by CFU/ml) during late-stationary phase was observed in the nos::gyrB mutant but not in the wildtype, starting just after 120 hours growth (Figure 2B). Because of the A227 frameshift mutation identified in the NP_RS12645 (NP_5136A) gene (predicted cation acetate symporter; Table A4 in Appendix 1) of the nos::gyrB mutant, extracellular acetate levels during growth were also compared between wild-type and mutant strains. During early exponential growth, acetate was present in both wild type and mutant, albeit at low concentrations, and disappeared by mid-exponential growth phase (Figure 2C). However, the nos::gyrB mutant accumulated acetate throughout stationary phase (96–144 h growth), whereas...
acetate was undetectable in the wildtype (Figure 2C). Since there was very little acetate detected in the medium at the time of inoculation (<0.1 mM), it is possible that the extracellular acetate detected in stationary phase nos::gyrB mutant cultures was due to increased acetate excretion (due to overflow metabolism) and/or an inability to reuptake excreted acetate due to the SNP mutation in the NP_5136A, a putative cation/acetate symporter. Interestingly, Nmn. pharaonis can sustain growth using acetate as a sole carbon source (Falb et al., 2005; Gonzalez et al., 2010). Comparison of growth in a Bioscreen C shaking microplate system in minimal medium (Gonzalez et al., 2010) with acetate as the sole carbon source (Figure 2D) revealed that the nos::gyrB mutant growth on acetate was overall reduced throughout exponential and early stationary growth phases compared to wildtype. This correlated with a decrease in maximum specific growth rate on acetate for the nos::gyrB mutant (0.029 ± 0.001 per hour) compared to wildtype (0.045 ± 0.004 per hour). Although these data suggest that growth is reduced in the nos::gyrB mutant when utilizing acetate as a carbon source, it is unclear whether this phenotype is specifically due to disruption of nos, and/or a result of the SNP detected in NP_5136A.

Interestingly, nos::gyrB mutant cultures grew better during exponential growth in Erlenmeyer flasks (Figure 2A,B), compared to growth in the Bioscreen (Figure 2D) relative to wild type when grown in modified DSM 205 medium. This may be due to altered aeration between the two growth methods (Biesta-Peters et al., 2010; Duetz & Witholt, 2004) and/or due to a slightly larger starter inoculum used in the Bioscreen experiment. To further characterize the effect of NO on the growth of the wildtype and nos::gyrB mutant, Bioscreen cultures were grown in modified DSM 205 with either the chemical NO donor DETA-NONOate or DETA alone (control) (Figure 2E). The OD$_{600}$ of the nos::gyrB mutant at the experimental endpoint was approximately 1.5-fold higher in the presence of NO donor relative to the DETA control, whereas a comparable increase was not observed in the wild-type strain. NO donor improved exponential-phase growth of both the wildtype and nos::gyrB mutant, but this effect was more pronounced in the nos::gyrB mutant, restoring it’s maximum specific growth rate from 0.072 ± 0 per hour (with DETA alone) to 0.107 ± 0.002 per hour (with NO donor), representing a ~1.49-fold increase. In comparison, the wild-type strain had a maximum specific growth rate of 0.108 ± 0.005 per hour in the DETA alone control culture, and 0.124 ± 0.007 per hour in the presence of NO donor, representing a ~1.15-fold increase.

It was also consistently observed in both flask-based and Bioscreen cultures that the nos::gyrB mutant visually displayed altered pigmentation relative to the wild type (Figure 3A). Pigment accumulation was therefore quantified using methanol-extracted pigments from cell samples collected from wild-type and mutant cultures at 120 h growth. Initial spectral analysis from 340–600 nm revealed peaks at 490 and 525 nm in both sets of cultures; however, the nos::gyrB mutant showed increased absorbance values at both of these wavelengths (Figure 3B). These peaks may reflect differences in bacterioruberin (525 nm) and/or its upstream precursor phytofluene (490 and 525 nm) (Ekiel et al., 1986; Falb et al., 2008).

