Supplementary Information

Genomic organization, gene expression and activity profile of *Marinobacter hydrocarbonoclasticus* denitrification enzymes

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S1 – List of denitrification genes identified in different bacteria

In Table S1 are listed the genes that have been identified to encode catalytic enzymes/subunits that catalyze the different steps of the denitrification pathway.

Table S1 – Genes that encode the catalytic subunits of the enzymes involved in the denitrification pathway.

Step	Enzyme	Gene encoding the catalytic subunit	Ref.
$NO_3^- \rightarrow NO_2^-$	Membrane nitrate reductase (NaR)	*narG*	(Ghiglione et al. 1999)
	Periplasmic nitrate reductase (NaP)	*napA*	(Gates et al. 2003)
$NO_2^- \rightarrow NO$	Nitrite reductase cytochrome cd_1 (cd_1NiR)	*nirS*	(Jungst et al. 1991)
	Copper nitrite reductase (CuNiR)	*nirK*	(Cantera & Stein 2007)
	Three domain T1Cu_CuNiR*	*Cu_nirK*	(Nojiri et al. 2007)
	Three domain cyt*c*_CuNiR**	*Cyt_nirK*	(Ellis et al. 2007)
$NO \rightarrow N_2O$	Cytochrome *c* nitric oxide reductase (*c*-NOR)	*cnorB*	(Arai et al. 1995)
	Quinol nitric oxide reductase (*q*-NOR)	*qnorB (norZ)*	(Cramm et al. 1999; Hendriks et al. 2000)
	CuA nitric oxide reductase (Cu_A-NOR)	*Not identified*	(Al-Attar & de Vries 2015)
$N_2O \rightarrow N_2$	Nitrous oxide reductase (N_2OR)	*nosZ*	(Viebrock & Zumft 1988)
	Cytochrome *c* nitrous oxide reductase (*c*N_2OR)	*cnosZ*	(Simon et al. 2004)

Notes: * This is a hexameric nitrite reductase isolated from *Hyphomicrobium denitrificans* that contains an additional N-terminal cupredoxin domain. ** The gene coding for this enzyme has been identified at the genome level and is proposed to present an additional C-terminal class I *c*-type cytochrome domain.

S2 - Bioinformatic analysis of denitrification gene cluster of *M. hydrocarbonoclasticus*

The identification of putative gene functions was performed by BLAST search using the web platforms of blastp and blastn suites (NCBI) (Altschul et al. 1997) (statistic data are provided in Table S1), gene and protein alignments using ClustalOmega (McWilliam et al. 2013), and comparison of gene organization with other denitrifying microorganisms.

Table S2 - Summary of the statistics for the homology of the gene clusters proposed to be involved in the denitrification of *Marinobacter hydrocarbonoclasticus*. The score is given for the higher score between each encoded protein in a search that only included the genomes of *Pseudomonas stutzeri* str. ZoBell 632 (taxid: 32042; Ps), *Pseudomonas aeruginosa* PAO1 (taxid:208964; Pa) and *Paracoccus denitrificans* PD1222 (taxid: 318586; Pd).

