

Research Article

Exploration of Nitrate Reductase Metabolic Pathway in *Corynebacterium pseudotuberculosis*

Sintia Almeida,¹ Cassiana Sousa,¹ Vinícius Abreu,² Carlos Diniz,¹ Elaine M. S. Dorneles,^{3,4} Andrey P. Lage,³ Debmalya Barh,⁵ and Vasco Azevedo¹

¹Institute of Biologic Sciences, Federal University of Minas Gerais, 31270-901 Belo Horizonte, MG, Brazil

²Center for Nuclear Energy in Agriculture, University of Sao Paulo, 13400-970 Piracicaba, SP, Brazil

³Escola de Veterinária, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

⁴Departamento de Medicina Veterinária, Universidade Federal de Lavras, Lavras, MG, Brazil

⁵Centre for Genomics and Applied Gene Technology, Institute of Integrative Omics and Applied Biotechnology (IIOAB), Nonakuri, Purba Medinipur, West Bengal, India

Correspondence should be addressed to Sintia Almeida; sintiaalmeida@gmail.com, Andrey P. Lage; aplage11@gmail.com, and Vasco Azevedo; vascoariston@gmail.com

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Based on the ability of nitrate reductase synthesis, *Corynebacterium pseudotuberculosis* is classified into two biovars: Ovis and Equi. Due to the presence of nitrate reductase, the Equi biovar can survive in absence of oxygen. On the other hand, Ovis biovar that does not have nitrate reductase is able to adapt to various ecological niches and can grow on certain carbon sources. Apart from these two biovars, some other strains are also able to carry out the reduction of nitrate. The enzymes that are involved in electron transport chain are also identified by in silico methods. Findings about pathogen metabolism can contribute to the identification of relationship between nitrate reductase and the *C. pseudotuberculosis* pathogenicity, virulence factors, and discovery of drug targets.

1. Introduction

Corynebacterium pseudotuberculosis is a Gram-positive facultative intracellular pathogen [1–11]. *C. pseudotuberculosis* can be classified into two biovars, based on their ability to convert nitrate to nitrite. The nitrate-positive biovar is Equi, which causes ulcerative lymphangitis in equines, while the nitrate-negative biovar is known as Ovis, which is the etiologic agent of caseous lymphadenitis in small ruminants [9]. Both diseases are globally distributed and cause large economic losses to goat, sheep, horse, and cattle farmers.

The nitrate reduction is associated with the bacterium's ability to breathe in the absence of oxygen and having two different metabolic pathways, (1) respiratory nitrate reductase and (2) dissimilatory nitrate reduction. In the first pathway, the denitrification process takes place where the nitrate is sequentially reduced to nitrite, nitric oxide, nitrous oxide, and finally to dinitrogen [12, 13]. In the second pathway

nitrate is directly converted into ammonia, which is secreted from the cell; this process can be performed by organisms with the *nrf* gene. This is a less common method of nitrate reduction than denitrification in most ecosystems [12–14]. There are four classes of nitrate reductases, one in eukaryotes and three are in prokaryotes. Prokaryotic nitrate reductases include a class of assimilatory enzymes and two classes of respiratory enzymes; all contain a guanylate molybdenum cofactor but differ in their substructures, cellular location, and requirement for cofactor. Variability among enzyme is also found into the classes [12, 13].

Among the Actinobacteria, respiratory nitrate reductase (Nar) is observed [15–18]. This class of enzymes is mostly membrane-bound and is having three different subunits: respiratory nitrate reductase from *Pseudomonas stutzeri* [19, 20], nitrate reductase A, and nitrate reductase Z from *Escherichia coli* K-12 [21–23]. In *Corynebacterium glutamicum* and *Mycobacterium tuberculosis* the nitrate reduction

TABLE 1: Strains used in phenotypic and genotypic analysis regarding the nitrate reductase activity.

| Strain | Biovar | Genome size (MB) | Nitrate test ^a | NCBI access | Ref |
|-----------|--------|------------------|---------------------------|---------------|----------|
| 1002 | Ovis | 2.33511 | Yes | NC_017300.1 | [26] |
| C231 | Ovis | 2.32821 | Yes | NC_017301.1 | [26] |
| FRC41 | Ovis | 2.33791 | Yes | NC_014329.1 | [27] |
| I19 | Ovis | 2.33773 | Yes | NC_017303.1 | [28] |
| PAT10 | Ovis | 2.33532 | Yes | NC_017305.1 | [29] |
| 42/02-A | Ovis | 2.33761 | Yes | NC_017306.1 | [30] |
| 3/99-5 | Ovis | 2.33794 | Yes | NC_016781.1 | [30] |
| 267 | Ovis | 2.33763 | Yes | NC_017462.1 | [31] |
| P54B96 | Ovis | 2.33794 | Yes | NC_017031.1 | [32] |
| CIP5297 | Equi | 2.32059 | Yes | NC_017307.1 | [33] |
| 1/06 -A | Equi | 2.27912 | Yes | NC_017308.1 | [34] |
| 316 | Equi | 2.31041 | Yes | NC_016932.1 | [35, 36] |
| 258 | Equi | 2.36982 | Yes | NC_017945.1 | [37] |
| 162 | Equi | 2.29346 | Yes | NC_018019.1 | [38] |
| 31 | Equi | 2.38969 | Yes | NC_017730.1 | [39] |
| 262 | Equi | 2.32575 | Yes | NZ_CP012022.1 | — |
| MB20 | Equi | 2.36309 | Yes | JPUV01 | [40] |
| E19 | Equi | 2.36796 | Unknown | NZ_CP012136.1 | — |
| CCUG27541 | Equi | 2.37942 | Unknown | JPJB01 | [41] |

