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High-affinity ammonium uptake system as an evolutionary constraint for growth rate and stress tolerance

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Abstract

Nitrogen is a most important nutrient resource for *Escherichia coli* and other bacteria that contain the *glnKamtB* operon, a high-affinity ammonium uptake system highly interconnected with cellular metabolism. Although this system confers an advantage to bacteria when growing under nitrogen-limiting conditions, little is known about the impact of these genes on microbial fitness under nutrient-rich conditions. Here, the genetically tractable *E. coli* BW25113 strain and its *glnKamtB*-null mutant (JW0441) were used to analyze the impact of GlnK-AmtB on growth rates and oxidative stress tolerance. Strain JW0441 showed a shorter initial lag phase, higher growth rate, higher citrate synthase activity, higher oxidative stress tolerance and lower expression of *serA* (involved in serine metabolism) than strain BW25113 under nutrient-rich conditions, suggesting a fitness cost to increase metabolic plasticity associated with amino acid biosynthesis. The overexpression of *serA* in strain JW0441 resulted in a decreased growth rate and stress tolerance in nutrient-rich conditions similar to that of strain BW25113, suggesting that the negative influence on bacterial fitness imposed by GnlK can be related to the control of serine biosynthesis. On this background, we also discuss the potential applications of *glnKamtB* in bioproduction processes.

73 **Introduction**

74

75 Natural environments normally exhibit an extremely high microbial diversity, nutrient
76 limitations, and a variety of abiotic stress conditions (Ayub *et al.*, 2004, Ayub *et al.*, 2009, Soto
77 *et al.*, 2012, Pascuan *et al.*, 2015, Stritzler *et al.*, 2018). Accordingly, the growth rate, nutrient
78 uptake and adaptation to abiotic stress are crucial parameters that reflect the fitness of
79 microbes in their natural habitat. Nitrogen is an elemental component of almost all
80 macromolecules in a microbial cell (Reitzer, 2003), including nucleic acids, proteins and cell
81 wall compounds. Consequently, prokaryotes possess complex control mechanisms to provide
82 an optimal nitrogen amount for cellular metabolism and to survive in nitrogen-limiting
83 conditions (Kustu *et al.*, 1984, van Heeswijk *et al.*, 2013).

84 Ammonium (found in an aqueous equilibrium between the protonated and the neutral
85 species, i.e. NH_4^+ and NH_3) is the optimal nitrogen source for prokaryotes that contain the
86 *glnKamtB* operon, including *Escherichia coli.* The *glnKamtB* operon encodes a sensitive
87 sensory system that regulates ammonium influx in response to external alterations in
88 ammonium availability (Coutts *et al.*, 2002). AmtB is an ammonium transporter whose activity
89 is negatively regulated by the reversible formation of a complex with GlnK (Javelle *et al.*, 2004),
90 a small cytosolic signal transduction protein that orchestrates the activities of the main
91 regulators of carbon and nitrogen metabolism (Burkovski, 2003, Kim *et al.*, 2012, van Heeswijk
92 *et al.*, 2013) and regulates the adenylylation state of glutamine synthase, a key enzyme for
93 nitrogen assimilation (Vo *et al.*, 2013, Gosztolai *et al.*, 2017).

94 Based on the genetic linkage and presence of the *glnK* and *amtB* genes in strains
95 belonging to the Bacteria and Archaea domains, it has been proposed that these genes
96 constitute an ancestral ammonium-sensing system of prokaryotes (Thomas *et al.*, 2000,
97 Javelle & Merrick, 2005). However, since horizontal gene transfer is a critical mechanism of
98 microbial evolution (Fox *et al.*, 2014, Pascuan *et al.*, 2015, Stritzler *et al.*, 2018), the presence
99 of this system in the Bacteria and Archaea domains does not necessarily imply that its
100 emergence occurred in the common ancestor of prokaryotes. In addition, although several
101 studies have demonstrated the importance of the ammonium-sensing system in the adaptation
102 of microbes to changes in nutrient availability, including starvation and nutrient-shock
103 conditions (Kustu *et al.*, 1984, Kidd & Wingreen, 2010, Gosztolai *et al.*, 2017), little is known
104 about the fitness cost of the occurrence of this nitrogen regulatory system.