Gene ID	Gene designation	Identity (%); organism	E-value	Coverage (%)
MARHY3014	*c-norB*	27, Pd	3e-25	61
	*q-nor*** (WP_055357926.1)	37, Gs	6e-163	95
MARHY3015	*moaA*	65, Ps	2e-152	98
MARHY3016	*nosL*	50, Ps	1e-57	92
MARHY3017	*nosY*	67, Ps	4e-105	99
MARHY3018	*nosF*	57, Ps	4e-107	97
MARHY3019	*nosD*	58, Pa	9e-159	85
MARHY3020	*nosZ*	76, Pa	0	97
MARHY3021	*nosR*	62, Ps	0	99
MARHY3022	Hypothetical protein (WP_003282777.1)	50, Ps	2e-44	99
MARHY3023	*dnr*	36, Ps	5e-39	86
MARHY3024	*Peptidylpropyl isomerase*	43, Ps	4e-57	93
MARHY3025	*narV, nar gamma*	57, Pd	1e-83	98
MARHY3026	*narJ*,	48, Pa	1e-57	78
MARHY3027	*narH (beta)*	81, Pa	0	98
MARHY3028	*narG (alpha)*	75, Ps	0	98
MARHY3029	*narK/nasA* family	31, Pa	2e-56	93
MARHY3030	*narX*	36, Ps	7e-107	99
MARHY3031	*narL*	57, Pa	2e-84	98
MARHY3053	*norC*	64, Ps	7e-66	100
MARHY3054	*norB*	77, Pa	0	96
MARHY3055	*ccoG*	26, Pd	1e-06	65
MARHY3056	*norD*	49, Ps	0	100
MARHY3057	*norE*	47, Ps	1e-45	98
MARHY3058	*norF* (see manuscript)*	60, Ps	0.006	32
MARHY3059	Hypothetical protein, no similarly	-	-	-
MARHY3060	*norQ*	72, Ps	2e-133	93
MARHY3061	Hypothetical protein, no similarly	-	-	-
MARHY3062	*nirF*	62, Pa	2e-171	95
MARHY3063	*nirC*	46, Ps	4e-21	72
MARHY3064	*nirS*	70, Pa	0	98
MARHY3065	*nirD*	53, Ps	3e-51	96
MARHY3066	*nirL*	53, Ps	3e-56	84
MARHY3067	*nirG*	59, Ps	8e-59	94
MARHY3068	*nirH*	60, Pa	9e-65	95
MARHY3069	*nirJ*	59, Ps	9e-168	94
MARHY3070	*nirE*	56, Ps	2e-93	91
MARHY3071	*nirN*	58, Ps	0	96
MARHY3072	Crp/Fnr family	32, Ps	2e-14	59
MARHY3073	*wrbA*	80, Ps	7e-117	100
MARHY3074	*yqfA*	44, Pa	5e-46	96
MARHY3075	*nnrS*	49, Ps	3e-50	64
MARHY3076	*narK2*	71, Ps	0	99
MARHY3077	Hypothetical protein (WP_049792410.1)	37, Pd	5e-19	83
MARHY3078	*narK/nasA* family	80, Ps	0	99
MARHY3079	Hypotherical protein (WP_003281670.1)	57, Ps	1e-53	97
MARHY3080	*narU (narK/nasA* family*)*	62, Pd	3e-166	94
MARHY0862	*fnrA*	53, Pa	2e-86	93

Note: Sequence homology with *Geobacillus stearothermophilus* (Gs) (taxid: 1422).

S3 - Score of the alignments of FNR and IHF motifs

Table S3 – FNR and IHF putative nucleotide motifs in the promoter regions of denitrification gene clusters of *M. hydrocarbonoclasticus*. PRODORIC database was used to identify the sequences.

PWM (species)	Promoter region	Sequence	Score
Fnr | *Escherichia coli* (strain K12) / Maximum score: 8.93	*narK*	**TTGA**GGATAG**TCAA**	7.14
	narGHJV	**TTGAT**CCCACA**CAA**	7.04
	narXL	**TTGA**CTATCC**TCAA**	7.92
	nirCF	**TT**A**AT**CAAT**ATCAA**	7.08
	nirSDLGHJEN	**TTGAT**ATTG**ATT**A**A**	7.30
	norCB3055DEF	**TTGAT**AATC**ATCAA**	8.47
	nosR	**TTGA**TTTTCC**TCAA**	8.16
	dnr	**TTGA**GCTAAG**TCAA**	7.27
IHF | *Escherichia* coli (strain K12) Maximum score: 7.67	*narK*	CCC**CAA**TGGT**TTA**TAA	6.39

Note: The nucleotides highlighted in bold match their consensus sequence. PWM- position weight matrix.

S4 – Composition of the Starkey oligoelement solution

The medium was supplemented with a Starkey oligoelement solution so that in the medium there would be 263 µM MgO, 20 µM $CaCO_3$, 22.3 µM $FeSO_4 \cdot 7H_2O$, 5 µM $ZnSO_4 \cdot 7H_2O$, 3.6 µM $MnSO_4 \cdot H_2O$, 1 µM $CuSO_4 \cdot 5H_2O$, 3.2 µM $CoSO_4 \cdot 7H2O$, 0.97 µM BO_3H_3, 0.08 µM $Mo_7(NH_4)_6 \cdot 4H_2O$, 0.14 µM $Ni(NO_3)_2 \cdot 6H_2O$, 0.12 µM Na_2SeO_3.

S5 - Oxygen profile and schematic representation of the bioreactor

Oxygen profile measured in the liquid phase in the microaerobic growth in the bioreactor

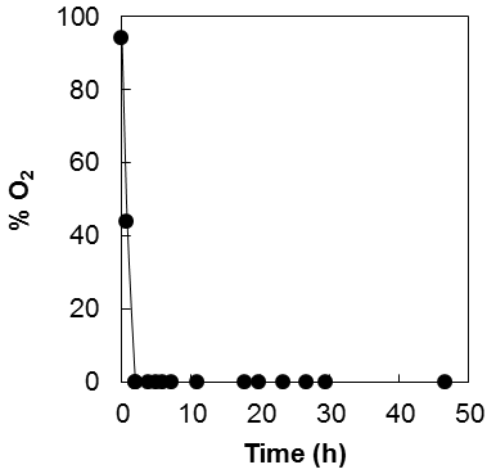

Figure S1 - Oxygen profile measured in the liquid phase of *M. hydrocarbonoclasticus* growth at pH 7.5 in the bioreactor during 48 h.

