Mutations at pipX Suppress Lethality of $P_{II}$-Deficient Mutants of Synechococcus elongatus PCC 7942

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The $P_{II}$ proteins are found in all three domains of life as key integrators of signals reflecting the balance of nitrogen and carbon. Genetic inactivation of $P_{II}$ proteins is typically associated with severe growth defects or death. However, the molecular basis of these defects depends on the specific functions of the proteins with which $P_{II}$ proteins interact to regulate nitrogen metabolism in different organisms. In Synechococcus elongatus PCC 7942, where $P_{II}$ forms complexes with the NtcA coactivator PipX, attempts to engineer $P_{II}$-deficient strains failed in a wild-type background but were successful in pipX null mutants. Consistent with the idea that $P_{II}$ is essential to counteract the activity of PipX, four different spontaneous mutations in the pipX gene were found in cultures in which $glnB$ had been genetically inactivated.

Cyanobacteria are phototrophic organisms that perform oxygenic photosynthesis. Autotrophic growth requires the constant assimilation of ammonium via the coordinated action of glutamine synthetase (GS) and glutamate synthase, also known as the GS-GOGAT cycle, resulting in consumption of 2-oxoglutarate (34). Due to the lack of 2-oxoglutarate dehydrogenase in cyanobacteria, synthesis of 2-oxoglutarate represents the final step in the oxidative branch of the trichloroacetic acid cycle and directly links 2-oxoglutarate levels to nitrogen assimilation (35). Thus, 2-oxoglutarate accumulates during nitrogen starvation, making this metabolite an excellent indicator of the intracellular carbon-nitrogen balance (12, 25).

In cyanobacteria, multiple metabolic and developmental processes are induced by nitrogen starvation. NtcA, the global regulator for nitrogen control, activates genes involved in nitrogen assimilation, heterocyst differentiation, and acclimation to nitrogen starvation (20, 30, 41). 2-Oxoglutarate, the signal of nitrogen deficiency, stimulates binding of NtcA to target sites (45), transcription activation in vitro (44), and complex formation between the global nitrogen regulator NtcA and its coactivator factor PipX, a regulatory protein conserved in cyanobacteria (5, 9). The interaction between PipX and NtcA is known to be relevant for maximal activation of NtcA-dependent genes under nitrogen limitation (9, 10). PipX-deficient cultures of Synechococcus elongatus PCC 7942 showed reduced activity of nitrogen assimilation enzymes, retarded nitrogen induction, a slower rate of nitrate consumption, and when subjected to nitrogen starvation, retarded phycobilisome degradation and faster reduction of the chlorophyll content (10).

The homotrimeric $P_{II}$ protein is one of the most conserved and widespread signal transduction proteins in nature and plays key roles in nitrogen assimilatory processes (28). $P_{II}$ proteins contain three binding sites (one per subunit) for 2-oxoglutarate and ATP, and their primary function is to regulate, by direct protein-protein interactions, the activity of proteins implicated in nitrogen metabolism (reviewed in reference 13). In cyanobacteria, several proteins are known to form complexes with $P_{II}$. The first two $P_{II}$ receptors were identified in S. elongatus: the enzyme N-acetyl-L-glutamate kinase (NAGK), a $P_{II}$ target conserved across domains of life during the evolution of oxygenic photosynthetic organisms (5, 6, 42), and the regulatory factor PipX (5, 9). The nonconserved membrane protein PamA was identified as a $P_{II}$ receptor in Synechocystis sp. strain PCC 6803 (37). Structural and functional details are only known for the $P_{II}$-NAGK complex (29). This complex consists of two polar $P_{II}$ trimers sandwiching one ring-like hexameric NAGK, with the flexible T loop, a key element for regulatory interactions, adopting a novel compact shape. Other $P_{II}$ functions for which direct protein-protein interactions have not been reported yet include the control of nitrate transport (23, 27), nitrate reductase (43), and the control of inorganic carbon transport (21).