While bacterioruberin is well known in Nmn. pharaonis as it forms a complex with the light-driven ion pump halorhodopsin (Sasaki et al., 2012), it can also serve as an antioxidant to protect halophiles from oxidative damage (Miller et al., 1996) and can reinforce membrane rigidity (Lazrak et al., 1988; Rodrigo-Baños et al., 2015). Mutation of bacterial nos has also been shown to impact cell membrane-associated carotenoid levels in S. aureus (Sapp et al., 2014), Staphylococcus xylosus (Ras et al., 2017) and D. radiodurans (Hansler et al., 2016).

### 3.5 Mutation of nos impacts Nmn. pharaonis intracellular RNS levels, cell respiration and stress resistance

Assessment of endogenous NO, respiratory, and stress resistance phenotypes were all performed in mid-late exponential-phase (24–48 h growth) shaking flask cultures. DAF-FM diacetate reacts with NO and/or other reactive nitrogen species (RNS) to form a fluorescent benzotriazole and was previously used to determine intracellular NO/RNS levels in S. aureus (Lewis et al., 2016; Sapp et al., 2014). DAF-FM diacetate staining was adapted here for use with Nmn. pharaonis. To first verify that DAF-FM diacetate was taken up by archaeal cells and responds to NO/RNS, exponential-phase
Halophilic archaea are exposed to strong levels of ultraviolet (UV) radiation in their natural environments [reviewed in (Jones & Baxter, 2017)]. Since UV stress resistance has been reported to be decreased in Deinococcus radiodurans nos mutants (Hansler et al., 2016; Patel et al., 2009), it is plausible that UV stress could have a more negative outcome for the *Nmn. pharaonis* in the absence of npNOS. *Nmn.* wildtype and nos::gyrB mutant were therefore assessed for viability and recovery when treated with 750 × 100 µJ/cm² of ultraviolet light (UV). In this assay, the *Nmn.* nos::gyrB mutant had significantly (*p* < 0.05) reduced survival rates immediately after UV treatment compared to wild type (Figure 5), with both strains.
displaying only a small amount of recovery in viability throughout the 24 hour period following initial UV exposure. Increased UV susceptibility has also been demonstrated in \textit{D. radiodurans nos} mutants, and while this was complemented both genetically and by supplementation with chemical NO donors, the exact mechanism remains unknown (Hansler et al., 2016; Patel et al., 2009).

4 | CONCLUSIONS

In this study, bioinformatic and biochemical approaches have confirmed that \textit{Nmn. pharaonis} ORF NP_1908A encodes a bacterial-like NOS protein which can catalyze NO production from NOHA. Furthermore, \textit{Nmn. pharaonis} NOS and/or its derived NO appears to serve a similar function to its bacterial counterpart with respect to protection against UV resistance, as the \textit{Nmn. pharaonis nos::gyrB} mutant displayed decreased viability following UV exposure. Moreover, NOS and/or derived NO modulates aerobic respiration in \textit{S. aureus} (Chaudhari et al., 2017; Kinkel et al., 2016; Mogen et al., 2017), and also appears to play a role in \textit{Nmn. pharaonis} aerobic respiration, as the nos::gyrB mutant experienced increased rates of oxygen-based respiration. Successful genetic manipulation of \textit{Nmn. pharaonis} as described herein opens many avenues for further investigation into its unique physiology necessary to thrive in extreme haloalkaliphilic environments.

