Role of NorR-like transcriptional regulators under nitrosative stress of the δ-proteobacterium, *Desulfovibrio gigas*

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ABSTRACT

NorR protein was shown to be responsible for the transcriptional regulation of flavorubredoxin and its associated oxidoreductase in *Escherichia coli*. Since *Desulfovibrio gigas* has a rubredoxin:oxygen oxidoreductase (ROO) that is involved in both oxidative and nitrosative stress response, a NorR-like protein was searched in *D. gigas* genome. We have found two putative *norR* coding units in its genome. To study the role of the protein designated as NorR1-like (NorR1L) in the presence of nitrosative stress, a *norR1L* null mutant of *D. gigas* was created and a phenotypic analysis was performed under the nitrosating agent GSNO. We show that under these conditions, the growth of both *D. gigas* mutants Δ*roo* and Δ*norR1-like* is impaired. In order to confirm that *D. gigas* NorR1-like may play identical function as the NorR of *E. coli*, we have complemented the *E. coli* Δ*norR* mutant strain with the *norR1-like* gene and have evaluated growth when nitrosative stress was imposed. The growth phenotype of *E. coli* Δ*norR* mutant strain was recovered under these conditions. We also found that induction of *roo* gene expression is completely abolished in the *norR1L* mutant strain of *D. gigas* subjected to nitrosative stress. It is identified in δ-proteobacteria, for the first time a transcription factor that is involved in nitrosative stress response and regulates the *rd-roo* gene expression.

1. Introduction

Sulfate-reducing bacteria (SRB) are prokaryote microorganisms that use sulfate as the terminal electron acceptor in their metabolism and thus play a key role in sulfur cycling and are involved in several processes such as biomineralization, biocorrosion and metal metabolism. SRB are also capable of reductively precipitate redox metals, being often referred to as promising tools for bioremediation of environments contaminated with heavy metals [1,2]. They have been described as strict anaerobe organisms having an extreme respiratory flexibility [3]. These bacteria have to sense and respond to the chemical changes in their surroundings in order to survive in ever-changing environments. As such they use several mechanisms to avoid the unfavorable habitats and to approach the favorable ones.

Desulfovibro gigas, a member of the δ-proteobacteria, is a model SRB that has the ability of surviving in the presence of oxygen in its natural habitats. Indeed, these microorganisms exist in oxic environments as for instance in the interface of microbial mats as well as in oxygenated areas of aquatic spaces [4]. *D. gigas* contains oxygen detoxifying enzymes such as desulfoferredoxins and neelaredoxins which were shown to have superoxide-scavenging activity either as superoxide dismutase or superoxide reductases [5]. Furthermore, SRB and in particular *D. gigas* were shown to reduce oxygen to water having oxygen-reducing systems [6–8]. In this system rubredoxin (Rd), a small mononuclear iron protein transfers electrons between a NADH oxidase (NRO) and the terminal oxidase rubredoxin:oxygen oxidoreductase (ROO) [9].

ROO was also found to be involved in the protective mechanisms against NO (nitric oxide) in several *Desulfovibrio* species (*D. gigas, Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans* among others) [10]. SRB has to cope with NO formed and released in the process of nitrite reduction by coexisting denitrifying microorganisms. Moreover, since *Desulfovibrio* organisms may inhabit the mammalian digestive tract, they are frequently challenged with NO, released by activated macrophages at the onset of infection and inflammatory processes [11,12]. Therefore bacterial response to NO is of considerable importance, occupying a central position in contemporary medicine, physiology, biochemistry and microbiology. A flavorubredoxin (Flrd) and its associated oxidoreductase (encoded by *norV* and

norW, respectively) detoxify NO to form N_2O (nitrous oxide) under anaerobic conditions in Escherichia coli [13]. In this bacterium, the transcription of these genes is activated under nitrosative stress by the σ54-dependent regulator, NorR, a member of the bacterial enhancer-binding protein family [14,15]. This factor binds to three sites upstream of the norV promoter that contains inverted repeats with core consensus [GT-(N7)-AC], being conserved amongst the β- and γ-proteobacteria [16,17]. The fact that upstream of the rd-roo polycistronic unit of D. gigas were found two putative consensus sequences for a NorR-binding box led us to search in its genome the potential NorR binding factors [18]. We have found two coding units designated norR1L and norR2L genes by using bioinformatic tools. We have generated the norR1L mutant strain of D. gigas of which the growth was assayed in the presence of GSNO, a NO generator. Our results show that the growth of D. gigas mutant strain is impaired under these conditions and have also shown that norR1L encoding gene of D. gigas complements the sensitive phenotype of E. coli norR mutant strain. Accordingly roo gene expression was examined in the norR1L mutant strain revealing a complete abolishment of its induced mRNA levels.