^aBiochemical test.

reaction is catalyzed by the operons *narKGHJI* and *narGHJI*, respectively [15, 16]. In these organisms, the first step in nitrate assimilation is nitrate reduction (NO_3^-) to nitrite (NO_2^-) and the second step is nitrite reduced (N_3^-) to ammonia (NH_4^+) [15, 16].

Nitrate respiration is associated with virulence and adaption of pathogenic organisms. In *Brucella suis* it helps in intramacrophagic multiplication, in *M. bovis* it allows survival inside the host, and in *M. tuberculosis* it confers resistance to stress [17, 24, 25]. However, the role of nitrate respiration is not known in *C. pseudotuberculosis*. We presume that Nar may be associated with pathogenicity in *C. pseudotuberculosis* biovar Equi, providing a generate energy pathway when it grows under oxygen deprived condition allowing the survival of *C. pseudotuberculosis* inside the host.

With the increase in genome sequencing projects, understanding of the biological capabilities of the organisms has also increased. The availability of the annotated genomes allows the information of metabolic pathways to be interpreted and used for computational reconstruction at genomic scale. Thus the reconstruction of metabolic pathways through comparative analysis can help identify potential targets and find the mechanisms that cause a disease.

In the present study, the genomes of *C. pseudotuberculosis* strains deposited in GenBank were used to identify the genes involved in the nitrate reduction pathway; a comparative genomic study was performed on 15 *C. pseudotuberculosis* strains of the biovars Ovis and Equi to identify the molecular bases of nitrate reduction in each *C. pseudotuberculosis* biovar Equi. Thus, we hope that understanding of Nar's role in *C. pseudotuberculosis* can help in identifying potent targets both for the development of more effective diagnostics and therapeutics and for the treatment and prevention of disease.

2. Material and Methods

2.1. Identification of Nitrate Reductase Genes, Metabolic Pathways, and Nitrate Reduction Enzymes. *C. pseudotuberculosis* genomes Table 1 were obtained from the NCBI GenBank. The sequence and arrangement analysis of nitrate reductase genes in *narKGHJI* operon was performed using the Artemis software (<http://www.sanger.ac.uk/Software/Artemis>) and manual curation based annotations are performed. Pathway Tools software developed by SRI International [45] and MetaCyc database [46] were used for computational prediction of metabolic pathways in *C. pseudotuberculosis*.

2.2. *narKGHJI* Operon Analysis. The families of proteins as well as the conserved protein domains present in the operon *narKGHJI* and adjacent genes were analyzed using InterProScan database (<https://www.ebi.ac.uk/interpro>). Prediction of transmembrane helices in protein was performed using TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). The similarity analysis of genes involved in the nitrate reduction pathway was performed using the UniProtKB database in category BLASTp. Clustal Omega was used to multiple sequence alignment between operon *narKGHJI* of *C. pseudotuberculosis* biovar Equi and homologous sequences of multiple organisms (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), and Jalview was used to analyze similarity (<http://www.jalview.org/>) among these sequences.

3. Results and Discussion

3.1. Correlation between Nitrate Reduction Phenotype and Genotype. Nineteen *C. pseudotuberculosis* strains that had genome information in GenBank are presented Table 1. To perform the phenotyping analysis, 18 strains that were

TABLE 2: Similarity sequence analysis of *narKGHJI* gene clusters of *C. pseudotuberculosis*.

