A specific role of the cyanobacterial PipX factor in the heterocysts of *Anabaena* sp. strain PCC 7120

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The PipX factor is a regulatory protein that seems to occur only in cyanobacteria. In the filamentous, heterocyst-forming *Anabaena* sp. strain PCC 7120, ORF *asr0485*, identified as the *pipX* gene, is mainly expressed under conditions of combined-nitrogen deprivation dependent on the global N regulator NtcA and the heterocyst-specific regulator HetR. Primer extension analysis detected three transcription start points corresponding to a canonical NtcA-activated promoter (to which direct binding of NtcA was observed), an NtcA- and HetR-dependent promoter and a consensus-type promoter, the latter with putative -35 and -10 determinants. Activation of *pipX* took place in cells differentiating into heterocysts at intermediate to late stages of the process. Accordingly, disruption of *pipX* led to impaired diazotrophic growth, reduced nitrogenase activity and impaired activation of the nitrogenase structural genes. The nitrogenase activity of the mutant was low under oxic conditions likely resulting form inefficient protection against oxygen. In line with this, the activation of *coxB2A2C2* and *coxB3A3C3* operons encoding heterocyst-specific terminal respiratory oxidases responsible for internal oxygen removal was deficient in the *pipX* mutant. Therefore, the *Anabaena* PipX factor shows a spatiotemporal specificity contributing to normal heterocyst function including full activation of the nitrogenase structural genes and genes of the nitrogenase protective features of the heterocyst.

Cyanobacteria conform a group of microorganisms characterized by a photoautotrophic mode of life relying on oxygenic photosynthesis, a process indeed developed by ancestors of extant cyanobacteria. Nowadays, cyanobacteria are important primary producers in the oceans, and thus play a crucial role in C and N cycling in our planet (32). Many cyanobacteria, both unicellular and filamentous, are able to carry out the fixation of N_2. To protect the N_2-fixation machinery against O_2, either coming from the external medium or produced inside the cells, some filamentous cyanobacteria, such as those of the *Anabaena*/*Nostoc* genera, produce cells specialized in the fixation of N_2 called heterocysts (41). In these organisms, diazotrophic growth requires the activity of two cell types: the vegetative cells, which fix CO_2 photosynthetically, and heterocysts, which fix N_2, and of intercellular exchange of the products of both processes (13). Heterocysts have specific features to minimize the concentration of oxygen in the cytoplasm, that may be either structural barriers to prevent external oxygen penetration including additional glycolipid and polysaccharide layers of the cell envelope and a narrowed septum of connection with neighboring vegetative cells, or metabolic features to avoid internal oxygen production and to remove traces of this gas, including lack of activity of the water-splitting photosystem II and of the Rubisco and ribulokinase enzymes involved in the photosynthetic fixation of CO_2 and the expression of special terminal oxidases to cope with residual O_2 (41).

Nitrogen metabolism in cyanobacteria, including the assimilation of nitrate, nitrite and urea in non-differentiated cells, as well as heterocyst differentiation and N_2 fixation, is regulated by the transcription factor NtcA, a member of the CRP class of bacterial regulators (15). NtcA together with 2-oxoglutarate (2-OG), a metabolic signal of N deficiency, binds to specific DNA sites with the sequence signature $GTAN_8TAC$ and

activates or represses the expression of regulated genes (21, 34, 38, 42). At canonical (Class II) activated promoters, which include an NtcA-binding site centred at about -41 nt from the regulated gene transcription start and a -10 box with the consensus TAN_3T, NtcA together with 2-OG induce the production of transcriptionally active open promoter complexes with RNA polymerase (RNAP) (35). In the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942, PipX has been identified as a protein interacting with the signal transduction P_{II} protein and with NtcA albeit not simultaneously (8, 20). Inactivation of the *pipX* gene lead to diminished NtcA-dependent activation of the *glnA*, *glnN* and *nblA* genes under N stress (8, 9). Thus, a role of PipX as a coactivator of NtcA has been proposed, on which PipX binding to the P_{II} protein would have an antagonistic effect. According to the model proposed, P_{II} would control the concentration of active PipX by sequestering it. The binding of 2-OG to P_{II} that would follow a rise in the cellular C/N ratio would release PipX, available then for interaction with NtcA loaded with 2-OG (8, 20, 43). The recent solution of the crystal structure of the NtcA-PipX complex has led to propose a role for PipX at stabilizing the active conformation of NtcA (20). Finally, besides NtcA, gene activation during heterocyst differentiation requires the participation of the differentiation-specific factor HetR (1, 12, 16, 26), which has been reported to bind DNA upstream of some regulated genes (17, 18).

This work represents the first study of the physiological role of the PipX factor in a filamentous, heterocyst-forming cyanobacterium. We have performed a detailed characterization of the expression of the *pipX* gene in *Anabaena* sp. PCC 7120, and have constructed strains with mutations in the *pipX* genomic region. We found that this gene is specifically activated in the cells that are undergoing differentiation at late stages of the process, and that it is required for attaining full nitrogenase activity, for efficient protection of this enzyme against O_2 and for normal diazotrophic growth.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 was grown in BG11 (containing $NaNO_3$ [33]), $BG11_0$ (free of combined nitrogen) or $BG11_0$ + ammonium ($BG11_0$ containing 4 mM NH_4Cl and 8 mM TES-NaOH buffer, pH 7.5) medium at 30 ºC in the light (75 μE m^{-2} s^{-1}), in shaken (80-90 r.p.m.) liquid cultures or in medium solidified with 1% Difco agar. Alternatively, cultures (referred to as bubbled cultures) were supplemented with 10 mM of $NaHCO_3$ and bubbled with a mixture of CO_2 and air (1% v/v). In this case, the ammonium-containing medium was supplemented with 6 mM NH_4Cl and 12 mM TES-NaOH buffer (pH 7.5). The *hetR* mutant, *Anabaena* sp. strain 216 (4), the *ntcA* mutant, strain CSE2 (14), and the mutant strains CSV6, CSV7, CSV6-53, CSV142 and CSV143 generated in this work were grown in $BG11_0$ + ammonium medium, supplemented with Sm and Sp in the case of strains CSE2, CSV6, CSAV142 and CSAV143, and Sm, Sp and Nm in the case of CSV6-53. Antibiotics were used at the following concentrations: Sm, 2-5 μg ml^{-1}; Sp, 2-5 μg ml^{-1} and Nm, 10-50 μg ml^{-1}.

To test growth in liquid medium, cells of the corresponding strains that had been grown in $BG11_0$ + ammonium medium (with antibiotics for the mutants) were harvested, washed with $BG11_0$ medium, and resuspended in $BG11_0$, BG11 or $BG11_0$ + ammonium media at 0.2 μg Chl·ml^{-1}. Chlorophyll *a* (Chl) content of cultures was determined by the method of Mackinney (22). After incubation for the indicated times, 0.2-ml samples were taken and their protein content was determined (23). The growth rate constant ($\mu = \ln 2/t_d$, where t_d is the doubling time) was calculated from the increase of the protein content of the cultures. To test growth of the mutants on solid medium, cell suspensions of the different strains with similar Chl content were prepared, drops of 10 μl were

spotted on plates of $BG11_0$, BG11 or $BG11_0$ + ammonium media, and the plates were incubated under culture conditions.

Escherichia coli DH5α and XL1Blue were used for plasmid constructions. They and strains HB101 and ED8654, used for conjugations with *Anabaena* sp., were grown in LB medium, supplemented when appropriate with antibiotics at standard concentrations (2).

