# Supporting Information

# Cytochrome $cd_1$ nitrite reductase NirS is involved in anaerobic magnetite biomineralization in *Magnetospirillum gryphiswaldense* and requires NirN for proper $d_1$ heme assembly

**Running title:** Nitrite reduction and magnetite biomineralization

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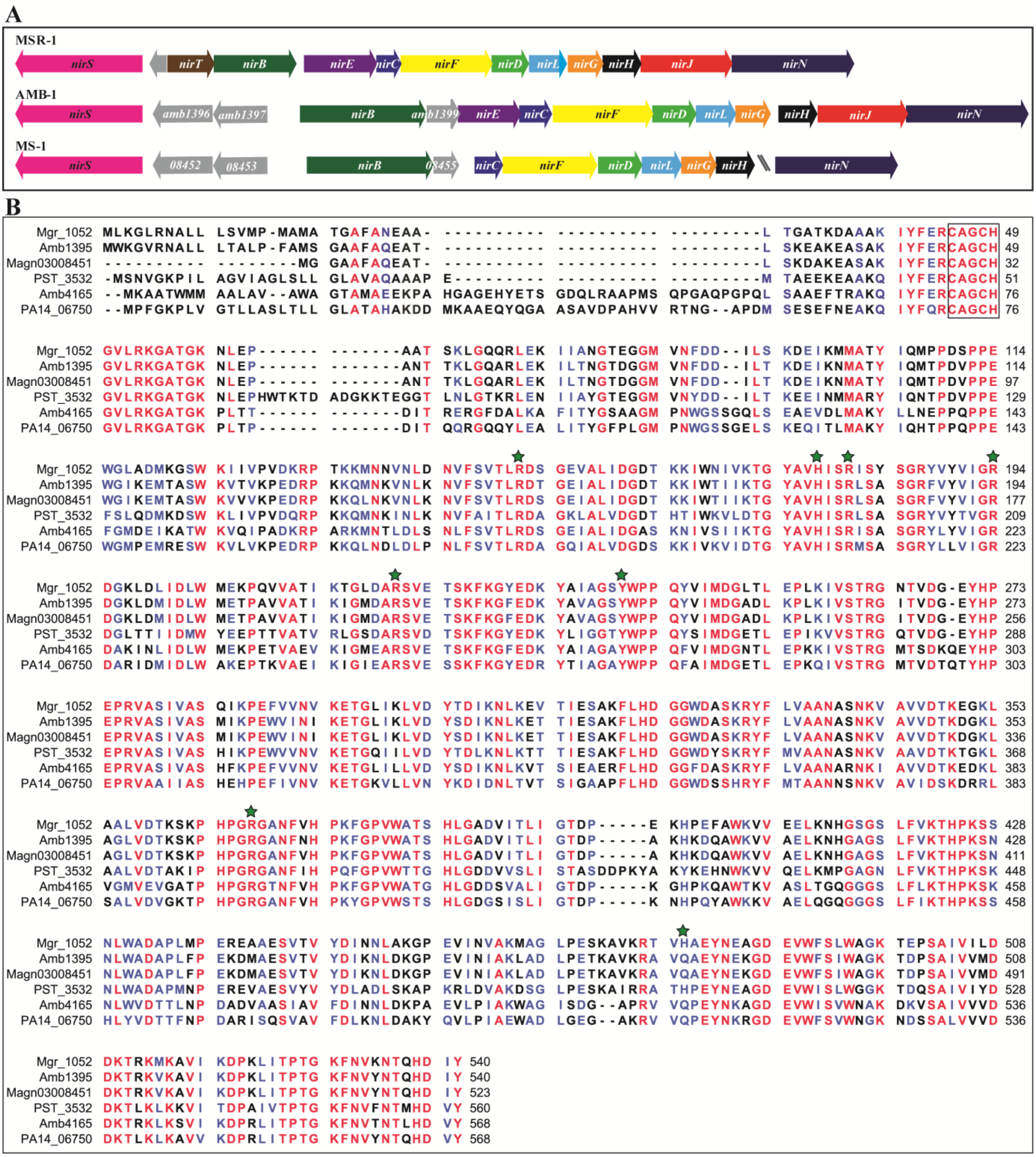

