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Identification and Transcriptional Control of the Genes Encoding the *Caulobacter crescentus* ClpXP Protease

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The region of the *Caulobacter crescentus* chromosome harboring the genes for the ClpXP protease was isolated and characterized. Comparison of the deduced amino acid sequences of the *C. crescentus* ClpP and ClpX proteins with those of their homologues from several gram-positive and gram-negative bacteria revealed stronger conservation for the ATPase regulatory subunit (ClpX) than for the peptidase subunit (ClpP). The *C. crescentus clpX* gene was shown by complementation analysis to be functional in *Escherichia coli*. However, *clpX* from *E. coli* was not able to substitute for the essential nature of the *clpX* gene in *C. crescentus*. The *clpP* and *clpX* genes are separated on the *C. crescentus* chromosome by an open reading frame pointing in the opposite direction from the *clp* genes, and transcription of *clpP* and *clpX* was found to be uncoupled. *clpP* is transcribed as a monocistronic unit with a promoter (P_{P1}) located immediately upstream of the 5′ end of the gene and a terminator structure following its 3′ end. P_{P1} is under heat shock control and is induced upon entry of the cells into the stationary phase. At least three promoters for *clpX* (P_{X1}, P_{X2}, and P_{X3}) were mapped in the *clpP-clpX* intergenic region. In contrast to P_{P1}, the *clpX* promoters were found to be downregulated after heat shock but were also subject to growth phase control. In addition, the *clpP* and *clpX* promoters showed different activity patterns during the cell cycle. Together, these results demonstrate that the genes coding for the peptidase and the regulatory subunits of the ClpXP protease are under independent transcriptional control in *C. crescentus*. Determination of the numbers of ClpP and ClpX molecules per cell suggested that ClpX is the limiting component compared with ClpP.

In *Caulobacter crescentus*, cell differentiation is an integral part of each cell cycle (18). Asymmetric cell division gives rise to two distinct progeny, only one of which, the stalked cell, is competent for a new round of DNA replication and cell division. The other daughter cell, the swarmer cell, has to go through an obligatory differentiation step into a stalked cell to regain its replicative ability. Recent studies have revealed that in this organism, specific proteolytic events are key control mechanisms for cell differentiation as well as for proper cell cycle progression (1, 6, 17, 20, 51). For example, CcrM, an essential DNA methyltransferase, is synthesized only in *C. crescentus* late predivisional cells, where it is required to fully methylate the newly replicated chromosomes (43, 44, 55). To avoid early methylation of the new chromosomes at the beginning of the S phase, DNA methylation activity is restricted to late predivisional cells. This restriction is accomplished by rapid degradation of CcrM, resulting in its presence in cells only when it is actively synthesized. The instability of CcrM and, thus, part of its temporal control are dependent on the *C. crescentus* Lon protease (51). Several other *C. crescentus* proteins have been shown to be degraded in a cell cycle-dependent way; these include the flagellar anchor protein FliF (17); the McpA chemoreceptor (1); CtrA, a cell cycle transcriptional regulator (6); and FtsZ, a tubulin-like GTPase required for cytokinesis (20). To understand how the proteolytic turnover of these key regulatory, metabolic, and structural proteins is controlled by the cell cycle, it is necessary to identify the corresponding protease(s) and to understand the regulation of its activity. However, none of the proteases responsible for the turnover of these proteins has been isolated so far.

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Short stretches of amino acids at the carboxyl termini of the CtrA, FliF, and McpA proteins have been identified as turnover signals and shown to be strictly required for the cell cycle-dependent degradation of these proteins (1, 6, 17). Similarly, in *Escherichia coli*, several proteins are tagged for degradation by the ATP-dependent ClpXP proteases by a short C-terminal domain (12, 24, 28). This possible parallel in the mechanism of substrate recognition led us to test the hypothesis that in *C. crescentus*, ClpXP might be responsible for the degradation of some of the proteins mentioned above. Here we report the isolation and characterization of the *C. crescentus* chromosomal locus that contains the genes for the peptidase and the ATPase regulatory subunits of the ClpXP protease. In a parallel study, we showed that both the *clpP* and the *clpX* genes are essential for growth, viability, and cell cycle progression (16). In addition, ClpXP was shown to be required for cell cycle-dependent degradation of the CtrA response regulator protein. To better understand the physiological role of the ClpXP protease in *Caulobacter*, the promoters and transcripts of the *clpP* and *clpX* genes were mapped and their transcriptional control was investigated. The analysis of heat shock induction as well as the cell cycle- and growth phase-dependent activities of the mapped promoters demonstrated that *clpP* and *clpX* are controlled differently at the transcriptional level. The significance of this finding is discussed with respect to the stoichiometry of ClpP and ClpX in cells and the role of the ClpXP protease in *Caulobacter* cell cycle progression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH10B and S17-1 were used as the host strain for molecular cloning experiments and as the donor strain for conjugation experiments, respectively. *E. coli* strains were cultured at 37°C in Luria-Bertani (LB) broth (39) supplemented with ampicillin (100 μg/ml), kanamycin (30 μg/ml), or tetracycline (10 μg/ml) as necessary. *C. crescentus* strains were

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Strains		
E. coli		
DH10B	F$^-$ *mcrA* Δ(*mrr hsdRMS mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR* Δ(*ara-leu*)*7697 araD139 galU galK nupG rpsL*	Gibco BRL
S17-1	M294::RP4-2 (Tet::Mu) (Kan::Tn*7*)	42
W3110	Wild-type K-12 strain; *sup*^{0}	34
SG22080	MC4100 Δ*clpX1*::Kan	11
SSN1	W3110 × P1 (SG22080); *sup*^{0} Δ*clpX1*::Kan	This study
C. crescentus NA1000	*syn-1000*; synchronizable derivative of *C. crescentus* wild-type strain	8
Plasmids		
pAR33	pBBR1 derivative containing the *C. crescentus rpoH* gene	36
pAS1	*Eco*RI-*Sal*I fragment containing the 3′ end of *tig* in pRKlac290	This study
pAS2	*Sal*I-*Xho*I fragment containing the *clpP* 5′ region in pRKlac290	This study
pAS3	*Sal*I fragment containing the *clpP* 5′ region in pRKlac290	This study
pAS4	*Sal*I-*Bam*HI fragment containing *clpP* and *cicA* in pRKlac290	This study
pAS5	*Xho*I-*Bam*HI fragment containing the *clpP* 3′ region and *cicA* in pRKlac290	This study
pAS6	*Bam*HI-*Fsp*I fragment containing the *clpX* 5′ region in pRKlac290	This study
pAS7	*Mlu*I-*Fsp*I fragment containing the *clpX* 5′ region in pRKlac290	This study
pAS8	*Eco*RI-*Bam*HI fragment containing the 3′ end of *tig*, *clpP*, and *cicA* in pRKlac290	This study
pAS9	*Eco*RI-*Eco*RV fragment containing the 3′ end of *tig* and *clpP* in pRKlac290	This study
pAS23	*Apa*I-*Fsp*I fragment containing the *clpX* 5′ region in pRKlac290	This study
pAS24	*Sal*I-*Fsp*I fragment containing *cicA* and the *clpX* 5′ region in pRKlac290	This study
pAS25	*Eco*RV-*Fsp*I fragment containing *cicA* in pRKlac290	This study
pAS26	*Apa*I-*Bam*HI fragment containing the *cicA-clpX* intergenic region in pRKlac290	This study
pAS27	*Sal*I-*Bam*HI fragment containing *cicA* in pRKlac290	This study
pAS29	*Sal*I-*Mlu*I fragment containing *clpP* in pRKlac290	This study
pAS63	*Apa*I-*Fsp*I fragment containing the *cicA* 5′ region in pRKlac290	This study
pAS64	*Apa*I fragment containing the internal *cicA* region in pRKlac290	This study
pBluescript SK(+)	Amp^r cloning vector	Stratagene
pCS225	*Nco*I fragment containing P_{xylX} in pRKlac290	32
pGB2ts	pSC101-based vector; thermosensitive for replication; Sp^r Sm^r	26
pGB2ts::*phd-doc*	Derivative of pGB2ts expressing *phd* and *doc*; Sp^r Sm^r	26
pMR10	RK2-based Kan^r broad-host-range vector	Rick Roberts and Chris Moor
pMR20	RK2-based Tet^r broad-host-range vector	Rick Roberts and Chris Moor
pSSN6	pMR20 containing $clpX_{Cc}$	This study
pSSN3	pMR20 containing $clpX_{Ec}$	This study
pRKlac290	RK2-based *lacZ* transcriptional fusion vector	10

[a] P_{xylX}, *xylX* promoter.

grown on either PYE complex medium (35) or M2 minimal glucose medium (7) supplemented with nalidixic acid (20 μg/ml), kanamycin (20 μg/ml), or tetracycline (2 μg/ml) as necessary. Synchronizable *C. crescentus* NA1000 was used as the wild-type strain in all experiments. Cell cycle synchronization was carried out with this strain by Ludox density gradient centrifugation as described previously (8).

