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An HcpR paralog of *Desulfovibrio gigas* provides protection against nitrosative stress

Sofia M. da Silva, Catarina Amaral, Susana S. Neves, Cátia Santos, Catarina Pimentel *, Claudina Rodrigues-Pousada *

Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal

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ABSTRACT

Desulfovibrio gigas **belongs to the group of sulfate reducing bacteria (SRB). These ubiquitous and metabolically versatile microorganisms are often exposed to reactive nitrogen species (RNS). Nonetheless, the mechanisms and regulatory elements involved in nitrosative stress protection are still poorly understood. The transcription factor HcpR has emerged as a putative regulator of nitrosative stress response among anaerobic bacteria. HcpR is known to orchestrate the expression of the hybrid cluster protein gene, *hcp*, proposed to be involved in cellular defense against RNS. According to phylogenetic analyses, the occurrence of *hcpR* paralog genes is a common feature among several *Desulfovibrio* species. Within the *D. gigas* genome we have identified two HcpR-related sequences. One of these sequences, *hcpR1*, was found in the close vicinity of the *hcp* gene and this finding prompted us to proceed with its functional characterization. We observed that the growth of a *D. gigas* strain lacking *hcpR1* is severely impaired under nitrosative stress. An *in silico* search revealed several putative targets of HcpR1 that were experimentally validated. The fact that HcpR1 regulates several genes encoding proteins involved in nitrite and nitrate metabolism, together with the sensitive growth phenotype to NO displayed by an *hcpR1* mutant strain, strongly supports a relevant role of this factor under nitrosative stress. Moreover, the finding that several *Desulfovibrio* species possess HcpR paralogs, which have been transmitted vertically in the evolution and diversification of the genus, suggests that these sequences may confer adaptive or survival advantage to these organisms, possibly by increasing their tolerance to nitrosative stress.**

1. Introduction

Sulfate reducing bacteria (SRB) are metabolically versatile microorganisms that can be found in a variety of anaerobic habitats from marine and freshwater sediments to the gastrointestinal tract of humans [1,2]. The ubiquity of SRB in nature and their presence in mixed communities makes them susceptible to the toxic effects of reactive nitrogen species (RNS), which are produced by other bacteria or by the human innate immune system.

RNS derive from nitric oxide (NO), a membrane-permeable gas, that can interact with and modify organic molecules [3]. NO is involved in several cellular functions such as signaling and defense mechanisms against pathogens [4]. The potent antimicrobial action of NO and RNS is due to the severe damage it causes on microbial cells by interacting with several targets such as thiols, metal centers, nucleotide bases and lipids [4]. Such NO-mediated cytotoxic effects on bacteria are attenuated by protective responses that detoxify NO or bypass its antimicrobial actions, a process often called nitrosative stress response [5,6].

Desulfovibrio is the most studied genus of SRB belonging to the group of δ-proteobacteria. Nonetheless, the mechanisms and regulatory elements involved in nitrosative stress response in these organisms are still poorly understood. Recently, a NorR-like transcription factor, belonging to the NtrC/Nif family of regulators, was identified in *D. gigas* and shown to control the expression of the *roo* gene under nitrosative stress conditions [7]. The *roo* gene codes for a rubredoxin oxygen reductase (ROO), a flavodiiron

Abbreviations: BI, Bayesian inference; BS, bootstrap; CRP/FNR, cAMP receptor protein/fumarate and nitrate reductase regulatory protein; Frdx, ferredoxin; GSNO, S-nitrosoglutathione; Hcp, hybrid cluster protein; HGT, horizontal gene transfer; ML, maximum likelihood; MP, maximum parsimony; NO, nitric oxide; PP, posterior probability; RNS, reactive nitrogen species; ROO, rubredoxin oxygen reductase; SRB, sulfate reducing bacteria

* Corresponding authors.
E-mail addresses: pimentel@itqb.unl.pt (C. Pimentel), claudina@itqb.unl.pt (C. Rodrigues-Pousada).

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protein involved in detoxification of molecular oxygen that was also shown to have NO reductase activity, contributing to the protection of *D. gigas* and other *Desulfovibrio* spp. from nitrosative stress [8–12]. Another protein that is thought to have a role in the protection against NO and nitrite stresses is the hybrid cluster protein (Hcp), however its exact mechanism of action is not yet elucidated [11,13,14]. Growing *in silico* and *in vivo* evidence suggests that the *hcp* gene is regulated by HcpR, a transcription factor that belongs to the CRP (cAMP receptor protein)/FNR (fumarate and nitrate reductase regulatory protein) family of transcriptional regulators [15–17]. In *Porphyromonas gingivalis*, an anaerobic periodontopathogen, Hcp expression is compromised in mutant strains lacking HcpR and these strains are unable to survive in host cells [18]. Also, a recent study indicates that in *Desulfovibrio vulgaris* Hcp expression is compromised in an *hcpR* knockout mutant [19]. Notwithstanding, the same mutant strain showed an improved fitness when growing in the presence of nitrite [19].

The aim of this work was to clarify whether *Desulfovibrio gigas* HcpR provides protection against nitrosative stress. To this end, we searched its genome for HcpR-related sequences and identified two HcpR paralogous genes [20]. Phylogenetic analyses on sequences of the HcpR and related regulators found in the *Desulfovibrio* genus indicate that several species possess two or more HcpR paralogs. Here we demonstrate that one of the HcpR genes (*hcpR1*) found in the *D. gigas* genome regulates Hcp and that its deletion impairs growth in the presence of NO donors, suggesting a role in nitrosative stress response.

2. Materials and methods

2.1. Search for HcpR homologs and HcpR1 putative targets

The *D. gigas* genome was searched for putative HcpR sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and a known sequence of HcpR from *D. vulgaris* Hildenborough. This search was then broadened to include all the available genomes of *Desulfovibrio* spp., related δ-proteobacteria, other proteobacteria, bacteroidetes and cyanobacteria.

The sequence alignment of HcpR1 and HcpR2 from *D. gigas* was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Additional editing of HcpR1 and HcpR2 protein sequence alignment was done using the GeneDoc software (http://www.nrbsc.org/gfx/genedoc/).

The *D. gigas* genome (NCBI accession number: CP006585) was scanned for putative HcpR1 binding sites by means of the Virtual Footprint software, version 3.0 (http://www.prodoric.de/vfp/index2.php), a tool from PRODORIC, and using the available HcpR position weight matrix (PWM) calculated for Desulfovibrionales in the RegPrecise database [21,22]. The default parameters for sensitivity, core sensitivity and size were changed to 1, 0.9 and 6, respectively, without the penalty for non-occurrence.