$P_{II}$ proteins bind 2-oxoglutarate and ATP synergistically. In S. elongatus and Synechocystis sp. strain PCC 6803, the $T$ loop is phosphorylated at a seryl residue (S49) located at the apex of the solvent-exposed T loop (12). The phosphorylation status of $P_{II}$ correlates with 2-oxoglutarate levels, both being maximal during nitrogen starvation. ATP in concert with elevated 2-oxoglutarate levels prevents complex formation of $P_{II}$ with either NAGK or PipX, suggesting that PipX-$P_{II}$ complexes could also have a function under nitrogen-sufficient conditions. In this context, previous analyses indicated that PipX is not required for $P_{II}$-dependent functions like ammonium inhibition of nitrate transport (10) or stimulation of NAGK activity (8). However, we show here that $P_{II}$ does, indeed, affect PipX functions. Our results indicate that $P_{II}$ is essential under standard growth conditions in S. elongatus and that the pipX gene is a target of suppressor mutations in $P_{II}$-deficient cultures.

MATERIALS AND METHODS

Strains and growth conditions. The strains and plasmids used in this work are listed in Table 1. Constructs and genomic mutations were analyzed by automated...
Reverse transcriptase (Fermentas) and primers 5'-H9262-H9262-phenol method. Reverse transcription (RT)-PCR analysis of centrifuged, and pellets were stored at the cultures for RNA extraction. The samples were rapidly chilled on ice and growth conditions, cells were grown under standard conditions until they reached

\[
\text{GMCT}^{+}(\text{glnB}:\text{CK2})\text{ Km'}
\]

This work

\[
\text{glnB}:\text{CK2 Ph}^{+}\text{ Sm'}\text{ Km'}
\]

This work

\[
\text{glnB}:\text{CK2 Ph}^{+}\text{ Sm'}\text{ Km'}
\]

This work

\[
\text{glnB}:\text{CS3}(-)\text{ Sm'}
\]

This work

\[
\text{glnB}:\text{CS3}(-)\text{ Sm'}
\]

This work

\[
\text{PipX}(\text{Con})[\lambda\text{Phi}(\text{CS3-pipX})]\text{ Km'}
\]

This work

\[
\text{PipX}(\text{pipX}:\text{CK1})\text{ Km'}
\]

This work

\[
\text{Plasmids}
\]

pBluescriptII SK(+) Cloning vector, Ap'

pPM128 glnB::CK2, Km'

pUAGC59 pBluescript SK(+) with 1.8 kb from pipX region, Ap'

pUAGC453 pBluescriptII SK(+) with CS3, Ap' Sm'

pUAGC700 pBluescriptII SK(+) with 1.3-kb glnB genomic region, Ap'

pUAGC701 pUAGC700 with CS3 into glnB [glnB:CS3(-)]. Ap' Sm'

pUAGC702 Same as pUAGC701, with CS3 in opposite orientation [glnB:CS3(+)]. Ap' Sm'