DNA manipulations and sequence analysis. DNA preparation and manipulation techniques used in this study were as previously described (2, 39). Transformations of *E. coli* were done by electroporation, and plasmids were transferred from *E. coli* S17-1 to *C. crescentus* by conjugation as described previously (7). *E. coli* clones for sequencing were obtained by subcloning specific DNA fragments into vector pBluescript SK(+) (Stratagene, La Jolla, Calif.). The DNA sequence of the *C. crescentus* chromosomal *clp* region was determined for both strands by the dideoxy chain termination method (40).

ClpX activity assay with *E. coli*. The *clpX* genes from *E. coli* and *C. crescentus* were amplified by PCR with specific primers (5′-CGA CTC TAG AGC ATA TGA CAG ATA AAC GCA AAG ATG GCT C-3′, 5′-CGC GGA TCC CCT TTT TGG TTA ACT TAT TGT ATG GG-3′, 5′-AAA CAT ATG ACG AAA GCC GCG AGC-3′, and 5′-AAA GGA TCC GCT TCG AAA GCA CGC GCT-3′) and cloned as *Nde*I-*Bam*HI fragments into pET21b to introduce a ribosome-binding site upstream of *clpX*. The resulting *Xba*I-*Bam*HI fragments were cloned into the low-copy-number vector pMR20, resulting in plasmids pSSN3 (*E. coli*) and pSSN6 (*C. crescentus*) with the *clpX* gene under the control of the *lacZ* promoter.

ClpX activity was determined by the cell killing assay based on the bacteriophage P1 plasmid addiction module Phd-Doc (25). The stable cellular toxin Doc is inhibited by the antidote protein Phd, which is unstable in *E. coli* due to its rapid degradation by the ClpXP protease (26). ClpX activity was determined by monitoring the growth of *E. coli* cells containing the *phd* and *doc* genes on a plasmid with a temperature-sensitive replicon (pGB2ts::*phd-doc*) after a shift to the nonpermissive temperature. While the progressive loss of the plasmid stops the new synthesis of Phd and Doc, functional ClpX will degrade the antidote protein Phd, allowing the toxic protein Doc to stop cell growth. In the absence of ClpX, both Phd and Doc are stable and growth is not affected.

For the plasmid addiction assay, combinations of plasmid pGB2ts or pGB2ts::*phd-doc* with plasmid pSSN3 or pSSN6 were introduced into strains W3110 and SSN1. Cultures in LB broth supplemented with the appropriate antibiotics were grown at 30°C and successively diluted so as to maintain logarithmic growth conditions. The assay was started by diluting the cultures into antibiotic-free LB broth at 40°C and monitoring cell growth by measuring the absorbance at 600 nm.

Promoter mapping and transcriptional activity assays. DNA fragments containing promoters for *clpP*, *clpX*, and *cicA* were identified by cloning restriction fragments spanning the entire chromosomal *clp* region (see Fig. 4) into vector pRKlac290, generating transcriptional fusions to the *lacZ* reporter gene. The resulting plasmids were transferred into *C. crescentus* NA1000 wild-type cells, and overnight cultures of the resulting strains were diluted in fresh PYE medium and grown to an optical density at 660 nm (OD_{660}) of 0.5 to 0.6. β-Galactosidase activity was then determined as described previously (33).

For primer extension assays, *C. crescentus* NA1000 was grown in PYE medium to an OD_{660} of 0.5, and total RNA was isolated as described previously (38). Residual DNA was removed by precipitating the RNA twice with 3 volumes of sodium acetate (pH 7, 3 M) at −20°C for 6 h before centrifugation for 15 min. Specific primers complementary to the 5′ end of the *clpP* gene (clpPPE1, 5′-TCG

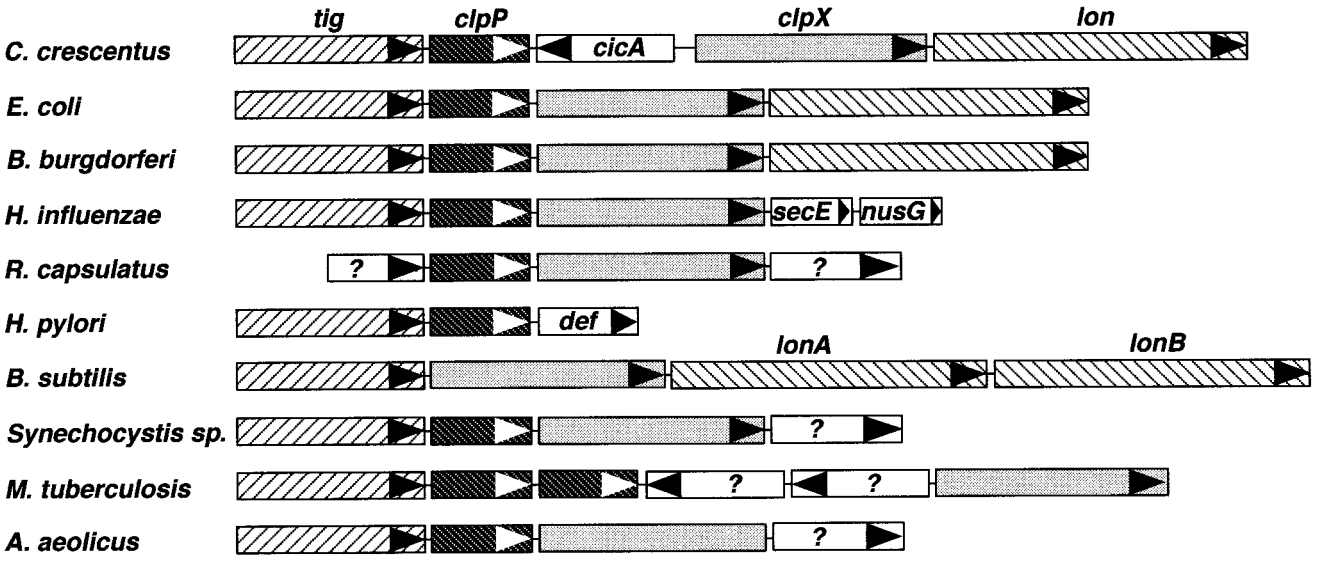


FIG. 1. Schematic diagram of the *clpP-clpX* regions of gram-positive and gram-negative bacteria. The genes coding for the trigger factor (*tig*), the ClpP peptidase subunit (*clpP*), the ClpX ATPase subunit (*clpX*), and the Lon protease (*lon*) are indicated. In most organisms analyzed so far, the gene order *tig-clpP-clpX* is conserved, with the *lon* gene located immediately downstream. In *C. crescentus*, the *clp* genes are separated by *cicA*, a gene of unknown function; in *Haemophilus influenzae*, the *lon* gene is not linked to the *clp* genes, which are followed by the *secE* (preprotein translocase subunit) and *nusG* (transcription antiterminator protein) genes (9); in *R. capsulatus*, neither *tig* nor *lon* is linked to the *clp* genes, which are flanked by genes of unknown function (?) (35a); in *H. pylori*, the *clpX* and *lon* genes, although linked, are not found at the same location as the *tig* and *clpP* genes, which are followed by the *def* (polypeptide deformylase) gene (45); and in *B. subtilis*, *clpP* is not linked to the *tig*, *clpX*, and *lon* genes. The *lon* gene has undergone duplication in *B. subtilis* (23); in *Synechocystis* and *Mycobacterium tuberculosis*, no gene coding for a homolog of the Lon protease is found. However, two *clpP* genes are present in *M. tuberculosis*, and four *clpP* genes are found in *Synechocystis*, one of them linked to the *tig* and *clpX* genes (19). Two genes are present between the *clpP* and *clpX* genes of *M. tuberculosis*, and the deduced sequences of their products are similar to those of a methyltransferase and a drug efflux protein (4). In *Aquifex aeolicus*, the *lon* gene is not linked to the *tig*, *clpP*, and *clpX* genes and is found elsewhere on the chromosome (5).