| <i>C. pseudotuberculosis</i> protein | Species | Length (aa) | ID (%) | Similarity (%) | Access number/UniProtKB |
|---|------------------------------------|-------------|--------|----------------|-------------------------|
| Nitrate reductase alpha subunit (NarG)—1240aa | <i>Corynebacterium diphtheriae</i> | 1,240 | 97 | 99 | Q6NJA9 |
| | <i>Corynebacterium glutamicum</i> | 1,248 | 77 | 87 | Q8NR68 |
| | <i>Mycobacterium tuberculosis</i> | 1,232 | 56 | 70 | P9WJQ3 |
| | <i>Escherichia coli</i> | 1,247 | 46 | 64 | P09152 |
| Nitrate reductase beta subunit (NarH)—533aa | <i>Corynebacterium diphtheriae</i> | 533 | 95 | 98 | H2G670 |
| | <i>Corynebacterium glutamicum</i> | 531 | 79 | 89 | S5Y1U5 |
| | <i>Mycobacterium tuberculosis</i> | 538 | 64 | 75 | A0A049E025 |
| | <i>Escherichia coli</i> | 512 | 54 | 72 | P11349 |
| Nitrate reductase gamma subunit (NarI)—259aa | <i>Corynebacterium diphtheriae</i> | 259 | 96 | 98 | H2HVN2 |
| | <i>Corynebacterium glutamicum</i> | 259 | 75 | 89 | S5YHX1 |
| | <i>Mycobacterium tuberculosis</i> | 241 | 48 | 68 | Q7D8Q6 |
| | <i>Escherichia coli</i> | 225 | 28 | 51 | P11350 |
| Nitrate reductase chaperone (NarJ)—209aa | <i>Corynebacterium diphtheriae</i> | 209 | 89 | 96 | H2H309 |
| | <i>Corynebacterium glutamicum</i> | 228 | 63 | 80 | Q6M5Z0 |
| | <i>Mycobacterium tuberculosis</i> | 206 | 37 | 49 | Q7D8Q7 |
| | <i>Escherichia coli</i> | 236 | 30 | 46 | P0AF26 |
| Nitrate/nitrite transporter (NarK)—443aa | <i>C. diphtheriae</i> | 443 | 93 | 97 | H2HG11 |
| | <i>C. glutamicum</i> | 445 | 66 | 78 | I0LIN6 |
| | <i>M. tuberculosis</i> | 395 | 22 | 35 | P9WJY7 |
| | <i>E. coli</i> | 463 | 33 | 51 | P10903 |

sequenced in our lab were used. All these strains were biochemically tested in order to verify their ability of nitrate reduction; see Table 1. Among the *C. pseudotuberculosis* strains, 31, 258, 262, and MB20 strains showed concordance between phenotypic and genotypic results. Strains 1/06-A, 316, 162, and CIP52.97 are found positive phenotype for reduction of nitrate; however they do not have genes involved in nitrate reduction in their genome.

3.2. Genes Involved in Respiratory Nitrate Reductase Metabolic Pathway. We first compared the autoassigned pathways by the Pathologic software with the pathways in databases such as KEGG (<http://www.genome.jp/kegg/>) and resolved possible discrepancies using manual curation. Comparative genome analysis revealed that *C. pseudotuberculosis* biovar Equi possess *narKGHJI* gene clusters that participate via the respiratory anaerobic process of the nitrate reduction similarly to *E. coli* (Figure S8) [47]. The *C. pseudotuberculosis narKGHJI* gene cluster showed great similarity with the protein sequences found in other Actinomycetes, such as *C. diphtheriae*, *C. glutamicum*, and *M. tuberculosis* (Table 2). All biovar Ovis strains do not present any gene of the *narKGHJI* operon in their genomes (Figure 1).

The nitrate locus that contains *narKGHJI* operon in *C. pseudotuberculosis* is composed of gene cluster encoding the molybdopterin *moeB*, *moeA*, *molB*, *molA*, *moeY*, *moeC*, *moeA*, and *moeA* (Figure 2). Molybdopterin is a cofactor that is indispensable for activity of nitrate reductase [48]. The organization and conservation of the *narKGHJI* gene cluster was assessed among *C. pseudotuberculosis* and *C. diphtheriae*,

C. glutamicum, *M. tuberculosis* and, *E. coli*. The analysis showed that *narKGHJI* gene cluster is conserved among these species; however gene orientation of the five genes is only conserved among *C. pseudotuberculosis*, *C. diphtheriae*, and *C. glutamicum*. In addition, in *C. pseudotuberculosis* we found *narT* gene is located at the upstream of the *narKGHJI* cluster. The *narT* gene encodes a nitrate transporter and is not present in *E. coli* and *C. glutamicum*.

3.3. Enzymes Involved in Electron Transport Chain. We found conserved functional domains of the genes present in the *narGHI* complex (involved in electron transport chain) in *C. pseudotuberculosis* biovar Equi (Table 3). To infer the functionality of *narGHI* complex, we used tridimensional structure from *E. coli* (PDB accession 1Q16) as a model for *nar* genes, as the structure of these enzymes is resolved in these bacteria.