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Magn03008451 ---------- --------MG GAAFAQEAT- ---------- ---------- ---------L SKDAKEASAK IYFERCAGCH 32
PST_3532     -MSNVGKPIL AGVIAGLSLL GLAVAQAAAP E--------- ---------- ---------M TAEEKEAAKQ IYFERCAGCH 51
Amb4165      --MKAATWMM AALAV--AWA GTAMAEEKPA HGAGEHYETS GDQLRAAPMS QPGAQPGPQL SAAEFTRAKQ IYFERCAGCH 76
PA14_06750   --MPFGKPLV GTLLASLTLL GLATAHAKDD MKAAEQYQGA ASAVDPAHVV RTNG--APDM SESEFNEAKQ IYFQRCAGCH 76

Mgr_1052     GVLRKGATGK NLEP------ -------AAT SKLGQQRLEK IIANGTEGGM VNFDD--ILS KDEIKMMATY IQMPPDSPPE 114
Amb1395      GVLRKGATGK NLEP------ -------ANT TKLGQARLEK ILTNGTDGGM VNFDD--ILT KDEIKNMATY IQMTPDVPPE 114
Magn03008451 GVLRKGATGK NLEP------ -------ANT TKLGQARLEK ILTNGTDGGM VNFDD--ILT KDEIKNMATY IQMTPDVPPE 97
PST_3532     GVLRKGATGK NLEPHWTKTD ADGKKTEGGT LNLGTKRLEN IIAYGTEGGM VNYDD--ILT KEEINMMARY IQNTPDVPPE 129
Amb4165      GVLRKGATGK PLTT------ -------DIT RERGFDALKA FITYGSAAGM PNWGSSGGQLS EAEVDLMAKY LLNEPPQPPE 143
PA14_06750   GVLRKGATGK PLTP------ -------DIT QQRGQQYLEA LITYGFPLGM PNWGSSGELS KEQITLMAKY IQHTPPQPPE 143

Mgr_1052     WGLADMKGSW KIIVPVDKRP TKKMNNVNLD NVFSVTLRDS GEVALIDGDT KKIWNIVKTG YAVHISRISY SGRYVYVIGR 194
Amb1395      WGIKEMTASW KVTVKPEDRP KKQMNKVNLK NVFSVTLRDT GEIALIDGDT KKIWTIIKTG YAVHISRLSA SGRFVYVIGR 194
Magn03008451 WGIKEMTASW KVVVKPEDRP KKQLNKVNLK NVFSVTLRDS GEIALIDGDT KKIWTIIKTG YAVHISRLSA SGRFVYVIGR 177
PST_3532     FSLQDMKDSW KLIVPVDQRP KKQMNKINLK NVFAITLRDA GKLALVDGDT HTIWKVLDTG YAVHISRLSA SGRYVYTVGR 209
Amb4165      FGMDEIKATW KVQIPADKRP ARKMNTLDLS NLFSVTLRDS GEIALIDGAS KNIVSIIKTG YAVHISRISA SGRYLYVIGR 223
PA14_06750   WGMPEMRESW KVLVKPEDRP KKQLNDLDLP NLFSVTLRDA GQIALVDGDS KKIVKVIDTG YAVHISRMSA SGRYLLVIGR 223

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Amb1395      DGKLDMIDLW METPAVVATI KIGMDARSVE TSKFKGFEDK YAVAGSYWPP QYVIMDGADL KPLKIVSTRG ITVDG-EYHP 273
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PST_3532     DGLTTIIDMW YEEPTTVATV RLGSDARSVD TSKFKGYEDK YLIGGTYWPP QYSIMDGETL EPIKVVSTRG QTVDG-EYHP 288
Amb4165      DAKINLIDLW MEKPETVAEV KIGMEARSVE TSKFKGFEDK YAIAGAYWPP QFVIMDGNTL EPKKIVSTRG MTSDKQEYHP 303
PA14_06750   DARIDMIDLW AKEPTKVAEI KIGIEARSVE SSKFKGYEDR YTIAGAYWPP QFAIMDGETL EPKQIVSTRG MTVDTQTYHP 303