2. Materials and methods

2.1. Bacterial strains and growth media

D. gigas wild-type (wt) ATCC 19364, Δroo and ΔnorR1-like mutants were grown at 37 °C, anaerobically in lactate–sulfate medium [19]. Kanamycin 50 µg/ml was added to the mutants. E. coli wt MC1000, ΔnorR MC1003 and ΔnorR MC1003 complemented with D. gigas norR1-like gene (amplified from D. gigas wt) were grown at 37 °C, aerobically in lysogeny broth medium as previously described [20] and anaerobically in the medium reported [21]. Kanamycin 50 µg/ml was added to ΔnorR and ampicillin 100 µg/ml to the complemented mutant.

2.2. norR1-like deletion

A null mutant for norR1-like gene of D. gigas was generated by gene replacement with kanamycin resistance gene by homologous recombination as previously described [10,20].

2.3. Phenotypic analysis of D. gigas and mutant strains

D. gigas wt, Δroo and ΔnorR1-like mutant strains were grown overnight in lactate–sulfate medium. Cells were diluted in the same growth medium to OD ∼0.05 and grown until early exponential phase (∼0.3 to ∼0.4 OD), when 10 µM of GSNO (NO donor) was added to the cultures. Growth of the cultures was monitored by OD_{600} measurement.

2.4. Functional complementation studies

D. gigas norR1-like gene was cloned into the expression vector pFLAG-CTC (Sigma) using restriction sites SmaI. The recombinant plasmid was confirmed by sequencing. Anaerobic cultures of E. coli ΔnorR mutant cells transformed with either the pFLAG-CTC or pFLAG-CTC(norR1-like gene) were grown without shaking at 37 °C in medium as described previously [21] with ampicillin (100 µg/mL).

E. coli wild-type, E. coli ΔnorR and E. coli ΔnorR complemented with plasmid pFLAG-CTC (norR1L gene) were grown overnight aerobically at 37 °C in LB medium and transferred to their growth medium and grown anaerobicaly at 37 °C until OD_{600} of 0.6. Cells were diluted in the same growth medium to OD_{600} of 0.05 and grown until early exponential phase, when were either untreated or exposed to 500 mM of GSNO. Growth of the cultures was monitored by OD_{600} measurement.

2.5. RNA isolation and real-time reverse transcription quantitative-PCR (qRT-PCR) analysis

D. gigas wt and D. gigas ΔnorR1-like were grown overnight in lactate–sulfate medium at 37 °C until they reached the early exponential phase. Cultures were then either untreated or treated with 10 µM of GSNO for several periods of time as indicated and harvested. Total RNA was extracted as previously described with minor modifications [10,18]. To eliminate the presence of DNA contamination in the RNA preparation, RNA samples were treated with turbo DNAse (Ambion) and total RNA (1 µg) was reverse transcribed with Transcriptor Reverse Transcriptase (Roche Diagnostics). Gene primer sequences used in the analysis of roo expression were 5′TCGATCCCAAAAAGATCGAC3′ and 5′GGCCAGTCCTTGTAATGGAA3′. qRT-PCR reactions were performed in a Light Cycler 1.5 Real-Time PCR System (Roche), using Light CyclerFast 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 Software 4.1. The housekeeping gene 16S rRNA was used as the reference gene.

2.6. Sequence analysis

Conserved domain searches were performed by means of CDD: a Conserved Domain Database for the functional annotation of proteins [22].

The multiple sequence alignments were constructed using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2) and edited and shaded in the program GENEDOC (http://www.nrbsc.org/gfx/genedoc). Phylogenetic analysis was conducted with MEGA version 5 [23], using the neighbor-joining method with Poisson correction.