In the *narGHI* complex in Figure 2, the *narG* gene is a member of a superfamily of enzymes that use a Mo-bisMGD cofactor (bisMGD) for their catalytic activity. Residues present in the active site of the molybdenum atom (Mo) are highly conserved in NarG subunits of Gram-negative and Gram-positive bacterial species [21]. The NarH belongs to the superfamily of electron transfer subunit (ferredoxins) of bacterial oxidoreductases. NarH has three clusters [4Fe-4S] (FS1, FS2, and FS3) and a [3Fe-4S] cluster (FS4) [21, 42, 44]. The general structure of NarH in *E. coli* is composed of a central region of a linker region that is inserted between the two subdomains in coordination of the iron-sulfur clusters and forms an extended connection between the NarG and

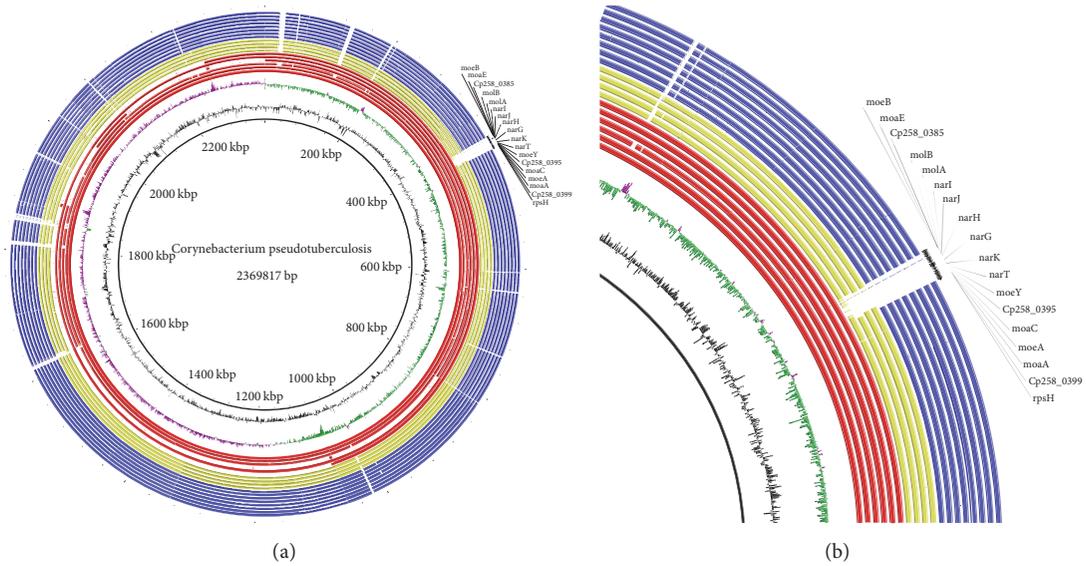


FIGURE 1: Graphical circular map showing BLAST between nineteen *C. pseudotuberculosis* strains. (a) From center to the outside: GC content in black and GC skew in pink and green; in red and yellow Equi strains and in blue Ovis strains. From the inner to outer circle on (a) and (b): the biovar Equi strains are the following: 258, 31, 262, MB20, E19, CCUG27541, CIP52.97, 1/06-A, 316, and 162, and the biovar Ovis strains are the following: 1002, C231, FRC41, I19, PAT10, 42/02-A, 3/99-5, 267, and P54B96. (b) Zoom to the nitrate locus, absent in the biovar Equi strains CIP52.97, 1/06 -A, 316, and 162 and all biovar Ovis strains.

