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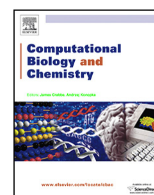

Research article

Genomic studies on nitrogen metabolism in *Halomonas boliviensis*: Metabolic pathway, biochemistry and evolution

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ABSTRACT

Halomonas boliviensis LC1^T = DSM 15516^T is a halophilic bacterium that copiously produces osmolytes and polyesters. The growth of *H. boliviensis* is restricted when glutamate or glutamine is not included in its culture medium. The concentration of glutamate in the medium can regulate the production of either osmolytes or polyesters. However, genomic studies on the nitrogen assimilation have not been performed on *H. boliviensis* and other members of the family *Halomonadaceae*. Glutamate metabolism in *H. boliviensis* was discerned based on genome sequence analysis. The genome sequences of other *Halomonadaceae* members revealed similar enzymes to those found in *H. boliviensis*. *H. boliviensis* and *H. elongata* DSM 2581^T acquired distinct glutamate dehydrogenase genes through horizontal gene transfer from a different bacterium. Two alleles of glutamine synthetase could be found in *H. boliviensis*, one of which was obtained from a thermophilic archaeon via horizontal gene transfer. Two subunits of glutamate synthase were also present in *H. boliviensis*. The small β-subunit had a molecular weight of 52 kDa and was phylogenetically closely affiliated to proteins of other halomonads and Gammaproteobacteria. The large (161 kDa) α-subunit of the halomonads gathered in a separate phylogenetic group, hence glutamate synthase α-subunits of halomonads may be included a novel group of enzymes. Furthermore, putative enzymes obtained from the genome of *H. boliviensis* should permit complete glutamate metabolism. A similar metabolism should be followed by other halomonads. However, some phenotypic differences between halomonads, such as the ability to assimilate ammonia, resulted as a consequence of horizontal gene transfer. Each enzyme that forms part of the glutamate metabolism in prokaryotes evolved following a different pattern. Yet, most enzymes of halomonads diverged in phylogenetic clusters composed of Proteobacteria, as might be expected.

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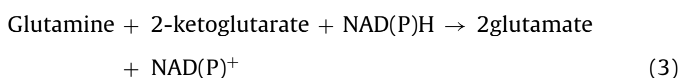
1. Introduction

Nitrogen is an essential component in cells. Ammonia is the most common inorganic nitrogen source assimilated by cells (Reitzer, 2003). The assimilation of ammonia into amino acids is initiated by three biochemical reactions that form part of glutamate metabolism (Forchhammer, 2007).

$$\text{2-Ketoglutarate} + NH_3 + NAD(P)H \leftrightarrow \text{glutamate} + NAD(P)^+ \quad (1)$$

$$\text{Glutamate} + NH_3 + ATP \rightarrow \text{glutamine} + ADP + Pi \quad (2)$$



$$\text{Glutamine} + \text{2-ketoglutarate} + NAD(P)H \rightarrow \text{2glutamate} + NAD(P)^+ \quad (3)$$

The first reaction is reversible and is catalyzed by glutamate dehydrogenases (GDHs), which can be divided in four classes with distinctive molecular weights (Harper et al., 2010). GDH-1 and GDH-2 are small hexameric enzymes (MW about 50 kDa) that utilize either NAD$^+$ (EC 1.4.1.2) or NADP$^+$ (EC 1.4.1.4) as a coenzyme, respectively (Antonopoulos et al., 2003; Consalvi et al., 1991). Larger tetrameric enzymes (115 kDa) were classified as GDH-3, and are dual coenzyme specific NAD(P)$^+$ (Veronese et al., 1974). Finally, enzymes of a fourth class (GDH-4) are approximately 180 kDa in size, hexameric and are NAD$^+$ specific (Miñambres et al., 2000). On the other hand, the sequential irreversible reactions (2) and (3) are catalyzed by glutamine synthetase (GS) and glutamate synthase (GOGAT), respectively (Forchhammer, 2007). The net result of reactions (2) and (3) is in essence the same as that of reaction (1); both routes produce one molecule of glutamate (Forchhammer, 2007). The difference is that the hydrolysis of ATP is required for the glutamine synthetase/glutamate synthase route. In organisms that are able to accomplish the three reactions, ammonia will generally be assimilated via the GDH reaction when its extracellular

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concentration is high, and via the GS/GOGAT route when it is scant (Forchhammer, 2007).