2.2. Phylogenetic analyses

Regarding HcpR sequences, we performed phylogenetic analyses in two separate data sets. Data set 1 includes HcpR-like sequences of *Desulfovibrio* and closely related δ-proteobacteria (plus an outgroup: *Porphyromonas*). Data set 2 is a larger matrix that includes HcpR, Dnr, Nnr and other CRP/FNR-like proteins from proteobacteria, bacteroidetes and cyanobacteria, as these sequences have been found to be closely related to the HcpR [16,23,24]. The cyanobacteria sequences were used to root the trees of data set 2, after being determined that these formed a separated group that was distantly related to the other sequences in our sampling (Supplementary data, Fig. S1). The characteristics of the sequence data sets that were analyzed in this study are presented in Supplementary data, Table S4. The 16S rRNA sequences were obtained from GenBank for the same strains that were sampled for the HcpR analysis. GenBank numbers of all the sequences and taxa used are listed in Supplementary data, Table S3. Sequences of the HcpR region were aligned using MUSCLE, version 3.8 [25,26], and the 16S rRNA sequences were aligned in ClustalX, version 2.1 [27]. Alignments were visually inspected to detect and manually correct misaligned positions. Poorly aligned and highly divergent positions of the HcpR data sets were excluded with the program Gblocks, version 0.91b [28,29]. Pairwise distances were computed in MEGA, version 6 [30], using p-distance and pairwise deletion, to obtain values that represent the proportion of positions that differ between every two sequences in our alignments. Three different approaches were used for phylogenetic reconstruction: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). MP was performed in PAUP*, version 4.0b10 [31]; ML analysis was performed in GARLI, version 2.01 [32]; BI was conducted in MrBayes, version 3.2.2 [33]. Prior to ML and BI, best-fit models of sequence evolution were selected with ProtTest, version 3.4 [34] for the HcpR alignments, and jModelTest, version 2.1.5 [35], for the 16S rRNA data set. A more detailed description of the methods used for phylogenetic analysis is provided in Supplementary data. Trees were rooted using the outgroup approach [36]. For the HcpR data sets, we performed preliminary analyses with a larger sampling of FNR-like sequences (Supplementary data, Table S3) to determine the position of sequences in unrooted trees and select as outgroups sequences that were clearly placed outside the clades we wanted to investigate.

2.3. Culture media and growth conditions

D. gigas WT and *ΔhcpR1* mutant strains were routinely grown at 37 °C, anaerobically, in modified Postgate medium C [37] containing 25 μM iron, sodium thioglycolate (0.88 mM) and ascorbic acid (0.57 mM) as reducing agents. Lactate (40 mM) and sulfate (17.6 mM) were used as electron donor and acceptor, respectively. For growth studies with nitrosative stress, both strains were grown in a medium with less yeast extract containing also lactate (40 mM) and sulfate (25 mM) as electron donor and acceptor, respectively [38]. Cells were grown in 100 ml flasks, containing half the volume of medium and a gas phase of 100% N_2, inoculated with 10% (v/v) fresh inoculum. Growth was monitored by optical density (OD) measurements at 600 nm (SmartSpec 3000, BioRad). For phenotypic analysis of WT and *ΔhcpR1* mutant strains, three independent cultures of each strain were grown until mid-exponential phase ($OD_{600} \approx 0.4$). Then, each culture was subdivided into two flasks and 10 μM GSNO or 100 μM of DETA NONOate (NO donors) was added to half the cultures while the other half remained unstressed (control). The concentration of GSNO was used as previously described [12]. DETA NONOate exhausted was prepared as in [39].

2.4. Mutant construction

The flanking regions upstream and downstream (2 kb and ~1 kb, respectively) of the *hcpR1* gene were amplified by PCR from *D. gigas* ATCC19364 wild-type (WT) genomic DNA, using NZYTaq DNA polymerase (NZYTech) and the respective primers (Supplementary data, Table S1). Kanamycin resistance gene (*kan*) was amplified from plasmid pJRD215 with *Phusion* high-fidelity DNA polymerase (Thermo Scientific) (Table S1). The PCR products were ligated and cloned into the vector YipLac211 according to the In-Fusion HD cloning kit (Clonetech) protocol. Kanamycin resistant clones were selected, plasmid DNA extracted (ZR Plasmid Mini-prep) and the construction was confirmed by analyzing the digestion pattern with *PstI* restriction enzyme. A culture of

200 ml of *D. gigas* WT cells was grown in lactate-sulfate medium until early-stationary phase and prepared to be transformed as previously described [40]. Prior electroporation the cells were mixed with 6 μg of the plasmid construct. Electroporation was done aerobically in a BioRad Gene Pulser Apparatus using a 0.1 cm cuvette in the following conditions: 200 Ω (resistance), 1.5 kV (voltage) and 25 μF (capacitance). Immediately after electroporation, cells were inoculated in lactate-sulfate medium and incubated overnight at 37 °C. Lactate-sulfate medium containing 50 μg/ml of kanamycin was inoculated with the overnight culture for 16 hours at 37 °C and these culture was next plated in lactate-sulfate-agar medium supplemented with kanamycin (50 μg/ml). Plates were kept inside an AnaeroPack Rectangular Jar 7L (Mitsubishi Gas Chemical Company, Inc.) with AnaeroPack System sachets (bioMérieux) at 37 °C for three weeks. The deletion of *hcpR1* was confirmed by PCR.

2.5. RNA isolation and quantitative RT-PCR analysis (qRT-PCR)

D. gigas WT and *ΔhcpR1* mutant strain were grown until mid-exponential phase ($OD_{600} \approx 0.4$) and were either untreated or exposed to 10 μM of GSNO for 1 h. Culture sampling was performed inside an anaerobic chamber (855-AC, Plas-Labs). Total RNA extraction was carried out as previously described [12,41]. RNA samples were treated with DNase (TURBO™ DNase-free; Ambion) according to the manufacturer's instructions and purified by on-column DNAse I digestion (RNase-Free DNase Set; Qiagen). Total RNA (1 μg) was reverse transcribed with Transcriptor Reverse Transcriptase (Roche Diagnostics). qRT-PCR reactions were performed in the LightCycler 480 Real-Time PCR System (Roche), using Light Cycler Fast Start DNA Master SYBR Green I (Roche). Relative standard curves were constructed for each gene, using triplicate serial dilutions of cDNA. The relative expression of the genes was calculated by the relative quantification method with efficiency correction, using the LightCycler 480 software 1.5. 16S RNA gene was used as a reference gene. The primers used in this assay are listed in Supplementary data, Table S2. qRT-PCR experiments were carried out using biological triplicates.

3. Results

3.1. Search for HcpR sequences in the D. gigas genome

Recent evidence suggests that *Desulfovibrio magneticus*, *Desulfovibrio salexigens* and *Desulfovibrio desufuricans* possess a second HcpR related sequence in their genome that was predicted to bind an 18-bp pseudopalindromic DNA sequence similar to the HcpR binding motif [23]. Interestingly, a search of the *D. gigas* genome [20] revealed the presence of two HcpR sequences that share 48.9% of similarity at the amino acid level, here designated by HcpR1 and HcpR2 (Fig. 1). These sequences are apart in the genome, but *hcpR1* is in the vicinity of the *hcp* gene (Fig. 6). HcpR1 and HcpR2 sequences exhibit the typical structural domain organization of the CRP/FNR family (Fig. 1).

3.2. Phylogenetic analyses of HcpR sequences

To understand the evolution of HcpR sequences in *Desulfovibrio* spp. and related δ-proteobacteria, we searched for orthologous sequences in the available genomes and performed phylogenetic analyses. The resulting trees of the HcpR and other CRP/FNR-like proteins are shown in Fig. 2 and Supplementary data, Fig. S1 (the latter includes a larger sampling of species and taxonomic groups).