3. Results and discussion

3.1. Search of NorR-like sequences in D. gigas genome

NorR is a σ54-dependent transcription factor that belongs to the NtrC/NifA family, whose members are formed by a modular structure containing three highly conserved domains: a variable N-terminal regulatory domain (GAF), a central ATPase domain that binds the σ54 factor and a C-terminal DNA binding region of the helix-turn-helix type [24–27]. In the absence of NO the catalytic activity of the central domain of NorR is repressed by the N-terminal regulatory domain that contains a non-haem iron centre [24]. The binding of NO to this center results in the formation of a mononitrosyl iron species, promoting the activation of ATPase activity. It was suggested that the highly conserved GAFTGA loop in the ATPase domain is a target for intramolecular repression by the regulatory domain [28].

Taking advantage of the privileged access to the data generated by the ongoing D. gigas genome sequencing project (our unpublished results) we used the E. coli NorR [19] as a query sequence, to search genes encoding NorR-like proteins in D. gigas genome. Our search retrieved two sequences with the typical structural domain organization of the NtrC/NifA family members, sharing 39% of identity at the amino acid level, designated NorR1-like (NorR1L) and NorR2-like (NorR2L) (Fig. 1A). The alignment of the deduced amino acid sequences of both D. gigas NorRLs with those of NorR from E. coli [19] and NifA from Klebsiella pneumonia [29], indicated that their overall primary structure is close to NifA (40% of
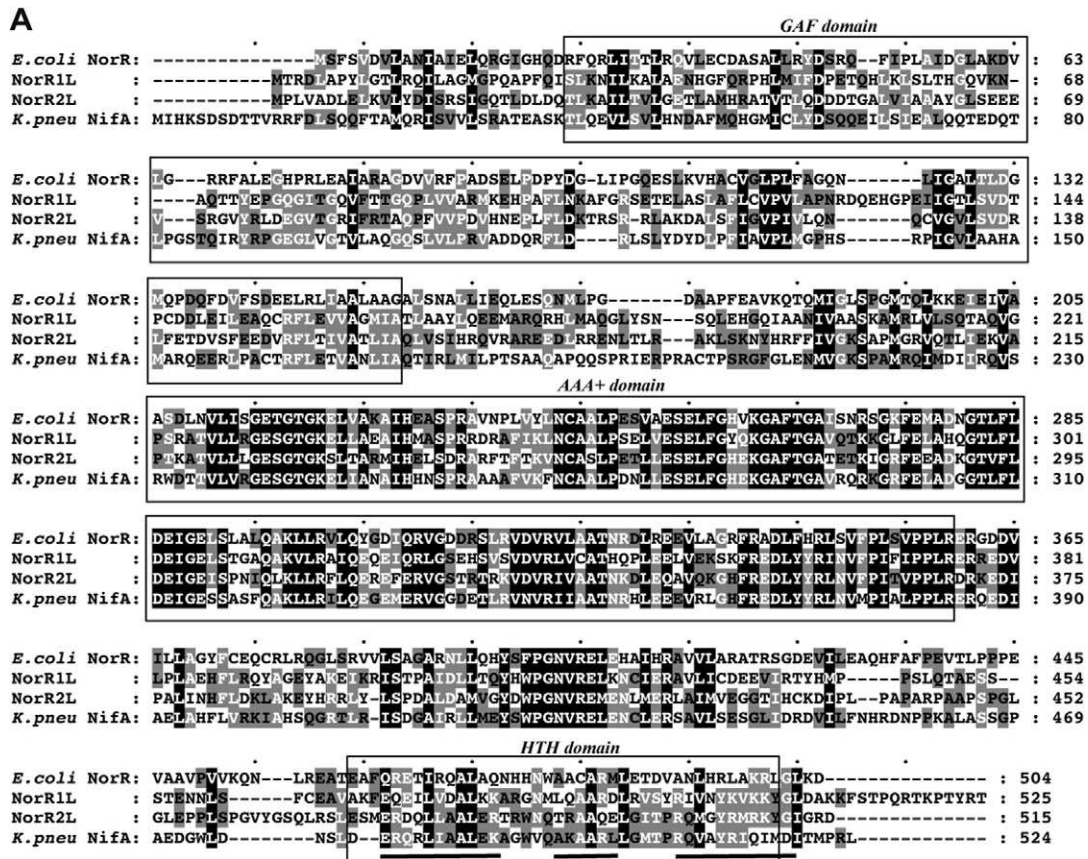
Fig. 1. Amino acid analysis and genetic organization of NorRLs. (A) Amino acid sequence alignment of NorR1L and NorR2L with NifA (*Klebsiella pneumonia*) and NorR (*Escherichia coli*). The three typical domains of the members of the Ntrc/NifA family are boxed. The amino acid residues of the HTH domain of NifA corresponding to the α-helical conformation are underlined. The four levels of shade correspond to 100% of similarity (black); 80–90% of similarity (grey with white symbols); 60–79% of similarity (grey with black symbols) and less than 60% of similarity (not shaded). (B) Gene organization in the surroundings of *norR1L*. ORF1 and ORF2 code for proteins that share a significant similarity with indolepyruvate ferredoxin oxidoreductase. In close vicinity of *norR2L* gene no further ORFS were found.