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CONFICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Silvia S Orsini: Conceptualization (equal); Formal analysis (equal); Funding acquisition (supporting); Investigation (equal); Methodology (equal); Writing-original draft (equal); Writing-review & editing (supporting). Kimberly L James: Conceptualization (supporting); Formal analysis (equal); Investigation (equal); Methodology (equal); Validation (equal); Writing-original draft (equal); Writing-review & editing (equal). Destiny J Reyes: Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Writing-original draft (supporting); Writing-review & editing (supporting). Ricardo L Couto-Rodriguez: Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Writing-original draft (supporting); Writing-review & editing (supporting). Miriam Kolog Gulko: Methodology (supporting); Writing-original draft (supporting). Angela Witte: Methodology (supporting); Resources (supporting); Writing-original draft (supporting); Writing-review & editing (supporting). Ronan K Carroll: Data curation (equal); Formal analysis (equal); Writing-original draft (supporting); Writing-review & editing (supporting). Kelly C Rice: Conceptualization (equal); Data curation (equal); Funding acquisition (lead); Project administration (lead); Writing-original draft (equal); Writing-review & editing (equal).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

All data in this manuscript are included as figures or in the Appendix. All DNA raw sequencing data files have been deposited to the National Center for Biotechnology Information's Sequence Read Archive (SRA), under BioProject PRJNA630572: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA630572.

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FIGURE 5 Nmn. nos::gyrB mutant displays reduced UV stress resistance. 48-hour cultures were split and treated with either 750 x 100 µJ cm\textsuperscript{-2} UV or no treatment, then recovered and plated. Data represented as percent survival of CFU ml\textsuperscript{-1} of treated to untreated cultures at each time point, n = 3 independent experiments. Error bars = SEM. *p < 0.05, Student's t test comparing WT to nos::gyrB mutant at each time point.
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**APPENDIX**

**ADDITIONAL METHODS**

**Recipes for modified DSM 205 and NVM+ media used in this study:**

| Modified DSM 205          |
|---------------------------|
| Casamino acids            | 15 g                     |
| Tri-Na citrate            | 3.0 g                    |
| Glutamic acid             | 2.5 g                    |
| MgSO4 × 7H2O              | 2.5 g                    |
| KCl                       | 2.0 g                    |
| NaCl                      | 204 g                    |
| H2O                       | 950 ml                   |

Autoclave, then add 12 ml of filter-sterilized 20% Na2CO3 to adjust pH to 8.5.

| NVM+                      |
|---------------------------|
| Casamino acids            | 8.8 g                    |
| Yeast extract             | 11.7 g                   |
| Tri-Na citrate            | 0.8 g                    |
| KCl                       | 2.35 g                   |
| NaCl                      | 235 g                    |
| H2O up to for plates      | 934 ml                   |
| Agar                      | 8 g                      |

After autoclaving, the medium or the agar was completed with:

| 0.57 M Na2CO3 filter sterilized | 65 ml |
| 1 M MgSO4 (autoclaved)          | 1 ml  |
| 20 mM FeSO4 filter sterilized   | 1 ml  |
| 20% Na2CO3 filter sterilized    | Until pH reaches 9.0     |

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**Bioinformatics analysis**

The *S. aureus* MRSA252 nos open reading frame (GenPept accession# CAG40992) was used to tBLASTn search (Altschul et al., 1990) the NCBI RefSeq genome database, restricting the search to Archaea (TAXID 2157). Clustal Omega (Sievers et al., 2011) was used to align the saNOS AA sequence from *S. aureus* MRSA 252 (CAG40992) to NOS AA sequences in the following organisms (GenPept accession #s indicated in brackets): *B. subtilis* 168 (AQR84931.1), *Nmn. pharaonis* DSM 2160 (CAI49045.1), *Natronobacterium gregoryi* SP2 (AFZ73990.1), *Natronomonibius egypytiacus* (ARS90434.1), *Halorientalis* sp. IM1011 (AQL41467.1), *Halobiforma nitratireducens* JCM 10879 (EMA47161.1), *Halostagnicola kamekurae* (SFS56640.1), *Haloplanus salinus* (RCU47147.1), *Halalkalicoccus pauchalophilus* (KYH24998.1), *Halonotius pteroides* (RJX51750.1), *Halovenus araneus* (SDK10415.1).