105 Thus, the aim of this study was to integrate phylogenetic and functional analyses to
106 characterize the emergence of the *glnK* and *amtB* genes in prokaryotes and to understand the
107 occurrence and conservation of these genes together with their possible impact of on microbial
108 fitness, focusing on growth rate and oxidative stress tolerance as key parameters.

Material and Methods

Bacterial strains and genomic analyses

Escherichia coli BW25113, a fast-growth model strain without auxotrophies, and its Δ*glnKamtB* derivative (strain JW0441, CGSC#: 8602), obtained from the Coli Genetic Stock Center (Baba *et al.*, 2006), were used in this study. The integrity of the *glnKamtB* operon in strain BW25113 and the knockout of this operon by the insertion of a kanamycin cassette in strain JW0441 were verified by sequencing and analyzing their genomes by means of Illumina Hiseq1500 technology and Geneious as indicated previously (Brambilla *et al.*, 2019).

Plasmid construction, bacterial transformation and gene expression

Plasmids pECglnKamtB and pNOD26 were constructed for homologous and heterologous complementation of ammonium uptake deficiency in strain JW0441, respectively. Firstly, a PCR was carried out to amplify a fragment containing the *glnKamt* operon of strain BW25113 by using primers OpFW (5'-ATCTGCAGGCCAGCGTGCGTGAAGAGGAAT-3') and OpRV (5'-ACCGTTCAGGAAGGGGTGATGCGTAAT-3'). The primers flanked a 1,955-bp fragment including the entire *glnKamt* operon and its intergenic regions. This amplification fragment was cloned into vector pGEM-t Easy (Promega #A1360) and its identity was verified by sequencing (Cuyeu *et al.*, 2013). The resulting plasmid was termed pECglnKamtB. Secondly, plasmid pNOD26, carrying the ammonium channel *NOD2*6 gene (Glyma08g12650) from *Glycine max* (Frare *et al.*, 2018), was constructed by introducing an amplified and XbaI-digested fragment of the full-length NOD26 cDNA into the *E. coli*-expressing vector pSEVA4413 (Silva-Rocha *et al.*, 2013). The primers used were 26FW (5'-CCTCTAGAATGGCTGATTATTCAGCAGG-3') and 26RV (5'-CCTCTAGATTATTTGGAGGCAGCACGGC-3') containing XbaI sites. The absence of mutations in the *NOD26* gene was verified by sequencing. Secondly, plasmid pNOD26, carrying the ammonium channel *NOD2*6 gene (Glyma08g12650) from *Glycine max* (Frare *et al.*, 2018), was constructed by introducing an amplified and XbaI-digested fragment of the full-length NOD26 cDNA into the *E. coli*-expressing vector pSEVA4413 (Silva-Rocha *et al.*, 2013). In addition, the *serA* gene from strain BW25113 was amplified using primers 5'-CCTCTAGAATGGCAAAGGTATCGCT-3' and 5'-CCAAGCTTTTAGTACAGCAGACGGG-3' and cloned in the expression vector pSEVA2513 (Silva-Rocha *et al.*, 2013) by using XbaI and HindIII restriction enzymes. The resulting plasmid was named pSEVA-serA. Plasmids pECglnKamtB, pNOD26 and pSEVAserA were introduced by transformation into competent cells of strain JW0441. Ampicillin-resistant (JW0441-pECglnKamtB), streptomycin-resistant (JW0441-pNOD26) and kanamycin-resistant (JW0441-

pSEVAserA) transformants were maintained on LB agar plus ampicillin (100 μg/L), streptomycin (100 μg/L) and kanamycin (50 μg/L). qRT-PCR experiments were performed according to (Setten *et al.*, 2013). 16S rRNA gene was chosen as the internal control gene.

Bacterial growth under nitrogen-limiting conditions

To test growth under nitrogen-limiting conditions, overnight cultures grown in lysogeny broth (LB) medium were washed twice in physiological solution and serially diluted to 10^{-6} in M9 minimal medium (47 mM Na_2HPO_4 Sigma cat#255793, 22 mM KH_2PO_4 Sigma cat#P9791, 20 mM NH_4Cl Sigma cat#213330, 8.5 mM NaCl Ciardelli cat#750325, 1.9 mM $MgSO_4$ Sigma cat#746452, 0.09 mM $CaCl_2$ ICN cat#195088, 27 mM glucose Biopack cat#9638.08, pH=7.4). Then, aliquots (0.1 mL each) were plated in solid M9 medium (M9 medium supplemented with 1.5% (w/v) Agar Sigma cat#A7921) varying both the concentration of nitrogen (from 0.01 mM to 20 mM NH_4Cl) and the pH values (5.5 or 7.4) in the last medium and incubated at 37°C for 60 days. As a control of the presence of bacterial cells in each sample, aliquots (0.1 mL each) were also plated onto LB agar. Experiments showing < 10 or > 200 colony-forming units (CFU) in this control were discarded.