**TABLE 1. Strains and plasmids used in this work**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>F' φ80lacZΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17(ryk- mcrB) 1 deor thi-1 supE44 gyrA96 relA1 K′</td>
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<td><em>Synechococcus</em> sp. strain PCC 7942</td>
<td>Wild-type strain</td>
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<td></td>
<td></td>
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<td><em>Synechococcus</em> sp. strain MP2-E</td>
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<td></td>
</tr>
<tr>
<td><em>Synechococcus</em> sp. strain GlnBK</td>
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</tr>
<tr>
<td><em>Synechococcus</em> sp. strain GlnB(S+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Synechococcus</em> sp. strain GlnB(S)</td>
<td></td>
<td></td>
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<tr>
<td><em>Synechococcus</em> sp. strain SA410</td>
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<td></td>
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<tr>
<td><em>Synechococcus</em> sp. strain SA591</td>
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<tr>
<td><em>Synechococcus</em> sp. strain SA410-GlnB(S)</td>
<td></td>
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<td><em>Synechococcus</em> sp. strain SA591-GlnB(S)</td>
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<tr>
<td>Plasmids</td>
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<td>pBluescriptII SK(+)</td>
<td>Cloning vector, Ap'</td>
<td>Stratagene</td>
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<td>pUAGC59</td>
<td>pBluescript SK(+) with 1.8 kb from pipX region, Ap'</td>
<td>9</td>
</tr>
<tr>
<td>pUAGC453</td>
<td>pBluescriptII SK(+) with CS3, Ap' Sm'</td>
<td>40</td>
</tr>
<tr>
<td>pUAGC700</td>
<td>pBluescriptII SK(+) with 1.3-kb glnB genomic region, Ap'</td>
<td>This work</td>
</tr>
<tr>
<td>pUAGC701</td>
<td>pUAGC700 with CS3 into glnB [glnB:CS3(-)]. Ap' Sm'</td>
<td>This work</td>
</tr>
<tr>
<td>pUAGC702</td>
<td>Same as pUAGC701, with CS3 in opposite orientation [glnB:CS3(+)].</td>
<td>This work</td>
</tr>
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</table>

*Note that some strains are heteroallelic for glnB and therefore unstable (see text for details).*
FIG. 1. Genetic inactivation of glnB. (A) Schematic representation of glnB null alleles. Relevant restriction sites in glnB (open arrows) are indicated. Positions of primers used to verify allele replacement are indicated by black arrows. (B) PCR verification of the correct integration of resistance cassettes with primers CS3-2F (S2F) or CK2-F (K2F) and GlnB-1R (1R) in a representative clone after transformation of S. elongatus with glnB::CK2 (lane K), glnB::CS3(−) (lane S−), and glnB::CS3(+) (lane S+). Lane L, size marker (GeneRuler 100-bp Plus DNA Ladder from Fermentas). PCR products corresponding to specific alleles are indicated at the right, and relevant marker sizes in base pairs are shown at the left. (C) Detection of glnB alleles by PCR with primers GlnB-1F (1F) and GlnB-1R (1R) in three independent clones after four consecutive transfers onto selective media. Other details are the same as in panel B.

In context, the persistence of wild-type alleles after transfers to new media under selective conditions indicates that the targeted gene is essential. That was the case with the three types of clones generated here, since after several consecutive transfers onto selective plates they remained heteroallelic for glnB or gave rise to gene rearrangements (see below). As shown in Fig. 1C, wild-type glnB alleles were clearly observed by PCR in all of the independent glnB::CK2 and glnB::CS3(+) transformants analyzed (lanes K and S+ and data not shown). These two types of clones retained wild-type glnB alleles after at least six transfers of colonies onto selective media. In contrast, some of the glnB::CS3(−) transformants apparently lost wild-type glnB alleles after the fourth transfer onto streptomycin-containing media (Fig. 1C, lanes S−, and data not shown). The viability of these apparently segregated GlnBS(−) clones was compromised, since they failed to grow when transferred from solid to liquid media (data not shown). However, additional subculturings of these glnB::CS3(−) transformants on plates was possible and allowed the detection of a longer PCR product (Fig. 2A). Sequence analysis of this unexpected PCR product revealed the presence of a 21-bp insertion into the AfeI site (allele glnB133_134insATAAAGCTTATCGATACCGTC, [hereafter, glnB133_134ins]). The rearrangement was probably generated by recombination between homologous sequences at the two junctions of the CS3 cassette with the glnB gene and resulted in seven extra amino acids between residues E44 and R45 (Fig. 2B), located at the flexible T loop of PII. In contrast to their predecessor GlnBS(−) clones, derivatives in which the glnB133_134ins allele was detected (strain GlnBS(−)T loop + 7) could be cultivated in liquid media. Detection of this spontaneously generated rearrangement of the glnB::CS3(−) allele suggested that the protein product, designated PII T loop + 7, conferred a selective advantage on S. elongatus cultures deficient in PII.