identity). At the level of the HTH domain, however, NorR1L shares the highest identity with the *E. coli* NorR (28%). Conversely, the DNA-binding domain of NorR2L is only 17% identical to the one of NorR, being close to the NifA corresponding domain (42% of identity). This observation prompted us to use NorR1L and not NorR2L in further studies.

Interestingly, some *Vibrio* species contain two *norR* paralogs that recognize slightly dissimilar *consensus* sequences and thus regulate different operons – *norVW* and *hcp-hcr* [30]. It was identified a member of the CRP/FNR family of transcription factors, HcpR, that regulates *hcp* and its associated ferrodoxin-like gene in *Desulfovibrio* species [31]. It seems therefore unlikely that either NorR1L or NorR2L factors regulate the *hcp-frdx* operon. Nevertheless, we cannot rule out the possibility that both NorR1L and NorR2L are functional and regulate different, yet unidentified, target genes.

The gene coding for NorR2L is monocistronic since no other coding units were found in its close vicinity. However, immediately upstream the *norR1L* gene we found three other putative ORFs (Fig. 1B). ORF1 and ORF2 encode proteins of 205 and 632

amino acid residues, respectively, with significant homology with an indolepyruvate ferredoxin oxidoreductase (IOR) from *Pyrococcus furiosus* [32]. IOR catalyzes the oxidative decarboxylation of aryl pyruvates generated by the transamination of aromatic amino acids, to the corresponding aryl acetyl-CoA [33]. The proximity of *norR1L*, ORF1 and ORF2, led us to admit that these genes are located in the same polycistronic unit, being thus subjected to the same transcriptional control. Northern blot analysis revealed however, that *norR1L* is transcribed as a monocistronic operon (data not shown).
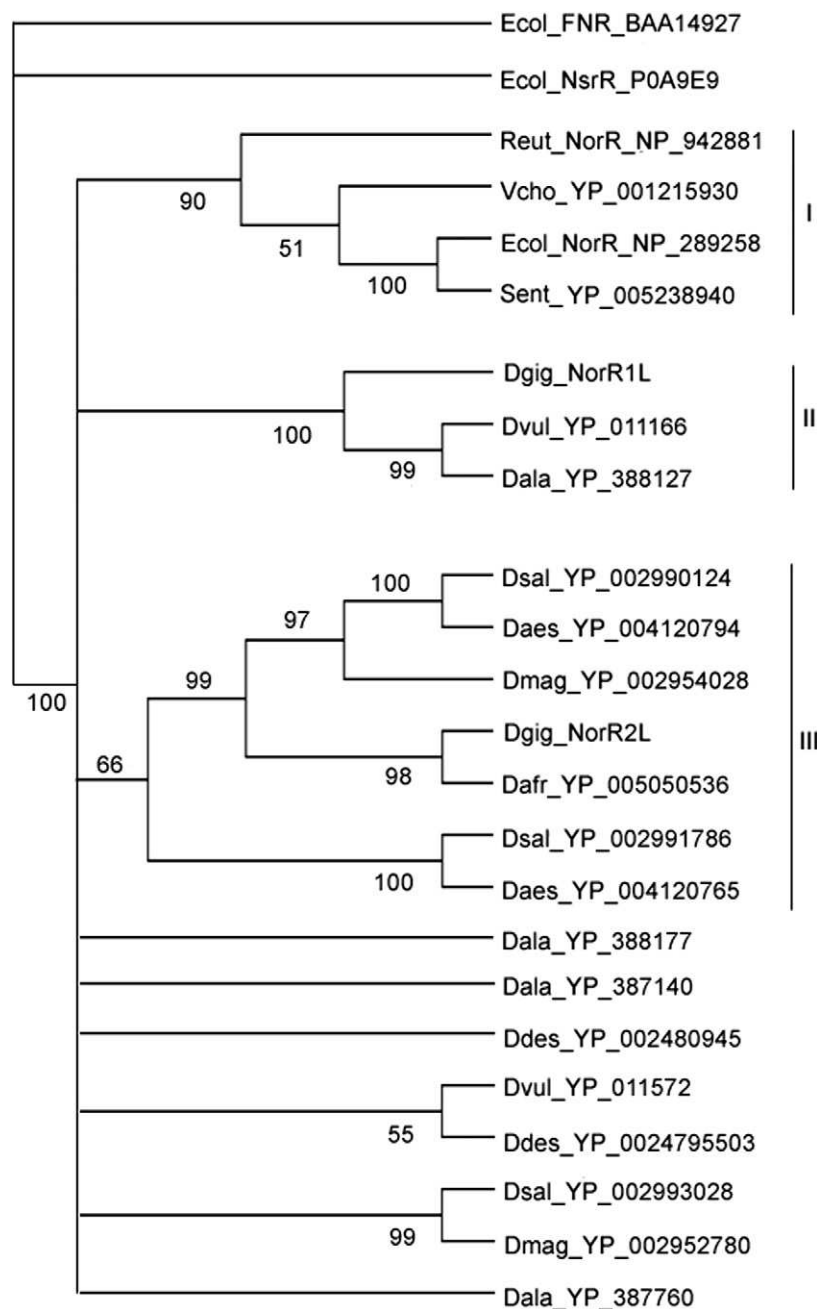
3.2. Phylogenetic analysis of NorR(-like) sequences from different species