Growth rate, citrate synthase activity and oxidative stress assays

Cultures were performed in 125-mL Erlenmeyer flasks containing 25 mL of LB medium, incubated at 37°C with shaking (250 rpm). Overnight cultures grown at 37°C were used to inoculate fresh media at an initial optical density (OD 580 nm) of 0.05. Bacterial growth, doubling time and citrate synthase activity of strains were assessed under this nutrient-rich environment. Growth was monitored by measuring optical density for 135 min, and doubling time was evaluated in exponentially growing cells, where citrate synthase activity was measured by the CSA kit (Sigma-Aldrich CS0720). Tolerance to H_2O_2 was measured as previously described (Ayub *et al.*, 2004), with slight modifications. Sterile Whatman No. 1 filter disks (5 mm) impregnated with 5 μL of 10% (w/w) H_2O_2 were placed on top of bacteria-seeded plates. Inhibition zones were measured after incubation at 37°C for 24 h.

Bioinformatic protein and phylogenetic analyses

GlnK (NP_414984) and AmtB (NP_414985) protein sequences of *Escherichia coli* were used as query to search against the genome database of NCBI (https://www.ncbi.nlm.nih.gov/) by using BLASTP software. Protein identities were calculated using MatGAT (Campanella *et al.*,

181 2003). The *glnK* and *amtB* genes are normally, but not always, co-localized in the genome
182 (Fig. S1). Bioinformatic studies were restricted to homologous proteins (> 25% amino acid
183 identity) as previously explained (Perez Di Giorgio *et al.*, 2014). Phylogenetic trees were
184 constructed using the Neighbor-Joining (NJ) method with genetic distances computed using
185 the p-distance model and bootstrap analysis of 500 resamples and root on midpoint, using the
186 MEGA software (Stecher *et al.*, 2020). A consensus tree of GlnK and AmtB proteins were
187 constructed using the SplitsTree4 software (Huson & Bryant, 2006) by setting standard
188 parameters as previously (Brambilla *et al.*, 2020).
189
190 **Results and Discussion**
191
192 To investigate the origins of the *glnK* and *amtB* genes in prokaryotes, we analyzed the
193 presence and the evolutionary relationships of these proteins from sequenced species
194 belonging to Bacteria and Archaea domains. Putative homologous proteins (>25% amino acid
195 identity), which contain conserved motifs of the GnlK and AmtB proteins, are ubiquitous in
196 Bacteria (thirty-two phyla) but almost absent (one phylum, Euryarchaeota) in Archaea (Fig. 1).
197 The phylogenetic analysis showed that the GlnK and AmtB proteins from the Bacteria and
198 Archaea domains are not divergent lineages and that these proteins from archaeal strains
199 cluster with different bacterial phyla (Fig. 1). The most parsimonious explanation for this result
200 is the emergence of the *glnK* and *amtB* genes in a common ancestor of Bacteria and their
201 occasional transfer to Archaea. Interestingly, we observed complete congruence (i.e., same
202 topology) between the GlnK-AmtB tree and the organismal evolution at the phylum level within
203 Bacteria (Fig. 1). This fact suggests that the *glnK* and *amtB* genes from strains belonging to
204 different bacterial phyla were inherited by vertical transfer. Since the number of possible
205 evolutionary trees grows exponentially with the number of taxa studied, the probability that the
206 congruent pattern observed in Bacteria occurs by chance is practically null (Perez Di Giorgio
207 *et al.*, 2014). Considering that our evolutionary analysis supported that the presence of *glnK*
208 and *amtB* is an ancestral feature of the Bacteria domain, the retention, long-term persistence,
209 and conservation of these genes suggest a robust contribution of this high-affinity ammonium
210 uptake system to the bacterial fitness.
211 To explore the potential impacts of the *glnKamtB* operon on metabolic plasticity, the
212 growth of the wild-type strain *Escherichia coli* BW25113 and its derived *glnKamtB* mutant strain
213 JW0441 was analyzed in solid M9 minimal medium using nitrogen-limiting conditions (Table
214 1). These experimental conditions included variable ammonium (NH_4^+ + NH_3) concentrations
215 (from 0.01 to 20 mM) and two pH values (5.5 and 7.4). NH_4^+ needs an ammonium channel to
216 enter the cell, while NH_3 crosses the cell membrane by unmediated diffusion (van Heeswijk *et*