In summary, attempts to generate S. elongatus PII-deficient cells with three different null alleles, glnB::CK2, glnB::CS3(+) and glnB::CS3(−), failed. The alleles or allelic pairs detected by PCR in particular cultures from transformant clones were glnB::CS3(−) and glnB::CS3(−)glnB133_134ins. Since cultures in which glnB::CS3(−) was apparently in homozygosis were invariably classified as glnB::CS3(−)glnB133_134ins in the next PCR test, it seems likely that viable cells in those cultures had already acquired the glnB133_134ins allele. Taken together, the results indicated that glnB is essential for culture growth, at least under our laboratory conditions.

glnB null mutants carry mutations in pipX. On several occasions, we noticed that our PII-deficient cultures (MP2 strains) carried secondary mutations affecting the pipX coding sequence. A point mutation (allele pipX160C>T, which encodes the substitution R54C) was found fortuitously while cloning glnB sequences for plasmid construction. Since PCR amplification of genomic DNA, followed by direct sequencing, confirmed that the pipX160C>T mutation was present in the MP2 strain used for DNA amplification, we obtained a “new” culture of the MP2 strain (a gift from K. Forchhammer) to perform further analysis. Although single-PCR amplification and subsequent sequencing demonstrated the presence of the wild-type pipX allele in this strain, after performing RT-PCR assays, we became aware that this strain also carried sequences with a 22-bp deletion in pipX (allele pipX25_46del) causing a frameshift after position N7. Subsequent PCR analysis confirmed that both alleles, pipX25_46del and wild-type pipX, were present in this MP2 strain that we had received from K. Forchhammer’s laboratory. Furthermore, a strain with the same or-
B

glnB 5'-GCC CAA ACG GAG CAT CAA GCT TTA TGC TTG TAA ACC GTT-3' CS3

CS3 5'-ACG GTT TAC AAG CAT AAA GCT TAT CGA TAC CGT CGC TAT-3' glnB

glnB 5'-GCC CAA ACG GAG CAT AAA GCT TAT CGA TAC CGT CGC TAT-3' glnB

FIG. 2. The spontaneously generated glnB rearrangement. (A) Schematic representation of the glnB133_134ins allele and detection of glnB and glnB133_134ins alleles with primers GlnB-1R (1R) and GlnB-1F (1F). Wild type, lane 2; GlnBS(-) T loop +7, lane 1. Relevant alleles are indicated at the left. Other details are as in Fig. 1. (B) Alignment of DNA sequences at the glnB-CS3 and CS3-glnB junctions from the glnB::CS3(-) allele and sequences present after the rearrangement. Predicted amino acids are shown in one-letter code. Nucleotides and predicted amino acids derived from the CS3 cassette are in bold.

predicted impacts on the amino acid sequence of the spontaneous mutations found at the pipX open reading frame or the pipX promoter region. The location of pipX-92delT, just upstream of the pipX open reading frame, suggested that it could be a promoter-down mutation. To obtain additional evidence of the impact of the pipX-92delT mutation on pipX expression, we compared pipX mRNA levels in strains carrying the wild-type or mutant allele by RT-PCR. As shown in Fig. 4, pipX mRNA levels were significantly reduced in the pipX-92delT (represented as XP\(^{-}\)1) mutant, while transcripts from unrelated controls were not altered, supporting the idea that the pipX-92delT mutation diminished the transcription of pipX. The effects of the R54C and L65Q mutations on PipX interactions will be analyzed elsewhere. Importantly, none of the pipX changes were found in wild-type S. elongatus strains, suggesting that the four mutations detected were bona fide suppressor mutations and that loss-of-function mutations at pipX alleviate the \(P_{II}\) deficiency phenotype.