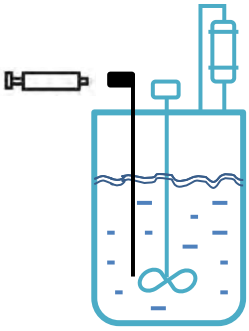

Figure S2 - Schematic representation of the bioreactor (blue). The location of sample collection is represented in black. Samples were collected from the bottom of the bioreactor through a syringe connected to a 0.22 µm filter to guarantee the sterility. Samples for RNA extraction were collected at flame and immediately frozen.

S6 - NO and N_2O reduction by the whole-cells - Data Analysis

The rate of NO and N_2O reduction by the whole-cells was indirectly measured through oxidation of methyl viologen. In the curve obtained, linear regressions were used to fit regions immediately before and after substrate addition. The slope of the fitting before substrate addition was subtracted to the slope obtained after substrate addition.

The rates were determined taking into consideration the calculated slope and the extinction coefficient of methyl viologen at 600 nm (ε_{600nm} = 11.4 mM^{-1} cm^{-1}) (Kristjansson & Hollocher 1980). We also consider that methyl viologen re-oxidation requires one electron while N_2O reduction involves two electrons and the reduction of two molecules of NO involves two electrons.

Note that in this assay, the subtraction of the slope prior to the addition of substrate is particularly important as other enzymes might also use the reduced methyl viologen, causing partial oxidation even before the addition of substrate.

Rates of NO and N_2O reduction were reported as micromoles of NO or N_2O reduced per minute per optical density ($\mu mol_{NO\ or\ N2O}$ min^{-1} OD^{-1}).

S7 – Genome organization of *M. hydrocarbonoclasticus* denitrification genes

A search in the genome of *M. hydrocarbonoclasticus* SP17 for genes involved in denitrification showed that several genes encoding proteins involved in the different steps of this pathway (see Figure S2). The *nosRZDFYL* gene cluster, containing the catalytic unit of N_2OR, encoded by *nosZ* (Philippot 2002; Zumft & Kroneck 2007). This gene cluster presents *nosR*, which encodes a transmembrane protein containing Fe-S centers and a periplasmic domain that binds a flavin (Cuypers et al. 1992; Wunsch & Zumft 2005). Although its function remains unclear, NosR seems to be important for *nosZ* transcription, as well as for enzyme full activity *in vivo*, as evidenced by mutational studies in *P. stutzeri* (Cuypers et al. 1992; Wunsch & Zumft 2005). The *nosD* gene encodes a protein that together with NosFY is proposed to form an ABC–transporter (NosDFY) and be involved in sulfur transport for CuZ center assembly (Wunsch et al. 2003; Zumft 2005a), while *nosL* encodes a putative copper chaperone (McGuirl et al. 2001).

Upstream *nos* cluster is the NaR gene cluster, *narLXKGHJV* (see Figure S2) containing the genes *narG*,

narH and *narV* (also designated as *narI*) that encode the α, β and γ subunits of the enzyme, respectively (Philippot 2002). The *narJ* gene encodes a chaperone-like component involved in the maturation and assembly of the enzyme complex (Lanciano et al. 2007), and *narXL* encode NarXL a two-component system. Several nark type genes have been identified in this genome, with one being associated with the *nar* cluster (*MARHY3029*). Two other *narK* genes are located upstream the *nir* operon, which correspond to *MARHY3076*, annotated as *narK2*, and *MARHY3078*, annotated as *narK*. Additionally, a *narU* (*MARHY3080*), encoding in *E. coli* a nitrate/nitrite transporter (Jia et al. 2009; Yan et al. 2013), is also found in this genome (see Figure S2).