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Amb1395      EPRVASIVAS MIKPEWVINI KETGLIKLVD YSDIKNLKET TIESAKFLHD GGWDASKRYF LVAANASNKV AVVDTKDGKL 353
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Amb4165      EPRVASIVAS HFKPEFVVNV KETGLILLVD YSDIKNLKVT SIEAERFLHD GGFDASKRYF LVAANARNKI AVVDTKEDKL 383
PA14_06750   EPRVAAIIAS HEHPEFIVNV KETGKVLLVN YKDIDNLTVT SIGAAPFLHD GGWDSSHRYF MTAANNSNKV AVIDSKDRRL 383

Mgr_1052     AALVDTKSKP HPGRGANFVH PKFGPVWATS HLGADVITLI GTDP-----E KHPEFAWKVV EELKNHGSGS LFVKTHPKSS 428
Amb1395      AGLVDTKSKP HPGRGANFNH PKFGPVWATS HLGADVITLI GTDP-----A KHKDQAWKVV AELKNHGAGS LFVKTHPKSN 428
Magn03008451 AGLVDTKSKP HPGRGANFVH PKFGPVWATS HLGADVITLI GTDP-----A KHKDQAWKVV AELKNHGAGS LFVKTHPKSN 411
PST_3532     AALVDTAKIP HPGRGANFIH PQFGPVWTTG HLGDDVVSLI STASDDPKYA KYKEHNWKVV QELKMPGAGN LFVKTHPKSK 448
Amb4165      VGMVEVGATP HPGRGTNFVH PKFGPVWATG HLGDDSVALI GTDP-----K GHPKQAWTKV ASLTGQGGGS LFLKTHPKSK 458
PA14_06750   SALVDVGKTP HPGRGANFVH PKYGPVWSTS HLGDGSISLI GTDP-----K NHPQYAWKKV AELQGQGGGS LFIKTHPKSS 458

Mgr_1052     NLWADAPLMP EREAAESVTV YDINNLAKGP EVINVAKMAG LPESKAVKRT VHAEYNEAGD EVWFSLWAGK TEPSAIVILD 508
Amb1395      NLWADAPLFP EKDMAESVTV YDIKNLDKGP EVINIAKLAD LPETKAVKRA VQAEYNEKGD EVWFSIWAGK TDPSAIVVMD 508
Magn03008451 NLWADAPLFP EKDMAESVTV YDIKNLDKGP EVINIAKLAD LPETKAVKRA VQAEYNEKGD EVWFSIWAGK TDPSAIVVMD 491
PST_3532     NLWADAPMNP EREVAESVYV YDLADLSKAP KRLDVAKDSG LPESKAIRRA THPEYNEAGD EVWISLWGGK TDQSAIVIYD 528
Amb4165      NLWVDTTLNP DADVAASIAV FDINNLDKPA EVLPIAKWAG ISDG--APRV VQPEYNKAGD EVWISVWNAK DKVSAIVVVD 536
PA14_06750   HLYVDTTFNP DARISQSVAV FDLKNLDAKY QVLPIAEWAD LGEG--AKRV VQPEYNKRGD EVWFSVWNGK NDSSALVVVD 536

Mgr_1052     DKTRKMKAVI KDPKLITPTG KFNVKNTQHD IY 540
Amb1395      DKTRKVKAVI KDPKLITPTG KFNVYNTQHD IY 540
Magn03008451 DKTRKVKAVI KDPKLITPTG KFNVYNTQHD IY 523
PST_3532     DKTLKLKKVI TDPAIVTPTG KFNVFNTMHD VY 560
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PA14_06750   DKTLKLKAVV KDPRLITPTG KFNVYNTQHD VY 568
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**C**