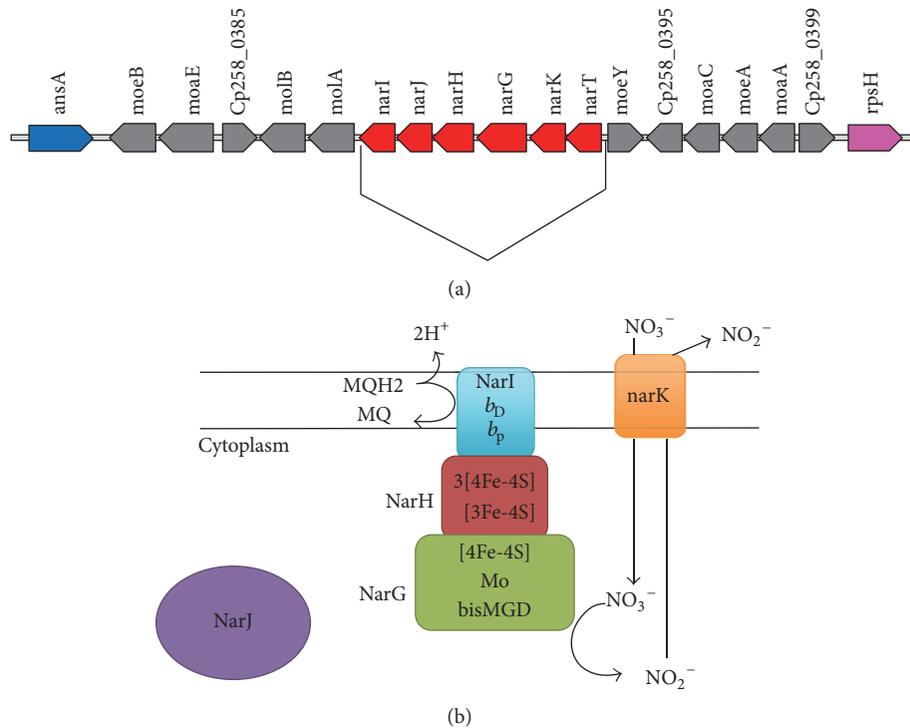


FIGURE 2: Nitrate locus from *C. pseudotuberculosis* biovar Equi. (a) This locus contains the following: genes encoding the nitrate reductase *narK*, *narG*, *narH*, *narJ*, and *narI*, and the genes encoding the molybdopterin *moeB*, *moaE*, *molB*, *molA*, *moeY*, *moaC*, *moaA*, and *moaA*. Insertion between *ansA* and *rpsH*: genes encoding the nitrate reductase are lacking in nitrate-negative *C. pseudotuberculosis* biovar Ovis strains. Arrows represent open reading frames and their orientations. (b) Bacterial nitrate respiration. The *NarG* and *NarH* bind to the membrane via the interaction with a hydrophobic patch of *NarH* and *NarI* which is buried within the membrane. The *NarJ* is a chaperone that is involved in folding, maturation, and molybdenum cofactor insertion of nitrate reductase.

TABLE 3: Residues analyzed regarding the conservation between the *narGHI* protein sequences of *E. coli* and *C. pseudotuberculosis*.

| <i>E. coli</i> | <i>C. pseudotuberculosis</i> | Conservation (%) | Function | REF |
|-----------------------|------------------------------|------------------|---|------|
| <i>Residues NarG*</i> | | | | |
| His49(H) | His55 | 100 | Mo ion coordination | [42] |
| Cys53(C) | Cys 59 | 100 | Mo ion coordination | [42] |
| Cys57(C) | Cys 63 | 100 | Mo ion coordination | [42] |
| Cys92(C) | Cys 98 | 100 | Mo ion coordination | [42] |
| Asp222 (D) | Asp 228 | 100 | Mo-bisMGD ligand | [42] |
| Val 578 (V) | Val 584 | 100 | Left relatively hydrophobic environment to allow binding within the active site, with one face of the side chain carboxylate exposed of Asp222. Optimal interaction with the Mo of the cofactor | [43] |
| Tyr 220 (Y) | Tyr 226 | 100 | Left relatively hydrophobic environment to allow binding to within the active site, with one face of the side chain carboxylate exposed of Asp222. Allowing optimal interaction with the Mo of the cofactor | [43] |
| His1092(H) | His 1099 | 100 | Form a hydrogen bond network that links the solvent interface with Asn ^{A52} and could be important for structural integrity and/or proton delivery to the active site | [42] |
| His1098(H) | His 1105 | 100 | Form a hydrogen bond network that links the solvent interface with Asn ^{A52} and could be important for structural integrity and/or proton delivery to the active site | [42] |
| His1163(H) | His 1170 | 100 | Form a hydrogen bond network that links the solvent interface with Asn ^{A52} and could be important for structural integrity and/or proton delivery to the active site | [42] |

TABLE 3: Continued.