Further upstream is *norBC*, encoding the two subunits of the short-chain membrane-bound *c*-NOR (see Figure S2). NorB (*MARHY3054*) is the catalytic subunit, while NorC (*MARHY3053*) is a small membrane bound *c*-type cytochrome functioning as an electron transfer subunit (Philippot 2002; Zumft 2005b). The accessory genes *norQ* (*MARHY3060*, annotated in other organisms as *napH*) and *norD* (*MARHY3056*), together with two other ORFs (*MARHY3057* and *MARHY3058*) are located upstream this operon. NorE and NorF, are predicted to be membrane associated, though their exact function remains unknown (Bergaust et al. 2014). It has been shown that NorEF are involved in denitrification as its inactivation slows nitrate reduction during denitrification, with accumulation of micromolar concentrations of nitric oxide (Bergaust et al. 2014; de Boer et al. 1996). In contrast, *norQ* and *norD* are always found linked to or in the vicinity of *norBC* (Zumft 2005b). The encoded proteins are suggested to be involved in the maturation of NorBC in either heme insertion, multisubunit assembly and/or insertion into the membrane (Zumft 1997; Zumft 2005b).

After the *norBC* genes, there is *nirFCSDLGHJEN*. The gene *nirS* encodes the cytochrome cd_1NiR, a homodimeric enzyme with *c*- and d_1-type hemes (Lopes et al. 2001). In recent years, it was identified that *nirDLGH* (Bali et al. 2011), *nirJ* (Bali et al. 2011), *nirE* (Zajicek et al. 2009) and *nirN* (Adamczack et al. 2014) encode enzymes involved in the biosynthesis of heme-d_1, all located in the cytoplasm, with the exception of NirN. NirF has been proposed to be a chaperone involved in the uptake and transport of dihydro-heme d_1 precursor from the cytoplasm to NirN located in the periplasm. The exact role of NirC remains unknown, but has been shown to be essential for the synthesis of heme-d_1 in *P. denitrificans* (Bali et al. 2014; de Boer et al. 1994). It is predicted to be a periplasmic *c*-type cytochrome, playing a role in the biosynthesis of heme-d_1 as an electron acceptor from NirN (Adamczack et al. 2014; Zajicek et al. 2009).

Finally, a gene annotated as *nnrS*, was identified upstream the *nir* cluster, which might be a membrane-bound NO sensor with a role in nitrosative stress, yet poorly explored (Stern et al. 2013).

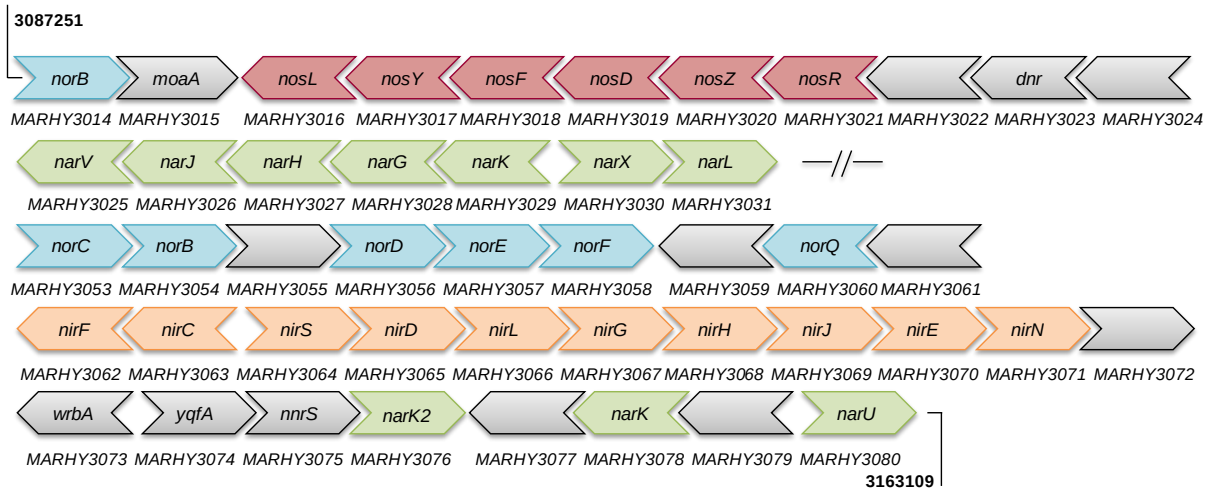

Figure S3 - Arrangement of denitrification genes in the genomic region 3087251-3163109 of *M. hydrocarbonoclasticus* SP17. Genes belonging to the nitrous oxide reductase cluster (*nos*) are shown in red, to the

nitrate reductase cluster (*nar*) are shown in green, to the nitrite reductase cluster (*nir*) are shown in orange and to the nitric oxide reductase cluster (*nor*) are shown in blue. Our own *in silico* analysis indicate that *MARHY3057* and *MARHY3058* are putative *norE* and *norF* homologues, respectively. Arrows show the direction of transcription. Genes are not drawn to scale.

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