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An essential protease involved in bacterial cell-cycle control

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Proteolytic inactivation of key regulatory proteins is essential in eukaryotic cell-cycle control. We have identified a protease in the eubacterium *Caulobacter crescentus* that is indispensable for viability and cell-cycle progression, indicating that proteolysis is also involved in controlling the bacterial cell cycle. Mutants of *Caulobacter* that lack the ATP-dependent serine protease ClpXP are arrested in the cell cycle before the initiation of chromosome replication and are blocked in the cell division process. ClpXP is composed of two types of polypeptides, the ClpX ATPase and the ClpP peptidase. Site-directed mutagenesis of the catalytically active serine residue of ClpP confirmed that the proteolytic activity of ClpXP is essential. Analysis of mutants lacking ClpX or ClpP revealed that both proteins are required *in vivo* for the cell-cycle-dependent degradation of the regulatory protein CtrA. CtrA is a member of the response regulator family of two-component signal transduction systems and controls multiple cell-cycle processes in *Caulobacter*. In particular, CtrA negatively controls DNA replication and our findings suggest that specific degradation of the CtrA protein by the ClpXP protease contributes to G_1-to-S transition in this organism.

Keywords: ATP-dependent protease/*Caulobacter*/cell cycle/Clp protease/proteolysis

Introduction

The cell cycle is an ordered series of events involving sequential chromosomal duplication, and chromosomal segregation followed by division of the cell. In eukaryotes, a conserved class of regulatory proteins [cyclin-dependent kinases (CDKs)] is responsible for the ordered progression of the cell through the periodic events of mitotic division (Nigg, 1995). Periodicity of the cell cycle also requires essential degradation processes (King *et al*., 1996). In particular, the G_1-to-S transition (Hoyt, 1997) as well as onset of anaphase and exit from mitosis (Hoyt, 1997) are associated with proteolysis of key regulators like cyclins or CDK inhibitors and structural proteins. The existence of checkpoint control and similar feedback mechanisms in bacteria (i.e. SOS system) suggests common strategies in the cell-cycle control of prokaryotes and eukaryotes (Murray, 1992; Walker, 1996). This has been underlined by recent findings obtained in a eubacterium, *Caulobacter crescentus*, which suggest that specific degradation events also play a key role in the bacterial cell cycle (Alley *et al*., 1993; Jenal and Shapiro, 1996; Domian *et al*., 1997).

During its biphasic life cycle, *C.crescentus* undergoes an asymmetric cell division that gives rise to progeny cells with different developmental programs (Figure 1). The sessile stalked cell (ST) is able to immediately initiate chromosome replication and subsequent cell division. The swarmer cell (SW) is motile and chemotactically active, but unable to replicate its chromosome and divide. To acquire its replicative capacity, the SW cell (G_1 phase) must first differentiate into a ST cell (start of S phase), a process during which the flagellum is ejected and the chemotaxis apparatus is lost. Thus, cell differentiation is an integral part of the *Caulobacter* cell cycle.

As in most eukaryotic cells, the periods of replication and chromosome segregation do not overlap in *C.crescentus*. Chromosome replication is initiated once per cell cycle and is followed sequentially by a segregation period, septum formation and finally cell division. While little data is available on how these processes are controlled and integrated, recent studies have revealed several proteins involved in *C.crescentus* differentiation and cell-cycle progression that are under spatial and temporal control involving both synthesis and proteolytic decay (Alley *et al*., 1993; Jenal and Shapiro, 1996; Quon *et al*., 1996; Wright *et al*., 1996; Domian *et al*., 1997).

One of these proteins, CtrA, is a member of the response regulator superfamily. CtrA modulates transcription of genes important for flagellar biogenesis, DNA methylation and cell division during the cell cycle (Quon *et al*., 1996; Wright *et al*., 1996; Kelly *et al*., 1998). Importantly, CtrA negatively controls initiation of chromosome replication in the SW cell (Quon *et al*., 1998). To restrict this inhibitory action to the motile SW cell, *Caulobacter* employs multiple mechanisms to control the activity and abundance of CtrA (Quon *et al*., 1996; Domian *et al*., 1997): (i) transcription of the *ctrA* gene occurs only in the PD cell; (ii) activation of CtrA by phosphorylation occurs in the early PD cell after initiation of DNA replication; and (iii) specific proteolysis clears CtrA from the ST compartment of the late PD cell and from the SW cell as it develops into a ST cell. Temporally and spatially appropriate degradation of CtrA is crucial for the G_1-to-S transition, thereby coupling the cell cycle to chromosome replication.

Four families of ATP-dependent proteases have been characterized in bacteria: Clp (ClpAP, ClpXP), HslUV (ClpYQ), AAA (FtsH) and the Lon family (Lon). The Clp proteins are assembled into structures similar to the eukaryotic proteasome (Yoshimura *et al*., 1993; Kessel *et al*., 1995, 1996; Rohrwild *et al*., 1996, 1997; Bochtler *et al*., 1997; Groll *et al*., 1997; Wang *et al*., 1997). A

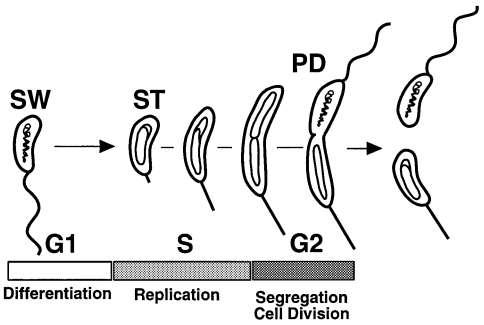

Fig. 1. Schematic diagram of the *C.crescentus* cell cycle. SW, swarmer cell; ST, stalked cell; PD, predivisional cell. The eukaryotic nomenclature has been adapted for the stages of the cell cycle. G_1 corresponds to the SW cell where initiation of DNA replication is inhibited by the CtrA protein (Quon *et al*., 1998). The S phase includes the ST cell, where DNA replication is initiated, and the early PD cell. G_2 corresponds to the late PD cell, where the newly replicated chromosomes are segregated to the cell poles and cell division gives rise to two daughter cells.