Strain constructions. To inactivate *asr0485*, two DNA fragments, one encompassing the 63 bp of the 5' end of *asr0485* and sequences upstream, and the other including the 39 bp of the 3' end of *asr0485* and 562 bp downstream, were amplified by standard PCR using as template DNA from strain PCC 7120 and oligonucleotide pairs PX1/PX2 and PX3/PX4, respectively (all oligodeoxynucleotide primers are listed in Table 1). The two DNA fragments were cloned in pGEM-T (Promega) and, after digestion, ligated in direct orientation separated by gene cassette C.S3 encoding Sm^r and Sp^r (6). The insert of the resulting plasmid, excised with XhoI was transferred to plasmid pRL278 carrying a Nm^r determinant and the *sacB* gene for positive selection (3), producing plasmid pCSV24, which was transferred by triparental mating (7) to *Anabaena* sp.

To inactivate *alr0486*, a DNA fragment of 486 bp internal to the ORF was amplified by standard PCR using as template DNA from strain PCC 7120 and oligonucleotide pair PX4/PX7. This DNA fragment was cloned into HincII-digested plasmid pCSV3 (which is a derivative of mobilizable vector pRL500 [6] in which the Ap^r gene has been excised with DraI and replaced by the DraI-ended gene cassette C.S3 encoding Sm^r and Sp^r in direct orientation) generating the suicide plasmid pCSV27, which was transferred by triparental mating (7) to *Anabaena* sp. strain PCC 7120.

To complement the *pipX* mutation of strain CSV6, a DNA fragment of 1504 bp from positions -654 to +850 with respect to the translational start of the gene was amplified by PCR using primers PX1 and PX4 and strain PCC 7120 DNA. This fragment was cloned in the mobilizable Nm^r-encoding vector pRL424 (6) producing plasmid pCSV53, which was transferred to strain CSV6 by conjugation. The genomic structure of the exconjugants in the *pipX* region was tested by PCR using primer pairs PX8/PX11 and PX8/CS3-1.

To fuse the genes *pipX* and *gfp*, a fragment of the *pipX* genomic region, encompassing positions from -605 to +276 with regard to the ORF was amplified with oligonucleotides PXgfp1 and PXgfp3 and cloned into ClaI/EcoRV digested plasmid pCSEL22a (28) producing plasmid pCSAV171, which encodes Sm^r/Sp^r and can be mobilized into *Anabaena* by conjugation. Plasmid pCSAV171 bears the *pipX* ORF, except for the last nucleotide, fused in frame to the *gfp* gene. This plasmid was transferred to strains PCC 7120 and 216 by conjugation, and selection was applied for resistance to Sm/Sp. The genomic structure of the exconjugants was analyzed by PCR with the oligonucleotide pair PX1/GFP4, and segregation with PX1/PX11 and PXgfp1/PX11. The accumulation of GFP (green fluorescent protein) was analyzed by laser confocal microscopy using a Leica HCX PLAN-APO 63X 1.4 NA objective and a Leica TCS SP2 microscope (Leica, Wetzlar, Germany) as described (28).

DNA and RNA isolation, manipulation and analysis. Isolation of genomic DNA (5) and of total RNA (2) from *Anabaena* sp. was done as described previously. For Northern blots, 10-20 μg of RNA were loaded per lane and electrophoresed in denaturing 1% agarose formaldehyde gels. DNA probes were generated by PCR using strain PCC 7120 DNA and oligonucleotide primers, as follows: PX9/PX10 for *pipX*; PXgfp2/PX22 for the *pipX* leader region; alr0486-7120-1F/alr0486-7120-4 for *alr0486*;

NA10/NA11 for *ntcA*; DB034/DB018 for *devB*; NH1/NH4 for *nifH*; CB2-4/CB2-5 for *coxB2* and CB3-6/CB3-7 for *coxB3*. Hybridizations were performed as previously described (31). As control of RNA loading and transfer efficiency, the filters were hybridized with a probe of the RNase P RNA gene (*rnpB*) from strain PCC 7120 amplified by PCR with primers Universal and Reverse and plasmid pT7-7120 as template (39). Probes were labeled with a DNA labeling kit (Ready to Go, Amersham Pharmacia Biotech) and [α-^{32}P]dCTP. Radioactive areas in Northern blot hybridizations were visualized with a Cyclone storage phosphor system (Packard).

For RT-PCR experiments, 10 µg of strain PCC 7120 total RNA was mixed with 40 pmol of oligonucleotide RT-1 or RT-2 in the presence of 10 mM Tris-HCl (pH 8.0), 150 mM KCl and 1 mM EDTA, heated for 10 min at 85 ºC and then at 50 ºC for 1 h for annealing. The extension reactions were carried out at 47 ºC for 1 h in a final volume of 32 µl containing the whole annealing reaction, 0.25 mM each deoxynucleoside triphosphate, 100 U of reverse transcriptase (Superscript II; Invitrogen) and the buffer recommended by the transcriptase provider. To control for the presence of contaminating DNA, samples containing 10 µg of RNA were processed as above but omitting reverse transcriptase (-RT control). PCR was carried out with 2-5 µl of retrotranscription mixture (or -RT control mixture) as the template and the following oligonucleotide pairs as the primers: alr0488-7120-1/alr0489-7120-1 (for the segment named A in Fig. 2), alr0487-7120-1/alr0488-7120-3 (segment B), alr0486-7120-1/alr0488-7120-3 (segment C), PX14/alr0488-7120-2 (segment D), PX14/RT-2 (segment E), and PX1/RT-2 (segment F). Samples containing the same oligonucleotides and strain PCC 7120 DNA as the template were run in parallel and used as controls. PCR was performed by standard procedures, and the PCR products were resolved by electrophoresis in 0.7% agarose gels.

195 Primer extension analyses were performed as previously described (25) with
196 oligonucleotide primers asr0485-7120-1, PX17, PX18 and PX21. DNase I footprinting
197 assays were performed as in (35) with purified NtcA. Radioactive areas of the gels were
198 visualized and quantified with a Cyclone storage phosphor system (Packard).
199 **Glycolipid analysis and nitrogenase activity.** For glycolipid analysis, filaments
200 of *Anabaena* sp. grown in BG11 medium (in the presence of Sm and Sp for the CSV6
201 mutant) were harvested, washed with nitrogen-free medium and incubated in $BG11_0$
202 bubbled cultures for 24 h. Filaments were then harvested and washed with buffer 1
203 containing 50 mM imidazol and 0.5 mM EDTA (pH 8.0), and lipids were extracted with
204 chloroform-methanol (2:1 v/v), concentrated under N_2, and chromatographed on thin
205 layers of silica gel (27). Heterocyst glycolipids were identified as described (40).
206 Nitrogenase activity was determined by the acetylene reduction technique in
207 filaments incubated for 24 h in $BG11_0$ medium as described (24). For assays under
208 microoxic conditions, 10 μM DCMU was added to the cell suspension, the flask
209 containing the cells was sealed with a rubber stopper, bubbled with argon for 3 min and
210 further incubated under culture conditions for 1 hour before the assay was started by
211 addition of acetylene.
212
213 **RESULTS**
214 **Identification and regulated expression of the *Anabaena pipX* gene.** A
215 similarity search using as query the sequence of the PipX protein from *Synechococcus* sp.
216 strain PCC 7942 against the whole genomic sequence of *Anabaena* sp. strain PCC 7120
217 (19) identified ORF *asr0485*. This ORF would encode a product of 92 amino acids
218 exhibiting 63% identity to the 89-amino acid *Synechococcus* PipX protein, but it did not
219 show similarity to any non-cyanobacterial gene product in the databases. In the genome

220 of strain PCC 7120, *asr0485* is flanked by ORFs *alr0484* and *alr0486*, both encoding
221 hypothetical proteins. The latter overlaps with *asr0485* by 4 nucleotides (Fig. 1A).