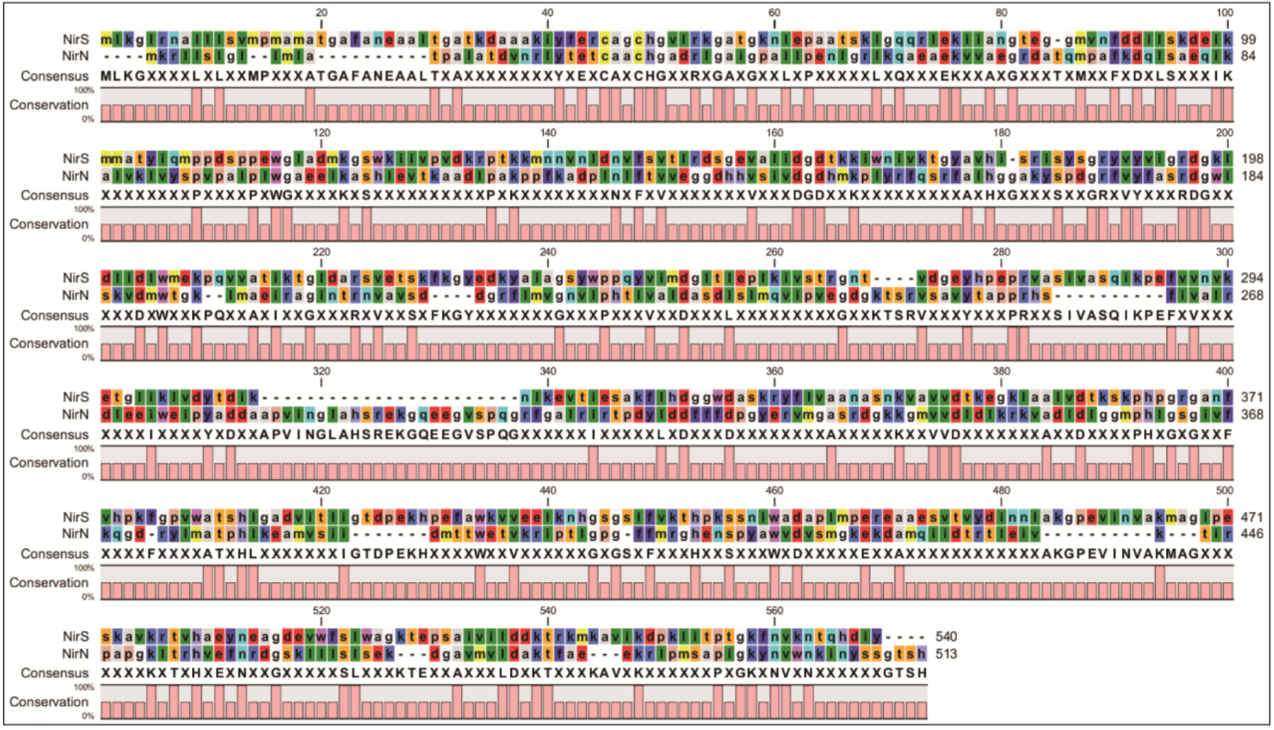


Fig. S1. (A) Molecular organization of identified *nir* genes in *Magnetospirillum magneticum* and *Magnetospirillum magnetotacticum*. Double backslash represents *nirN* and other *nir* genes are found in different contigs from the incomplete genome assembly in *M. magnetotacticum*. (B) Sequence alignment of NirS with selected homologs from MTB and non-MTB. PST_3532 and PA14_06750 refer to NirS from *Pseudomonas stutzeri* A1501 and *Pseudomonas aeruginosa* UCBPP-PA14, respectively. Conserved, homologous, and nonconserved residues among the various species are shown in red, blue, and black, respectively. Residues involved in $d_1$ heme binding site are indicated by green star. Heme *c* binding site is shown in a black box. (C) Sequence alignment of NirS and NirN from MSR-1. The level of conserved amino acid residues among the various species are shown below the sequences. The residues of the alignment are color-coded according to the Rasmol colour scheme (http://life.nthu.edu.tw/~fmhsu/rasframe/COLORS.HTM#aminocolors).

Compared to MSR-1, a similar *nir* cluster was observed in both *M. magneticum* and *M. magnetotacticum* (Fig. S1A). While neither *nirS* nor other *nir* genes are present in *Magnetococcus marinus* MC-1 (1), two homologs of *nirS* (*amb1395* and *amb4165*) are present in *M. magneticum* (Table S1), thus being the only known bacterium having more than one *nirS* gene. As in MSR-1, *amb1395* is part of a larger *nir* cluster and shares high amino acid similarity (91%) with NirS of both MSR-1 (Mgr_1052) and *M. magnetotacticum* (Magn03008451), respectively (Table S1), whereas *amb4165* is more divergent (78% similarity to NirS of MSR-1) and not adjacent to other *nir* genes. Also, the N-terminus of Amb4165 containing the heme *c*-binding region differs from that of other magnetobacterial NirS homologs, but rather resembles NirS from *Ps. aeruginosa*, whereas in NirS from *Ps. stutzeri* this region is absent (Fig. S1B) (2).