ACC ACC ATC GGC ACC AGG TTC AT-3′; clpPPE2, 5′-ATG ATC CGT TCC TTC AAC AGG CGC GA-3′) and the *clpX* gene (clpXPE1, 5′-GGC TTT CGT CAT GAT CGC TTC TCA CA-3′; clpXPE2, 5′-GCT TGC GCA CCT CAT GTT GGC TCT TT-3′) were end labeled with [γ-^{33}P]ATP (370 MBq/ml; Amersham) and T4 polynucleotide kinase (NEN BioLabs). Radiolabeled primer (5×10^5 dpm) was annealed to 40 μg of *C. crescentus* total RNA, and the extension reaction was carried out with a SuperScript II kit (Bethesda Research Laboratories). A ^{35}S-labeled DNA sequencing reaction with the desired primer and pUJ138 DNA as a template was carried out with a ThermoSequenase cycle sequencing kit (Amersham) and served as a standard to identify the transcriptional start site.

The promoter activities of *clpP* and *clpX* promoters throughout the cell cycle and following heat shock were determined by pulse-labeling of cultures harboring plasmid-encoded *lacZ* reporter gene fusions. Pulse labeling and immunoprecipitation of β-galactosidase were carried out as described previously (32).

Immunoblotting. Immunoblotting was performed as described previously (17). Polyclonal sera against ClpP or ClpX were diluted 1:10,000 and 1:5,000, respectively, before use. Secondary antibody (goat anti-rabbit immunoglobulin G [IgG] coupled to horseradish peroxidase [HRP]) was used at a 1:10,000 dilution. Immunoblots were developed with a Renaissance kit from DuPont NEN by following the manufacturer's instructions. The cellular levels of ClpP and ClpX were estimated by comparison to a dilution range of purified His-tagged Clp proteins (16). Protein concentrations were determined by the method of Lowry with a Bio-Rad DC protein assay kit and bovine serum albumin as the standard. Signals from the immunoblot analysis were quantitated by use of scanned images and ImageQuant software.

Nucleotide sequence accession number. The DNA sequence for *clp* has been deposited in the EMBL nucleotide sequence database under accession no. AJ010321.

RESULTS

Cloning of the genes for the subunits of the *Caulobacter* ClpXP protease. We isolated the *C. crescentus clpP* and *clpX* genes on the basis of their proximity to the *lon* gene, coding for the Lon protease (51). In *E. coli*, the *clpP* and *clpX* genes are located immediately upstream of the *lon* gene (Fig. 1) (11). Assuming a conserved gene order, we isolated and analyzed the chromosomal region upstream of the *C. crescentus lon* gene. The genes coding for the ClpP and ClpX homologues were identified together with the *tig* gene, coding for the homologue of the *E. coli* trigger factor (Fig. 1). The gene order *tig-clpP-clpX* is conserved in 7 of 10 bacteria analyzed so far, with the exception of *Rhodobacter capsulatus*, *Helicobacter pylori*, and *Bacillus subtilis*, in which one of the three genes is located elsewhere on the chromosome (Fig. 1). The *C. crescentus clp* locus contains an additional piece of DNA of about 1 kb between *clpP* and *clpX*; this piece is not present in any of the organisms listed in Fig. 1. This region contains an open reading frame, *cicA* (*clp* intergenic region in *Caulobacter*), transcribed in the direction opposite that of the *clp* genes. Database searches revealed a weak similarity between the deduced amino acid sequence of *cicA* and that of a family of bacterial proteins with unknown function (data not shown).

It is interesting to note that the ClpX ATPase subunit seems significantly more conserved in bacteria than the ClpP peptidase subunit. The *C. crescentus* ClpP amino acid sequence showed between 31.6 and 65.6% similarity and the ClpX amino acid sequence showed 49.9 to 78.3% similarity with homologues from other bacteria. For both proteins, the weakest similarity was observed with ClpX and ClpP of the spirochete *Borrelia burgdorferi*, while the strongest match was with ClpX and ClpP of *R. capsulatus*, like *C. crescentus* a member of the α-purple group of gram-negative bacteria. The similarity of the *Caulobacter* ClpP protein with its counterparts extends over the entire protein sequence, and the Ser, His, and Asp residues of the catalytic triad (31, 49) are conserved (Fig. 2A). Similarly, the ClpX sequences are conserved throughout the entire protein length, with the highest homology around the ATP-binding boxes (48) and the C-terminal signature sequences (41) (Fig. 2B). A Zn finger-like motif, CXXC(X_{18})CXXC, is also conserved in all ClpX sequences shown in Fig. 2B (11). The

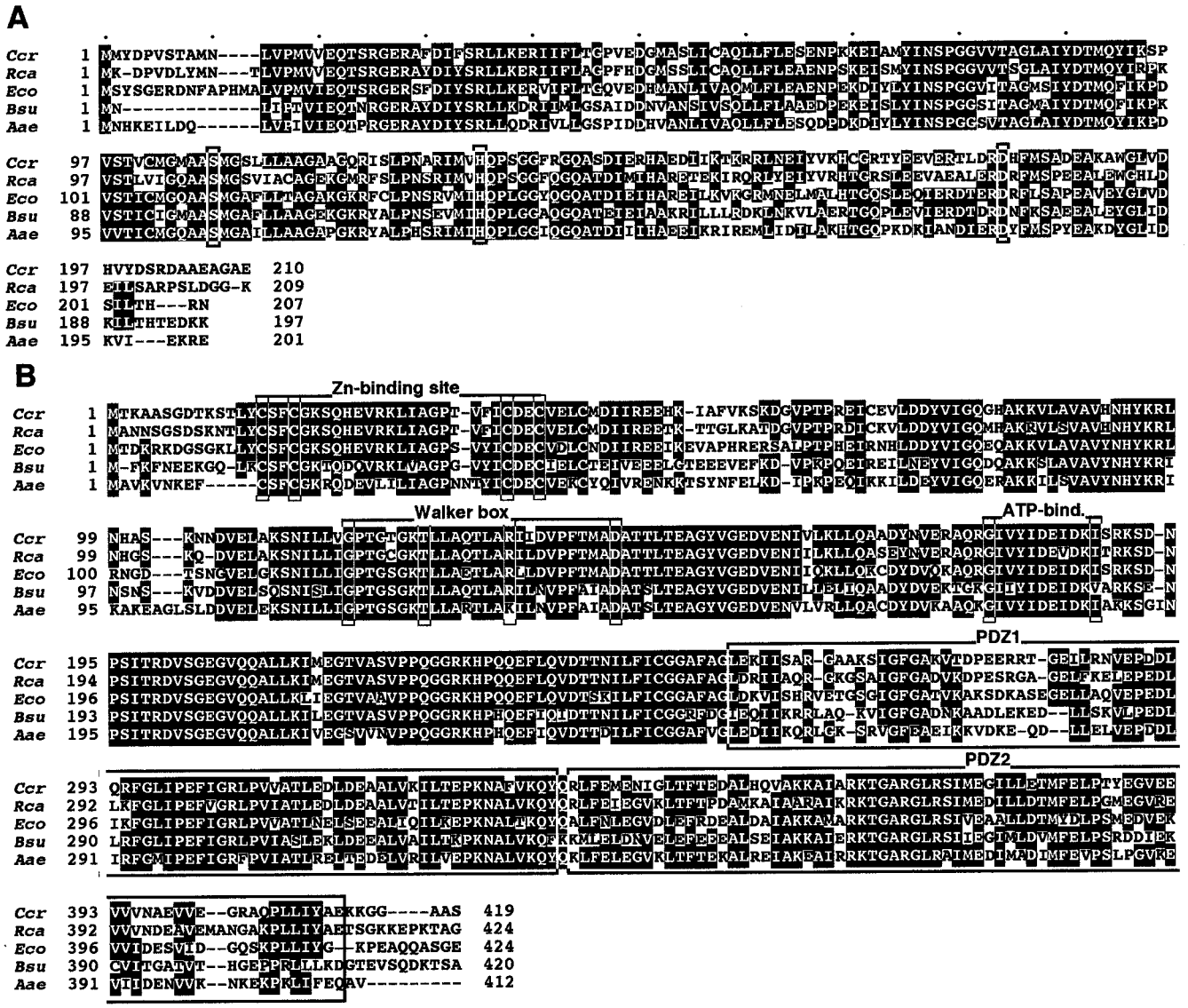


FIG. 2. Sequence alignment of ClpP (A) and ClpX (B) from *C. crescentus* (*Ccr*) with homologous sequences from *R. capsulatus* (*Rca*) (35a), *E. coli* (*Eco*) (11, 30), *B. subtilis* (*Bsu*) (23), and *A. aeolicus* (*Aae*) (5). The amino acid residues identical to those of ClpP or ClpX from *C. crescentus* are indicated by white letters on a black background. A putative Zn finger-binding site, the ATP-binding (Walker box and ATP-bind.) motifs, and the proposed PDZ-like domains of ClpX (27) are marked. The serine, histidine, and aspartate residues involved in ClpP activity (31, 49) are boxed. Gaps are represented by dashes and were introduced to maximize the alignment. The alignment was generated with the Megalignment program of the DNAstar program package (DNAstar, Madison, Wis.).

weakest similarity among all ClpX amino acid sequences is found in the tandem C-terminal PDZ-like (PSD-95, Dlg, and ZO-1 proteins, where the domain was first identified) domains that were proposed to specifically bind target proteins (Fig. 2B) (27).