222 We analized the expression of the *Anabaena* gene cluster depicted in Fig. 1A by
223 RT-PCR using 2 distinct primers for retrotranscription and different primer pairs for
224 PCR to determine the transcriptional units in this genomic region. The pattern of
225 amplification products shown in Fig. 1B indicates the existence of transcripts
226 overlapping *pipX* and *alr0486*, on one hand, and *alr0487*, *alr0488* and *alr0489* on the
227 other hand. Evidence for transcripts overlapping *alr0484* and *pipX* or *alr0486* and
228 *alr0487* was not obtained.

229 The pattern of expression, as well as nitrogen regulation of *pipX* was studied by
230 Northern blot analysis using RNA isolated from filaments of strain PCC 7120 and of an
231 *ntcA* mutant (strain CSE2; 14) and a *hetR* mutant (strain 216; 4) grown with ammonium
232 and incubated in the absence of combined nitrogen (i.e., in the absence of any nitrogen
233 source other than atmospheric nitrogen; Fig. 2). In the wild-type strain, the *pipX* probe
234 detected RNA species of ca. 0.5, 0.8, 1.2 and, weakly, 1.4 kb (*pipX* ORF is 279 nt long),
235 whose abundance was very low in the ammonium-grown cultures and increased upon
236 combined nitrogen deprivation. Whereas the 0.5-kb transcript seems to accumulate early
237 after ammonium withdrawal, the accumulation of the larger (0.8, 1.2 and 1.4 kb)
238 transcripts was higher after 9-12 h of combined-nitrogen deprivation, and was impaired
239 in both the *ntcA* and *hetR* mutant strains. When a probe covering the whole ORF
240 *alr0486* was used, RNA species of ca. 1.4 and 1.2 kb, whose abundance increased upon
241 combined nitrogen deprivation, and faintly of ca. 0.9 kb were detected (Fig. 2B). Since
242 *pipX* and *alr0486* are respectively 279 and 668 nt long, the regulated 1.4 and 1.2 kb
243 bands, which hybridize with both probes, may include the messages of both *pipX* and, at
244 least in part, *alr0486*, whereas the regulated transcripts of ca. 0.5 and 0.8 kb hybridizing

to *pipX* and the transcript of ca. 0.9 kb hybridizing to *alr0486* would represent independent transcripts of those ORFs, respectively. Given that the transcripts of 1.4 and 1.2 kb are longer than the two ORFS together, we checked whether they covered additional sequences upstream of *pipX*. When a DNA fragment covering positions from -415 to -109 bp with respect to the *pipX* ORF (see below) was used as a probe (probe up-*pipX*) in Northern assays (Fig. 2A), the four RNA species previously detected with the probe of the *pipX* ORF were seen. This observation indicates that these transcripts include leader sequences upstream of *pipX*. Additionally, a smaller RNA species, faintly detected with the probe from within the *pipX* ORF, was clearly seen with the upstream probe. This one could correspond to an abortive transcript, to a degradation product of RNAs initiated upstream of *pipX* or to a small RNA from the *pipX* leader region.

In order to analyze the molecular basis of the regulation of the expression of *pipX*, the location of putative transcription start points (tsp) was sought by primer extension analysis using RNA extracted from cultures of strains PCC 7120, CSE2 and 216 grown on ammonium and incubated in the absence of combined nitrogen. In the wild type, several 5' transcript ends were detected using primers upstream or within the 5' region of *pipX*. A 5' end corresponding to the C located 436 nucleotides upstream of the ORF was detected with primers PX18 (Fig. 3A) and PX21 (not shown). Its signal increased upon combined nitrogen deprivation, and it was detected in the *hetR* strain but not in the *ntcA* mutant.

Direct binding of purified NtcA to a DNA fragment (from -588 to -282 bp with regard to the ORF) including sequences just upstream of the 5' end at -436 was assayed by DNase I footprinting. Fig. 3B shows changes in the digestion pattern produced by the presence of NtcA with or without 2-OG. These changes included a general protection of the sequence between 23 and 52 nucleotides upstream of the -436 tsp (a

region that includes a putative NtcA-binding site with the sequence GTAGCAATGCAGAC) and hypersensitivity of the thymine located 45 nucleotides upstream of that tsp. Indeed, NtcA-induced hypersensitivity at T of the GTA triplet of the NtcA recognition site has been previously noted (e.g. 29; see below). Thus, results of primer extension analysis and NtcA-binding indicate the operation of a promoter directly activated by NtcA governing N-regulated transcription of *pipX* initiated at position -436.

With the same oligonucleotides, a second 5' transcript end was identified corresponding to a G at position -388. As in the case of the -436 one, the abundance of transcripts with this end increased upon combined nitrogen deprivation and was not detected in the *ntcA* mutant but, in contrast to the -436 one, was also not detected in the *hetR* mutant (Fig. 3A). With primers PX17 (not shown) and asr0485-7120-1 (Fig. 3C), a third 5' end was detected corresponding to G at position -193, which depended on NtcA (not shown). However, we cannot discern whether it represents a true tsp or a degradation product of transcripts originating from the upstream regulated promoter(s). Additionally, with primers PX17 and asr0485-7120-1, three other 5' RNA ends were detected corresponding to positions -107, which was detected under all tested conditions (Fig. 3C), -54 and -23 (not shown). Again, the -54 and -23 ends could originate from additional promoters or from processing.

Expression of the *pipX* gene in different cell types. Transcription of *pipX* from NtcA- and HetR-regulated promoters suggested differential expression of this gene in different cell types. Expression of *pipX* along the filament was studied using GFP as a reporter. The *gfp-mut2* gene was inserted, maintaining the translational frame, just before the stop codon of *pipX* in the chromosome of strains PCC 7120 and 216, the latter of which does not show heterocyst differentiation (see Materials and methods).

295 The selected clones (strains CSAV142 and CSAV143, respectively) had incorporated
296 the construct by single crossover, thus keeping an intact copy of the P_{pipX}-*pipX* region.
297 Fluorescence from GFP was analyzed in ammonium-grown filaments subjected to
298 different periods of combined nitrogen deprivation (Fig. 4). In the two strains, very low
299 fluorescence was detected in the ammonium-grown filaments (t=0 h). In strain
300 CSAV142, fluorescence was observed to increase from ca. 6 to 14 h after combined-
301 nitrogen step down, mainly confined to specific, semi-regularly spaced cells identified
302 as proheterocysts (Fig. 4A). No comparable increase in fluorescence was observed in
303 strain CSAV143. In this strain, some fluorescence increase was observed at later times
304 in some filaments, but without apparent spatial specificity (Fig. 4B).

305 **Inactivation of *pipX*.** In order to study the function of *pipX* in *Anabaena*, the
306 construction of strains carrying inactivated versions of this ORF was undertaken.
307 Plasmid pCSV24 was constructed (Materials and methods) consisting of two
308 discontiguous fragments of the *pipX-alr0486* genomic region of strain PCC 7120 joined
309 by gene-cassette C.S3 encoding resistance to Sm and Sp, cloned in the conjugative
310 vector pRL278 (3). This vector carries an Nm^r determinant and the *sacB* gene
311 conferring sensitivity to sucrose (Suc) for positive selection. Clones exhibiting Sm^r/Sp^r,
312 resistance to Suc and sensitivity to Nm are expected to have incorporated the transferred
313 construct by double crossover with the recipient's chromosome, replacing the wild-type
314 *pipX* gene by a mutant version in which 177 bp from inside the reading frame were
315 substituted by gene-cassette C.S3. PCR and restriction analyses showed a number of
316 clones exhibiting such genomic structure. One of those clones, which according to
317 Southern analysis (not shown) exhibited no wild-type chromosome, was selected and
318 named strain CSV6. Consistent with Southern analysis, no transcript of *pipX* could be
319 detected by Northern analysis in strain CSV6 (Fig. 2B). It should be pointed out,

however, that a faint band that could correspond to the wild-type allele was observed when the genomic structure of strain CSV6 was analyzed by prolonged (35 cycles) PCR, even after repeated rounds of filament fragmentation by sonication and growth in the presence of ammonium, Sm and Sp. Thus, we cannot ascertain that strain CSV6 is completely devoid of wild-type *asr0485* alleles but, in any case, expression of this gene was negligible in the mutant.