To determine if the groups (clades) recovered in the HcpR phylogenetic trees reflect the organismal phylogeny, we estimated a phylogeny for the genus *Desulfovibrio* and related taxonomic groups using sequences of the 16S rRNA gene (Fig. 3). The 16S rRNA tree includes a higher number of species, sequences, and taxonomic groups compared to the HcpR tree (Fig. 2). Nevertheless, the HcpR protein data set includes sequences with divergence up to 85.3% (Supplementary data, Table S4), in contrast to a sequence divergence up to 28.6% in the 16S rRNA data set, showing that the HcpR and HcpR-like sequences are evolving at a must faster rate than the 16S rRNA gene.

Based on phylogenetic analysis, we can define two main groups within *Desulfovibrio* HcpR sequences: I and II (Fig. 2). Group I is strongly supported and includes HcpR sequences of *Desulfovibrio desulfuricans* ATCC 27774, *Desulfovibrio piger*, two yet unidentified species and the opportunist pathogen *Bilophila wadsworthia*. Group II includes all the other sampled *Desulfovibrio* spp. and is further subdivided into two groups (clades II.a and II.b). Group II.a includes *D. vulgaris*, *Desulfovibrio alaskensis*, *Desulfovibrio africanus* and one unidentified species. Both HcpR sequences of *D. gigas* are placed in clade II.b, but in two distinct subgroups: HcpR1 is found in clade II.b.1 and HcpR2 in clade II.b.2.

It should be noted that several species of *Desulfovibrio* and other δ-proteobacteria include two or more paralogs of HcpR-like proteins. For example, the genomes of *D. alaskensis* and *D. vulgaris* include only one HcpR version; those of *D. desulfuricans* ATCC 27774, *D. gigas* and *D. magneticus* have two HcpR paralogs; whereas the genomes of *D. aespoeensis* and *D. desulfuricans* ND132 have three versions of HcpR-like sequences, and that of

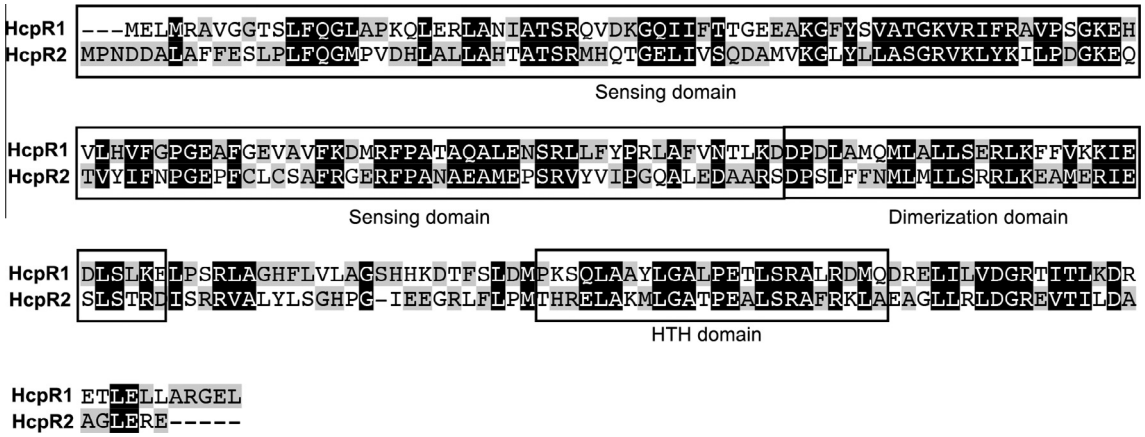

Fig. 1. Amino acid sequence alignment of *D. gigas* HcpR paralogs. The three typical domains of the members of the CRP/FNR family are boxed according to the structure of DNR regulator from *Pseudomonas aeruginosa* [65]. Black shading of the amino acid residues corresponds to 100% of similarity.