**DNA isolation and genome re-sequencing**

Mid-exponential phase aerobically grown cultures of wild type and nos mutant A2 were harvested by centrifugation and genomic DNA was isolated from each strain using Qiagen Genomic-tip 100/G columns and manufacturers protocols for bacterial DNA isolation. Genomic DNA was submitted to the UF-ICBR NextGen DNA sequencing for barcoding, library construction, and subsequent Illumina MiSeq sequencing (2x300 cycles, paired-end format) with v3 Illumina sequencing reagents. Whole-genome sequencing data was analyzed using the CLC Genomics Workbench software package (Qiagen) as previously described (Mashruwala et al., 2016). In brief, sequencing reads were aligned to the *Nmn. pharaonis* DSM 2160 reference genome (NC_007426.1). Quality-based variant detection was performed to identify polymorphisms in each sequenced strain. SNPs with a minimum 40% frequency of detection were reported in this study.
| Strain or plasmid | Description | Source or references |
|------------------|-------------|---------------------|
| E. coli DH5α     | dlacZ Delta M15 Delta(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 | Hanahan (1983) |
| E. coli JM110    | rpsL (Strr) thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44 Δ(lac-proAB) | Yanisch-Perron et al. (1985) |
| E. coli BL21(DE3) | F- ompT hsdSB (rBmB-) gal dcm (DE3) | Novagen |
| Nmn. pharaonis   | Wild-type DSM 2160 | ATCC 35678 |
| Nmn. pharaonis A2 | nos::gyrB mutation | This publication |
| Hfx. volcanii H26 | ΔpyrE2 derivative of Hfx. volcanii D70 | Allers et al. (2004) |
| pUC19            | bla (AmpR) | Norrander et al. (1983) |
| pCRBlunt         | KanR, MCS | Invitrogen |
| pUC19-npNos      | NP-1908A (nos) cloned into pUC19 at EcoRI and HindIII sites | This publication |
| pJAM809          | Source of gyrB gene | Humbard et al. (2009) |
| pUC19nos::gyrB   | bla (AmpR), gyrB (NovR), Nmn. pharaonis nos disrupted | This publication |
| pET24b           | N-terminal T7•Tag, C-terminal His•Tag, KanR | Novagen |
| pET24b-npNOS     | Natronomonas pharaonis nos with C-terminal His•Tag, KanR | This publication |
| pET24b-saNOS     | Staphylococcus aureus nos with C-terminal His•Tag, KanR | This publication |
| pROS             | φCh1 and CoIE1 origins of replication; bla (AmpR), gyrB (NovR) | Mayrhofer-Iro et al. (2013) |