The phenotype of strain CSV6 was analyzed with regard to growth with different nitrogen sources, heterocyst differentiation and expression of nitrogenase activity. In the presence of combined nitrogen, either nitrate or ammonium, growth of strain CSV6 was similar to that of strain PCC 7120 both in liquid and solid media. However, in the absence of combined nitrogen strain CSV6 showed a yellowish colour indicative of N deficiency, being its growth rate in liquid medium about half that of the wild-type strain (Fig. 5). When observed under the optical microscope, diazotrophic cultures of strain CSV6 showed many short filaments frequently with one terminal heterocyst, and detached cells, which are rare in the wild type. Some longer filaments were also evident, but in them the vegetative cell intervals between heterocysts were longer than in the wild-type filaments. Under the electron microscope, mature heterocysts of strain CSV6 appeared similar to wild-type heterocysts (not shown). Consistently, heterocyst-specific glycolipids could be detected by thin layer chromatography in strain CSV6 after 24 h of incubation in the absence of combined nitrogen (not shown).

Nitrogenase activity was measured under oxic and microoxic conditions in strain CSV6 in comparison to the wild type. Both under microoxic and oxic conditions, activity levels were lower in strain CSV6 than in the wild type (e.g. 15 and 27 nmol ethylene · μg Chl^{-1} · h^{-1}, respectively, in a representative experiment under microoxic

345 conditions), but the difference was considerably larger under oxic conditions (2 and 19
346 nmol ethylene · μg Chl^{-1} · h^{-1} for strains CSV6 and PCC 7120, respectively). The low
347 nitrogenase activity of strain CSV6 under oxic conditions can explain its impaired
348 diazotrophic growth.

349 Strain CSV6 was complemented with a wild-type copy of the *pipX* gene cloned
350 in the Km^r-encoding mobilizable plasmid pRL424 (6). Km^r exconjugant CSV6-53 had
351 integrated into the chromosome the whole transferred plasmid by a single
352 recombination event, keeping the inactivated version of *pipX* present in strain CSV6
353 (not shown). Although still somewhat lower than in the wild type, strain CSV6-53
354 exhibited an ability for diazotrophic growth considerably higher than that of its parental
355 strain CSV6 (Fig. 5C). Thus, the defect in strain CSV6 can be ascribed to the mutation
356 introduced in the *pipX* gene.

357 On the other hand, to check for possible polar effects of the mutation introduced
358 in strain CSV6, expression of the downstream ORF *alr0486* was studied in this strain in
359 comparison to the wild type. Fig. 2B shows that in strain CSV6, the RNA bands of ca.
360 1.4 and 1.2 kb, previously interpreted as originated upstream of *pipX* in the wild type,
361 were not produced. Instead, the band of ca. 0.9 kb was stronger in strain CSV6. Thus, in
362 the *pipX* mutant, ORF *alr0486* is expressed albeit with a pattern different from that
363 taking place in the wild type.

364 To evaluate the possibility that the phenotype of strain CSV6 results from the
365 observed alteration in the expression pattern of ORF *alr0486*, a knock out mutant of this
366 gene was generated. Plasmid pCSV27 was constructed (see Materials and methods)
367 consisting of an internal fragment of *alr0486* of strain PCC 7120 cloned in the pCSV3
368 plasmid, which includes gene-cassette C.S3 encoding resistance to Sm and Sp. Plasmid
369 pCSV27 was transferred to strain PCC 7120 by conjugation with selection for resistance

370 to Sm and Sp in the presence of ammonium. Sm^r/Sp^r clones were expected to have
371 incorporated the transferred construct into *alr0486* by single recombination with the
372 recipient's chromosome splitting the *alr0846* sequence in two halves at both sides of the
373 integration point. Southern analysis (not shown) showed that all of a number of selected
374 clones exhibited such genomic structure keeping no wild-type chromosome. One of
375 them was named strain CSV7. Growth rate of strain CSV7 was similar to that of the
376 wild type with nitrate or ammonium, and considerably higher than that of strain CSV6
377 under diazotrophic conditions (Fig. 5B). Nitrogenase activity of strain CSV7 was
378 similar to that of strain PCC 7120 under microoxic conditions (29 and 27 nmol ethylene
379 $\cdot$ μg $Chl^{-1} \cdot h^{-1}$, respectively, in a representative experiment), and considerably higher
380 than that of strain CSV6 (2, 14 and 19 nmol ethylene $\cdot$ μg $Chl^{-1} \cdot h^{-1}$ for strains CSV6,
381 CSV7 and PCC 7120, respectively) under oxic conditions. Thus, the phenotype of strain
382 CSV6 could not be explained by a polar effect resulting in impaired expression of
383 *alr0486.*

384 **Role of PipX in the expression of genes involved in heterocyst**
385 **differentiation.** Because *pipX* was preferentially expressed in the heterocysts, and the
386 strain CSV6 showed impaired diazothophic growth, we checked the expression of a
387 number of genes involved in heterocyst maturation and function. As shown in Fig. 6A,
388 no significant differences in *ntcA* expression were observed between the wild type and
389 the CSV6 strain. The *devBCA* operon, which encodes an ABC-type exporter involved in
390 deposition of the heterocyst envelope (10, 11) also showed similar expression profiles in
391 the PCC 7120 strain and in the CSV6 mutant (Fig. 6A). In contrast, the expression of
392 the nitrogenase structural genes *nifHDK* was retarded in the mutant and reached lower
393 levels than in the wild type (20% those of the wild type after 24 h of combined nitrogen
394 deprivation in the representative expreriment shown in Fig. 6A). This is consistent with

the lower nitrogenase activity of the mutant. On the other hand, expression of *nifHDK* in strain CSV7 was similar to that in the wild type (not shown) confirming that the low activity observed in strain CSV6 is due to the inactivation of the *pipX* gene and not to a polar effect on the downstream gene. Finally, the expression of the *coxB2A2C2* and *coxB3A3C3* operons, which encode heterocyst-specific terminal respiratory oxidases that help to maintain a microoxic ambient in the cytoplasm by active oxygen consumption (36), was impaired albeit not to the same extent. Activation of *coxB2A2C2* was significantly retarded in the mutant as compared to the wild-type strain although at 24h of combined nitrogen deprivation they both reached similar levels (Fig. 6A). Activation of *coxB3A3C3* was severely retarded showing 50 and 80% of wild-type levels at 12 and 24 h, respectively, of combined nitrogen deficiency (Fig. 6B).

DISCUSSION

In the genome of *Anabaena* sp. strain PCC 7120, *asr0485* encodes a homolog of the PipX factor of the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942. PipX homologs are widespread in cyanobacteria but do not exist outside this phylum. Physiological differences between *Anabaena* and *Synechococcus* are relevant, especially those concerning nitrogen metabolism and adaptation to different nitrogen regimes. For instance, while *Synechococcus* may be confronted to prolonged periods of nitrogen stress due to deficiency in combined nitrogen sources, for *Anabaena,* nitrogen starvation is transient instead, lasting for the time that heterocysts take to differentiate. Therefore, important differences are also expected in the operation of the elements involved in signal transduction of nitrogen stimuli. Our results indicate that in *Anabaena* sp. strain PCC 7120 (i) *pipX* expression is induced by combined nitrogen deficiency and occurs preferentially in differentiating heterocysts, (ii) transcription of

420 *pipX* takes place from a complex promoter region containing promoters controlled by
421 NtcA and HetR, and (iii) inactivation of the *pipX* gene provokes slow diazotrophic
422 growth and low nitrogenase activity due to impaired expression of at least the
423 nitrogenase structural genes and of the gene clusters *coxB2C2A2* and *coxB3C3A3*
424 encoding terminal respiratory oxidases involved in nitrogenase protection against
425 oxygen in the heterocyst.