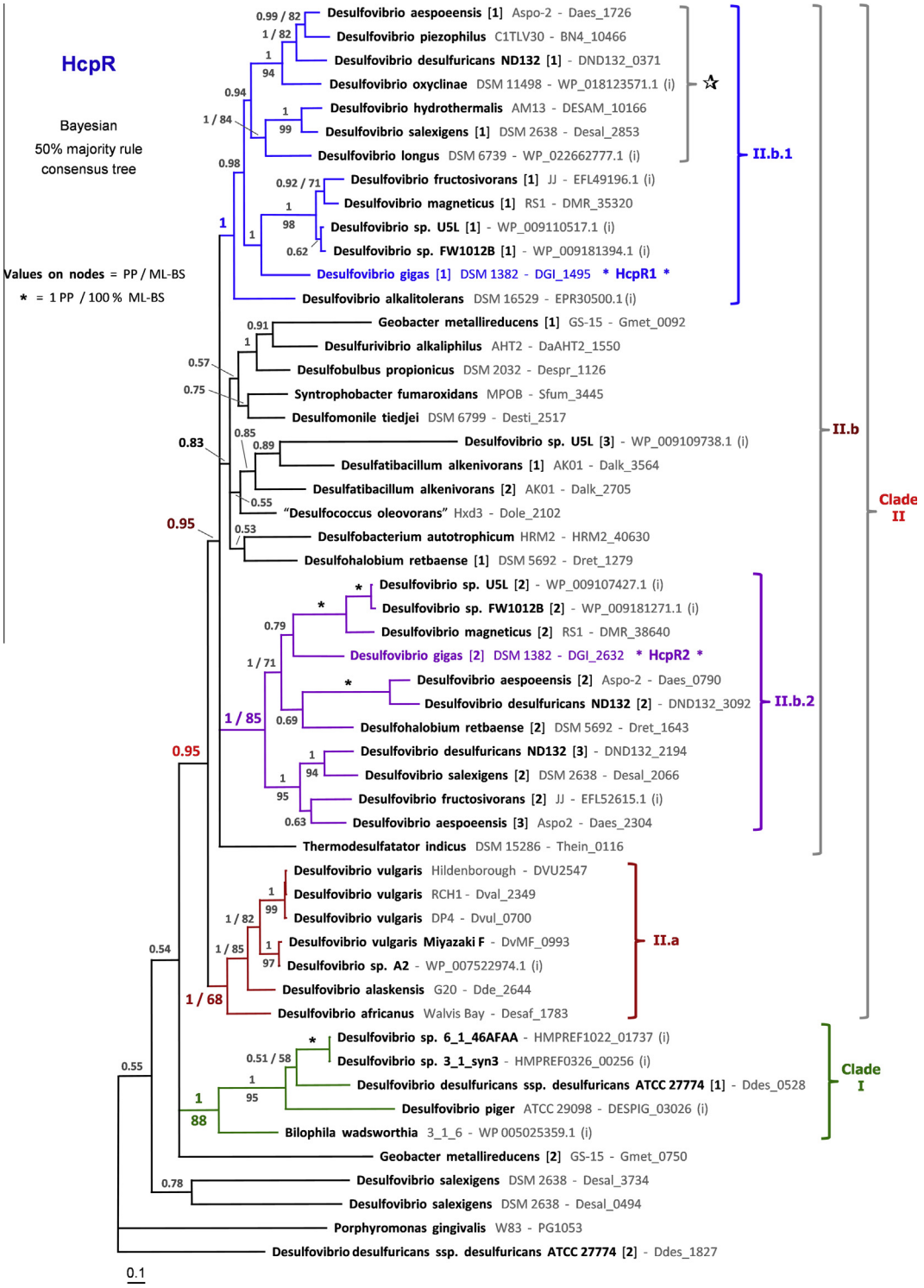

Fig. 2. Phylogenetic tree of HcpR and HcpR-like proteins of *Desulfovibrio* and related δ-proteobacteria. The tree is a Bayesian 50% majority rule consensus tree, with associated branch-lengths. Values on nodes refer to posterior probabilities (PP) and maximum-likelihood bootstrap (ML-BS). Clade support can be regarded as high (ML-BS ⩾ 70% and PP ⩾ 0.95), moderate (either ML-BS ⩾ 70% or PP ⩾ 0.95) or low (ML-BS < 70% and/or PP < 0.95). When only one value is given in a node it refers to PP. Sampling also included HcpR-like sequences from a species of Thermodesulfobacteria and from *Porphyromonas gingivalis* (outgroup). The group marked with a "star" is an example of the clades that are recovered with similar topology in the 16S rRNA tree (see Fig. 3), the notation (i) refers to sequences obtained from genomes that are not completely sequenced.

D. salexigens has four. Some of these paralogs are sequences that are highly divergent, both in sequence and length, and some of them also appear separated from the main HcpR clades (Fig. 2), such as two of the HcpR paralogs of *D. salexigens* (Desal_0494, Desal_3734), and paralogs number 2 of *D. desulfuricans* ATCC 27774 (Ddes_1827) and *Geobacter metallireducens* (Gmet_0750).

We next evaluated whether HcpR phylogeny-derived groups could be correlated with the corresponding HcpR DNA binding domain. The C-terminal HTH domain of HcpR comprises two residues of arginine (R) and glutamate (E), known to be essential for DNA binding specificity [16,42]. However, these residues are variable, especially arginine which sometimes is replaced by a proline

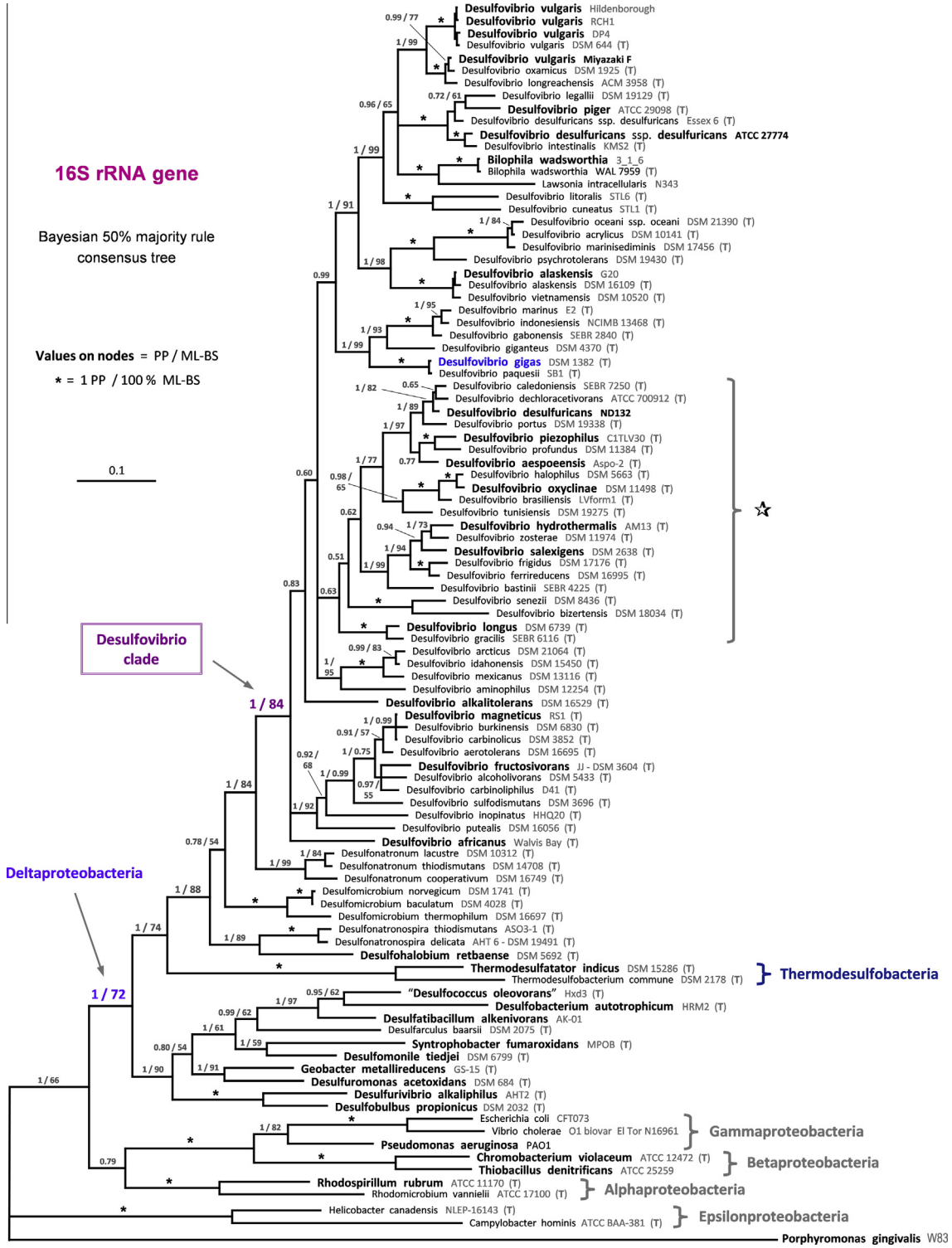

Fig. 3. Phylogenetic tree of the 16S rRNA gene in *Desulfovibrio* and other proteobacteria. The tree is a Bayesian 50% majority rule consensus tree, with associated branch-lengths. Values of clade support (PP and ML-BS) are explained in Fig. 2. The group marked with a "star" is one of the clades that is recovered with similar topology in the HcpR trees (Fig. 2 and Supplementary data, Fig. S1).

or a serine [16]. We found that there is a good correlation between the well-supported groups defined in the phylogenetic analysis and these two residues (Fig. 4). In addition, we found that besides proline and serine, arginine can also be replaced by glutamine (Fig. 4, clade II.b.1).