Inactivation by pipX suppresses the lethality associated with \(P_{II}\) deficiency. The finding of different putative suppressor mutations at pipX suggested that the lethality phenotype associated with \(P_{II}\) deficiency was due to an excess of active PipX protein, harmful to \(S.\) elongatus cells unless counteracted by \(P_{II}\). If that were the case, inactivation of glnB alleles would be facilitated by elimination of PipX. To test this idea, we separately introduced the glnB::CS3(+) and glnB::CS3(-) alleles into a PipX\(^{-}\) strain (SA591) in which the CK1 cassette replaced part of the pipX coding sequence. As an additional control, the glnB::CS3(+) and glnB::CS3(-) alleles were introduced in parallel into kanamycin-resistant strain SA410 [PipX-(Con)], where the pipX gene is transcribed from a constitutive promoter present in the CK1 cassette. The only phenotypic features noticed in the PipX(Con) strain were a small increase in the nitrogen induction of NtcA-dependent promoters (10)

TABLE 2. Spontaneous mutations found at pipX in strains carrying glnB::CK2 alleles in homozygosis

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Strain origin</th>
<th>(P_{II}) protein expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>pipX25_46del</td>
<td>MP2</td>
<td>None</td>
</tr>
<tr>
<td>pipX160C&gt;T</td>
<td>MP2</td>
<td>None</td>
</tr>
<tr>
<td>pipX194T&gt;A</td>
<td>MP2</td>
<td>None</td>
</tr>
<tr>
<td>pipX92delT</td>
<td>MP2-A</td>
<td>(P_{II})</td>
</tr>
<tr>
<td>pipX194T&gt;A</td>
<td>MP2-E</td>
<td>(P_{II})</td>
</tr>
</tbody>
</table>

\(^{a}\) The mutation position is the distance, in nucleotides, from the start codon to the mutation site.
and a level of PipX protein twice that of the wild type (data not shown).

PCR analyses confirmed the correct integration of the CS3(+) and CS3(−) cassettes within glnB for each of the two strains used (Fig. 5A). After several transfers onto selective media, wild-type glnB alleles were observed in the PCR analysis of transformants from PipX(Con) (Fig. 5B, lanes X′S− and X′S+). Thus, as previously observed with the wild-type strain (Fig. 1C), the presence of active pipX alleles prevented homozygosis of the glnB::CS3(+) and glnB::CS3(−) alleles. In contrast, homozygosis of both the glhB::CS3(+) and glhB::CS3(−) alleles was easily achieved in the PipX− background, as shown by the absence of glnB alleles in these PipX− recombinants (Fig. 5B, lanes X′S− and X′S+). These results confirmed that the presence of functional pipX genes interfered with the inactivation of glnB, implying that an intact glnB gene is required for viability only in the presence of an active pipX gene.

DISCUSSION

Genetic inactivation of the genes that encode PII proteins in many microorganisms leads to severe growth defects or death (2, 7, 32, 33, 48). In cyanobacteria, growth defects and death associated with PII deficiency have also been reported, but depending on the model system, particular laboratory strains, or culture conditions, the glhB gene was considered essential or not essential. In S. elongatus PCC 7942 and Synechocystis sp. strain PCC 6803, glhB null mutants have been reported (14, 21, 24). In Nostoc punctiforme PCC 73102, glhB null mutants could not be obtained and the glhB gene was reported to be essential (17). In Anabaena sp. strain PCC 7120, previous attempts to inactivate glnB were unsuccessful (26). However, that report was followed by two others in which the obtainment of glnB null mutants was described (38, 49). In the most recent of these, the authors showed that successful inactivation of glnB could only be achieved when the expression pattern of downstream genes of unknown function was altered (38). In the present work, we show the importance of PII proteins for viability in the model system of S. elongatus, a cyanobacterium where successful inactivation of glnB was previously reported. Attention is called to the occurrence of suppressor mutations at the pipX gene of PII-deficient strains.