$ClpX_{Cc}$ is functional in *E. coli*. *C. crescentus* ClpX ($ClpX_{Cc}$) showed 66.8% similarity to *E. coli* ClpX ($ClpX_{Ec}$). To test if $ClpX_{Cc}$ is a functional homologue of the $ClpX_{Ec}$ chaperone (50), we tried to complement the *E. coli clpX* mutant SSN1 with the gene for $ClpX_{Cc}$ ($clpX_{Cc}$). For this purpose, the $clpX_{Cc}$ coding region was fused to the *E. coli* P_{lac} promoter with efficient translational start signals (see Materials and Methods), and the fusion was cloned into the low-copy-number vector pMR20. As a test system for functionality, we used the cell killing assay based on the bacteriophage P1 plasmid addiction module Phd-Doc (25) (see Materials and Methods). The growth of *E. coli* with the temperature-sensitive replicon that does not contain the *doc* and *phd* genes (pGB2ts) was unaffected. While wild-type strain W3110 with pGB2ts::*phd-doc* stopped growing at a nonpermissive temperature (Fig. 3A), the growth of *clpX* mutant strain SSN1 was not affected by the loss of plasmid pGB2ts::*phd-doc* (data not shown), demonstrating that the basis of plasmid stabilization by the Phd-Doc module is the degradation of Phd by the ClpXP protease (26). Growth was affected, however, when the *clpX* mutant strain was complemented with a plasmid-encoded copy of the $ClpX_{Ec}$ gene ($clpX_{Ec}$) (pSSN3). Similarly, expressing $clpX_{Cc}$ from plasmid pSSN6 in a *clpX* mutant background resulted in growth inhibition after a temperature shift (Fig. 3A). Growth inhibition in cells expressing $ClpX_{Cc}$ was slightly less severe than that in cells expressing the homologous $ClpX_{Ec}$ protein (Fig. 3A). This result could be due to reduced activity of the $ClpX_{Cc}$ protein in the heterologous host, as immunoblot analysis with an anti-ClpX antibody (16) confirmed that $ClpX_{Ec}$

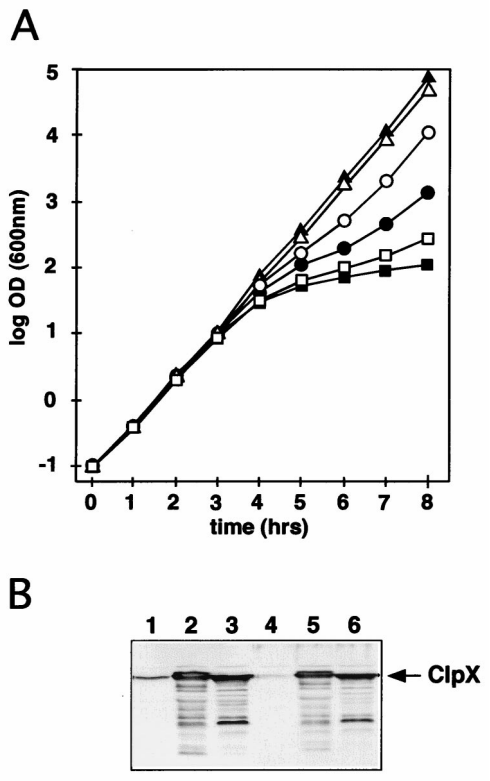


FIG. 3. $clpX_{Cc}$ is able to complement an *E. coli clpX* mutant. (A) The activity of ClpX was monitored by its ability to degrade, together with ClpP, the antidote protein Phd of the P1 plasmid addiction module Phd-Doc (26). Loss of the *phd* gene results in Doc-dependent cell killing if the Phd protein is degraded by the ClpXP protease. Growth of cultures containing the *phd* and *doc* genes on a plasmid with a temperature-sensitive replicon (pGB2ts::*phd-doc*) was monitored after a shift to the nonpermissive temperature. Growth is shown as the log OD_{600}. The time after the temperature increase is indicated in hours. Cessation of growth 3 to 5 h after the temperature shift was an indicator of the rapid disappearance of the antidote protein Phd and thus of ClpX activity. The following plasmids were used: plasmid pGB2ts is temperature sensitive for replication; pGB2ts::*phd-doc* is identical to pGB2ts except that it contains the plasmid addiction genes *phd* and *doc*; pSSN6 contains $clpX_{Cc}$; and pSSN3 carries $clpX_{Ec}$. Growth of the following *E. coli* strains was monitored: W3110/pGB2ts/pSSN6 plus isopropyl-β-D-thiogalactopyranoside (IPTG) (▲; negative control); W3110/pGB2ts::*phd-doc*/pSSN6 plus IPTG (■; positive control); SSN1/pGB2ts/pSSN6 plus IPTG (△); SSN1/pGB2ts::*phd-doc*/pSSN3 plus IPTG (□); SSN1/pGB2ts::*phd-doc*/pSSN6 plus IPTG (●); and SSN1/pGB2ts::*phd-doc*/pSSN6 (○). (B) Immunoblot analysis with an anti-ClpX serum and extracts of *E. coli* strains expressing $ClpX_{Cc}$ and $ClpX_{Ec}$. Equal amounts of total protein from the following strains were analyzed: W3110/pMR20 (lane 1); W3110/pSSN3 (lane 2); W3110/pSSN6 (lane 3); SSN1/pMR20 (lane 4); SSN1/pSSN3 (lane 5); and SSN1/pSSN6 (lane 6). The band corresponding to ClpX is marked by an arrow.

and $ClpX_{Cc}$ were present in comparable amounts in the *clpX* mutant test strain (Fig. 3B). These results suggest that $ClpX_{Cc}$ not only is able to recognize the Phd protein but also can interact with the proteolytic ClpP component of *E. coli*. Interestingly, while a plasmid-encoded copy of $clpX_{Cc}$ (pSSN6) was able to support the growth of a *C. crescentus* mutant with a disrupted chromosomal *clpX* copy, $clpX_{Ec}$ (pSSN3) could not rescue the mutant phenotype (data not shown). This result suggested that $ClpX_{Ec}$ is not able to interact with the *C. crescentus* ClpP subunits, potential substrates, or accessory proteins.

The *clpP* and *clpX* genes are transcribed from independent promoters. In *E. coli*, the *clpP* and *clpX* genes are transcribed as an operon from the same promoter, indicating that the syntheses of the regulatory and proteolytic subunits of the ClpXP protease are coupled (11). Three GC-rich inverted repeats followed by several thymidine residues, located downstream of the *tig* (T1, 5′-GGCGCGGCTCGCGAGGGCCGC GCCTTTTT-3′), *clpP* (T2, 5′-ACAAAGCCGCCGGCCAGG AGGTCGGCGGCTTTTTT-3′), and *clpX* (T3, 5′-CGCCATC ATCGGATGGCGCGCTTTT-3′) genes, could act as rho-independent transcriptional terminators, implying that these genes are expressed independently from each other (Fig. 4). Also, the large intergenic region between *clpP* and *clpX* (Fig. 4) suggested that *clpP* transcription and *clpX* transcription are not coupled in *C. crescentus*.

To analyze the transcription of the *clp* genes and to localize their promoter regions, we cloned several fragments of the *clp* locus into vector pRKlac290, generating transcriptional fusions between potential promoter fragments and the *lacZ* reporter gene. The transcriptional activities of the corresponding fragments were determined by introducing the resulting constructs into *C. crescentus* NA1000 wild-type cells and measuring β-galactosidase activities. A *Sal*I-*Xho*I fragment (pAS2; Fig. 4) containing the 5′ end of *clpP* and its upstream region generated 1,809 Miller units. This fragment most likely contains the promoter for *clpP* (P_{P1}; see below). The 3′ part of the *tig* gene generated only background levels of β-galactosidase activity and thus does not carry a promoter (pAS1; Fig. 4). However, we cannot exclude the possibility that some transcriptional activity originating upstream of *tig* reads through the putative terminator structure T1 located immediately downstream of the *tig* gene (Fig. 4). Comparison of the Miller units generated by constructs pAS3 and pAS29 (Fig. 4) suggested that the majority of the transcripts that originate at P_{P1} are terminated by the stem-loop structure T2 downstream of the *clpP* gene. Thus, P_{P1} does not contribute significantly to the expression of downstream genes.