3.3. Effect of NO stress in the growth of *D. gigas* Δ*hcpR1* strain

Previous experimental evidence suggests that Hcp is involved in bacterial defense against nitrosative stress [11,13,14]. As in *D. gigas* genome *hcpR1* is close to *hcp*, probably sharing the same promoter

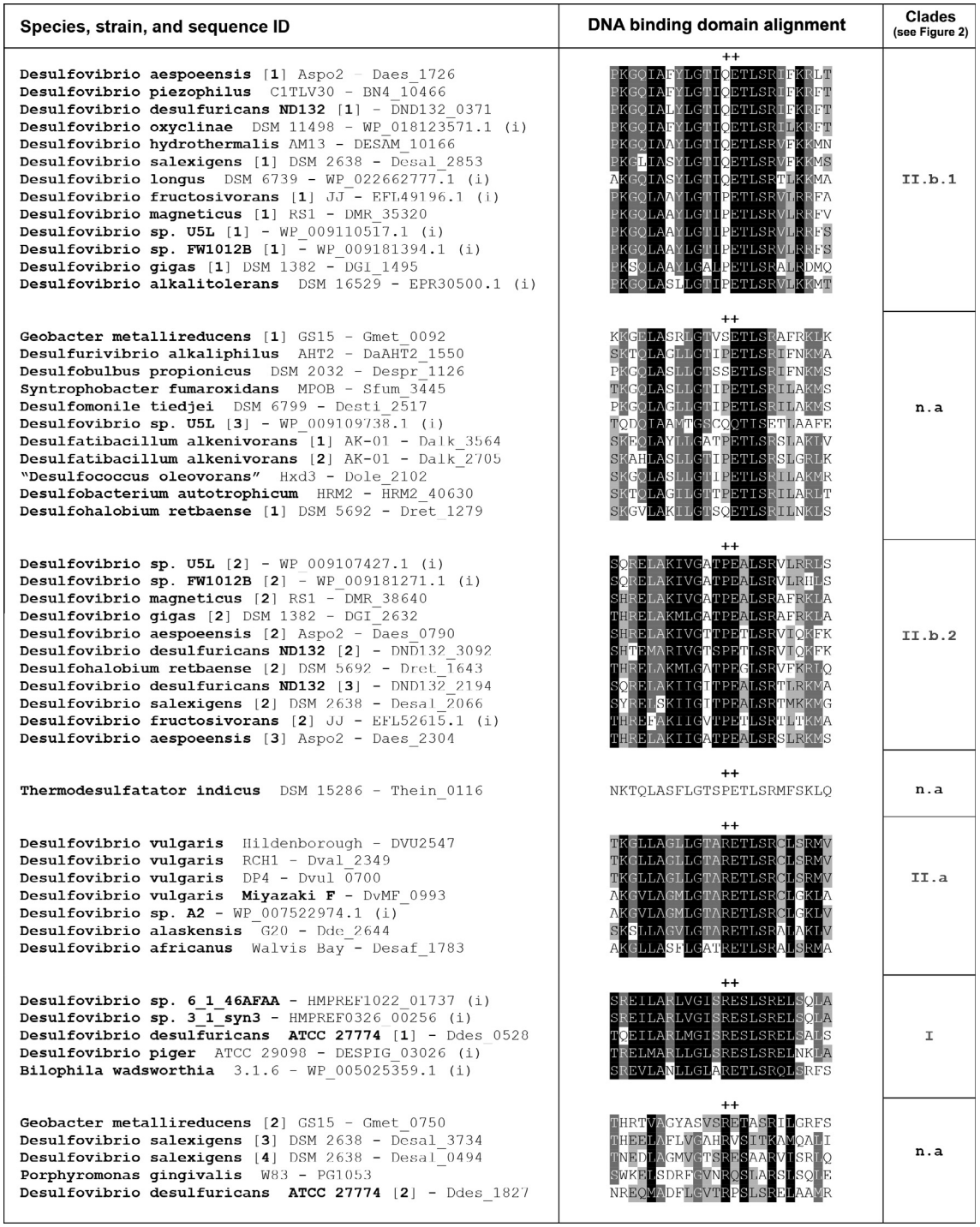

Fig. 4. Alignment of the HcpR DNA binding domain (helix-turn-helix domain) of each HcpR phylogeny-derived group. The two amino acid residues previously shown to interact with DNA (16) are indicated by "+". Species and strains are presented in the order they appear in the phylogenetic tree of Fig. 2; the notation (i) refers to sequences obtained from genomes that are not completely sequenced. n.a-not assigned. The four levels of shade correspond to 100% of similarity (black); 80–90% of similarity (gray with white symbols); 60–79% of similarity (gray with black symbols) and less than 60% of similarity (not shaded).

(Fig. 6), we decided to proceed with the functional characterization of this *hcpR* gene. As such we generated a *null* mutant for this regulator, *ΔhcpR*1. To this end, the *hcpR1* gene was replaced by the gene conferring resistance to kanamycin and gene disruption was confirmed by PCR.

The growth phenotype of the mutant was next analyzed in the presence of 10 μM of S-nitrosoglutathione (GSNO), an NO donor, and compared to the wild-type strain (WT). Under physiological conditions, the WT and *ΔhcpR1* strains exhibited similar growth profiles (Fig. 5A). However, the growth of the mutant strain was severely impaired in the presence of GSNO, while the WT strain resumed growth 7 h after drug addition. To confirm the behavior of *ΔhcpR1* mutant towards NO, we have used another structurally distinct NO donor – DETA NONOate (Fig. 5B). We observed that the growth of *ΔhcpR1* mutant was impaired by treatment with 100 μM of DETA NONOate, whereas the WT strain is insensitive to this