217 *al.*, 2013). At pH 5.5, ammonium (pK_a = 9.25) is mostly protonated (NH_3 only accounts for
218 0.02% of the total ammonium), and then, nitrogen limitation is stronger for cells lacking
219 ammonium channels (Vo *et al.*, 2013, Frare *et al.*, 2018). As expected, strains BW25113 and
220 JW0441 showed similar vigorous growth at high ammonium concentrations (≥ 0.5 mM),
221 independently of the pH of the medium, and strain JW0441 exhibited slower growth than strain
222 BW25113 at low ammonium concentrations (≤ 0.25 mM) (Table 1). Specifically, at low
223 ammonium (≥ 0.1 mM) and low pH (5.5), strain JW0441 was unable to grow (Table 1). In this
224 extreme condition, the behaviour of the complemented strain JW0441/pECglnKamtB
225 (homologous complementation) was similar to that of the wild type strain BW25113 (Fig. 2),
226 further supporting a critical role of the ammonium channel AmtB under nitrogen-limiting
227 conditions. Complementation of the mutant strain JW0441 with plasmid pNOD26 carrying the
228 ammonium channel *NOD26* from *Glycine max* (heterologous complementation) also restored
229 the ability of the mutant cells to grow under these strict nitrogen-limiting conditions (Fig. 2).
230 Taken together, these results verify the relevance of an ammonium channel to enable a high
231 metabolism plasticity.
232 In addition to its contribution to the uptake of ammonium under nitrogen-limiting
233 conditions, the occurrence of the *glnK* and *amtB* in bacterial cells also implies an integration
234 of the modulation of carbon and nitrogen metabolisms (Kustu *et al.*, 1984, Kidd & Wingreen,
235 2010). This co-occurrence could potentially affect other important parameters reflecting the
236 fitness and competitiveness of bacteria such as the maximal growth rate. The last point is
237 particularly important because the intrinsic constraints of bacterial maximal growth rate
238 remains unclear (Zhu & Dai, 2018). In this context, we studied the growth and oxidative stress
239 tolerance of strains either containing or lacking the *glnKamtB* operon in rich liquid LB medium,
240 where growth rate is usually unrestricted. The mutant strain JW0441 showed a shorter initial
241 lag and a higher duplication time than its parental strain BW25113 (Fig. 3). The results
242 observed for the complemented strain JW0441/pECglnKamtB were similar to those described
243 for the wild type strain (Fig. 3), supporting the notion that the *glnKamtB* operon plays a role on
244 growth phenotypes.
245 The lag phase is a stress period that prepares bacteria for cell division, and its
246 extension is positively correlated with the control of cellular damage (Bertrand, 2019).
247 Specifically, oxidative stress is a distinctive feature of the lag phase (Bradley *et al.*, 2007, Cuny
248 *et al.*, 2007) and genetic modifications that increase oxidative stress tolerance of bacteria
249 notably reduce the duration of lag periods (Ayub *et al.*, 2009). In this context, we decided to
250 explore the impact of the lack of the *glnKamtB* operon on the resistance to oxidative stress.
251 Strain JW0441 exhibited a higher oxidative stress tolerance than strain BW25113 (Fig. 4a).
252 Complementation of the mutant strain JW0441 with the pECglnKamtB plasmid suppressed the

253 stress-tolerant phenotype of strain JW0441 (Fig. 4a), a fact that verifies the negative impact of
254 this ammonium uptake system in abiotic stress tolerance and that provides a mechanism to
255 explain the lag phase reduction in strain JW0441.

256 Citrate synthase (CS) catalyzes the first reaction of the tricarboxylic acid cycle (TCA),
257 playing a critical roles in central carbon and energy metabolism. Developmental roles of CS
258 have been described in divergent bacterial lineages (Ireton *et al.*, 1995, Viollier *et al.*, 2001,
259 Vornhagen *et al.*, 2019, Zalis *et al.*, 2019) and some lower eukaryotes (Kim *et al.*, 1986,
260 Ruprich-Robert *et al.*, 2002). Thus, we decided to evaluate the effect of the lack of the
261 *glnKamtB* operon on the activity of CS as a marker of metabolic robustness. Strain JW0441
262 showed increased CS activity compared to strains BW25113 and JW0441/pECglnKamtB in
263 exponentially growing cells (Fig. 4b), suggesting that the loss of GlnK and AmtB correlates
264 with improve the growth rate connected, at least partially, with enhanced catabolism in the
265 acetyl-coenzyme A node and TCA cycle.