central cavity is formed by multimeric rings of either ClpP or ClpQ stacked on top of each other, with ATP-dependent chaperones (ATPases; ClpA, ClpX, ClpY) regulating entry to the channel at both ends. The proteolytic activity resides in the ClpP or ClpQ subunits, while it is hypothesized that access to the channel depends on specific recognition and unfolding of a substrate protein by the ATPase. One of these ATPases, the *Escherichia coli* ClpX protein, interacts specifically with disordered C-terminal peptides of its substrate proteins (Laachouch *et al*., 1996; Levchenko *et al*., 1997a,b). The C-terminus of the *Caulobacter* CtrA protein is required for cell-cycle-dependent proteolysis (Domian *et al*., 1997) and shares sequence homology with the C-terminal *E.coli* peptides that are specifically recognized by ClpX. We have recently isolated the genes encoding the proteolytic and regulatory subunits of the *C.crescentus* ClpXP protease (Figure 2A; M.Osteras, A.Stotz, S.Schmid Nuoffer and U.Jenal, manuscript in preparation). Here we show that ClpXP is responsible for the cell-cycle-dependent degradation of the CtrA protein. The ClpXP protease is strictly required for growth, survival and normal cell division of *C.crescentus*. Loss of either ClpP or ClpX results in cell-cycle arrest. Thus, the role of *Caulobacter* ClpXP in the control of cell-cycle progression in this prokaryote is similar to that of its structurally conserved eukaryotic counterpart, the proteasome.

Results

The ClpXP protease is essential in Caulobacter

We have previously isolated the genes encoding the subunits of the *C.crescentus* ClpXP protease (Figure 2A). To analyze the function of the ClpXP protease in *Caulobacter*, we attempted to generate null mutations in *clpP*, the gene for the proteolytic subunit, and *clpX*, the gene for the regulatory subunit, by replacing the chromosomal genes with copies disrupted by an interposon carrying a spectinomycin-resistance cassette (Figure 2A). The disrupted genes were introduced into the chromosome by homologous recombination on a plasmid that also contained a kanamycin resistance marker (*nptI*) and the *sacB* gene from *Bacillus subtilis* conferring sucrose sensitivity (Figure 2A, 1. rec.). Colonies that had lost the plasmid by a subsequent second recombination step (Figure 2A, 2. rec.) were isolated on the basis of their resistance to sucrose and analyzed for the presence or absence of the

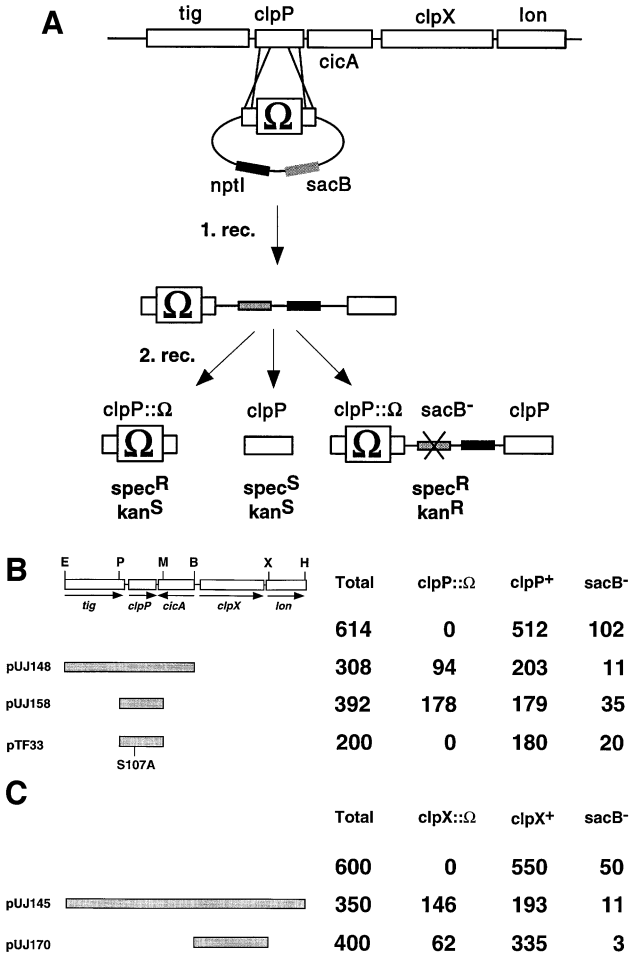

Fig. 2. The *C.crescentus clpP* and *clpX* genes are essential for growth. (**A**) The chromosomal wild-type copy of *clpP* was exchanged via a two-step homologous recombination reaction with a gene copy disrupted by the insertion of an interposon (Ω) (see Materials and methods). The interposon carries a spectinomycin- and streptomycin-resistance cassette and was introduced into the chromosome on a plasmid that also contained a kanamycin-resistance marker (*nptI*) and the *sacB* gene from *B.subtilis* conferring sucrose sensitivity (1. rec.). Colonies that had lost the plasmid by a subsequent second recombination step (2. rec.) were isolated on the basis of their resistance to sucrose. Sucrose-resistant colonies were analyzed by their antibiotic-resistance profile for the presence or absence of the interposon in the *clp* locus, and for mutational inactivation of the *sacB* gene. A similar approach was used to mutagenize *clpX*. *cicA* is a gene of yet unknown function, that has been identified in the chromosomal region between *clpP* and *clpX* (M.Osteras, P.Wiget, T.Fuchs and U.Jenal, unpublished). The total numbers of sucrose-resistant colonies that were screened for *clpP* (**B**) or *clpX* mutants (**C**) are indicated in the first column (Total). The numbers of isolated colonies with a disrupted *clpP* (clpP::Ω) or *clpX* gene (clpX::Ω) in the chromosome are listed in the second column, and the third column shows the numbers of isolated colonies with a restored wild-type *clp* locus after the second recombination ($clpP^+$, $clpX^+$). Colonies that had acquired sucrose-resistance through *sacB* inactivation are listed in the fourth column ($sacB^-$). The plasmids carrying *clpP* (pUJ148, pU158) (B) or *clpX* (pUJ145, pUJ170) (C) are indicated on the left and the cloned fragments of the *clp* locus are shown schematically for each construct. The *clpPS107A* allele carried by plasmid pTF33 (B) contains a mutated codon for the active site serine of the ClpP protein. E, *Eco*RI; P, *Pst*I; M, *Mlu*I; B, *Bam*HI; X, *Xho*I; H, *Hin*dIII.