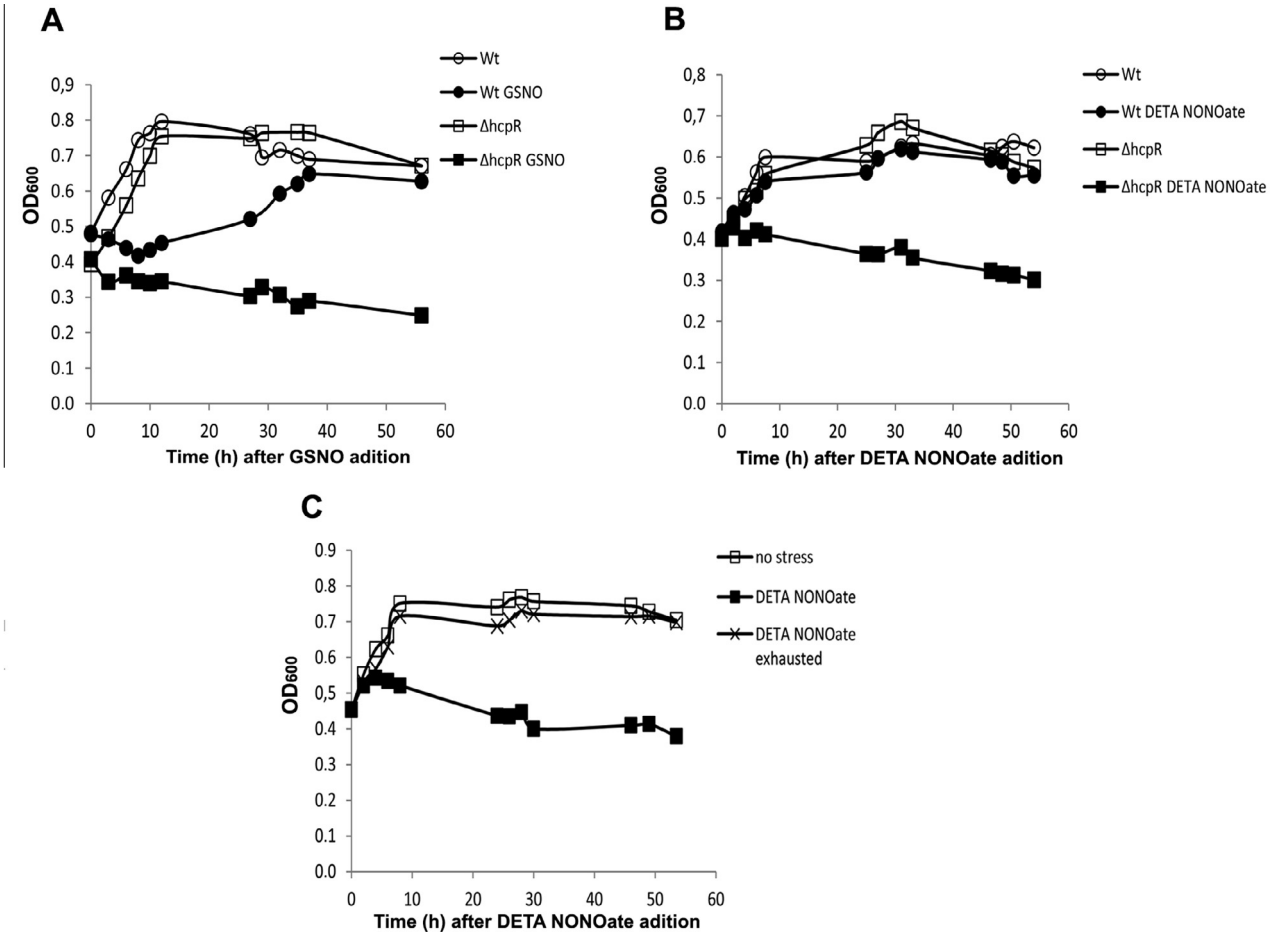

Fig. 5. Growth curves of *D. gigas* wild-type and *D. gigas hcpR1* mutant under nitrosative stress conditions. (A) Wild-type (squares) and mutant (circles) strains were treated (closed symbols) or not treated (open symbols) with 10 μM GSNO. (B) Wild-type (squares) and mutant (circles) strains were treated (closed symbols) or not treated (open symbols) with 100 μM DETA NONOate. (C) The Δ*hcpR1* mutant was left untreated (open squares) or treated with 100 μM DETA NONOate (black squares) or with 100 μM of exhausted DETA NONOate (cross symbols). Results are the mean of three independent experiments ± standard deviation.

Table 1
Possible targets and DNA binding sequences of *D. gigas* HcpR1.

Target	Consensus sequence	Position
Hcp	TTTTGATATGTGTCAAGG	−103
Ferredoxin	TCTTGACGTGCGTCAACG	−53
NrfA	TCTTGACCTACATCAAGG	−139
DGI_2602	TTTTGATGCGTATCAAGG	−64
DGI_1194	TGTTGACACACATCAAGG	−34
DGI_2097	TCTTGACCTGCATCATGG	−65
DGI_3367	TCTTGACCTGCATCATGG	−65
DGI_0682	TCTTGACATAATTCAGGG	−114
DGI_1469	TTTTGACTGCCGTCAACA	−106

compound concentration. Since different NO donors had the same effect on the mutant growth, it is highly likely that the observed growth defect was due to NO release. This assumption was further corroborated by the fact that exposure to the DETA nucleophile alone (ie, exhausted DETA NONOate) had no inhibitory effect in the growth of *ΔhcpR1* mutant (Fig. 5C).

These data clearly indicate that HcpR1 in *D. gigas* promotes cell survival under nitrosative stress conditions.

3.4. Search for HcpR targets in the D. gigas genome

The putative regulon of HcpR and the corresponding binding motif in *Desulfovibrio* spp. were previously determined *in silico* and are publicly available in the RegPrecise database [15,16,22,23]. HcpR1-like proteins were predicted to recognize an 18-bp pseudopalindromic consensus binding sequence, nttTGACnnnnTCAaag, found in the promoter region of its putative targets [23].

After a search in the genome of *D. gigas* [20] for genes that may be regulated by HcpR1, fifteen putative targets were found to contain the described consensus binding site within the respective promoter regions. Among the fifteen candidate targets, those annotated in the *D. gigas* genome as hypothetical proteins were disregarded, as well as those whose consensus binding site was located more than 250 bp upstream of the initiation codon. We then proceeded to analyze the remaining nine putative targets that are listed in Table 1.

Five out of the nine putative HcpR1 targets found (Table 1) have been previously assigned to the HcpR regulon of Desulfovibrionales [23] and include the genes coding for the 'hybrid cluster protein' (Hcp), ferredoxin (frdx), a putative membrane-bound polyferredoxin (DGI_1194), the operon of the dissimilatory cytochrome *c* nitrite reductase (NrfHA) and a gene encoding a protein containing a cupin domain (DGI_2602). In addition, four new putative targets were identified: a gene annotated as a pseudogene (DGI_0682), a gene coding for a porin (DGI_1469) and two identical genes encoding a protein with a 4Fe-4S cluster region (DGI_2097/DGI_3367).

The structural organization of the *D. gigas* HcpR1 regulon appears to be unique when compared to all other *Desulfovibrio* spp. analyzed so far. Indeed, in the genomes of other

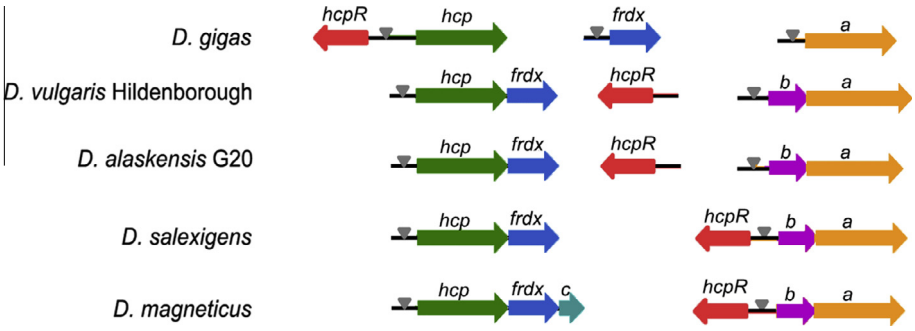

Fig. 6. Genomic localization of *hcpR1* among different *Desulfovibrio* spp. *hcp*, hybrid cluster protein; *frdx*, ferredoxin; a, membrane-bound polyferredoxin; b, oxidoreductase; c, cupin 2 barrel domain. The dark gray arrow indicates the position of HcpR1 putative binding sites.

Desulfovibrio spp. the HcpR regulon appears to include two conserved operons, one containing *hcp* and *frdx* genes and another one containing an oxidoreductase and a membrane-bound polyferredoxin [23]. The *D. gigas hcp* gene is, however, part of a monocistronic operon and the *frdx* gene appears several bp upstream of the *hcp* operon (Fig. 6). Contrary to other *Desulfovibrio* spp., the membrane-bound polyferredoxin (DGI_1194) is also monocistronic (a in Fig. 6), and no oxidoreductase gene ortholog (b in Fig. 6) was found in the *D. gigas* genome.