We searched the NorR-like orthologs encompassing the typical tri-modular structure in several *Desulfovibrio* genomes, by using the same strategy as described above. We found 2 paralog genes in the genomes of *D. vulgaris*, *D. desulfuricans*, *Desulfovibrio aespoeensis* and *Desulfovibrio magneticus*; 3 paralog genes in the genome of *Desulfovibrio salexigens* and 4 paralog genes in *Desulfovibrio*

alaskensis genome. Next, NorR-like amino acid sequences of *Desulfovibrio* genus were compared with those of gamma-bacteria and with two other *E. coli* nitrosative or nitrite/nitrate responsive factors – Nsr and FNR [34]. We found three distinct groups within the NorR-like sequences, on the basis of the resulting phylogenetic tree (Fig. 2). Group I comprises the best-studied NorRs from *E. coli* and *Ralstonia eutropha* [19,27]; group II includes NorR1L and two NorR-like sequences from *D. alaskensis* and *D. vulgaris* and group III encompasses NorR2L and NorR orthologs found in *D. aespoeensis*, *D. magneticus*, *D. salexigens* and *Desulfovibrio africanus* genome. The topology of the latter strongly suggests that the divergence of a common ancestor gene after speciation occurred rather than a convergent evolution, according to the 16S rRNA gene-based phylogeny of the genus *Desulfovibrio* as described by Gilmour et al. [35].

3.3. NorR1L functionally complements NorR from E. coli

In order to further confirm the functional relationship between *E. coli norR* and *D. gigas norR1L*, we carried out the complementation of *E. coli norR* mutant strain by expressing the plasmid-borne *norR1L* gene. The growth phenotypes of these strains were then assayed in presence of 500 mM GSNO. Our results revealed that wild-type and mutant strains of *E. coli* grow similarly under physiological conditions whereas in presence of 500 μM GSNO, the growth of *E. coli* Δ*norR* mutant strain is abrogated. However, after 6hr of GSNO treatment the growth of *E. coli* Δ*norR* mutant complemented with *norR1L* gene of *D. gigas* was resumed as illustrated in Fig. 3. Nevertheless it is worth noting that the complemented mutant takes longer time to reach the growth of the wildtype strain.