266 Previous studies have shown that the *glnK* mutation leads to an increase in the
267 expression of the nitrogen assimilation control (NAC) protein under both nitrogen-limiting and
268 nitrogen-excess conditions (Blauwkamp & Ninfa, 2002) and that NAC mediates the repression
269 of the expression of *serA* under nitrogen-rich conditions (Blauwkamp & Ninfa, 2002). The *serA*
270 gene encodes D-3-phosphoglycerate dehydrogenase, an enzyme that catalyzes the first and
271 rate-limiting step of the L-serine biosynthesis pathway from intermediates of glycolysis and the
272 TCA cycle (2-ketoglutarate) (Zhao & Winkler, 1996, Zhang *et al.*, 2017). In addition to its link
273 to carbon metabolism, L-serine is also a precursor for synthesis of important metabolites such
274 as other amino acids (e.g. glycine and cysteine) and purines (Grant, 2018). Considering this
275 background and our results, we propose that GlnK can moderate the growth rate and stress
276 tolerance under nitrogen-rich conditions through the indirect induction of *serA* expression. In
277 accordance with this hypothesis, the mutant strain JW0441 exhibited lower expression of the
278 *serA* gene than its parental strain BW25113 and complemented strain JW0441/pECglnKamtB
279 (Fig. 5a). Transformation of the JW0441 mutant with plasmid pSEVA2513-SerA, containing
280 the *serA* gene under the control of a constitutive promoter, reversed the rapid growth (Fig. 5b)
281 and stress-tolerant (Fig. 5c) phenotype of this strain.

282 Regardless of the evolutionary mechanism of the maintenance of the *glnK* and *amtB*
283 genes in bacteria, which is probably related to recurrent exposures to nitrogen-limiting
284 conditions in natural environments, fitness costs associated with the presence of these genes
285 in optimal growth conditions could be a serious obstacle to their conservation in bacterial
286 populations. Thus, the reduced *glnKamtB* expression under nutrient-rich conditions (Atkinson
287 *et al.*, 2002) could be interpreted as a mechanism to mitigate the cost of the maintenance of
288 these genes under favourable environmental conditions. Under controlled conditions, the

289 presence of the *glnK* and *amtB* genes could negatively affect the efficiency of recombinant
290 bacteria as cell factories for the biosynthesis of natural and artificial products. In fact, the
291 growth rate and general stress resistance are critical factors, for example, in the efficiency of
292 *E. coli* in fermentation processes (Liu *et al.*, 2015, Yang *et al.*, 2020). Specifically, oxidative
293 stress resistance in *E. coli* plays a critical role in the production of biofuels (Koppolu & Vasigala,
294 2016, Chen *et al.*, 2018). Thus, the experimental design of metabolically engineered *E. coli* for
295 the production of different compounds can include the knockout of the *glnK* and *amtB* genes
296 to maximize its efficiency. Further studies involving other bacterial species and *glnKamtB*
297 mutant strains are necessary to determine whether the alteration in growth and stress
298 tolerance via knockout of these genes, as that described in this work, is a general mechanism
299 to produce improved microbial cell factories.

300

301 **Conclusions**

302

303 The benefits of possessing a high-affinity ammonium uptake system highly
304 interconnected with the carbon and nitrogen metabolisms for coordination of microbial growth
305 under variable nutrient availability have been known since the identification of the *glnK* and
306 *amtB* genes in bacteria. However, the origin and fitness cost of this evolutionary innovation
307 have not yet been analyzed. In this study, we found evidences of the emergence and
308 conservation of these genes in the Bacteria domain, and confirmed their importance under
309 certain nitrogen-limiting conditions. Interestingly, we showed that the presence of this
310 ammonium uptake system implies an intrinsic constraint on the growth rate and stress
311 tolerance of bacteria. To our knowledge, this is the first report describing a nutrient uptake
312 system as a factor that limits the potential maximal growth rate of microbes.

313

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315

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