3.5. In vivo validation of the putative HcpR1 targets

In order to validate the *in silico* found HcpR1 targets, their expression was evaluated by qRT-PCR in the WT and mutant strains, both in the absence and after treatment with GSNO for 1 h as we observed *hcp* to be maximum induced under this conditions in the WT strain (Fig. S2). The expression of five genes, *hcp*, *frdx*, *nrfA*, DGI_1194 and DGI_2602, was dependent on HcpR1 after GSNO addition (Fig. 7A–E). Although only residual transcript levels of these targets were observed under normal growth conditions, a significant upregulation was registered in WT cells after GSNO treatment. The nucleotide sequences of DGI_2097 and DGI_3367 genes are 100% identical. As such, the increased expression observed after 1 h of treatment with GSNO may reflect the upregulation of one or of both genes (Fig. 7F).

The two genes identified in the *D. gigas* genome with the *locus* tag DGI_0682 and DGI_1469 were downregulated by GSNO, but independently of HcpR (Fig. 7G and H). The gene identified as DGI_0682 was annotated in the *D. gigas* genome as a 'pseudogene'. However, our results indicate that DGI_0682 is expressed and as such is not a pseudogene [20].

4. Discussion

The search for HcpR homologs in the *D. gigas* genome revealed the presence of two HcpR-related sequences. The dissimilarities observed in the alignment of the HTH domain of both sequences (Fig. 1) suggest that these regulators may have different DNA binding affinities, as previously proposed [16]. Alternatively, as HcpR binds its target DNA as a dimer [43], the formation of HcpR heterodimers in these species cannot be ruled out.

To understand whether the occurrence of HcpR paralogs is a common feature among *Desulfovibrio* spp., we conducted phylogenetic analyses of the HcpR and other CRP/FNR-like sequences. The topology of the phylogenetic trees revealed several well-supported clades (Fig. 2 and Supplementary data, Fig. S1), including two main clades, I and II, whose relationship to each other is unresolved. Clade I is strongly supported and includes HcpR from *Desulfovibrio* spp. commonly present in human digestive tract and often associated with gastrointestinal diseases or other opportunistic infections [44–46]. This group also comprises the HcpR from *Bilophila wadsworthia*, a Desulfovibrionaceae closely related to *Desulfovibrio* and that is a recognized opportunistic pathogen found in several anaerobic infections [47,48]. Clade II has moderate support and includes the majority of δ-proteobacteria *hcpR* genes sequenced to date. The two main clades (I and II) in the HcpR tree have likely resulted from a duplication event of an ancestral HcpR sequence. A second gene duplication may have originated the group designated as clade II.b.2, which corresponds to HcpR2-like sequences, including that of *D. gigas*. Within clade II.b.2, there was a third *hcpR* duplication event. After duplication, resulting genes (paralogs) evolve independently, and may even acquire new functions. It may happen that all the paralogs are preserved in some lineages, but quite often some of the paralogs are lost, and the sequences that survive in separate lineages are not always the same (e.g. [49]). The pattern of duplication and unequal loss, explains why we found *Desulfovibrio* species with two or even three genes of HcpR-like sequences, whereas others had only one version. It also explains why species like *D. piger* (clade I) and *D. vulgaris* (clade IIa), which appear closely related in the 16S rRNA phylogeny (Fig. 3), and have only one HcpR sequence, appear in very distinct clades in the *hcpR* gene tree (Fig. 2). These results suggest that the common ancestor of *D. piger* and *D. vulgaris* had (at least) two distinct HcpR copies (paralogs), subsequently, one of the copies (but not the same) was lost in each of the lineages that gave rise to the two species. Therefore, the HcpR sequences of *D. piger* and *D. vulgaris* are paralogs, not orthologs.

The relationships shown in the 16S rRNA phylogeny (Fig. 3) generally agree with the results of previous studies in Desulfovibrio (e.g. [50–52]). Comparison of the topological relationships of the *Desulfovibrio* species in the 16S rRNA tree (Fig. 3) to those in the HcpR tree (Fig. 2 and Supplementary data, Fig. S1) indicates great similarity between some clades. The similar or identical topological relationships found in the trees for group II.b.I clearly suggest that the HcpR (paralog 1) was transmitted vertically from the common ancestor that gave rise to this group. As the relationships among the main clades of the HcpR are unresolved or poorly supported, it is not possible to indicate with certainty in which nodes of the HcpR tree the sequences may have duplicated. Nevertheless, we can suggest that paralogs 2 and 3 of the HcpR-like sequences, as seen in clade II.b.2, derived from a single duplication event that occurred before the separation of the lineage that originated *D. aespoensis* and *D. desulfuricans* ND132. The position of the HcpR sequence 'copy 2' of *Desulfovibrio fructosivorans* in clade II.b.2 is not in agreement with the species phylogeny as suggested by the 16S rRNA tree. *D. fructosivorans* belongs to the same clade of *D. magneticus*, which appears to be distantly related