Fig. 2. Phylogenetic analysis of NorR-like amino acid sequences from different species. The phylogenetic analysis was carried out by the neighbor-joining method using MEGA. One thousand bootstrap replicates were calculated and bootstrap values are shown at each node. Nodes were collapsed to a single horizontal line whenever statistical support was less than 55%. The first letters in the sequence label indicate the species. The following characters indicate the sequence accession number.
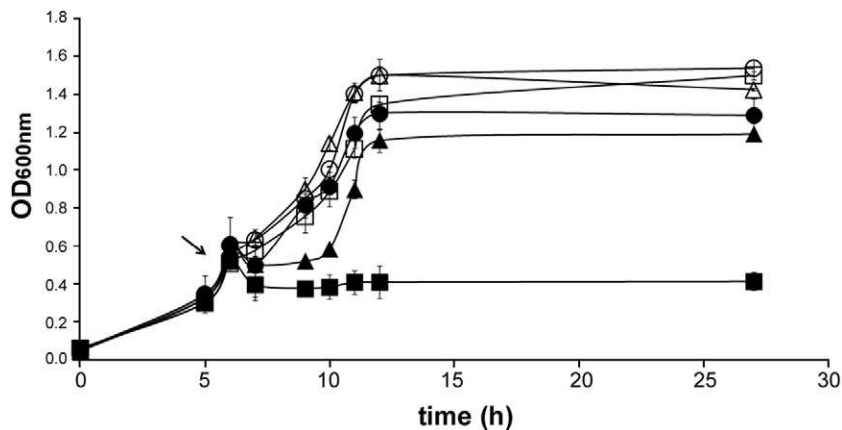
Fig. 3. Anaerobic growth of the *E. coli* strains MC1000 (wild-type), MC1003 (Δ*norR*) and MC1003 expressing a plasmid borne *D. gigas norR1L*, in the presence of GSNO. *E. coli* wild-type (circles), *E. coli* Δ*norR* (squares) and *E. coli* Δ*norR* complemented with pFLAG-CTC plasmid containing *D. gigas norR1L* (triangles) were induced (solid symbols) or not induced (open symbols) with 500 μM of GSNO (the arrow indicates the induction time). Each data point is the average of three independent growth experiments.

Our results obtained by complementation experiments strongly suggest that NorR1-like factor of *D. gigas* is playing the same role under NO stress as its counterpart of *E. coli*.

3.4. Genotypic and phenotypic analysis of D. gigas □norR1-like mutant

To clearly evaluate the NorR1L function *in vivo*, we generated and characterized a *D. gigas* □*norR1L* mutant. To this end, a plasmid containing the kanamycin resistance gene and the flanking regions of *norR1L* was constructed and used to transform the wt *D. gigas* as described in Material and Methods. After transformation and isolation of the colonies grown in presence of kanamycin, the *norR1L* deletion was confirmed by both PCR and Southern blot analysis. The results obtained by both techniques confirmed the deletion of *norR1L* gene via its replacement by the kanamycin resistance gene (Fig. S1).

We have next determined the growth phenotype of the *D. gigas* □*norR1L* mutant strain, by assaying their growth in the presence of GSNO (as described in Materials and Methods). Our previous studies showed that 10 μM of GSNO inhibited the growth of the *D. gigas* Δ*roo* mutant [12], thus this was the concentration of nitrosating agent used in the present work.

We have verified that under standard growth conditions no differences were observed in *D. gigas* wild-type and the *D. gigas* mutant strain Δ*norR1L*. On the contrary, in the presence of 10 μM GSNO it is clear that growth of the mutant strains *D. gigas* Δ*norR1L*

is severely impaired (Fig. 4). This is another indication that NorR1L factor is responsible for NO detoxification in *D. gigas*.

3.5. NorR1-like factor regulates the roo gene expression

E. coli NorR regulates *norVW* operon, which encodes for the nitric oxide reductase flavorubredoxin (NorV) and its associated reductase (NorW) [15,16]. NorR is activated in the presence of nitrosative stress under anaerobic conditions driving the induction of its target genes [17,18]. We therefore evaluated whether *D. gigas* NorR1L regulates the expression of *rd-roo*. Indeed, the *rd-roo* gene promoter contains two σ54 promoter motifs, CC**GGC**CAACCGGTG-**GA**G, at the positions 166 and 207 upstream of the *rd* ATG resembling the canonic sequence of σ54 found in several genes of pathogenic bacteria [36]. Remarkably, the σ54 sequence has a G + C content higher than the canonic one, which is in agreement with the high G + C content of *D. gigas* genome. Moreover, two putative consensus sequences for *norR1-like* gene [GT-(N7)-AC] box are found upstream the *rd-roo* promoter, respectively at 253 and 269 bp upstream of the *rd* ATG. The promoter of *norVW* operon of *E. coli* contains three functional boxes identical to the one we detected in *rd-roo* operon [37].