In *M. magneticum* and *M. magnetotacticum*, *nirCFDLGH* genes also show synteny. However, compared to MSR-1 their *nir* clusters also display some differences (Fig. S1A): First, in *M. magnetotacticum* and *M. magneticum nirT* and *nirB* genes are fused into a single gene designated as *nirB*. Second, in *M. magneticum* and *M. magnetotacticum* an additional gene (*amb1399* and *magn03008455*) encoding a copper-binding protein of plastocyanin/azurin family is present between *nirB* and *nirE*. Third, *nirJ* is missing in *M. magnetotacticum*, probably due to the incompleteness of its genome assembly. Fourth, *nirE* encoding an S-adenosylmethionine (SAM)-dependent uroporphyrinogen methyltransferase is absent from *M. magnetotacticum*. The monocistronic organization of *nirS* encoding a cytochrome $cd_1$ nitrite reductase in MSR-1 and other magnetospirilla is unique compared to all other non-magnetic denitrifyers in which *nirS* is part of a single operon along with other *nir* genes. In magnetospirilla we found all other *nir* genes (*nirTBECFDLGHJN*) within a closely adjacent, but distinct cluster, which encodes a set of proteins likely required for synthesis of $d_1$ heme. This different organization might reflect a somewhat distinct regulation or function of magnetobacterial nitrite reduction proteins.