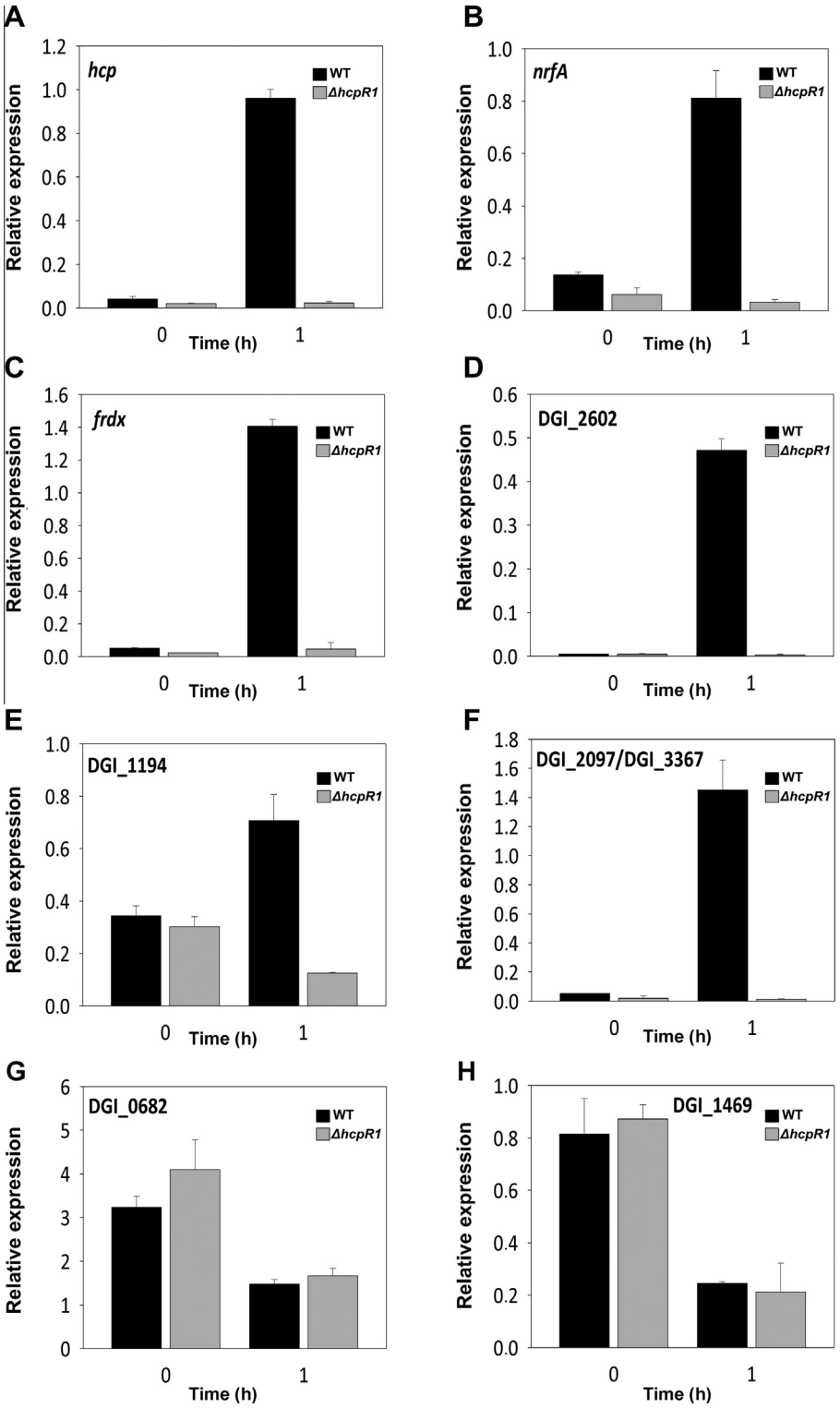

Fig. 7. Expression of HcpR1 putative targets under nitrosative stress. *D. gigas* wild-type and Δ*hcpR1* mutant cells were exposed to 10 μM GSNO for 1 h. The expression of the indicated genes was assessed by qRT-PCR in both treated and untreated samples. Each panel shows the relative expression of A. *hcp* gene, B. *frdx* gene, C. *nrfA* gene, D. DGI_2602, E. DGI_1194, F. DGI_2097/DGI_3367, G. DGI_0682 and H. DGI_1469. The black bars represent the relative expression of each gene in the wild-type strain and the gray bars the relative expression in the *hcpR1* mutant strain. The two bars on the left refer to the relative expression in the untreated samples and the two bars on the right refer to the relative expression in the samples after 1 h exposure to GSNO. 16S RNA gene was used as a reference gene. Values are the mean of biological triplicates ± standard deviation.

to the clade that includes *D. aespoeensis*, *D. salexigens* and *D. desulfuricans* ND132 (Fig. 3). Therefore, it is possible that the HcpR copy 2 of *D. fructosivorans* was acquired via horizontal gene transfer (HGT) from a species that belongs to the *D. aespoensis–D. salexigens* clade.

The features of protein and DNA alignments are not directly comparable, but the information on sequence divergence of the HcpR and 16S rRNA data sets (Supplementary data, Table S4) clearly shows that the HcpR regulator and its paralogous sequences are evolving at a fast rate. The fast mutation rate of HcpR sequences

is in agreement with findings that transcription regulators evolve rapidly in bacteria [53]. Although HcpR proteins have very low sequence conservation (Supplementary data, Table S4), there is still structural similarity in most of these sequences. However, some of the highly divergent sequences in our data sets, such as genes 3 and 4 of *D. salexigens* (Desal_0494 and Desal_3734), and paralog 2 of *D. desulfuricans* ATCC 27774 (Ddes_1827), show a much lower degree of sequence and structural similarity, suggesting that these proteins may have different functions or are evolving with relaxed functional constraints. None of the HcpR-like sequences we analyzed had stop codons or other features that could suggest that they are pseudogenes [54]. However, some of the more divergent HcpR-like sequences of *Desulfovibrio* had several insertions (1–9 aa in length), and such indels associated with higher rate of sequence divergence may, in some situations, indicate the start of a pseudogenization process (loss of function).

In previous studies, *hcp* gene was shown to be upregulated in *D. vulgaris* upon nitrate and nitrite stresses [14,55,56]. Nonetheless, *D. vulgaris hcpR* mutant was shown to have an improved fitness when growing in the presence of nitrite, although *hcp* was clearly downregulated in the *ΔhcpR* mutant strain [19]. In an attempt to clarify the role of HcpR in nitrosative stress response we have generated a mutant strain for the *hcpR*1 gene, which is localized in the proximity of *hcp* in the *D. gigas* genome. The *ΔhcpR1* strain was shown to exhibit an impaired growth in the presence of GSNO and DETA NONOate, two NO generators (Fig. 3), suggesting an important role of HcpR1 in cellular protection during nitrosative stress. We next sought to identify the HcpR1 regulon under nitrosative insult, by performing an *in silico* search for HcpR1 target genes, within the genome of *D. gigas*. Our analysis retrieved nine candidate genes whose expression under nitrosative stress was experimentally evaluated. The expression of two of these genes appeared to be independent of HcpR upon stress. Three of the HcpR-induced genes, *hcp*, *frdx* and *nrfA* are known to participate in nitrogen metabolism [57,58]. However, the only gene product with a well-established function is NrfA, an enzyme catalyzing the reduction of nitrite to ammonia and present in organisms able to perform nitrate or nitrite ammonification [57]. NrfA was also shown to reduce hydroxylamine and nitric oxide thus assuming a detoxifying function even in non-ammonifying microorganisms [56,59–62]. In this context, the upregulation of *D. gigas nrfA* after GSNO addition is in agreement with its NO-reductase activity (Fig. 7B). The genes encoding Hcp and Frdx were previously shown to be upregulated by nitrite and nitrate in *D. vulgaris* [14,55,56,63]. Hcp was also implicated in cell survival under nitrite stress and in cell protection during macrophage infection [10,11,13]. Concurrently, we observed the HcpR1-dependent upregulation of *hcp* and *frdx* genes under such conditions. Contrary to other *Desulfovibrio* spp, in *D. gigas*, *hcp* and *frdx* belong to different operons, but are nevertheless regulated by HcpR1. The remaining genes DGI_1194 and DGI_2097/DGI_3367 encode metalloproteins containing iron-sulfur clusters, whose function remains unknown. Gene DGI_2602 encodes a putative cupin 2 barrel domain-containing protein. Cupin domain-containing proteins belong to a superfamily whose members perform diverse cellular functions, including response to stress conditions [64]. Expression analysis revealed that this gene is barely expressed under physiological conditions, but highly expressed upon treatment with GSNO in an HcpR dependent manner, suggesting a putative role of the encoded protein in the response to nitrosative stress.

5. Conclusions

The absence of HcpR1 renders *D. gigas* sensitive to NO. This regulator orchestrates the expression of several genes encoding proteins involved in nitrite and nitrate metabolism, strongly suggesting its involvement in NO detoxification. The fact that several *Desulfovibrio* species possess HcpR paralogs, which have been transmitted vertically in the evolution and diversification of the genus, indicates that these sequences may confer adaptive or survival advantage to these organisms, possibly by increasing their tolerance to nitrosative stress. Detailed structural and functional analysis of those sequences need, however, to be performed to fully understand their role in stress response. Further work in this context is in progress, with the aim to clarify the putative role of *D. gigas* HcpR2 under nitrosative stress conditions.

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SMS, CP and CRP conceived and designed the experiments. SMS, CA, CS and CP performed the experiments. SMS, CP and CRP analyzed the data. SSN performed phylogenetic analyses. SMS, SSN, CP and CRP wrote the paper. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2015.07.001.

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