**TABLE A2** Primers used in this study

| Primer          | Sequence (5′-3′)               |
|-----------------|--------------------------------|
| NpNOS_Ndel_F    | CATATGCAGAGCCGCAAG              |
| NpNOS_NotI_R    | TGCGGCGGAGCCGATTGCCGCTCT        |
| Sanos_Ndel_F    | CATATGTTATTTAAGAGGGCTAAAGC     |
| Sanos_XhoI_R    | CTGGAGGCCAGTGGCTTTCTACTCAT      |
| NpNOS_EcoRI_F   | AGTGAATTCATGCACGAGCCGCGCGAG    |
| NpNOS_HindIII_R | GCCAAGCTTTTACGGCGCCGAGCGA      |
| NpNOS_promoter_F| GTTATATGTTACGAAGTGCTCCA         |
| NpNOS_check_R   | TCAAGGCAGAAGGGATTC             |
| gyrB_check_R    | TCTCCACGAGTCCCTTTGAC           |
| Description | Query cover | Order | E-value | %Identity | Genome accession |
|-------------|-------------|-------|---------|-----------|------------------|
| *Natronobacterium gregoryi* SP2, complete genome | 96% | Natrialbales | $3.00 \times 10^{-154}$ | 50.99% | NC_019792.1 |
| *Halobiforma nitratireducens* JCM 10879 contig_3, whole-genome shotgun sequence | 96% | Natrialbales | $2.00 \times 10^{-110}$ | 49.44% | NZ_AOMA01000003.1 |
| *Natronobacterium texcocoense* strain DSM 24767, whole-genome shotgun sequence | 96% | Natrialbales | $5.00 \times 10^{-110}$ | 50.00% | NZ_FNLC01000002.1 |
| *Natronolimnobaebus* sp. AArc1 chromosome, complete genome | 95% | Natrialbales | $3.00 \times 10^{-106}$ | 48.72% | NZ_CP024047.1 |
| *Natronomonas* sp. F20-122 NODE_2_length_675769_cov_15.592506, whole-genome shotgun sequence | 96% | Halobacteriales | $7.00 \times 10^{-106}$ | 49.86% | NZ_QKNX01000002.1 |
| *Natronolimnobaebus* sp. AArc-Mg chromosome, complete genome | 95% | Natrialbales | $9.00 \times 10^{-106}$ | 48.72% | NZ_CP027033.1 |
| *Natronolimnobaebus aegyptiacus* strain JW/NM-HA 15 chromosome, complete genome | 98% | Natrialbales | $4.00 \times 10^{-104}$ | 45.00% | NZ_CP019893.1 |
| *Halostagnicola kamekurae* strain DSM 22427, whole-genome shotgun sequence | 96% | Natrialbales | $2.00 \times 10^{-103}$ | 48.58% | NZ_TOZS01000001.1 |
| *Natronomonas pharaonis* DSM 2160 complete genome | 95% | Halobacteriales | $3.00 \times 10^{-103}$ | 48.43% | NC_007426.1 |
| *Halostagnicola* sp. A56 14, whole-genome shotgun sequence | 96% | Natrialbales | $6.00 \times 10^{-103}$ | 48.30% | NZ_JMIP02000014.1 |
| *Halorientalis* sp. IM1011, complete genome | 98% | Halobacteriales | $2.00 \times 10^{-101}$ | 46.67% | NZ_CP019067.1 |
| *Haloplanus salinus* strain JCM 18368 contig00001, whole-genome shotgun sequence | 96% | Halofacerales | $7.00 \times 10^{-101}$ | 47.32% | NZ_QPHM01000001.1 |
| *Halalkalicoccus paucihalophilus* strain DSM 24557 HAPAU_contig000010, whole-genome shotgun sequence | 98% | Halobacteriales | $8.00 \times 10^{-101}$ | 46.67% | NZ_LTAZ0100001.1 |
| *Halovenus aranensis* strain IBRC-M10015, whole-genome shotgun sequence | 96% | Halobacteriales | $4.00 \times 10^{-96}$ | 44.89% | NZ_FNFC01000018.1 |
Figure A1  CLUSTAL O (1.2.4) multiple sequence alignment of representative archaeal NOS homologs with *S. aureus* and *B. subtilis* NOS. Blue = conserved dimerization residue in bacterial NOS; black underline = dimerization regions (saNOS); green = heme-binding residues (saNOS); Red = active-site residues (saNOS); orange = predicted bacterial NOS interaction site with reductase domain (line the pocket where heme edge is exposed); conserved sequence (*), conservative mutations (:), semi-conservative mutations (.), and non-conservative mutations ( ). Conserved residues denoted in color above have been reported in (Alderton et al., 2001; Bird et al., 2002; Crane et al., 2010; Fischmann et al., 1999)

Figure A2  Intracellular NO/RNS detection by DAF-FM diacetate. *Hfx. volcanii* and *Nmn. pharaonis* early exponential-phase cells were stained with 5 µM DAF-FM diacetate as described in Section 2. Cells were then incubated in buffer alone or buffer containing 100 µM diethylamine-NONOate donor. DAF-FM fluorescence was measured after 45 min using a Biotek Synergy microplate reader. DAF-FM fluorescence reported as relative fluorescent units (RFU) per OD of each sample. Data representative of *n* = 3 independent experiments, error bars = SEM
TABLE A4  SNP analysis of re-sequenced wild type and nos::gyrB mutant to published Nmn. pharaonis DSM 2160 reference genome (NC_007426.1).