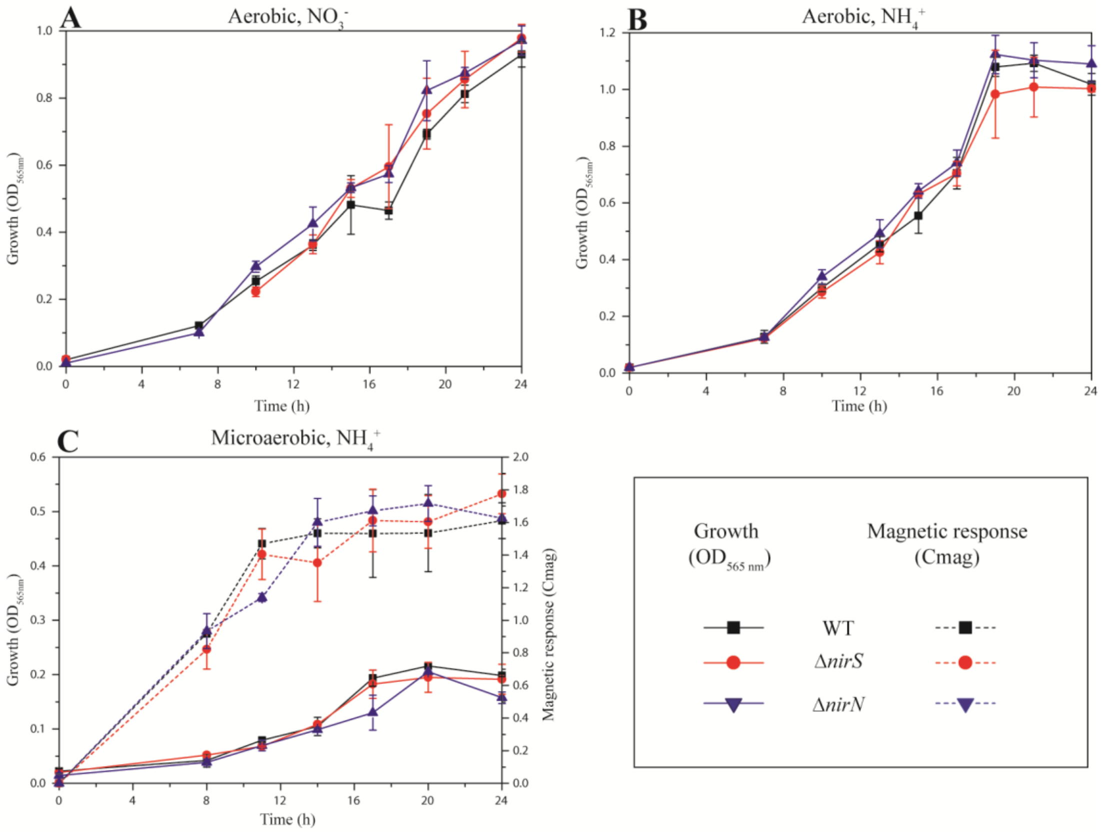
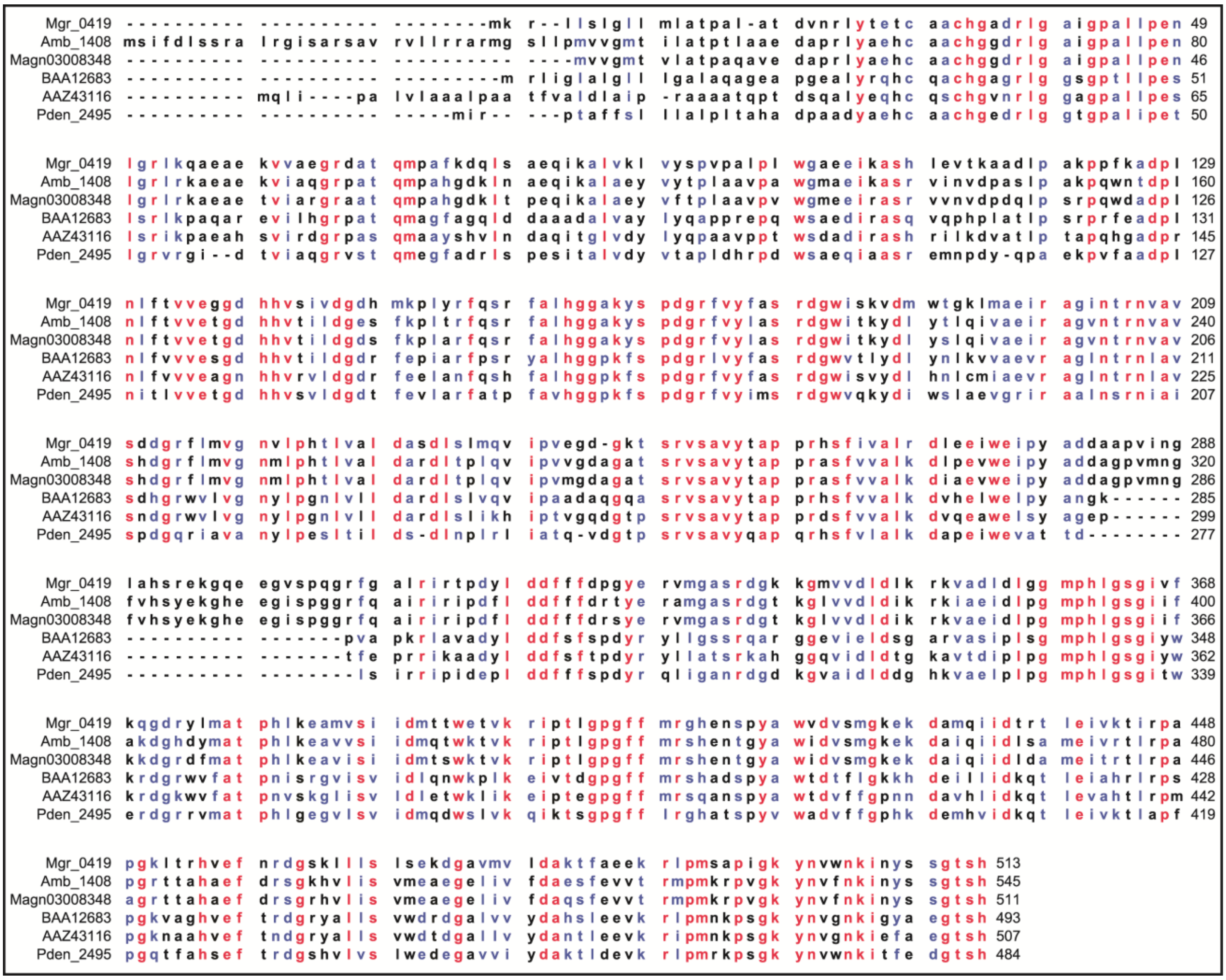


Fig. S2. Growth ($OD_{565\ nm}$) and magnetic response ($C_{mag}$) of MSR-1 WT, Δ*nirS*, and Δ*nirN* under different conditions at 30°C. Under aerobic conditions, the $C_{mag}$ values were always zero and not shown. (A) Aerobic, nitrate medium; (B) Aerobic, ammonium medium; (C) Microaerobic, ammonium medium. Results from representative experiments were measured in triplicate, and values are given as means and standard deviations.