interposon in the *clp* locus. Approximately 600 sucrose-resistant colonies were analyzed both for *clpP* and *clpX* but none of them contained a chromosomal null mutant (Figure 2B and C). Most of the colonies tested (512 out of 614 for *clpP* and 550 out of 600 for *clpX*; Figure 2) had reverted to the wild-type genotype. Since this was a strong indication that both *clp* genes are essential for growth of *C.crescentus*, we asked if chromosomal mutations could be obtained in the presence of an additional, plasmid-encoded, functional copy of *clpP* or *clpX*. Two constructs, pUJ148 and pUJ158, that contained either *clpP* alone or *clpP* with flanking regions, allowed the isolation of chromosomal *clpP* mutants when present in strain LS2037 (Figure 2B). Similarly, in the presence of plasmids pUJ145 or pUJ170, carrying either the entire *clp* region or *clpX* alone, chromosomal *clpX* mutants were readily obtained from strain UJ127 (Figure 2C). This suggested that both *clpP* and *clpX* are essential genes in *C.crescentus* and that the proteolytic activity of ClpXP is strictly required for growth or survival of *Caulobacter* cells.

To confirm that the proteolytic activity of ClpP is essential, we changed the codon for the active site serine residue of *clpP* (S107) (Maurizi *et al*., 1990) to an alanine codon and tested the ability of the *clpP*S107A allele to rescue a chromosomal *clpP* mutation. Out of 200 sucrose-resistant colonies tested for strain LS2037/pTF33, none had a mutated *clpP* copy in the chromosome (Figure 2B). Since chromosomal *clpP* mutants could easily be isolated in the presence of plasmid pUJ158 (Figure 2B), which differs from construct pTF33 only in the *clpP* codon for Ser107, we conclude that the essential nature of ClpP is based on its proteolytic activity.

To study the phenotype of *clpP* and *clpX* mutants, strains were constructed with a single functional copy of either *clpX* or *clpP* under the control of the xylose-dependent promoter, P_{xylX} (Meisenzahl *et al*., 1997). Translational fusions between either *clpP* or *clpX* and the first six codons of *xylX* were created (pUJ174 and pUJ175) and introduced by homologous recombination into the chromosomal *xylX* locus of strains LS2037 and UJ127, respectively (see Materials and methods). A sucrose-selection step (see above) was carried out with the resulting strains in order to isolate mutants with a disrupted *clpP* or *clpX* copy at the chromosomal *clp* locus and the corresponding wild-type gene integrated at the *xylX* locus. Because these mutants should be strictly dependent on xylose for their growth, the sucrose selection was done on plates containing both sucrose and xylose. Since addition of glucose was shown to inhibit P_{xylX} activity (Meisenzahl *et al*., 1997) we also performed a control experiment where the same selection was carried out on plates containing sucrose and glucose. Loss of the functional copy of the *clpP* or *clpX* gene at the *clp* locus was only possible when xylose was present in the selective medium. On medium containing glucose all sucrose-resistant colonies either had the wild-type *clp* locus restored by recombination or contained an inactivated *sacB* gene (data not shown).

ClpP and ClpX are required for normal cell division, growth and viability in C.crescentus

The growth of both strains isolated, UJ199 (NA1000 *clpP*::Ω *xylX*::*clpP*) and UJ200 (NA1000 *clpX*::Ω *xylX*::*clpX*), was strictly dependent on xylose (Figure 3A). The few colonies of strains UJ199 and UJ200 which were able to grow on PYE complex medium containing glucose (PYEG; Figure 3A) represent spontaneous mutations that either relax the xylose dependence of P_{xylX} activity or suppress the strict requirement of the cells for ClpP and ClpX, respectively (U.Jenal, unpublished). Strains UJ199 and UJ200 are tetracycline-resistant due to a resistance gene carried by the inserted constructs pUJ174 and pUJ175, respectively. In order to propagate plasmids with a tetracycline-marker in these strains, we also generated identical *clpP* and *clpX* mutants that had a kanamycin-resistance marker by exchanging plasmids pUJ174 and pUJ175 in strains UJ199 and UJ200 with plasmids pUJ167 and pUJ168 (see Materials and methods). The resulting strains UJ270 (*clpP* mutant) and UJ271 (*clpX* mutant) were also xylose-dependent showing no growth on PYEG plates (data not shown). Introduction of plasmid pTF3 carrying a functional *clpP* gene abolished xylose dependence of strain UJ270; thus the interposon insertion in *clpP* had no polar effect on the expression of the downstream gene *clpX* (Figure 3A). Introduction of the *clpP*S107A allele on plasmid pTF33, did not stimulate growth of strain UJ270 on PYEG (Figure 3A).

To demonstrate the absence of ClpP and ClpX in the mutant strains UJ199 and UJ200 grown under restrictive conditions, immunoblots were carried out with antibodies (see Materials and methods) prepared against either ClpP or ClpX (Figure 3B). Both antibodies reacted with a single protein band of the expected size present in wild-type extracts (Figure 3B, lanes 1 and 5). The same proteins were present in strain UJ199 and UJ200, respectively, at approximately wild-type concentrations when grown in the presence of xylose (Figure 3B, lanes 2 and 6), but were absent in extracts of the same mutant strains that were grown in media containing glucose (Figure 3B, lanes 3 and 7). Thus strains UJ199 and UJ200 are depleted for the ClpP or ClpX proteins when grown overnight under non-permissive conditions. The ClpP protein was present in strain UJ200 grown under restrictive conditions (Figure 3B, lane 4) demonstrating that the absence of the ClpX protein in this strain had no effect on the level of expression of the ClpP protein. Similarly, the chromosomal *clpP* mutation in strain UJ199 had no effect on the expression of the ClpX protein (Figure 3B, lane 8).