| Reference Position | Type | Leng | Reference | Allele | Count | Coverage | Frequency | Forwards/ reverses balance | AVERAGE QUALITY | Protein Description | Locus Tag | Coding region change | Amino acid change |
|--------------------|------|------|-----------|--------|-------|----------|-----------|-----------------------------|----------------|---------------------|-----------|---------------------|-----------------|
| 1061               | SNV  | 1    | G         | A      | 494   | 499      | 99        | 0.48                        | 34.22          | Predicted restriction endonuclease | NP_00022A | WP_011321731.1:p.Pro16Ser |                   |
| 177659             | SNV  | 1    | C         | G      | 542   | 545      | 99.45     | 0.49                        | 34.72          | hypothetic protein            | NP_0338A | WP_011321898.1:p.Gly93Ala |                   |
| 501766             | SNV  | 1    | C         | T      | 471   | 472      | 99.79     | 0.48                        | 34.64          | F0F1 ATP synthase subunit C   | NP_1022A | WP_011322237.1:p.Ala227Val |                   |
| 504445             | SNV  | 1    | G         | A      | 511   | 511      | 100       | 0.5                         | 34.67          | ATP synthase subunit A        | NP_103GA | WP_011322241.1:p.Leu137Ser |                   |
| 1017698            | SNV  | 1    | G         | T      | 444   | 445      | 99.78     | 0.49                        | 35.93          | CheY family response regulator | NP_2102A | WP_01132270.1:p.Glu23* |                   |
| 1174954            | SNV  | 1    | T         | A      | 400   | 403      | 99.26     | 0.49                        | 33.62          | hydroxymethylglutaryl-CoA reductase (NADPH) | NP_2422A | WP_011322928.1:p.Leu1Met |                   |
| 1213764            | SNV  | 1    | A         | G      | 424   | 429      | 98.83     | 0.5                         | 35.03          | DUF5591 domain-containing protein | NP_2506A | WP_011322970.1:p.Leu1Met |                   |
| 1375984            | SNV  | 1    | T         | C      | 74    | 153      | 48.37     | 0.17                        | 22.47          | hypothetic protein            | NP_14125* | WP_083761695.1:p.Met45Val |                   |
| 1376089            | SNV  | 1    | T         | C      | 70    | 152      | 46.05     | 0.23                        | 17.63          | hypothetic protein            | NP_RS14585* | WP_083761695.1:p.Met45Val |                   |
| 1376101            | SNV  | 1    | T         | C      | 80    | 165      | 48.48     | 0.24                        | 18.66          | hypothetic protein            | NP_RS14585* | WP_083761695.1:p.Met45Val |                   |
| 1376116            | SNV  | 1    | T         | C      | 75    | 173      | 43.35     | 0.2                         | 17.08          | hypothetic protein            | NP_RS14585* | WP_083761695.1:p.Met45Val |                   |
| 1376467            | SNV  | 1    | T         | C      | 81    | 170      | 47.66     | 0.35                        | 18.4           | hypothetic protein            | NP_RS14585* | WP_083761695.1:p.Met45Val |                   |
| 1467124            | SNV  | 1    | A         | C      | 408   | 410      | 99.51     | 0.48                        | 34.31          | hypothetic protein            | NP_3046A | WP_011322236.1:p.Leu137Ser |                   |
| 2494576            | Deletion | 1 | C  | -    | 477   | 478      | 99.79     | 0.5                         | 34.59          | VC_2705 family sodium/solute symporter | NP_5136A | WP_04993988.1:p.Ala227fs |                   |
| 2405308            | SNV  | 1    | T         | C      | 111   | 254      | 43.7      | 0.48                        | 34.9           | putative selenidependent hydroxylase | NP_4954A | WP_01132180.1:p.Glu136Ala |                   |

Blue: SNP unique to nos::gyrB mutant; yellow: SNP common to re-sequenced wild type and nos::gyrB mutant; orange: SNP unique to re-sequenced wild type.

*Original Gene Locus Tag not available.