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 Magn03008348 ---------- ---------- ---------- ----mvvgmt vlatpaqave daprlyaehc aachggdrlg aigpallpen 46
     BAA12683 ---------- ---------- ---------m rliglalgll lgalaqagea pgealyrqhc qachgagrlg gsgptllpes 51
     AAZ43116 ---------- mqli----pa lvlaaalpaa tfvaldlaip -raaaatqpt dsqalyeqhc qschgvnrlg gagpallpes 65
    Pden_2495 ---------- ---------- -----mir-- ---ptaffsl llalpltaha dpaadyaehc aachgedrlg gtgpalipet 50

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 Magn03008348 fvhsyekghe egispggrfq airiripdfl ddfffdrsye rvmgasrdgt kglvvdldik rkvaeidlpg mphlgsgiif 366
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Fig. S3. Sequence alignment of NirN with selected homologs from MTB and non-MTB. PST_3538, PA14_06650, and Pden_2495 refer to NirN from *Pseudomonas stutzeri* A1501, *Pseudomonas aeruginosa* UCBPP-PA14, and *Paracoccus denitrificans* PD1222, respectively. Conserved, homologous, and nonconserved residues among the various species are shown in red, blue, and black, respectively.

**Table S1** Bacterial strains used in this work

| Strain | Important feature (s) | Source or reference |
|---|---|---|
| *E. coli* strain DH5α | F’ Φ80d*lacZ*ΔM15Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsd*R17 ($r_k$-, $m_k$+) *pho*A *sup*E44 λ-*thi*-1 *gyr*A96 *rel*A1 | Invitrogen |
| *E. coli* strain BW29427 | *dap* auxotroph derivative of *E. coli* strain B2155 | K. Datsenko and B. L. Wanner, unpublished |
| *Ps. stutzeri* | Wild type | H. Jung |
| MSR-1 WT | Wild type R3/S1, but $Rif^r$, $Sm^r$ | (3) |
| Δ*nirS* | R3/S1 Δ*nirS* | This study |
| Δ*nirN* | R3/S1 Δ*nirN* | This study |

**Table S2** Plasmids used in this work

| Plasmid | Important feature (s) | Source or reference |
|---|---|---|
| pCM184 | Broad-host-range allelic exchange vector, Amp$^r$, Km$^r$, Tc$^r$ | (4) |
| pBBR1MCS-2 | Km$^r$, mobilizable broad-host-range vector | (5) |
| pBBR1MCS-3 | Tc$^r$, mobilizable broad-host-range vector | (5) |
| pK19mobGII | - | (6) |
| pFM208 | Km$^r$ | F. D. Müller, unpublished data |
| pRU1 | Km$^r$ | R. Uebe, unpublished data |
| pCM157 | Tc$^r$, Cre recombinase expression vector | (4) |
| pLYJ06 | pCM184 plus *nirS* 2-kb downstream region | This study |
| pLYJ08 | pLYJ06 plus *nirS* 2-kb upstream region | This study |
| pLYJ20 | pCM184 plus *nirN* 2-kb upstream region | This study |
| pLYJ23 | pLYJ06 plus *nirN* 2-kb downstream region | This study |
| pLYJ36 | pBBR1MCS-2 plus *nirS* promoter region | This study |
| pLYJ51 | pBBR1MCS-2 plus *nirS* with its own promoter | This study |
| pLYJ52 | pBBR1MCS-2 plus *nirS* without its candidate signal peptide sequence | This study |
| pLYJ59 | pLYJ52 with *nirS* promoter and signal peptide sequence | This study |
| pLYJ64 | pLYJ59 plus *mcherry* | This study |
| pLYJ74 | pRU1 plus *nirN* | This study |
| pLYJ86 | pCM157 plus *nirS* promoter region from pLYJ36 | This study |
| pLYJ87 | pBBR1MCS-2 plus *nirS* promoter and *cre* fusion from pLYJ86 | This study |
| pLYJ88 | pLYJ36 plus *amb1395* from *M. magneticum* | This study |
| pLYJ89 | pLYJ36 plus *amb4165* from *M. magneticum* | This study |
| pLYJ94 | pLYJ97 plus *nirS* promoter region | (7) |
| pLYJ95 | pLYJ36 plus *nirS* from *P. stutzeri* | This study |
| pLYJ97 | pBBR1MCS-2 plus *gusA* from pK19mobGII | (7) |
| pLYJ104 | pLYJ97 plus *nirTBECFDLGHJN* promoter region | This study |
| pLYJ113 | pRU1 plus *nirN* from *M. magneticum* | This study |
| pLYJ124 | pRU1 plus *nirN* from *P. stutzeri* | This study |