Strains UJ199 and UJ200 required exogenously added xylose for continuous growth both in complex and minimal media. Upon removal of xylose, both strains showed a severe reduction in biomass accumulation and cell viability (Figure 4A and B). Biomass accumulation of UJ199 and UJ200 was measured under non-permissive conditions after overnight cultures were shifted from a xylose-based medium to a medium containing glucose. Both mutant strains increased their biomass exponentially during ~4 generations with and without xylose in the growth medium. After 4 or 5 generations both mutant strains stopped accumulating biomass in glucose medium while continuing to grow normally in medium containing xylose (Figure 4A). The increase in cell numbers of both mutant strains under permissive conditions (PYEX) again reflected exponential growth kinetics (Figure 4B). In sharp contrast, the increase of viable cell counts stopped only 1 or 2 generations after the media shift and subsequently dropped

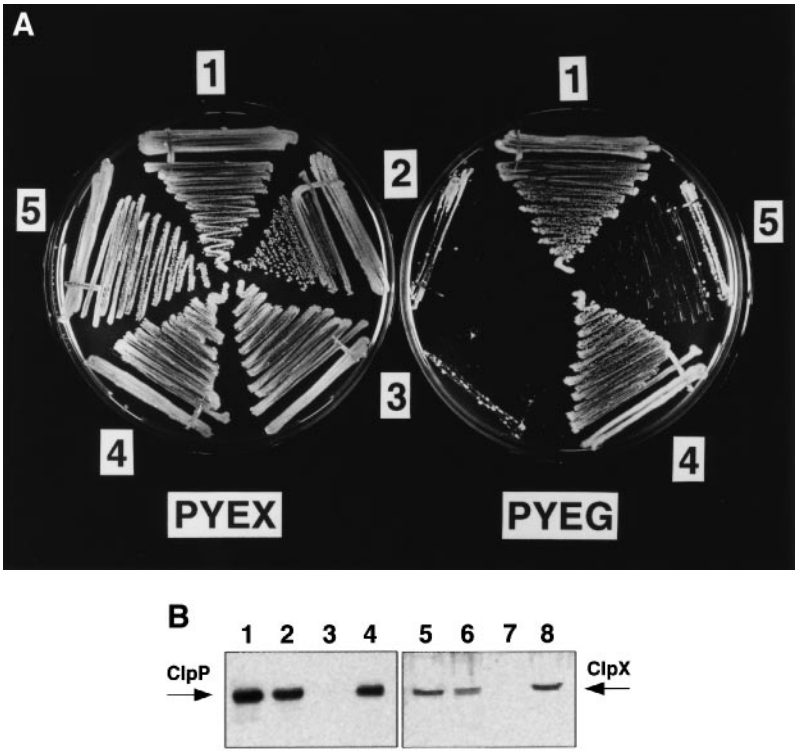

Fig. 3. Conditional *clpP* and *clpX* mutants. Strains were constructed that carry a disrupted *clpP* or *clpX* gene in the chromosomal *clp* locus and a functional copy of either *clpP* (UJ199) or *clpX* (UJ200) inserted in the *xylX* locus under transcriptional control of P_{xylX} (see Materials and methods). (**A**) The *clpP* and *clpX* mutant strains and are strictly dependent on xylose for growth. Colonies of the following strains were streaked onto plates containing complex media supplemented with 0.1% of either xylose (PYEX) or glucose (PYEG): 1, NA1000 (wild-type); 2, UJ199 (NA1000 *clpP*::Ω *xylX*::*clpP*); 3, UJ200 (NA1000 *clpX*::Ω *xylX*::*clpX*); 4, UJ270 / pTF3 [NA1000 *clpP*::Ω *xylX*::*clpP* / pTF3 (*clpP*)]; 5, UJ270 / pTF33 [*clpP*::Ω *xylX*::*clpP* / pTF33 (*clpP*S107A)]. A wild-type copy of *clpP* (pTF3) but not the *clpP*S107A allele (pTF33) was able to rescue growth of strain UJ199 on glucose. (**B**) Conditional expression of ClpP and ClpX from the *xylX* promoter. Crude extracts of strain NA1000 (wild-type, lanes 1 and 5), UJ199 (NA1000 *clpP*::Ω *xylX*::*clpP*) grown on PYEX (lane 2), UJ199 grown on PYEG for 12 h (lane 3 and 8), UJ200 (NA1000 *clpX*::Ω *xylX*::*clpX*) grown on PYEG for 12 h (lane 4 and 7), and UJ200 grown on PYEX (lane 6) were stained with an anti-ClpP (lanes 1–4) or anti-ClpX antibody (lanes 5–8) as described in Materials and methods.

drastically for both *clp* mutant strains grown under restrictive conditions (PYEG). After 8 or 9 generations of growth on media without xylose, the viable cell numbers of the *clpP* and *clpX* mutants had dropped 4–5 orders of magnitude compared with the same strains supplemented with xylose (Figure 4B). It is interesting to note that loss of ClpX (UJ200) had a more drastic effect on both growth and viability than depletion of ClpP (UJ199) (Figure 4). This could be due to either a higher persistence of the ClpP protein at the restrictive conditions (see below) or to a stricter requirement for the ClpX protein during growth.

ClpP antibodies and ClpX antibodies were used to monitor the concentrations of the proteins in strains UJ199 and UJ200, after removal of xylose (Figure 4C). Most of the cellular ClpP and ClpX had disappeared 6 h later (corresponding to about four cell doublings), demonstrating that there is a clear correlation between the concentrations of the ClpP and ClpX proteins and the phenotypic behavior of the xylose-dependent mutant strains. We conclude that loss of growth and viability is a direct effect of decreasing cellular concentrations of ClpP or ClpX.

Mutations in clpP or clpX result in cell-cycle arrest

Both *clp* mutant strains, UJ199 and UJ200, accumulated biomass in the absence of the inducer for up to 7 generation-time equivalents, although a decrease of viable cell numbers was detected by 2 generations after the shift to restrictive conditions. This suggests that removal of ClpP or ClpX resulted in an inhibition of cell division long before affecting the increase of biomass. In agreement with this, the cells of strains UJ199 and UJ200 were filamentous when grown in a xylose-free medium for 12 h. Figure 5 shows light microscopy images of strains UJ199 and UJ200 grown under permissive and restrictive conditions. In the absence of xylose both strains show a drastically elongated and filamentous morphology indicative of an inhibition of cytokinesis (Figure 5B and D). In most cells division had not initiated or had stopped at an early stage. A fraction of the cells, which apparently had undergone initiation of division, remained attached to each other via a short stalk-like structure suggesting another role for ClpP or ClpX during a later stage of cell division. Both mutant strains showed a normal morphology when grown on complex medium containing xylose (Figure 5A and C) confirming that the abnormal cell morphology was a result of the absence of ClpP or ClpX.