The region between the *clpP* and *clpX* genes generated a total transcriptional activity corresponding to 3,443 Miller units and reading into the *clpX* gene (pAS24; Fig. 4). This total activity could be assigned to at least three promoter regions represented by the constructs pAS64 (P_{X3}, 690 Miller units), pAS26 (P_{X2}, 1,422 Miller units), and pAS6 (P_{X1}, 1,399 Miller units). Since the activities of these constructs can be added up to the activity found for the entire *clp* intergenic region (pAS24), we assume that the transcripts originating in all three promoter regions extend into the *clpX* gene. In addition, construct pAS23 (3,005 Miller units; Fig. 4) combined the transcriptional activities of P_{X1} and P_{X2}, and the activity generated by construct pAS27 was equal to the sum of the transcriptional activities of P_{X2} and P_{X3} (2,246 Miller units; Fig. 4). The expression of *clpX* in *C. crescentus* is therefore controlled by at least three different promoter regions located in the *clpPX* intergenic region. It is not clear if and to what extent these promoters contribute to the expression of the adjacent *lon* gene. *clpX* is followed by a putative rho-independent terminator (T3) that could uncouple the expression of the *clpX* and *lon* genes (Fig. 4).

The *clpPX* intergenic region contains several potential open reading frames, the longest, *cicA*, pointing in the direction opposite that of *clpP* and *clpX* (Fig. 4). To identify a potential promoter region for *cicA*, we created a *lacZ* fusion to the *Apa*I-*Fsp*I fragment in the direction opposite that in construct pAS23. While this piece of DNA contains P_{X1} and P_{X2}, generating about 3,000 Miller units reading into *clpX*, it also gives rise to about 2,100 Miller units reading in the opposite direction (pAS63; Fig. 4). Speculating that this activity drives the expression of *cicA*, we call this promoter region P_{cicA} (Fig. 4). In contrast to that generated by construct pAS63, the activity generated by construct pAS25 was only marginally above the

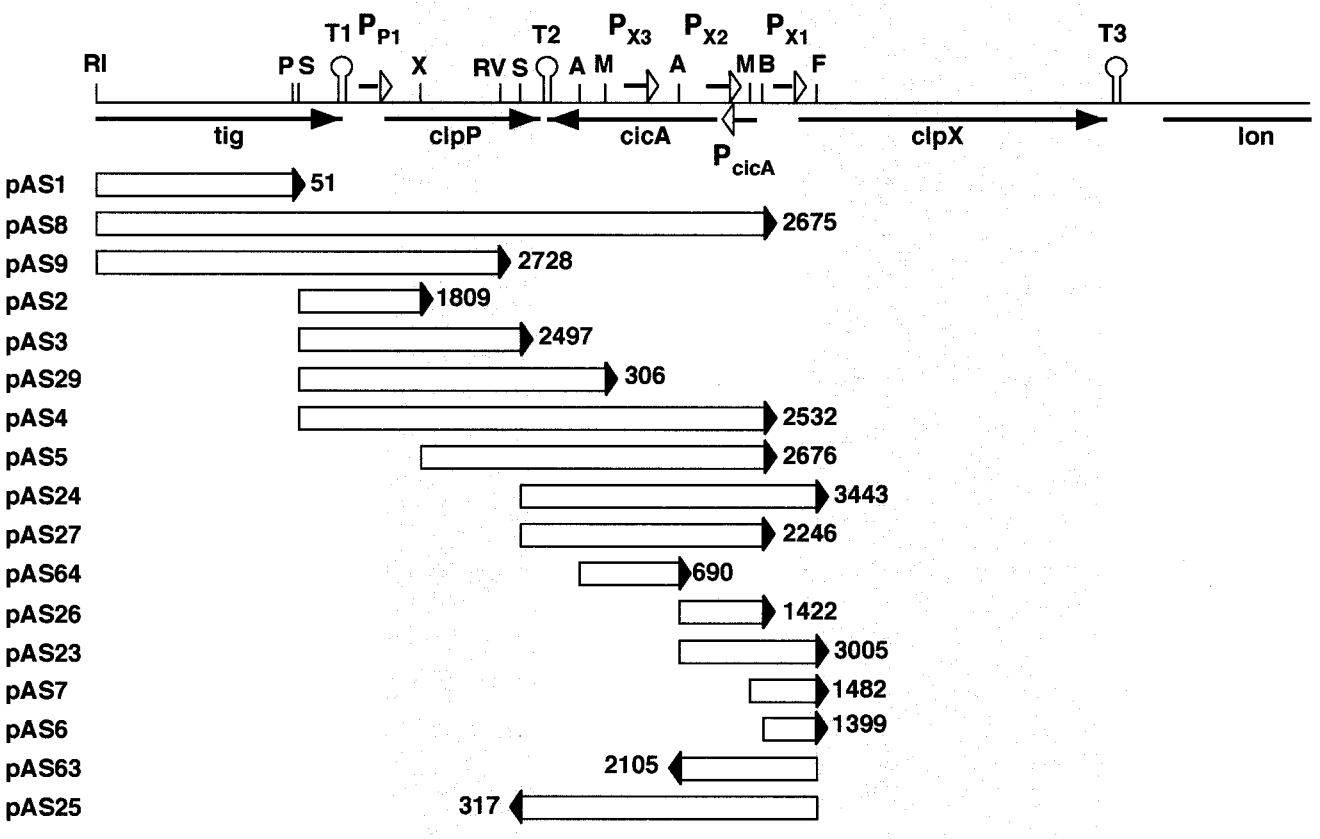
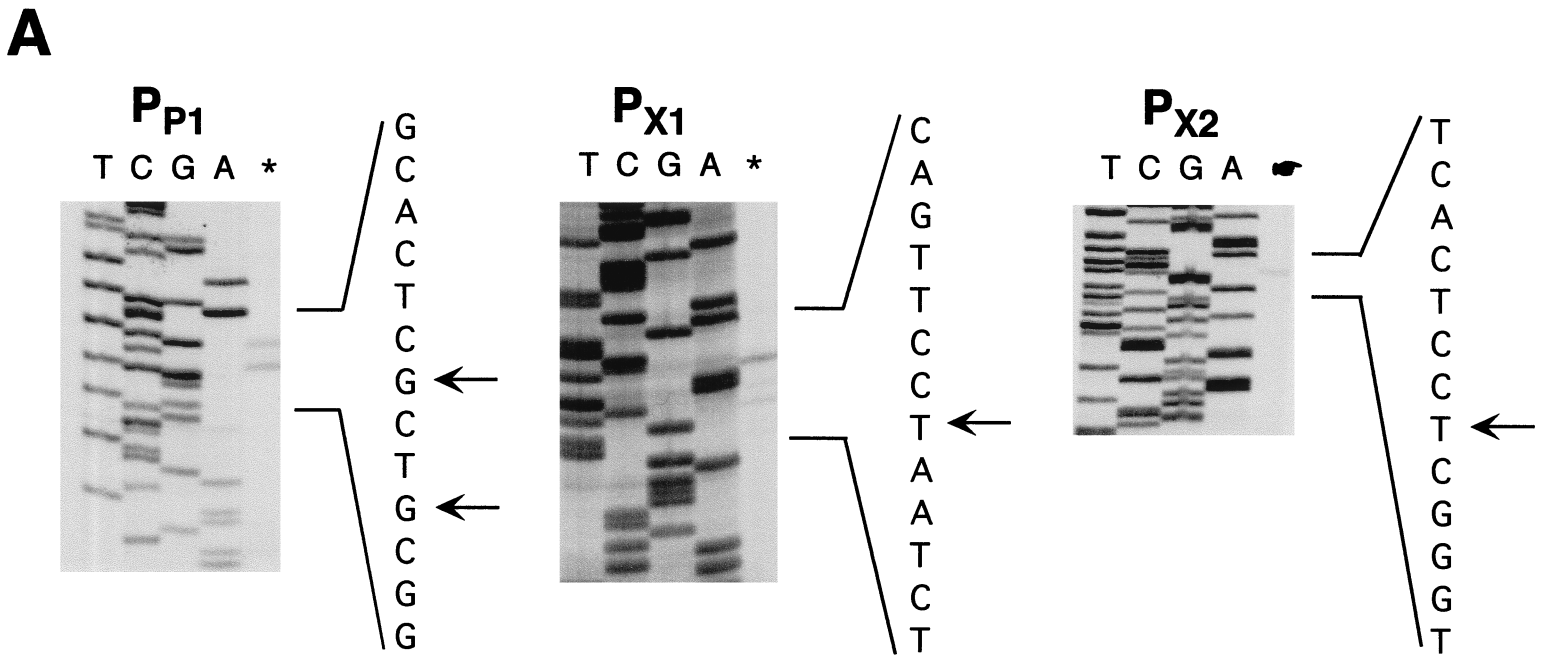