426 Information on the transcription profile of *pipX* was not available for unicellular
427 or heterocyst-forming cyanobacteria. Here we have shown that in *Anabaena* sp. strain
428 PCC 7120, the expression of *pipX* is activated upon combined-N deprivation influenced
429 by the global N regulator NtcA and the heterocyst-differentiation regulator HetR (Fig.
430 2). Using the GFP as a reporter of *pipX* expression, activation is observed to take place
431 mainly in specific, semi-regularly spaced cells in the wild-type strain but not in the *hetR*
432 mutant (Fig. 4). Taken together, these results indicate that expression of *pipX* takes
433 place mainly in an NtcA- and HetR-dependent manner at intermediate to late stages of
434 the differentiation process (under our laboratory conditions, heterocyst differentiation is
435 completed within 24 h of incubation in the absence of combined nitrogen). Similar to a
436 number of other *Anabaena* genes expressed during heterocyst differentiation (12), *pipX*
437 appears to be expressed from a complex promoter region encompassing several in
438 tandem promoters. In addition to a tsp (-107) preceded by recognizable -10 and -35
439 determinants, thus representing a consensus-type promoter, two N-regulated tsps could
440 be identified upstream from the *pipX*-coding sequence (Fig. 3D). The use of the -436 tsp
441 requires NtcA and the DNA sequence upstream of it conforms to a canonical Class II
442 NtcA-activated promoter (16, 21). Indeed, purified NtcA has been shown to bind and
443 protect from DNase I digestion sequences upstream from this tsp. As other Class II
444 NtcA-activated promoters of *Anabaena* sp. strain PCC 7120 (16), this *pipX* promoter

445 does not show a requirement for HetR. In addition, an NtcA- and HetR-dependent tsp
446 has been identified at position -388. This tsp is expected to contribute to the increase of
447 *pipX* expression observed after ca. 9 h of N deprivation (Fig. 2), which would be mainly
448 localized to proheterocysts (Fig. 4). As is the case in other *Anabaena* promoters
449 directing localized gene expression in proheterocysts, no promoter determinants could
450 be recognized here other than an imperfect -10 box (see Fig. 3D). On the other hand, no
451 consensus -10 box or NtcA-binding sequence could be recognized upstream from
452 position -193. Thus, the detected regulated RNA species with 5' end at this position
453 (Fig. 3C) could result from processing of transcripts initiated at the regulated upstream
454 promoters.

455 A schematic of the transcription profile of the *pipX-alr0486* cluster of *Anabaena*
456 sp. strain PCC 7120, which is consistent with the RT-PCR, Northern and primer
457 extension analyses included in this work, is presented in Fig. 2C. The *pipX* gene, which
458 is very weakly expressed in the presence of ammonium, is activated upon N deprivation
459 according to a time-specific cascade of promoter utilization that generates transcripts of
460 different lengths. Transcripts differ in their 5' end, depending on the promoter from
461 which they originate or on processing, but may also differ in their 3' end, depending on
462 premature termination.

463 Strain CSV6 bearing an inactivated *pipX* gene is impaired specifically in
464 diazotrophic growth. The phenotype of this strain results from disruption of the *pipX*
465 gene, and not from a polar effect on the downstream gene since the phenotype of CSV6
466 is not reproduced by an insertion in *alr0486* and besides, the wild-type phenotype can
467 be rescued by complementation of CSV6 with the *pipX* gene. Filaments of strain CSV6
468 grown diazotrophically show lower levels of nitrogenase activity under anoxic
469 conditions, what is consistent with the diminished expression of the *nifHDK* genes with

respect to the wild type. The deficiency in nitrogenase activity levels in the CSV6 mutant is more severe under oxic than under anoxic conditions additionally indicating an inefficient oxygen protection of the nitrogenase. However, we have not found evidence for the structural barriers of the heterocysts against oxygen to be altered, including the cell envelope, which shows a normal appearance in electron micrographs (not shown), the glycolipids content of the heterocyst cell wall that seems to be normal when analyzed by thin layer chromatography (not shown) or the expression of the *devBCA* operon encoding a glycolipid exporter, which does not show significant differences in CSV6 with respect to PCC 7120. In contrast, the expression of the *coxA2B2C2* and the *coxA3B3C3* operons encoding heterocyst-specific terminal respiratory oxidase complexes (36), which are involved in the removal of internal oxygen by reduction, is impaired (albeit to a different extent). Both *cox* operons are specifically expressed in the heterocyst and although none of them is essential, a double mutant is unable of diazotrophic growth and does not exhibit nitrogenase activity under oxic conditions (36). Concomitant impairment of both operons in the CSV6 mutant would likely compromise oxygen protection of the nitrogenase leading to a partial inactivation of the enzyme in oxic conditions. It is worth noting that *cox3*, whose expression is more severely affected in CSV6, has probably a more important role than *cox2* in oxygen protection of the nitrogenase in the heterocysts (37).

In *Synechococcus* sp. strain PCC 7942 PipX has been proposed to be an accessory protein for NtcA-mediated transcription activation (8, 20). Indeed, several genes or activities subjected to NtcA regulation show a reduced activation in a *pipX* mutant (8, 9). However in the presence of 2-OG, NtcA is able to bind DNA and activate transcription in vitro in the absence of PipX both at *Synechococcus* (34) and *Anabaena* promoters (35). We show here that in vivo expression of two *ntcA*-dependent genes,

495 *devB* and *ntcA*, is not affected in the CSV6 mutant, whereas three operons, *nifHDK,*
496 *cox2,* and *cox3* are impaired. This observation suggests that, at least in *Anabaena* sp.
497 PCC 7120, some *ntcA*-dependent genes would require PipX for full activation whereas
498 others would be independent of this factor.

499 As we have shown here, the *Anabaena pipX* gene is mainly expressed in the
500 (pro)heterocysts. Because in these cells the expression of the *glnB* gene (encoding the
501 P_{II} regulator) is turned down (30), an effect of PipX sequestration by P_{II} would be
502 minimized. This would leave a mostly active PipX protein in the differentiating cells,
503 consistently with the phenotype of the CSV6 mutant defective in heterocyst function.
504 Thus, in filamentous heterocyst-forming cyanobacteria the function of the PipX factor
505 appears adapted to the distinct physiology of these organisms, reinforcing the
506 expression of late heterocyst-specific genes to allow full levels of nitrogen fixation
507 under oxic environments.
508

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514

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646
647

FIGURE LEGENDS

Fig. 1. RT-PCR analysis of the expression of the *alr0484-alr0489* gene cluster. (A) The gene cluster is depicted with the indication of the *Anabaena* ORF names (19). (B) Retrotranscription was carried out with oligonucleotide primers RT-1 or RT-2 (initial positions indicated by the start of the corresponding arrow) and RNA isolated from bubbled ammonium-grown cultures incubated during 6 h in the absence of combined nitrogen. The positions of the primers used for amplification corresponded to the ends of the segments indicated in (A), which are depicted in black, indicating amplification by RT-PCR, or in gray, indicating no amplification. + or –, RNA samples subjected or not to retrotranscription; g, total genomic DNA used as template for amplification with the corresponding primers.