Genes encoding GDHs are ubiquitous in nature and have been subject of extensive horizontal gene transfer (HGT) (Andersson and Roger, 2003). These genes have been transferred among prokaryotes and from prokaryotes to eukaryotes (Andersson and Roger, 2003). Nevertheless, it has been recognized that most bacteria harbor genes that express GDH-1, whereas most archaea include GDH-2. GDH-3 enzymes are mainly found in eukaryotes and higher organisms, although these dehydrogenases are present in some bacteria as well (Andersson and Roger, 2003; Forchhammer, 2007; Miñambres et al., 2000). GDH-4 enzymes were more recently discovered in bacteria (Miñambres et al., 2000). On the other hand, glutamate synthases are found in three distinct forms: NADPH–GOGAT (EC 1.4.1.13) that is usually found in prokaryotes (Jongsareejit et al., 1997; Oliver et al., 1987), ferredoxin–GOGAT (EC 1.4.7.1) and NADH–GOGAT (EC 1.4.1.14) that are generally obtained from higher organisms (Forchhammer, 2007; Suzuki and Knaff, 2005). However, NADH–GOGATs are also found in cyanobacteria, yeasts and algae (Muro-Pastor et al., 2005). NADPH–GOGATs purified from *Escherichia coli* and *Azospirillum brasilense* are composed of two subunits of approximately 135 kDa (large α-subunit) and 53 kDa (small β-subunit) (Oliver et al., 1987). The phylogenetic delineation of the different type of GOGATs among organisms seems to reflect a lesser influence of HGT on genome evolution than that observed for GDHs. On the whole, evolution has retained the synthesis of glutamate and glutamine in all living organisms so that they have became the major donors of nitrogen for all other components in a cell (Andersson and Roger, 2003; Forchhammer, 2007). Glutamine is the biosynthetic precursor of purines, pyrimidines, a number of amino acids, glucosamine and ρ-benzoate, whilst glutamate provides nitrogen for most transaminases and participates in the formation of about 85% of the nitrogenous compounds in a cell (Fisher, 1999).

Halomonas boliviensis is a halophilic (salt loving) bacterium and is able to accumulate polyesters when a nitrogen source restricts cell growth and when a carbohydrate is in excess in a culture medium (Quillaguamán et al., 2004, 2008). This bacterium is included in the family *Halomonadaceae* that forms part of the Gammaproteobacteria. All members of the family *Halomonadaceae*, herein referred as halomonads, are halophilic or halotolerant microorganisms (Oren, 2008). *H. boliviensis* is also able to produce ectoines that are used as intracellular osmolytes that help to maintain cell turgor pressure, cell volume, and concentration of electrolytes in the cell when the salt (NaCl) concentration in the culture medium is high (Van-Thuoc et al., 2010). However, the synthesis of ectoines requires the presence of nitrogen in the culture medium to attain high yields (Van-Thuoc et al., 2010). Bioprocesses that led to high yields and volumetric productivities of ectoines, polyesters, and the combination to these products were designed based on the nitrogen source availability for the cells (Guzmán et al., 2009; Van-Thuoc et al., 2010). The culture medium used for optimization of the production processes was based on a defined medium used for *Halomonas elongata*; the medium contains NH_4Cl as the sole nitrogen source (Sauer and Galinski, 1998). However, unlike *H. elongata*, cell growth of *H. boliviensis* in such a medium is limited (Quillaguamán et al., 2008). In this respect, amino acids soluble in water were included in the medium composition for *H. boliviensis*. Only three amino acids (aspartic acid, glycine, and glutamine) were found to improve the growth of *H. boliviensis* (Quillaguamán et al., 2008). Glutamine is an amino acid that favors the growth of *H. boliviensis*. Replacement of glutamine by sodium glutamate does not affect the cell concentration attained by *H. boliviensis* (Quillaguamán et al., 2008). Microbial polyesters and osmolytes are used in biotechnological applications in medicine, pharmacy, and food technology (Philip et al., 2007). Biopolyesters can be used, for instance, as biodegradable tissue and bone implants, whereas osmolytes such as ectoines are employed as enzyme, DNA, and whole cell protectants against freezing, heating, and drying (Pastor et al., 2010).