FIG. 4. Identification of promoter regions in the *C. crescentus clp* locus. A schematic of the chromosomal *clp* region is shown at the top. The *tig* (trigger factor), *clpP* (ClpP peptidase), *cicA* (unknown function), *clpX* (ClpX ATPase), and *lon* (Lon protease) genes are indicated by black arrows. The approximate locations and orientations of the promoters identified (P_{P1}, P_{X1}, P_{X2}, and P_{X3}) are marked by short open arrows, and putative transcriptional terminator structures (T_1, T_2, and T_3) are indicated as stem-loop outlines. Fragments that were cloned into the *lacZ* reporter plasmid pRKlac290 (see Materials and Methods) are indicated as open bars below the schematic, with the filled triangles marking the location and orientation of the *lacZ* reporter gene. The names of the corresponding constructs are on the left, and the number on the right indicates the β-galactosidase activity (Miller units) generated by each fusion construct. All measurements were determined in triplicate, and average numbers are presented. The following abbreviations are used for recognition sites of restriction enzymes: RI, *Eco*RI; P, *Pst*I; S, *Sal*I; X, *Xho*I; RV, *Eco*RV; A, *Apa*I; M, *Mlu*I; B, *Bam*HI; and F, *Fsp*I.

background of about 200 Miller units found for vector pRKlac290 alone. This result suggested that the transcripts originating from P_{cicA} do not read into the *clpP* gene but are terminated at the stem-loop structure T2 (Fig. 4). T2 is localized about 20 bp downstream of the *clpP* stop codon and 30 bp downstream of the stop codon for the potential *cicA* open reading frame. This observation and the observation that the T2 inverted repeat is flanked at both ends by several thymidine residues on the transcribed strain indicate that this structure could act as a bidirectional transcriptional terminator.

On the basis of the genetic mapping of the promoter regions, we performed primer extension experiments (see Materials and Methods) to precisely localize the transcriptional initiation sites for *clpP* and *clpX*. With primers clpPPE1 and clpPPE2 (see Materials and Methods), two close start sites were found to be positioned 59 and 62 bp upstream of the ATG start codon of *clpP* (Fig. 5). A −10 box and a −35 box designating P_{P1} were identified upstream of the transcriptional initiation sites for *clpP* (Fig. 5B). Two separate transcriptional start sites were found directly upstream of *clpX* by use of two different primers, clpXPE1 and clpXPE2 (see Materials and Methods). One was positioned 103 bp upstream of the proposed translational start codon of *clpX* and immediately downstream of the *Bam*HI site (Fig. 4 and 5). Two weaker start signals seen in Fig. 5A (P_{X1}) were observed only with the clpXPE1 primer, suggesting that they are artifacts. The second transcriptional start site was located 196 bp upstream of the *clpX* ATG start codon and immediately upstream of the *Mlu*I site (Fig. 4 and 5). Consensus sequences for −10 and −35 promoter boxes were identified upstream of both transcriptional start sites (Fig. 5B). The locations of these two transcriptional start sites downstream of the *Bam*HI site and upstream of the *Mlu*I site suggested that the former corresponds to P_{X1} (pAS6 and pAS7; Fig. 4) and that the latter corresponds to P_{X2} (pAS26; Fig. 4). It is difficult to unambiguously assign the identified promoter regions to *C. crescentus* promoter consensus sequences. As depicted in Fig. 5B, the −10 and −35 boxes of P_{P1}, P_{X1}, and P_{X2} show some similarity to the consensus sequences of both σ^{32}-dependent heat shock promoters (37, 53) and σ^{73}-dependent housekeeping promoters (29).

Heat shock control of *clpP* and *clpX* promoters. The components of the ClpXP protease in *E. coli* (22) and the ClpP peptidase in *B. subtilis* (15, 47) are induced by heat shock, implying a role for the corresponding proteases in the degradation of unfolded and misfolded proteins. To examine if the *C. crescentus clpP* and *clpX* genes are also under heat shock control, we assayed the activities of the *clpP* and *clpX* promoters as well as the relative concentrations of the ClpP and ClpX



B

-35 -10 +1

P_{P1}	CGTTTCAGGCCGCGTCGCGCGCTCCTTGCAACCTGTTAAGGGGAACGCGATATGCGTGAGCGACGCCACG	– N_{52} – ATG
P_{X2}	CCGTAAGGGCCTAGGCGCACGGCGCTTGCACGAGGGGGCCAATCGCCCAATTTAGTGAGGAGCCCATGAC	– N_{186} – ATG
P_{X1}	CACGGCCGAAAGGATCCGGCGAGGCTTGTGAACCTGGCCC GGATCGGGGAATGTCAAGGATTAGACAAC	– N_{93} – ATG
	σ^{73} consensus TTGaCG[G/C] GCtANA[A/T]C	
	σ^{32} consensus GNNTT[G/C]CNNG C[GG/CC]CATAT	

FIG. 5. Promoter analysis of the *C. crescentus clpP* and *clpX* genes. (A) Primer extension products obtained with the *clpX*- and *clpX*-specific primers are shown in the rightmost lanes. Sequencing reactions generated with the same primers are shown in lanes T, C, G, and A. The relevant sequence of the coding strand is shown to the right of each gel, and the positions of the major extension products are indicated by arrows. (B) Sequence alignment of P_{P1}, P_{X1}, and P_{X2}. The −35 and −10 (boxed) and +1 (circled) regions are indicated, and the distance between the transcriptional start site and the presumed translational start codon is shown (N_x). The *C. crescentus* consensus sequences for σ^{73}-dependent (29) and σ^{32}-dependent (37, 53) promoters are shown in boldface below the three *clp* promoters.

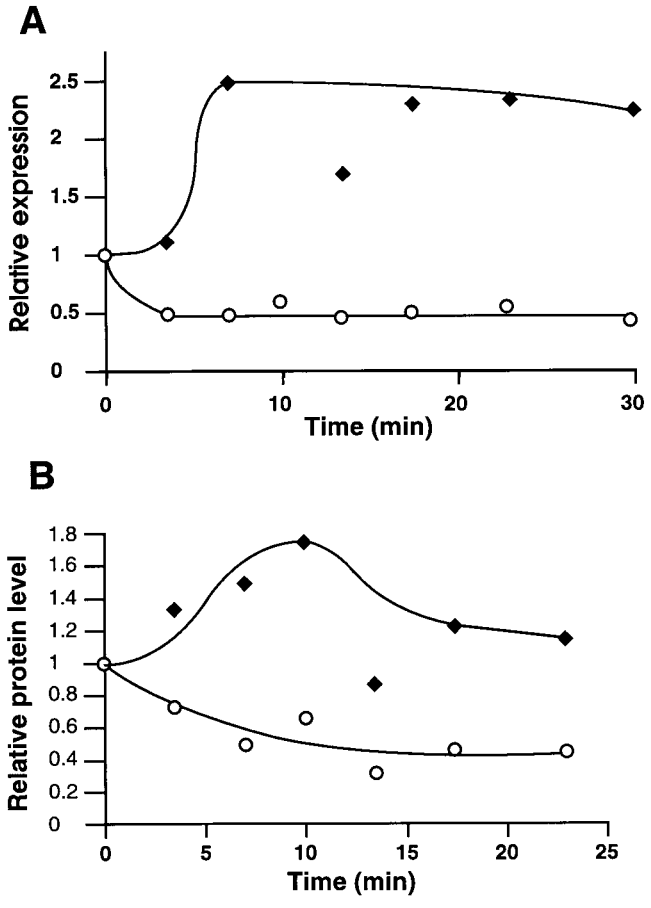


FIG. 6. Heat shock control of ClpP and ClpX expression. (A) The transcription of *clpP* (◆) and *clpX* (○) was determined with cultures of NA1000/pAS2 (*clpP*::*lacZ*) and NA1000/pAS24 (*clpX*::*lacZ*) by [^{35}S]methionine labeling, β-galactosidase immunoprecipitation, and quantitation at different times after a shift in the temperature from 30 to 42°C. (B) Cellular concentrations of ClpP (◆) and ClpX (○) after the temperature shift from 30 to 42°C, as determined by immunoblot analysis for the same samples as those analyzed in panel A. The values are relative to the level of transcription or protein at 30°C.

proteins after shifting the temperature from 30 to 42°C. Cells of wild-type strain NA1000 carrying plasmid pAS2 (P_{P1}-*lacZ*) or plasmid pAS24 (P_{X1}-P_{X2}-P_{X3}-*lacZ*) were grown to the mid-log phase at 30°C, and aliquots of the cultures were shifted to the higher temperature. Promoter activity was assayed by pulse labeling of cells with [^{35}S] Met at different times after the temperature shift and subsequent immunoprecipitation of the β-galactosidase synthesized. The activity of P_{P1} increased about 2.5 times during the initial 5 to 10 min after heat shock, with transcriptional activity remaining high even after prolonged exposure of the cells to heat (Fig. 6A). In contrast, *clpX* promoter activity very rapidly decreased by about 50% after heat shock (Fig. 6A). The levels of the ClpP and ClpX proteins followed a similar trend, as the ClpP protein level increased about two times upon heat shock induction, while the ClpX protein level decreased to below 50% its initial value (Fig. 6B). These results suggested that P_{P1} is under heat shock control and possibly dependent on the *C. crescentus* sigma factor RpoH (36, 53). Since the *clpP* gene has recently been shown to be essential for the growth and viability of *C. crescentus* (16), a requirement for RpoH to activate P_{P1} could explain the finding that even at low temperatures, the *C. crescentus rpoH* gene could not be inactivated (36).