**Table S3** BlastP analysis results of denitrification genes in MTB and non-MTB using MSR-1 as a query.

| Gene in MSR-1 | Encoded gene product (aa, kDa, pI) | *M. magneticum* (e-value, similarity) | *M. magnetotacticum* (e-value, similarity) | Best hit in non-MTB (e-value, similarity) |
|---|---|---|---|---|
| *mgr_1052** | Nitrite reductase, NirS<br>(540, 59.26, 8.81) | *amb1395* (0, 91%)<br>*amb4165* (0, 78%) | *magn03008451*<br>(0, 91%) | *Dechlorosoma suillum* PS<br>(0, 90%) |
| *mgr_1053* | NirT<br>(199, 22.62, 8.38) | *amb1398*<br>(6e-110, 89%) | *magn03008454*<br>(2e-108, 91%) | *Endoriftia Persephone*<br>(2e-93, 79%) |
| *mgr_1055* | NirB<br>(341, 36.22, 8.41) | *amb1398*<br>(3e-161, 81%) | *magn03008454*<br>(1e-149, 77%) | *Endosymbiont of Riftia pachyptila*<br>(2e-99, 67%) |
| *jn634764* | NirE<br>(304, 32.12. 6.86) | *amb1400*<br>(1e-91, 77%) | – | *Pectobacterium wasabiae* WPP163<br>(3e-85, 69%) |
| *mgr_0426* | NirC<br>(104, 11.05, 7.83) | *amb1401*<br>(4e-29, 73%) | *magn03008456*<br>(2e-31, 76%) | *Dechloromonas aromatica* RCB<br>(2e-30, 70%) |
| *mgr_0425* | NirF<br>(387, 42.85, 6.27) | *amb1402*<br>(0, 82%) | *magn03008457*<br>(0, 82%) | *Thiobacillus denitrificans*<br>(7e-179, 79%) |
| *mgr_0424* | NirD<br>(149, 16.39, 5.22) | *amb1403*<br>(2e-56, 74%) | *magn03008458*<br>(6e-59, 77%) | *Pseudomonas aeruginosa* PAO1<br>(4e-47, 68%) |
| *mgr_0423* | NirL<br>(165, 18.51, 8.97) | *amb1404*<br>(2e-69, 77%) | *magn03008459*<br>(5e-66, 75%) | *Dechloromonas aromatica* RCB<br>(3e-54, 71%) |

| | | | | |
|---|---|---|---|---|
| *mgr_0422* | NirG<br>(148, 16.33, 5.41) | *amb1405*<br>(7e-59, 73%) | *magn03008460*<br>(3e-56, 72%) | *Beggiatoa sp.* PS<br>(3e-61, 74%) |
| *mgr_0421* | NirH<br>(161, 17.60, 9.29) | *amb1407*<br>(2e-70, 80%) | *magn03008461*<br>(3e-71, 78%) | *Rubrivivax gelatinosus* IL144<br>(5e-63, 71%) |
| *mgr_0420* | NirJ<br>(375, 41.28, 7.16) | *1530431-1531552 bp*<br>(2e-155, 72%) | – | *Dechloromonas aromatica* RCB<br>(1e-75, 76%) |
| *mgr_0419** | NirN<br>(513, 56.40, 6.72) | *amb1408*<br>(0, 83%) | *magn03008348*<br>(0, 84%) | *Dechloromonas aromatica* RCB<br>(0, 73%) |

*indicate genes deleted.

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