Fig. 2. Northern analysis of the expression of *asr0485* (*pipX*)-*alr0486*. (A and B) RNA isolated from bubbled cultures of *Anabaena* sp. strain PCC 7120, strain CSE2 (*ntcA*), strain 216 (*hetR*), and strain CSV6 (*pipX*) grown with ammonium (0) and incubated in the absence of combined nitrogen for the times indicated in hours was electrophoresed and hybridized with the probes indicated at the right, which were generated by PCR (see Materials and methods). Deduced sizes of some apparent transcripts are indicated at the left. Hybridization with an *rnpB* gene probe was used as a loading and transfer control. (C) Schematic transcription profile of the *asr0485-alr0486* gene cluster. Transcription start sites are indicated by arrows, whereas the -193 position is proposed to correspond to a processed 5' RNA end. Transcripts are indicated by grey horizontal lines, in which a dashed end indicates an ambiguity in the determination of its precise location.

672

673 Fig. 3. Primer extension analysis and NtcA DNaseI footprinting on the promoter
674 region of the *pipX* gene. (A) Primer extension analysis was carried out with primer
675 PX18 and RNA isolated from bubbled cultures of *Anabaena* sp. strain PCC 7120, strain
676 CSE2 (*ntcA*) and strain 216 (*hetR*) grown with ammonium (0) and incubated in the
677 absence of combined nitrogen for the times indicated in hours. The positions of the -436
678 and -388 RNA 5' ends are indicated. (B) DNase I protection assays were carried out
679 with purified NtcA (90 or 180 nM) and a DNA fragment of the *pipX* promoter region
680 amplified by PCR using oligonucleotides PX18 (unlabeled) and PX19 (^{32}P labelled), in
681 the presence or absence of 0.6 mM 2-OG. The sequence of the NtcA-protected region is
682 indicated including the GTA/GAC triplets (underlined). (C) Primer extension analysis
683 carried out with primer asr0485-7120-1 and RNA isolated from bubbled cultures of
684 *Anabaena* sp. strain PCC 7120 grown with ammonium (0) and incubated in the absence
685 of combined nitrogen for the times indicated in hours. The positions of the -193 and
686 -107 5' RNA ends are indicated. A sequence ladder of the same DNA region is shown
687 at the left. (D) DNA sequences upstream from three identified *pipX* tsps (see the text).
688 The NtcA-protected region upstream of tsp -436, as well as putative -10 and -35 boxes
689 are indicated.

690

691 Fig. 4. Spatiotemporal expression of *pipX*. Transmited light and GFP
692 fluorescence images of filaments of strains CSAV142 (*pipX-gfp* in strain PCC 7120)
693 (A) and CSAV143 (*pipX-gfp* in strain 216) (B) grown with ammonium and incubated in
694 the absence of combined nitrogen for the times indicated in hours. The arrows indicate
695 (pro)heterocysts.

696

Fig. 5. Growth of strains CSV6, CSV7 and CSV6-53 with different N sources. (A), (C) Cell suspensions of *Anabaena* sp. strain PCC 7120, strain CSV6 and strain CSV6-53, as indicated, grown with ammonium (with antibiotics in the case of the mutants) and washed with combined-nitrogen-free medium were used to inoculate (A, 12 ng Chl per spot; C, 100 ng Chl per spot) plates of BG11 (NO_3^-) or of $BGll_0$ medium supplemented (NH_4^+) or not (N_2) with ammonium that were incubated for 14 days (A) or 10 days (C) under culture conditions (see Materials and methods for details). (B) Cell suspensions of *Anabaena* sp. strain PCC 7120 and strains CSV6 and CSV7 grown with ammonium (and Sm and Sp in the case of the mutants) and washed with combined-nitrogen-free medium were used to inoculate liquid cultures in Erlenmeyer flasks containing 25 ml media with the indicated N source, which were incubated under culture conditions. Aliquots were withdrawn from the cultures at different times to determine their protein content. The figures are specific growth rate constants (in day^{-1}), average of two (CSV6 and CSV7) or four (PCC 7120) independent experiments with similar results.

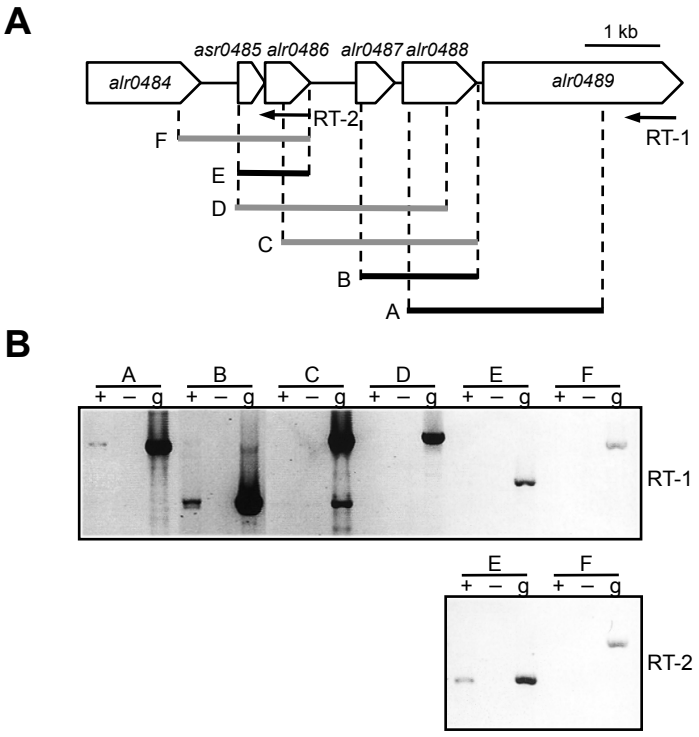
Fig. 6. Expression of several genes involved in heterocyst differentiation in strain CSV6 as compared to strain PCC 7120. Northern assays were carried out with RNA isolated from *Anabaena* sp. strain PCC 7120 or strain CSV6 (*asr0485*::C.S3) grown with ammonium (0) and incubated in the absence of combined nitrogen for the times indicated in hours and probes for the indicated genes generated by PCR (see Materials and methods). Size markers (kb) are indicated at the right. Hybridization with an *rnpB* gene probe was used as a loading and transfer control. A and B report hybridization with the same RNA preparations on different filters.