| <i>E. coli</i> | <i>C. pseudotuberculosis</i> | Conservation (%) | Function | REF |
|-----------------------|------------------------------|------------------|--|------|
| Arg 94(R) | Arg 100 | 100 | Forms a hydrogen bond with the Cys92 ligand of FS0 | [43] |
| <i>Residues NarH*</i> | | | | |
| Cys196 (C) | Cys 196 | 100 | Binding of iron atoms of FS4 cluster [3Fe-4S] | [44] |
| Cys217(C) | Cys 217 | 100 | Binding of iron atoms of FS4 cluster [3Fe-4S] | [44] |
| Cys223(C) | Cys 223 | 100 | Binding of iron atoms of FS4 cluster [3Fe-4S] | [44] |
| Cys184(C) | Cys 184 | 100 | Binding of iron atoms of FS3 cluster [4Fe-4S] | [44] |
| Cys187(C) | Cys 187 | 100 | Binding of iron atoms of FS3 cluster [4Fe-4S] | [44] |
| Cys192(C) | Cys 192 | 100 | Binding of iron atoms of FS3 cluster [4Fe-4S] | [44] |
| Cys 227(C) | Cys 227 | 100 | Binding of iron atoms of FS3 cluster [4Fe-4S] | [44] |
| Cys26(C) | Cys 26 | 100 | Binding of iron atoms of FS2 cluster [4Fe-4S] | [44] |
| Cys244(C) | Cys 244 | 100 | Binding of iron atoms of FS2 cluster [4Fe-4S] | [44] |
| Cys247(C) | Cys 247 | 100 | Binding of iron atoms of FS2 cluster [4Fe-4S] | [44] |
| Cys259(C) | Cys 259 | 100 | Binding of iron atoms of FS2 cluster [4Fe-4S] | [44] |
| Cys16(C) | Cys 16 | 100 | Binding of iron atoms of FS1 cluster [4Fe-4S] | [44] |
| Cys19(C) | Cys 19 | 100 | Binding of iron atoms of FS1 cluster [4Fe-4S] | [44] |
| Cys22(C) | Cys 22 | 100 | Binding of iron atoms of FS1 cluster [4Fe-4S] | [44] |
| Cys263 (C) | Cys 263 | 100 | Binding of iron atoms of FS1 cluster [4Fe-4S] | [44] |
| <i>Residues NarI*</i> | | | | |
| His66 (H) | His66 | 100 | Iron atoms coordination of heme b_D | [43] |
| His187 (H) | His190 | 100 | Iron atoms coordination of heme b_D | [43] |

TABLE 3: Continued.

| <i>E. coli</i> | <i>C. pseudotuberculosis</i> | Conservation (%) | Function | REF |
|----------------|------------------------------|------------------|---|------|
| His56 (H) | His56 | 100 | Iron atoms coordination of heme b_p | [43] |
| His205 (H) | His208 | 100 | Iron atoms coordination of heme b_p | [43] |
| Arg112 | Arg113 | 100 | Hydrogen bond network electron transfer between the redox center b_p , FS4, and NarG | [43] |
| Arg202 | Arg205 | 100 | Hydrogen bond network electron transfer between the redox center b_p , FS4, and NarG. | [43] |
| Ser39 | Ser39 | 100 | Hydrogen bond network electron transfer between the redox center b_p , FS4, and NarG. | [43] |
| Ser40 | Ser40 | 100 | Hydrogen bond network electron transfer between the redox center b_p , FS4, and NarG. | [43] |
| Tyr213(Y) | Tyr216 | 100 | Involved in electrostatic interactions and hydrogen bond, are involved in the formation of NarGHI heterotrimer | [43] |
| Arg216(R) | Arg219 | 100 | Involved in electrostatic interactions and hydrogen bond, are involved in the formation of NarGHI heterotrimer | [43] |
| Arg222(R) | Arg225 | 100 | Involved in electrostatic interactions and hydrogen bond, are involved in the formation of NarGHI heterotrimer | [43] |
| Ser201(S) | Ser204 | 100 | A strong link at Ser201 of NarI allows distinctly shorter distances between the hemes in NarI than the distances observed between the hemes in the cytochrome bcl complex | [43] |

* Shown is the position of the residues at *E. coli* and *C. pseudotuberculosis* sequences and as well as the function of each residue in the *E. colinarGHI* structure.

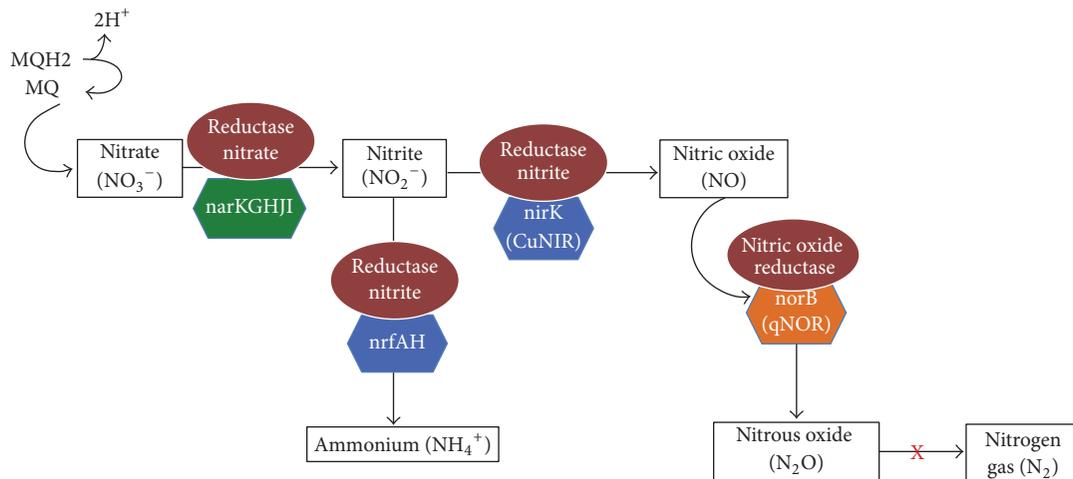


FIGURE 3: Model building of nitrate reductase from *C. pseudotuberculosis* biovar Equi. Shown here is respiratory nitrate reduction to nitrite; incomplete denitrification of nitrite in which nitrous oxide is the final product; and Nrf-dependent ammonification.