These features of *rd-roo* promoter together with the previous results led us to monitor the *roo* gene expression in *D. gigas* □*norR1L* mutant by real time quantitative RT-PCR in the presence of GSNO. We found that *roo* expression is constitutively expressed
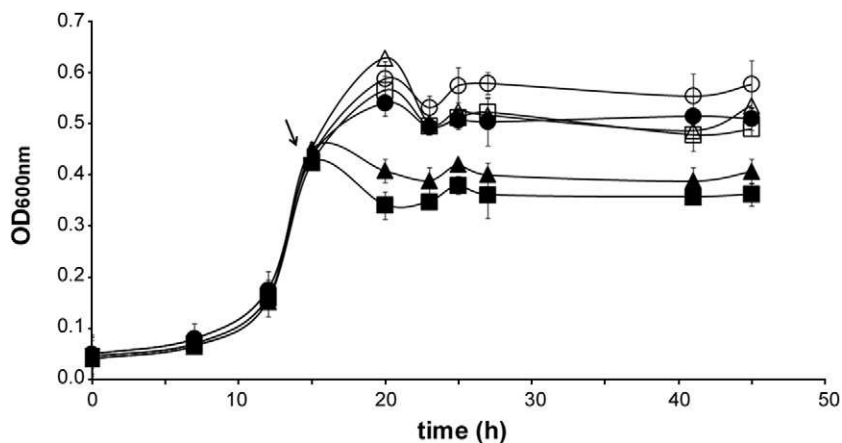
Fig. 4. Growth curves of *D. gigas* wild-type and *D. gigas* Δ*norR1L* in the presence of GSNO. Wild-type (circles) and mutant (squares) were treated (solid symbols) or not treated (open symbols) with 10 μM of GSNO (the arrow indicates the induction time). Each data point is the average of three independent growth experiments.

Table 1
Effect of 10 µM GSNO on *D. gigas roo* gene expression.

	Fold variation[a]		
	1 h	2 h	3 h
wt	1.17 ± 0.18	1.71 ± 0.36	1.32 ± 0.40
DnorR1L	1.00 ± 0.12	1.00 ± 0.02	1.00 ± 0.20

[a] Fold variation was calculated using the expression levels of *roo* immediately before and 1, 2 and 3 h after GSNO addition.

in wt strain and induced upon GSNO treatment. This induction is completely abolished in the Δ*norR1L* mutant strain (Table 1). It should be pointed out that as illustrated in Fig. 4 the Δ*roo* mutant strain exhibits a growth phenotype similar to the one of Δ*norR1L*.

Altogether our results show that *roo* gene induction is completely dependent on the regulation by NorR1L transcription factor under nitrosative stress playing an essential role in NO detoxification.

In the present work we have identified in the *D. gigas* genome, a transcription factor, NorR1L, which belongs to the Ntrc/Nif family and closely resembles the NorR factor from *E. coli*. The gene encoding NorR1L factor is able to functionally complement the growth phenotype sensitivity of *E. coli* D*norR* mutant. In addition, NorR1L factor controls the expression of *roo* gene upon nitrosative stress. Another Ntrc/Nif factor was identified, NorR2L, of which the function is unknown. The *roo* gene besides being involved in nitrosative stress is also activated by oxidative stress [38]. Although Victor et al. by using molecular dynamics simulation reported that ROO does not exhibit any significant preference to accommodate both O_2 and NO [39] it might be possible that *roo* gene expression is regulated by different transcription factors depending on the stressors. In this context it is well possible that NorR2L factor would play a role in *roo* gene expression under oxidative stress conditions. Nevertheless we cannot exclude that NoR2L is regulating the expression of yet unidentified genes involved in nitrosative stress.

Acknowledgments

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.bbrc.2012.12.130.

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