TABLE 1. Oligodeoxynucleotides used in this study

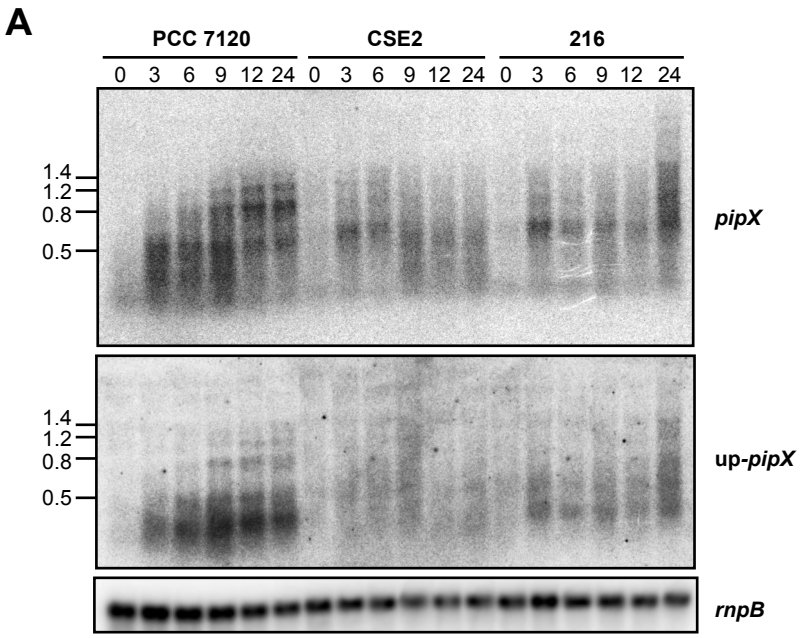
Oligonucleotide	Sequence (5'-3')	Positions relative to the translation start of the corresponding gene
PX1	GCCTCCTCGAGTATATTCTG	-654 to -637 (*pipX*)
PX2	CATGCGGATCCGATATAG	+70 to +52 (*pipX*)
PX3	CAGGAGTAGGATCCGCTTC	+232 to +251 (*pipX*)
PX4	CAACTGACCCTCGAGAATATG	+576 to +556 (*alr0486*)
PX7	GACTGAAGTTATTCGTGCTGC	+90 to +110 (*alr0486*)
PX8	CTACCTACAAGGATGAGATCAAGG	-165 to -142 (*pipX*)
PX9	GAAGGTGCGTTGGAAAACAC	+276 to +252 (*pipX*)
PX10	CTACTCGATGGATCCATGAATCCAG	-10 to +8 (*pipX*)
PX11	GCAATTAGGTCGACTGAAGGCG	+71 to +50 (*alr0486*)
PX14	GAAAGCCAGGATTTGTTCACAAC	+76 to+98 (*pipX*)
PX17	CTATCAATCGAGTAGGAGAATC	-1 to -22 (*pipX*)
PX18	CAACGCTGAAAATCCCTGATGCG	-282 to -304 (*pipX*)
PX19	CTTACAAGCTAGAAGAAGCAG	-588 to -568 (*pipX*)
PX21	GTTTATTTGCGGTGTCCAG	-304 to -322 (*pipX*)
PX22	GCAAATTTTACCCTTAAATG	-109 to -128 (*pipX*)
PXgfp1	GCAATCGATTTTAATAGCTATATGAACTTAC	-614 to -584 (*pipX*)
PXgfp2	ATCGATCGAAGGATAATTATGGCAACATGG	-415 to -392 (*pipX*)
PXgfp3	GATATCTTGGAAGGTGCGTTGGAAAACACTC	+276 to +252 (*pipX*)
asr0485-7120-1	GCAGATCCGATATAGCAAACC	+66 to +46 (*pipX*)
alr0486-7120-1	ATGATTAGTTCGATCAACGAAC	+1 to +22 (*alr0486*)
alr0486-7120-4	GGATCCGAGTAATATTGCTTAAGTACG	+687 to +661 (*alr0486*)
RT-1	CTGGTTTGAGCATCAATTCTG	+1849 to +1829 (*alr0489*)
RT-2	CTCTAATTATCCGGTTAATGCTTAATG	+1015 to +989 (*alr0486*)
alr0487-7120-1	CAATTTGCAGAAGGAATTCAACTAAATG	-25 to +3 (*alr0487*)
alr0488-7120-1	GCAGATGCAGTATTCACAGACCATTC	+253 to +278 (*alr0488*)
alr0488-7120-2	GAATGGTCTGTGAATACTGTATCTGC	+278 to +253 (*alr0488*)
alr0488-7120-3	CGATTAAGGCTGAACGGAATC	+766 to +746 (*alr0488*)
alr0489-7120-1	GTGGGTTTGCAGTAAGTTCAAAC	+1341 to +1318 (*alr0489*)
DB034	ATGTCAAGGGTGACGGAAG	+1 to +19 (*devB*)
DB018	ATTTATTAATGTCAACCACTACC	+1423 to +1400 (*devB*)
CB2-4	CACTCTGGACTCTAATTGCTG	+20 to +40 (*coxB2*)
CB2-5	GACGTTGTATGCAATGTCTC	+1127 to +1108 (*coxB2*)
NH1	GTACTGCAAGGGGCGTGTGGC	-334 to -314 (*nifH*)

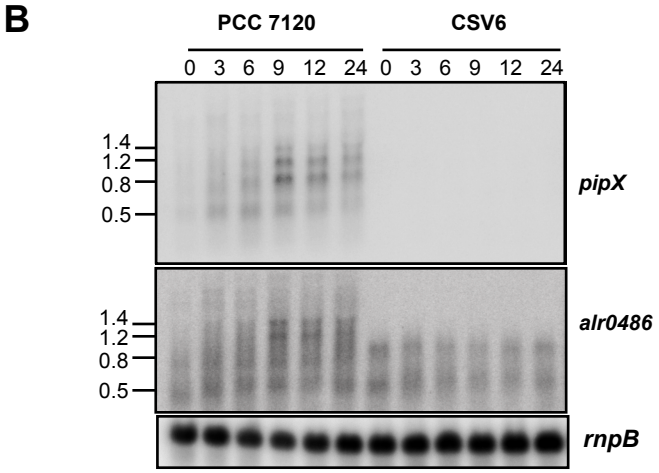
NH4	CCTATTGGTAGCTTCTGCGGGC	+885 to +864 (*nifH*)
NA10	TAGGATCCTGTTATTCCGGCATTGGGTAGG	-39 to -10 (*ntcA*)
NA11	CAGGATATCAGTATGGGTTCACCGTCAC	+809 to +782 (*ntcA*)
CS3-1	GGATGACCTTTGAATGACC	-518 to -537 (*Sm/Sp*)
GFP4	CAAGAATTGGGACAACTCC	+46 to +28 (*gfpmut2*)
CB3-6	CGATCGCTGTGACTATTACCAG	+47 to +68 (*coxB3*)
CB3-7	AACCAGGGTAATTAACCAAAGG	+913 to +892 (*coxB3*)