NarH subunits and an extended C-terminus. In *C. pseudotuberculosis* it was observed that the nine cysteine residues responsible for coordinating three clusters [4Fe-4S] and one cluster [3Fe-4S] are conserved in all analyzed sequences. NarI is the transmembrane subunit that anchors NarGH to the cytoplasmic side of the membrane and provides the connection between the quinone and the site of oxidation, referred to as "Site Q." This subunit coordinates two hemes, one of which is located towards the side of cytoplasmic end of NarI and is referred to as the proximal heme (heme b_p), while the other is located towards the periplasmic side of the same and is referred to as the distal heme (heme b_D). Such hemes act as mediators in electron transfer from Site Q to the clusters [Fe-S] in NarH. NarI of *C. pseudotuberculosis* presented five transmembrane helices S1 Figure, which is in agreement with the literature when compared to the *E. coli* crystal structure [49, 50]. This C-terminal segment presents highly conserved residues in the family NarI proteins and also is present in *C. pseudotuberculosis*. It is interesting to note that NarI is considerably preserved in relation to its primary sequence between orthologous proteins found in species of the *Corynebacterium* genus, particularly in relation to *C. diphtheriae*, which has 96% identity and 98% similarity. These results suggest that the enzymatic complex *narGHI* is probably very similar with respect to its three dimensional structure among the species of *C. pseudotuberculosis* biovar Equi and *C. diphtheriae* in view of the high identity found among NarG, NaH, and NarI subunits of these species. The *C. pseudotuberculosis* biovar Equi presents 48% and 43% identity in relation to the orthologous proteins from *M. tuberculosis* and *B. subtilis*, respectively.

3.4. NarJ (Molybdenum-Cofactor-Assembly Chaperone). The NarJ is a chaperone that is involved in folding, maturation, and molybdenum cofactor insertion of nitrate reductase in *E. coli* [51, 52]. The similarity values found between *C. pseudotuberculosis* biovar Equi and other sequences of orthologous proteins by BLAST, including the genus *Corynebacterium*,

were slightly lower than those found in other proteins (NarG, NarH, and NarI) that form the three subunits of the enzyme nitrate reductase (Table 2). However, *narJ* remained conserved between *C. pseudotuberculosis* biovar Equi strains, and results of biochemical tests (Table 1) indicate that its function has remained preserved, since NarJ is important for nitrate reductase expression in others organisms [53].

3.5. Nitrate and Nitrite Transporters. The nitrate and nitrite export is mediated by nitrate and nitrite transporters that are members of the major facilitator superfamily (MFS) [54]. In bacteria such carriers are the *narK* family represented by NarK and NarU proteins in *E. coli* that transport both nitrate and nitrite [47]. However, NarK is expressed at higher levels than NarU and it remains unclear till date if NarK is a nitrate/nitrite antiporter or symporter [55]. In *C. pseudotuberculosis* biovar Equi, we found that the two genes *narK* and *narT* are responsible for coding two probable nitrate and nitrite carriers and in *C. pseudotuberculosis* biovar Equi is present at the upstream of the *narGHJI* operon (Figure 2). Both proteins are found to be composed of 12 transmembrane helices similar to other members of the superfamily of bacterial transporters; see S2 and S3 Figures. In *C. glutamicum* the *narK* is in close proximity to *narG*, *narH*, *narJ*, and *narI*, which together form the *narKGHJI* operon [56], but in *E. coli*, *narGHJI* operon is transcribed individually and *narT* is upstream of the operon *narKGHJI* [57]. Since we did only the in silico analysis, we cannot state the precise transcription and regulation of these genes in vivo. We presume that this *narKGHJI* cluster in *C. pseudotuberculosis* biovar Equi may be regulated together as found in *C. glutamicum*. However, experimental confirmation is required.