The inhibition of cell division could be a direct and immediate effect of the absence of the ClpXP protease. Alternatively, ClpXP could be required for an earlier event, which is acting as a checkpoint for processes occurring later during cell division. Since later steps in the cell cycle such as cytokinesis are dependent on the completion of chromosome replication (Osley and Newton, 1977), we tested whether depleting cells for either ClpP

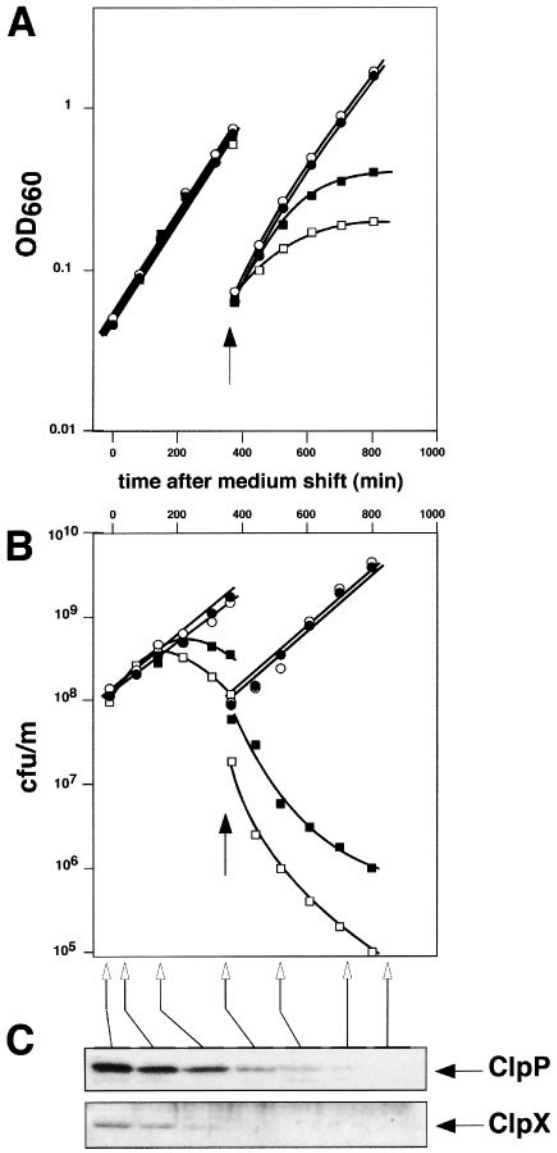

Fig. 4. *clpP* and *clpX* are required for growth and viability of *C.crescentus*. Biomass accumulation (monitored as optical density at 660 nm) (**A**) and viable cell counts (measured as colony forming units per ml) (**B**) were determined for strains UJ199 (*clpP*::Ω *xylX*::*clpP*) (■) and UJ200 (*clpX*::Ω *xylX*::*clpX*) (□) after medium shift from PYE supplemented with 0.1% xylose (PYEX) to PYE supplemented with 0.1% glucose (PYEG). As a control, growth and viability of UJ199 (●) and UJ200 (○) were also monitored after resuspending in xylose-containing medium. To prevent cultures from reaching stationary phase they were diluted 1:10 into fresh PYEX or PYEG medium (marked by arrows). (**C**) The relative concentration of ClpP in strain UJ199 (top panel) and ClpX in strain UJ200 (bottom panel) was monitored immunologically (see Figure 3) after medium shift from PYEX to PYEG. Open arrows indicate the time after the medium shift when culture samples were removed for Western blot analysis.

or ClpX would result in a block in DNA replication. For this purpose cells were stained with the DNA-specific dye YO-PRO-1 and the DNA content per cell was measured by flow cytometry (Winzeler and Shapiro, 1995). Control experiments confirmed that SW cells (G_1 phase) contain a single chromosome (Figure 6B), and late PD cells (G_2 phase) contain two chromosomes (Figure 6C). A mixed culture includes G_1 and G_2 cells, and actively replicating cells (ST and early PD; S phase) with between one and two chromosomes (Figures 7A). Wild-type cells that had been treated with rifampicin for 3 h contained either one or two chromosomes but no actively replicating DNA (Figure 6D). To assay the chromosome content of the *clp* mutants, strains UJ199 and UJ200 were grown on PYEX and then shifted to PYEG medium for 12 h. Complete depletion for either ClpP or ClpX resulted in a clear reduction of cells containing more than one chromosome. In the ClpP-depleted culture a dominant peak corresponding to one chromosome was observed although a significant fraction of the cells had a higher chromosome content (Figure 6F). The latter could account for cells that had not completely separated in the absence of the ClpP protein (see Discussion). In a ClpX depleted culture, G_1 cells predominated (Figure 6H). Under permissive conditions the chromosome pattern of the mutant strains (Figure 6E, G) was comparable to wild-type cells (Figure 6A).

Thus, depletion of *C.crescentus* cells for either ClpP or ClpX resulted in a defect in initiation of chromosome replication, most prominent in the *clpX* mutant. To confirm the replication initiation defect of UJ200 mutant cells, DNA synthesis was monitored by incorporation of radioactively labeled dCTP over the course of one cell cycle. When UJ200 was grown under non-permissive conditions (glucose) for 6 h (see below), DNA synthesis was strongly reduced, as compared with cells that were allowed to proceed through the cell cycle in the presence of the inducer (data not shown).