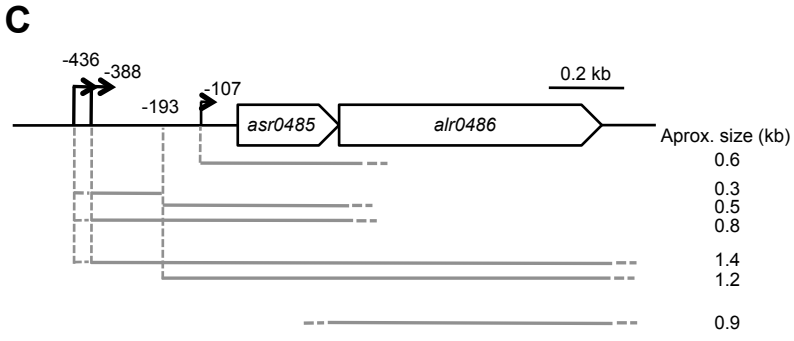
Valladares et al., Fig. 1



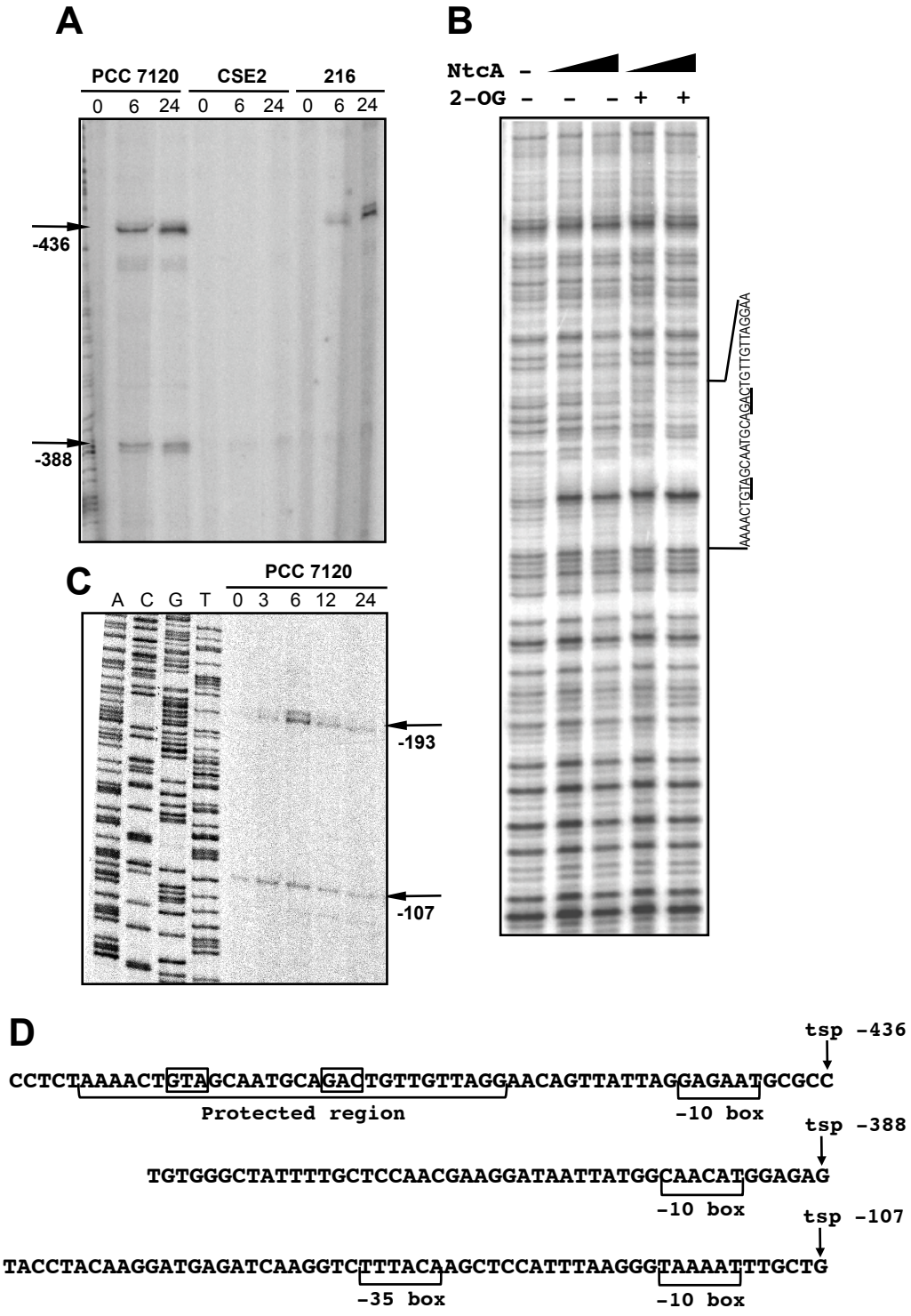
Valladares et al., Fig. 2



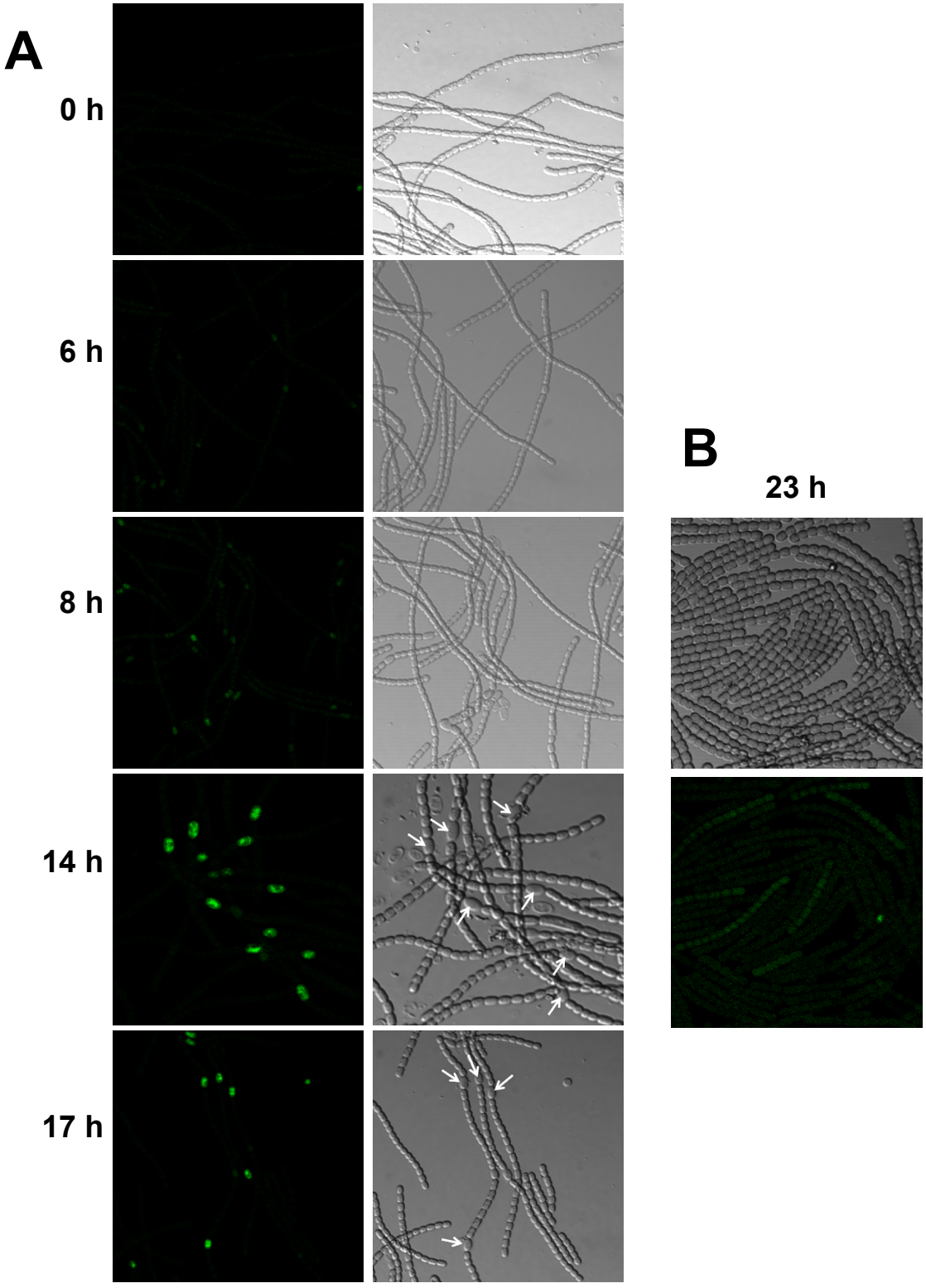




Valladares et al., Fig. 3

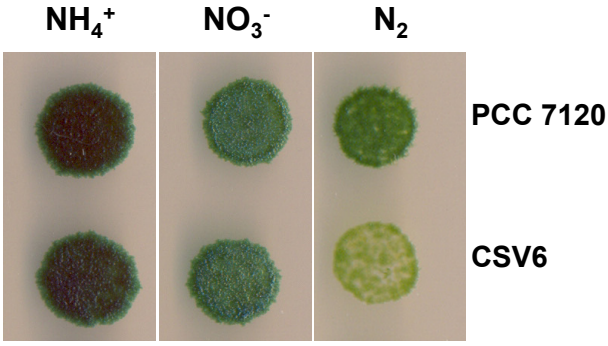


Valladares et al., Fig. 4



Valladares et al., Fig. 5

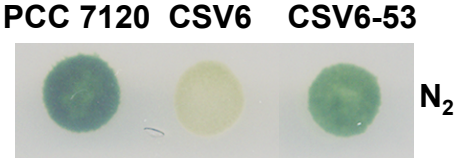
A



B

Strain	NH_4^+	NO_3^-	N_2
PCC 7120	0.51	0.62	0.37
CSV6	0.47	0.58	0.20
CSV7	0.55	0.60	0.32

C



Valladares et al., Fig. 6