The genome sequence of *H. boliviensis* was recently obtained (Guzmán et al., 2012). Evolutionary studies on several genes of *H. boliviensis* revealed that various genes involved in carbohydrate transport and metabolism have been acquired by HGT (Guzmán et al., 2012). Gene transfer from other bacterium had a profound effect on *H. boliviensis* – 45% of the genes related to carbohydrate uptake and their metabolic assimilation were obtained from other bacteria (Guzmán et al., 2012). Moreover, genes attainment from thermophilic and halophilic archaea was also discovered, although in a lesser extent (Guzmán et al., 2012). Enzyme polymorphism was also found in the pathways of glycolysis and gluconeogenesis of *H. boliviensis* (Guzmán et al., 2012). However, glutamate metabolism in *H. boliviensis* and its link to these catabolic routes have not been studied.

This research work presents the evolution of the glutamate metabolism in *H. boliviensis* based on analysis of supernetworks that were built from cluster of orthologous proteins (COGs). The COGs were obtained from the genome sequences of 90 microorganisms and *H. boliviensis*. Similarities between the protein evolution of *H. boliviensis* and other halomonads were also determined. Moreover, this report depicts the phylogeny of enzymes that catalyze reactions (1)–(3), describes and models the primary, secondary and tertiary structures of GDHs and GOGATs in *H. boliviensis*.

2. Methods

2.1. Genome sequences studied

Four genome sequences corresponding to strains of the family *Halomonadaceae* were selected for our studies. The strains were *H. boliviensis* $LC1^T = DSM\ 15516^T$ (Guzmán et al., 2012), *H. elongata* DSM 2581^T (Schvibbert et al., 2010), *Halomonas* sp. TD01 (Cai et al., 2011), and *Chromohalobacter salexigens* DSM 3043^T (Oren et al., 2005).

2.2. Determination of biochemical pathways

Halomonas boliviensis was taken as the reference strain for the determination of biochemical pathways. The functional annotation of enzymes involved in the glutamate pathway was accomplished by analysis of protein sequences. Genes of *H. boliviensis* were aligned to others in databases to attain its corresponding functional annotation. To ensure the biological meaning, only high-quality information was chosen for the annotation of the genes from the many results available. BLAST was used to accomplish functional annotation combined with different databases. BLAST version: blastall 2.2.21 software (provided by the National Center for Biotechnology Information) was used for these studies. Alignment results were obtained using the KEGG, COG, SwissProt, TrEMBL, and NR databases. A BLAST search was also used to find enzymes in *H. elongata*, *C. salexigens*, and *Halomonas* sp. TD01 that share high identities with those annotated for *H. boliviensis.*

2.3. Evolutionary analysis and structure models of proteins

A total of 6901 alignments of clusters of orthologous proteins (COGs) of 90 microorgansims, as classified in COGs (Tatusov et al., 2003) and EggNOG (Jensen et al., 2008) databases, were obtained from that described by Puigbò et al. (2009). The protein sequences of these 90 microorganisms were used as reference for the evolutionary analysis. Protein sequences of strains of the family *Halomonadaceae* were included in the alignments with the

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