ClpXP is required for degradation of a replication control protein during G_1-to-S phase transition

The replication block of SW cells is released during the G_1-to-S transition by dephosphorylation and specific degradation of the CtrA response regulator protein (Quon *et al*., 1998). Expression of a mutant CtrA protein that is both constitutively active and stable results in cells arrested in G_1 similar to results obtained for the *clpP* and *clpX* mutant strains (Domian *et al*., 1997). This prompted us to test whether ClpXP is required for the cell-cycle-dependent degradation of the CtrA protein. Immunoblot analysis of strains UJ199 and UJ200 revealed that CtrA levels were slightly increased in cells grown on glucose as compared with cells grown on xylose (data not shown). To analyze if this was due to a lack of CtrA turnover during G_1-to-S transition we assayed CtrA levels as a function of the cell cycle in the presence or absence of the ClpP and ClpX proteins. Cultures of strains UJ199 and UJ200 were grown on xylose-containing medium (PYEX) and subsequently shifted to restrictive conditions (PYEG) for 6 h (4 generations). At this stage the cells had lost most of their ClpP (UJ199) or ClpX (UJ200) proteins (Figure 4C) but enough SW cells could still be isolated for the subsequent cell-cycle experiment. Purified SW cells were resuspended in complex medium containing either xylose or glucose and allowed to proceed through one cell cycle. The timing of stalk formation and cell elongation was comparable to wild-type cells and was independent of the medium supplement suggesting that the ClpXP protease is not required for the SW-to-ST cell transition. However, while most of the cells of both UJ199 and UJ200 grown in the presence of xylose initiated septation, developed motility, and had divided after 90

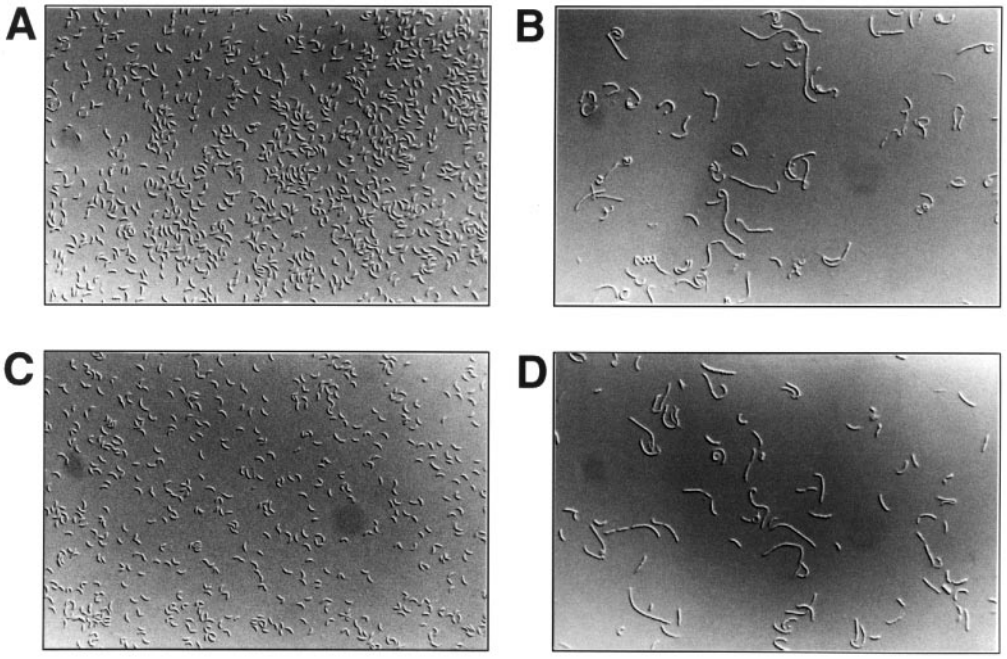

Fig. 5. *clpP* and *clpX* mutants are defective in cell division. Cultures of strains UJ199 (**A** and **B**) and UJ200 (**C** and **D**) were grown in xylose-containing complex medium (PYEX) and shifted to permissive conditions (PYEX) (A and C) or restrictive conditions (PYEG) (B and D) for 12 h. Cell morphology was then analyzed and photographed on a Olympus AX70 microscope with DIC (Nomarski) optics.

min, the majority of the cells grown without xylose arrested as elongated cells without initiating septation even after an extended period (data not shown). Immunoblot analysis of cell extracts from UJ199 and UJ200 using an anti-CtrA antibody (Domian *et al*., 1997) revealed that both strains degraded CtrA normally when going through the cell cycle in a medium containing xylose (Figure 7A and B). However, CtrA was not degraded during the SW-to-ST cell transition in either strain grown without xylose. Immunoblots with antibodies against ClpP and ClpX confirmed that both proteins were absent in the synchronized SW cells but were resynthesized rapidly in the mutant strains grown in the presence of xylose (Figure 7A and B). In contrast, synchronized cells of both mutant strains that had been resuspended in glucose containing medium did not synthesize ClpP or ClpX (Figure 7A and B). Thus, there is a clear correlation between the presence of the Clp proteins and the cell-cycle-dependent degradation of CtrA.

The results shown in Figure 7 suggest that the ClpXP protease is directly responsible for degradation of the cell-cycle transcriptional regulator CtrA during the G_1-to-S transition. This is supported by the observation that overexpression of the *ctrA* wild-type gene from a high copy number plasmid caused a slightly filamentous phenotype in strains UJ199 and UJ200 even when grown under permissive conditions (data not shown). In a wild-type background, overexpression of the *ctrA* gene was shown to have no visible effect (Domian *et al*., 1997). The activity of the CtrAD51E mutant protein is independent of phosphorylation and its inactivation upon entry into S phase relies entirely on proteolysis (Domian *et al*., 1997). When the *ctrAD51E* allele (Quon *et al*., 1996), was overexpressed in *clpP* and *clpX* mutant strains, the cells had a very strong cell division defect at the permissive conditions (PYEX). This phenotype was identical to that of the