Cell cycle- and growth phase-dependent transcription of *clpP* and *clpX*. Both *clpP* and *clpX* are essential genes in *C. crescentus*, and their product, the ClpXP protease, is involved in cell cycle control in this organism (16). To examine a possible cell cycle-dependent activity pattern of the *clpP* and *clpX* promoters, all four *clpP* and *clpX* promoter fragments fused to the *lacZ* reporter gene (pAS2, pAS6, pAS26, and pAS64; Fig. 4) were assayed by pulse labeling at different short intervals during the cell cycle. Subsequent immunoprecipitation with an anti-β-galactosidase antibody allowed us to quantitate promoter activity at any given time of the cell cycle. Cell cycle fluctuations of promoter activity were observed only for P_{X1} and P_{X3} (Fig. 7A). The activity of P_{P1} and P_{X2} did not change significantly during the cell cycle. The activity of P_{X1} and P_{X3} peaked in predivisional cells (Fig. 7A). However, while P_{X1} activity was low in stalked cells and high in swarmer cells, the pattern for P_{X3} activity was the opposite (Fig. 7A). Even though these activity patterns are reproducible, their significance is not clear. Immunoblot analysis with anti-ClpP and anti-ClpX antibodies had revealed that the concentrations of both proteins did not fluctuate significantly during the cell cycle (16).

During the analysis of the *clp* promoter fragments, we found that the strength of the *clp* promoters was dependent on the growth phase of the cells. To determine the influence of the growth phase on the activity of the *clpP* and *clpX* promoters, β-galactosidase production from constructs pAS2 and pAS24 (Fig. 4) was monitored through the logarithmic and stationary phases. When cells from an overnight culture were diluted into fresh complex medium, the activity of P_{P1} was initially high but decreased to about 50% during exponential growth (Fig. 7B). When the cells reached the late logarithmic phase, the activity of P_{P1} increased coincidently with a decrease in growth rate. Eventually, when the cells entered the stationary phase, the activity of P_{P1} reached its original value (Fig. 7B). A similar behavior was also observed for the *clpX* promoters, although the initial decrease occurred more rapidly (Fig. 7B). As a control, the activity of the *xylX* promoter (32) was assayed as a function of growth phase in the presence of the inducer xylose. In contrast to that of the *clp* promoters, the activity of the *xylX* promoter did not change over the course of exponential growth and the stationary phase, demonstrating that promoter induction in the stationary phase is not a general phenomenon in *C. crescentus*. The levels of the ClpP and ClpX proteins showed a similar, but more pronounced, fluctuation (Fig. 7C). These results suggested that in *C. crescentus*, the *clpP* and *clpX* promoters are induced in the late exponential and stationary phases, resulting in increased ClpP and ClpX levels in cells.

Determination of the number of ClpP and ClpX molecules per cell. Specific antibodies against ClpP and ClpX were used for an approximate determination of the number of molecules per cell for both components of the protease (see Materials and Methods). Different concentrations of purified His-tagged ClpP and ClpX proteins (16) were analyzed in immunoblot experiments, and the signals obtained were compared quantitatively with the signals from total cellular protein (Fig. 8). On the basis of the assumption that both antibodies react similarly with the full-length His-tagged and native proteins, the numbers of molecules per cell could be calculated from the data. Averages of 23,000 and 41,000 molecules per cell were determined for ClpP in logarithmically growing cells and in the stationary phase, respectively. The corresponding numbers for ClpX were 5,000 molecules per cell in the logarithmic growth phase and 9,000 molecules per cell in the stationary phase. Under the condition that all of the protein pools in the cell are assembled into stable tetradecamers (ClpP) (21, 49) and hex-

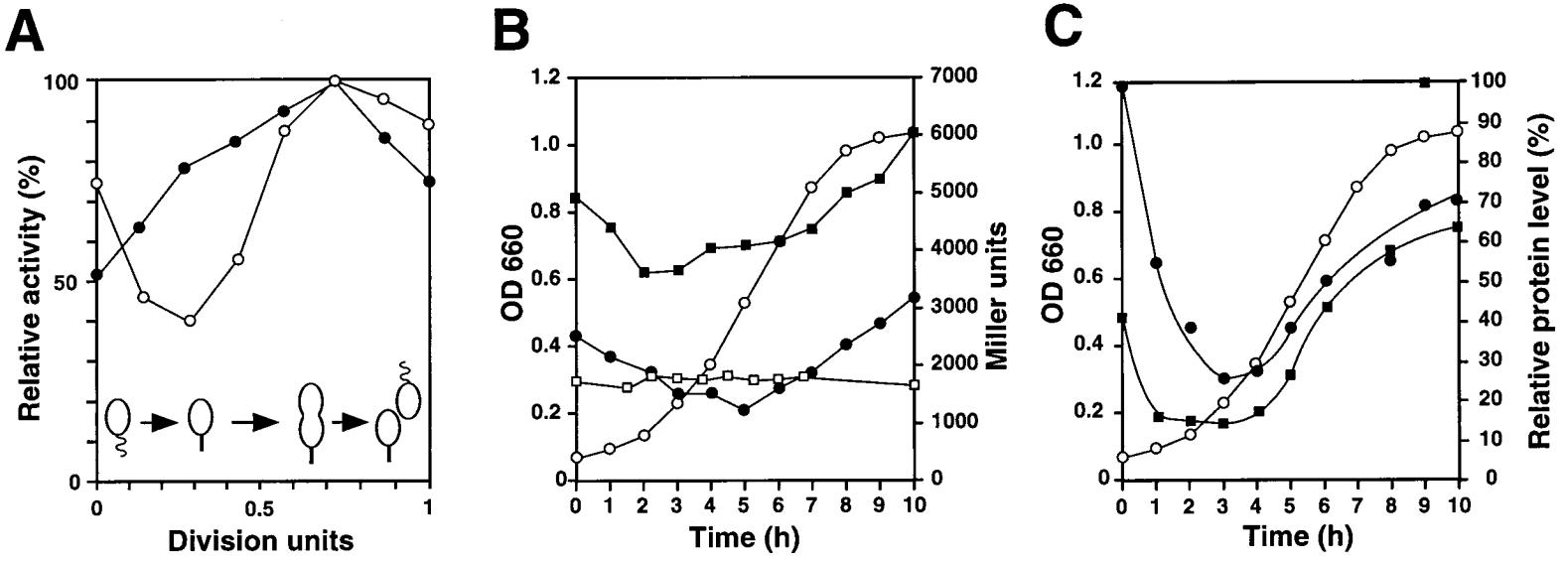


FIG. 7. Cell cycle- and growth phase-dependent expression of *clpP* and *clpX*. (A) The relative activities of P_{X1} (○) and P_{X3} (●) were determined during the cell cycle by pulse labeling of synchronized cultures of strain NA1000 containing plasmids pAS6 (P_{X1}) and pAS64 (P_{X3}), respectively, with [^{35}S]methionine for 5 min at different intervals and determining β-galactosidase synthesis during the pulse time by immunoprecipitation (see Materials and Methods). The promoter activity is shown relative to maximal activity. Progression of the cell cycle is indicated schematically at the bottom. (B and C) Growth phase-dependent expression (B) and cellular levels (C) of ClpP and ClpX. (B) Stationary-phase overnight cultures were diluted in fresh PYE complex medium (NA1000, NA1000/pAS2, and NA1000/pAS24) or PYE complex medium plus 0.2% xylose (NA1000/pCS225), and growth was monitored by determining the OD_{660} (○). At different intervals, samples were removed from the cultures, and β-galactosidase activity (Miller units) was determined as described in Materials and Methods for the following strains: NA1000/pAS2 (P_{P1}) (●), NA1000/pAS24 (P_{X1}, P_{X2}, and P_{X3}) (■), and NA1000/pCS225 (*xylX* promoter) (□). (C) Relative cellular levels of ClpP (●) and ClpX (■) in wild-type strain NA1000 were determined as a function of the growth phase by immunoblot analysis. ○, OD_{660}.