3.6. Nitrite Reductase in *C. pseudotuberculosis*. Genes in *C. pseudotuberculosis* are found to encode two different types of nitrite reductase enzymes. One is the nitric oxide-forming enzyme that is encoded by *nirK* gene and shown to be a copper dependent enzyme. The second groups of enzymes

are ammonium nitrite reductase that are encoded by *nrfA* gene that encodes catalytic subunit and *nrfH* that encodes for a membrane associated electron transfer subunit. In all *C. pseudotuberculosis* strains we found NrfA and NrfH subunits of nitrite reductase NrfAH complex that catalyze the reduction of nitrite to ammonium. Beside *C. pseudotuberculosis*, such genes are found only in *C. ulcerans* and are absent in all other species from this genus. In *C. pseudotuberculosis*, the two genes *nrfA* and *nrfH* are located in a “cluster” of genes, S4 Figure, responsible for coding biogenesis system I proteins cytochrome c that appears to be a common feature among bacterial genomes. *C. pseudotuberculosis* also has a nitrite reductase enzyme likely dependent on copper (CuNIR). The gene for CuNIR is *nirK* that encodes a protein composed of 882 residues and is conserved in all strains of *C. pseudotuberculosis*; see Figure S5 of the Supplementary Material available online at <https://doi.org/10.1155/2017/9481756>. The sequence similarity analysis of *nirK* was performed using the BLAST and it was observed that such protein has an N-terminal region of about 500 amino acids, S6 Figure, compared to the most homologous proteins that have been experimentally well characterized [58–61]. Within the genus *Corynebacterium*, the CuNIR proteins are found to have similar sizes to that of *C. pseudotuberculosis*, such as *Corynebacterium durum* (WP_006063127.1) and *Corynebacterium vitaeruminis* (WP_051483598.1), with 40% identity between the sequences; see Table S1. Furthermore, it was found that the N-terminal region of about 500 residues is probably composed of 13 transmembrane helices in *C. pseudotuberculosis*, S7 Figure, which indicates that it can be a membrane associated protein. To verify the residues conservation, we used *G. kaustophilus* CuNIR proteins as a reference sequence because its crystal structure has recently been determined (pdb|3WIA|A). All residues responsible for binding to the copper atoms and the catalytic site were analyzed. It is observed that the C-terminal region is 100% conserved in *C. pseudotuberculosis*; see S6 Figure.

3.7. Reconstruction of *C. pseudotuberculosis* Biovar Equi Metabolic Network. A metabolic network reconstruction is assembled piece-by-piece by compiling data on known enzymes, and annotated genome together with data from the literature is used to assemble a network reconstruction [62]. In *C. pseudotuberculosis* after the similarity analysis we observed that genomic sequences participating in the nitrate reduction pathway are conserved, and the residues were analyzed, indicating a possible conserved function. Thus we can infer that *C. pseudotuberculosis* biovar Equi is different from that occurring in *C. glutamicum*, where nitrate is excreted out of the cells as the major end product under the conditions of anaerobic respiration, and nitrate and nitrite are not used as sources of nitrogen for aerobic or anaerobic growth [18] so *C. pseudotuberculosis* biovar Equi has a likely route of denitrification composed of the nitrate reductase Nar, nitrite reductase dependent copper, and nitric oxide reductase quinol-dependent, and this is a way of incomplete denitrification, in which nitrous oxide is the final product, and, therefore, there is no presence of nitrous oxide reductase enzyme and subsequent reduction to nitrogen gas; see Figure 3. Furthermore *C. pseudotuberculosis*

biovar Equi also shows the pathway of ammonification in dissimilatory nitrate reduction to ammonium, what has been proposed as catalyzed by the enzyme nitrate reductase Nar and nitrite reductase complex NrfAH, a membrane-associated cytochrome; see Figure 3. *E. coli* not only reduce nitrite to ammonia but also catalyze the reduction of nitrite to NO, which can be used as an electron acceptor for anaerobic respiration [63]. The presence of enzymes and pathways of nitrate reduction proposals was also compared between biovar Ovis and biovar Equi strains and with respect to enzymes, the main difference was the presence of the nitrate locus that contains the genes encoding the molybdopterin and the genes encoding the nitrate reductase and a region situated upstream of the *ansA* gene and this locus has a 19,606 pb length.

4. Conclusions

In this study, we found that *C. pseudotuberculosis* strains 1/06-A, 316, 162, and CIP52.97 are able to do nitrate reduction but in their genomes they do not have genes associated with nitrate reduction, warning us about resequencing of these genomes. The *C. pseudotuberculosis* nitrate locus is composed of *narKGHI* gene cluster and by genes encoding the molybdopterins *moeB*, *moaE*, *molB*, *molA*, *moeY*, *moaC*, *moeA*, and *moa* genes are similar to the other bacteria under Actinomycetes and all the biovar Ovis strains that lack the nitrate locus in their genomes. The *narGHI* complex in *C. pseudotuberculosis* biovar Equi shows conserved functional domains. *narK* and *narT* genes in *C. pseudotuberculosis* biovar Equi encode two putative nitrate and nitrite carriers, respectively. Finally, the *nirK*, *nrfA*, and *nrfH* encoding nitrite reductase enzymes are conserved in *C. pseudotuberculosis*. Therefore, the study of pathogen metabolism can contribute to the identification of pathogen virulence, discovery of drug targets, host response, and generating hypotheses for experimental investigation.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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