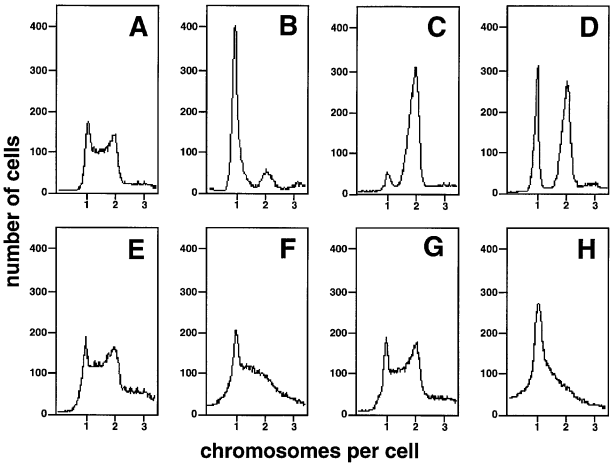

Fig. 6. Replication initiation is affected in *clpP* and *clpX* mutants. The DNA content of wild-type cells (A–D), *clpP* mutant strain UJ199 (E and F), and *clpX* mutant strain UJ200 (G and H) was measured by flow cytometry. A mixed population (**A**), G_1 cells (**B**), G_2 cells (**C**) and rifampicin-treated cells (**D**) of wild-type strain NA1000 were stained and analyzed as controls. Strains UJ199 and UJ200 were grown for 12 h on PYEX (permissive) (**E** and **G**, respectively) or PYEG medium (restrictive) (**F** and **H**, respectively) before they were fixed with ethanol and stained with YO-PRO-1 and analyzed. G_1 cells contain one chromosome, G_2 cells contain two chromosomes, and actively replicating S phase cells contain between one and two chromosomes.

clp mutants grown for an extended period under non-permissive conditions (Figure 5B and D). When strains UJ199 and UJ200 containing the *ctrAD51E* allele were shifted to the non-permissive conditions (PYEG), they arrested growth immediately (data not shown) indicating a strict requirement of ClpP and ClpX for CtrAD51E removal.

The degradation of CtrA by ClpXP during the cell

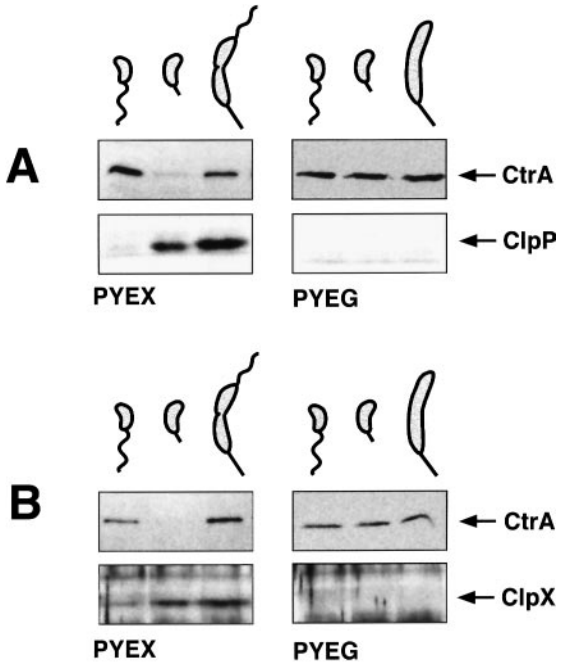

Fig. 7. ClpX and ClpP are required for cell-cycle-dependent proteolysis of the replication control protein CtrA. CtrA turnover was assayed in *clpP* mutant strain UJ199 (**A**) and in *clpX* mutant strain UJ200 (**B**) under permissive (PYEX) and restrictive conditions (PYEG). Cultures were depleted for either ClpP or ClpX and synchronized. SW cells were resuspended in PYEX or PYEG and allowed to proceed through the cell cycle. Levels of CtrA, ClpP, and ClpX were determined in SW cells, ST cells, and in PD cells. Immunoblots of cell extracts from UJ199 and UJ200 were stained with CtrA-specific antibody (A and B, upper panels), ClpP-specific antibody (A, lower panels), and ClpX-specific antibody (B, lower panels).

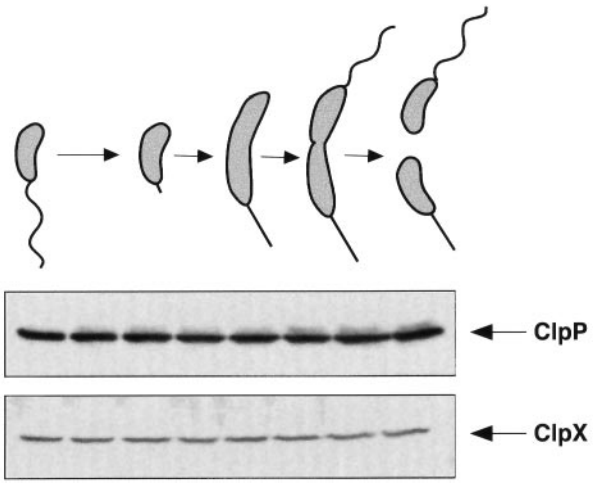

Fig. 8. ClpP and ClpX levels do not fluctuate during the cell cycle. Steady-state levels of ClpP and ClpX were determined in synchronized *C.crescentus* wild-type (NA1000) populations throughout the cell cycle. Immunoblots of cell extracts were stained with ClpP-specific (upper panel) or ClpX-specific antibody (lower panel). The stages of the cell cycle are indicated schematically.

cycle must be a tightly controlled event. Several possible mechanisms exist for degradation control. One possibility is that the cell simply restricts the expression of one or both components of the ClpXP protease to only the period of the cell cycle or the compartment of the PD cell where their activity is needed. To test if the ClpP or ClpX protein levels fluctuate during the cell cycle we monitored their concentration during the cell cycle of NA1000 wild-type cells by immunoblot. We found that neither the ClpP nor the ClpX protein concentration changed during the course of one cell cycle (Figure 8). These results exclude a mechanism of temporal separation of substrate and protease.