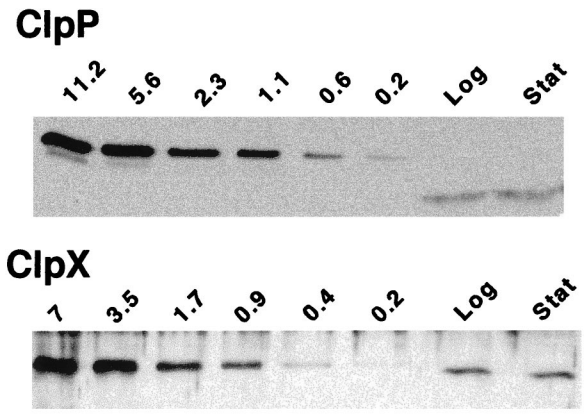


FIG. 8. Estimation of the number of ClpP and ClpX molecules per cell. Immunoblot analysis was done with anti-ClpP and anti-ClpX sera and with specific amounts (picomoles) of purified His-tagged ClpP and ClpX proteins, respectively, and total proteins from NA1000. The band intensities of total proteins correspond to the amounts of ClpP and ClpX present in 2×10^7 cells and 1×10^8 cells, respectively, during exponential growth (Log) and in 1×10^7 cells and 5×10^7 cells, respectively, during the stationary phase (Stat).

amers (ClpX) (14), the numbers of complexes were about 1,650 ClpP structures and 830 ClpX rings in the logarithmic growth phase and about 3,000 ClpP structures and 1,500 ClpX rings in the stationary phase.

DISCUSSION

We have isolated the genes coding for the ClpXP protease in *C. crescentus*. ClpXP is a two-component protease with a peptidase subunit (ClpP) and an ATP-dependent regulatory chaperone subunit (ClpX). The peptidase by itself does not have any substrate specificity and requires ClpX for substrate recognition and interaction. Thus, the presence of the regulatory subunit is crucial for the specific activity of the protease. In *E. coli*, an alternative ATPase, ClpA, is able to interact with and compete for ClpP subunits (14), with ClpXP and ClpAP having distinct substrate spectra (13). However, the molecular basis for substrate selection by the regulatory proteins is not known. In some cases, substrate-specific mediator proteins are required for the interaction between the Clp ATPase and the substrate protein (46, 54). In other cases, a direct substrate interaction is mediated by PDZ-like domains in the C-terminal part of the chaperone (27). In support of this notion, the ClpX C termini are strongly conserved in all bacterial ClpX sequences, indicating a conserved substrate recognition mechanism for ClpX homologues in different organisms. The results of our complementation experiments are in agreement with such a prediction. A copy of the *C. crescentus clpX* gene can functionally substitute for *clpX* in *E. coli*. This fact strongly suggests that the $ClpX_{Cc}$ protein is able to interact with at least some of the $ClpX_{Ec}$ substrates and with the ClpP peptidase in *E. coli*. On the other hand, $clpX_{Ec}$ was not able to complement a $clpX_{Cc}$ mutation. Unlike $clpX_{Ec}$, $clpX_{Cc}$ has been shown to be essential for cell cycle progression and thus for growth (16). Thus, one or several substrate proteins for which ClpXP-dependent degradation is indispensable must exist in *Caulobacter* (16). The failure of $ClpX_{Ec}$ to support growth in *Caulobacter* could be due to its inability to interact with $ClpP_{Cc}$ or to inadequate recognition of some or all of the ClpXP substrates. Alternatively, lack of proper control of the $ClpX_{Ec}$-substrate interaction might be the reason for the deficient function of $ClpX_{Ec}$ in *C. crescentus*. Since ClpXP plays a role in controlling the *C. crescentus* cell cycle, tight temporal control of its activity and thus ClpX substrate accessibility is postulated.

In *E. coli*, the *clpP* and *clpX* genes are coregulated at the transcriptional level (11). In particular, heat shock results in a twofold induction of transcription of the *clpPX* operon (11), suggesting a role for the ClpXP protease in the stress response in *E. coli*. In contrast, we found that in *C. crescentus*, transcription of the *clpP* and *clpX* genes is not linked and only the *clpP* gene is subject to heat shock induction. P_{P1} was mapped by primer extension analysis and shown to resemble the previously identified σ^{32}-dependent heat shock promoters (3, 36, 37, 52, 53). However, based on the proposed consensus sequences (29, 37, 53), it is difficult to unambiguously assign P_{P1} to either the σ^{32}-dependent or the σ^{73}-dependent family of promoters. We have observed a twofold increase of P_{P1} activity in the presence of an additional, plasmid-encoded copy of the *rpoH* gene (data not shown), indicating that σ^{32} at least contributes to P_{P1} activity.

Most transcripts that originated at P_{P1} terminated immediately downstream of the *clpP* gene, probably at a transcription terminator-like structure. Thus, heat-inducible P_{P1} is not responsible for the transcription of the *clpX* gene. The transcription of *clpX* is directed by at least three independent promoters in the *clpP-clpX* intergenic region. This region of about 1 kb was shown to code for an open reading frame (*cicA*) pointing in the direction opposite that of *clpP* and *clpX*. Data obtained recently have confirmed that *cicA* codes for a protein that, like ClpP and ClpX, is essential for the growth of *C. crescentus* (50a). Two of the three *clpX* promoters were localized to the region between the 5′ ends of *cicA* and *clpX*. Both show some similarity with the postulated consensus sequence for σ^{73}-dependent promoters (29). The third *clpX* promoter was located further upstream, in the coding region of *cicA*. In contrast to the data for *clpP*, heat shock did not result in an increase but rather resulted in a decrease in total *clpX* transcription. This finding clearly emphasizes the independent control of the *clpP* and *clpX* promoters. Since the change in the concentrations of ClpP and ClpX after heat shock was very similar to the change in the transcription of both genes, we concluded that heat shock control of these genes is exerted primarily at the transcriptional level. It is not clear which mechanism is responsible for the reduction of *clpX* transcription following heat shock. Since downregulation did not occur for the *xylX* promoter (15a), the observed reduction of *clpX* transcription is likely to be the result of a specific regulatory mechanism rather than a general phenomenon. Thus, unlike $ClpX_{Ec}$, $ClpX_{Cc}$ does not appear to be involved in the heat shock response. A reduction of ClpX synthesis after heat shock would allow the use of the available ClpP pool more specifically for stress-associated functions. One way to accomplish this would be via an exchange of ClpP-associated ATPases, in which ClpX is replaced by another regulatory ATPase subunit, i.e., ClpA. In vitro, the affinities of the *E. coli* ClpA and ClpX proteins for ClpP have been shown to be comparable, suggesting that ClpA and ClpX compete for ClpP in vivo (14). The $clpA_{Cc}$ gene has recently been cloned and shown to be dispensable for growth at a normal temperature (34a). We are currently testing the hypothesis that ClpA is involved in the heat shock response in *C. crescentus*.

The fact that *clpX* has three promoters with different cell cycle controls suggested that *Caulobacter* very carefully regulates the expression of this gene and thereby the cellular concentration of the ClpX protein. While the physiological significance and the individual control of each promoter remain to be elucidated, tight regulation of the *clpX* gene is consistent with our finding that both the depletion and the overexpression

of the ClpX protein are highly toxic for the cell and result in cessation of growth and loss of viability (16). In contrast, the overexpression of the ClpP protein has no obvious physiological consequences for the cell. Assessment of the molar ratio of ClpP to ClpX has revealed that ClpP is present in a molar excess in *Caulobacter*. Under the assumption that both ClpP and ClpX exist predominantly in their complexed form, about 1,650 assembled ClpP double rings and 830 ClpX rings are present in a cell. Since each ClpX hexamer can complex with two opposite sides of a ClpP tetradecamer in *E. coli* (14) and assuming that ClpX and ClpP assemble with the same stoichiometry in *C. crescentus*, the available ClpX pool can saturate at most one-fourth of the theoretically accessible peptidase sites. In comparison, *E. coli* ClpP has been shown to be limiting compared with ClpX and ClpA combined (14), a finding that is reasonable in view of *clpP* and *clpX* being coexpressed and thus most likely present in similar amounts. In *Caulobacter*, tight control of ClpX expression therefore might be necessary because ClpX is the limiting factor for the degradation of one or several key proteins by the ClpXP protease (16). Alternatively, it is conceivable that ClpX, in addition to its essential role in protein degradation, fulfills critical cellular ClpP-independent functions as a chaperone (50). One of the main future challenges will thus be to characterize the different activities of ClpX in *Caulobacter* and to identify the proteins with which it is able to interact.

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