The metabolic basis of the defect of glnB null mutants in the studied microorganisms appears to be diverse. In E. coli, glnB null mutants show elevated activity of the nitrogen response regulator NtrC that results in increased activity of the Nac protein which, in turn, represses serA to levels insufficient for normal growth (4). This regulatory cascade is not even conserved in other enterobacteria (36). In Rhodospirillum rubrum, the other model system for which the metabolic defect is known, the poor-growth phenotype is due to an excess of GS activity (47). In S. elongatus, the gene that encodes GS (glnA) is activated by NtcA and GS activity is associated with PII deficiency in S. elongatus. However, none of the MP2 strains analyzed here carried mutations in the glnA or ntcA gene. Since mutations inactivating the ntcA gene have been reported (46), the impaired phenotype of S. elongatus glnB null mutants appears to be related to an unknown PipX activity, thus raising questions of whether glnB null mutants obtained in other cyanobacteria contain compensatory changes at pipX and whether previous inactivation of pipX would facilitate recovery of glnB null mutants in species that have been so far recalcitrant to glnB inactivation.

Previous studies with PII-deficient strains carrying the glnB::CK2 allele (strains MP2) suggested that PII is required to stimulate NtcA activity under conditions of nitrogen deprivation (11, 39) and has an inhibitory role with nitrate as a nitrogen source (1, 11). Structural determination of the NAGK-PII complexes (29) provided a rationale for the negative impact that mutations at Ser49, the phosphorylatable residue of PII, have in interactions with NAGK (5, 18, 29). Although no differences among PII, PII∗S49A, and PII∗S49E were found regarding interactions with PipX (5) or in vitro interactions with the effectors 2-oxoglutarate and ATP (27), Ser49 has nevertheless been implicated in the regulation of NtcA activity. Two studies carried out with MP2 strains claimed that substitution of PII∗S49A, but not PII∗S49E, for PII prevented the induction of NtcA-dependent genes (27, 39). Since the authors assumed that mutations S49A and S49E may mimic, respectively, the nonphosphorylated and phosphorylated states of PII, the inference was that PII phosphorylation was important for NtcA function (27, 39). Since we cannot safely exclude the possibility...
that the pipX mutations detected in strains MP2-A and MP2-E (Table 2) were already present in the particular cultures used then, inferences about NtcA-dependent activities in MP2 derivatives should be made with caution. Isogenic strains are needed to reevaluate the effect of PII point mutations on NtcA function.

The effect of 2-oxoglutarate on PipX-PII and PipX-NtcA complexes suggests that these protein associations are physiologically relevant at low and high C/N ratios, respectively (9), but little is known about the physiological role of PipX-PII complexes. As a starting point to get insights into the function of PipX-PII complexes, we contemplated three simple scenarios that are not necessarily incompatible: (i) PipX regulates PII functions, (ii) PII regulates PipX functions, and (iii) the complex itself has its own functions. The possibility that PipX-PII complexes bind to additional proteins, fed by recent reports of complexes binding to additional proteins, (ii) PII regulates PipX functions, and (iii) the complex itself has its own functions. The possibility that PipX-PII complexes bind to additional proteins, fed by recent reports of complexes themselves suggest that PII deficiency is best suppressed by partial rather than complete loss-of-function mutations at pipX, that 2-oxoglutarate may have an effect on PipX in bacteria, and that PipX is required for the PII-dependent inhibition of nitrate transport by ammonium (10), stimulation of NAGK activity (8), or PII phosphorylation (data not shown) and obtained negative responses in all of the cases examined. However, the results presented here strongly support a role for PII in counteracting unknown but toxic functions of PipX in S. elongatus.

The features and occurrence of the spontaneous mutations at pipX suggest that PII deficiency is best suppressed by partial rather than complete loss-of-function mutations at pipX, and some gene dosage (or specific activity) of pipX may improve the fitness of MP2 strains. This would explain the genetic heterogeneity noticed in pipX alleles. The coexistence of wild-type and mutant pipX alleles in several PII-deficient cultures, and particularly the apparent stability of the allelic combination pipX/pipX25_4del, may be an adaptive mechanism for PII-deficient cultures of S. elongatus, raising questions about the extent of genetic heterogeneity in cyanobacteria.

ACKNOWLEDGMENTS

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