Discussion

We show here that ClpXP, an essential bacterial protease, is required for the cell-cycle-dependent turnover of CtrA, an inhibitor of G_1-to-S transition in the eubacterium *C.crescentus*. Mutant *C.crescentus* strains that either lack the regulatory or the proteolytic subunit of the ClpXP protease are blocked in the cell division process; flow cytometry experiments indicated that the mutants arrested in G_1 before initiation of chromosome replication. The involvement of ClpXP in temporally controlled degradation of a key cell-cycle regulator and its requirement for cell-cycle progression emphasize the crucial role of this protease in *C.crescentus* cell-cycle control. The nature of this control is remarkably similar to the mechanism that regulates the G_1-to-S transition in the budding yeast *Saccharomyces cerevisiae*. In eukaryotes, different types of CDK complexes trigger the transition from one phase of the cell cycle to the next (Nigg, 1995). Highly specific proteolytic events remove the cyclin proteins at stages when they are no longer required or deleterious (King *et al*., 1996; Hoyt, 1997). In *S.cerevisiae*, the Sic1 cyclin inhibits CDKs which promote S phase. Sic1 is specifically removed at the end of G_1 to allow initiation of the S phase and DNA replication (Schwob *et al*., 1994). Specificity is conferred by a ubiquitin-conjugating complex that targets Sic1 for degradation by the 26S proteasome (Hoyt, 1997). Similarly, CtrA inhibits the G_1-to-S transition in *Caulobacter* and is also specifically targeted for degradation to allow cell-cycle progression. Thus, as is the case for eukaryotic cyclins, oscillation of CtrA levels in *Caulobacter* is the result of combined transcriptional and proteolytic regulation (Quon *et al*., 1996; Domian *et al*., 1997; this paper).

CtrA belongs to the superfamily of response regulators, most members of which control transcriptional activity of target promoters in response to aspartyl phosphorylation by a cognate sensory kinase (Parkinson and Kofoid, 1992). CtrA has been shown to control the expression of cell-cycle-regulated genes encoding flagellar proteins, a DNA methyltransferase and cell division proteins (Quon *et al*., 1996; Kelly *et al*., 1998). CtrA is also a critical element of DNA-replication control in *C.crescentus* (Quon *et al*., 1998).

CtrA activity is temporally and spatially controlled at several levels: synthesis, phosphorylation and degradation (Quon *et al*., 1996; Domian *et al*., 1997). Specific degradation of CtrA occurs during the G_1-to-S transition and in the ST compartment of the late PD cell (Domian *et al*., 1997). This observation immediately poses an important question: how is CtrA degradation restricted to a specific compartment of the *C.crescentus* cell and to a distinct period of its cell cycle? The finding that ClpXP is involved in CtrA turnover prompts us to consider this problem in terms of how the protease recognizes its substrate and

how this activity is controlled? With the *E.coli* ClpXP protease complex, the ATPase subunit is responsible for substrate recognition, disassembly and presentation to the protease active sites (reviewed in: Gottesman *et al*., 1997a,b). Specificity is based on selective interactions between PDZ-like domains in ClpX and disordered C-terminal tails of the substrate proteins (Levchenko *et al*., 1997a). As with some of the C-termini of known ClpXP substrates in *E.coli*, the C-terminus of CtrA is essential for degradation (Domian *et al*., 1997). Even though a direct interaction between CtrA and ClpXP remains to be demonstrated, we hypothesize that the CtrA C-terminus is one part of the protein that is selectively recognized by ClpX. A more indirect role for ClpXP in CtrA abundance cannot, at this point, be ruled out completely. However, the observation that constitutive overexpression of CtrA did not alter its degradation pattern during the cell cycle (Domian *et al*., 1997), strongly argues against ClpXP controlling expression of CtrA.

Flow cytometry studies show that most of the ClpP- or ClpX-depleted filamentous cells contain a single chromosome, suggesting that initiation of DNA replication is disrupted in these cells. Evidence for such a defect after ClpP- or ClpX-depletion was also obtained from directly measuring rates of DNA synthesis during the cell cycle. Thus, CtrA stabilization at the G_1-to-S boundary coincides with a clear reduction of DNA replication initiation. However, replication is not completely abolished in these cells. It is possible that the low levels of ClpP or ClpX observed still allow some residual replication initiation activity but are not sufficient to degrade CtrA. Stabilizing CtrA alone is not sufficient to cause a G_1 arrest (Domian *et al*., 1997). This argues against CtrA degradation being the only essential function of the ClpXP protease. Therefore, we propose that the cell-cycle arrest observed in ClpX- or ClpP-depleted cells must be caused by the stabilization of one or several additional proteins (Figure 9). It is intriguing to speculate that members of the phosphorelay system that activates CtrA (Shapiro and Losick, 1997; Wu *et al*., 1998) could be degraded by ClpXP during the cell cycle in a CtrA-like manner. In this case mutational disruption of ClpXP would not only lead to stabilization of CtrA but, due to an increased level of phosphorelay components, could also result in an increased flow of phosphate through the relay. This in turn would lead to an increased phosphorylation status of a stabilized CtrA protein, a situation that has been shown to result in a block of chromosomal replication initiation and G_1 arrest, and thus could explain the cell-cycle phenotype observed in our *clp* mutants. Alternatively, stabilization of other ClpXP substrates which do not affect CtrA activity could contribute to the cell-cycle arrest.

Our observation that the block in initiation of DNA replication was more pronounced after depletion of ClpX as compared with ClpP-depletion, could be explained by a ClpP-independent role for ClpX in replication control. In *E.coli* both ClpA and ClpX have been shown to function as chaperones involved in assembly and disassembly of DNA replication and transposition complexes (Wickner *et al*., 1994; Levchenko *et al*., 1995; Wawrzynow *et al*., 1995; Kruklitis *et al*., 1996; Konieczny and Helinski, 1997). In each of these cases it has been shown that the chaperone disassembles multimeric complexes of the substrate proteins into active monomers *in vitro*. It is intriguing to speculate that ClpX in *C.crescentus* might also be involved in remodelling of protein substrates. Disaggregation of CtrA multimers bound to the Cori (origin of replication) prior to their degradation could be one such activity. It is possible that while the chaperone activity of ClpX is required for CtrA disassembly, CtrA degradation is a non-essential consequence of that former event. A protease-independent role for ClpX in *C.crescentus* is supported by the finding that *C.crescentus* does not tolerate increased concentrations of the ATPase, while overexpression of ClpP has no consequences for the cells (U.Jenal, unpublished). The fact that fluctuations in ClpX levels have deleterious effects on the cell suggests that the ClpX concentration needs to be tightly controlled. In agreement with this, we have found that in *C.crescentus*, unlike in *E.coli*, *clpX* is not under heat-shock control, and is not transcribed from the same promoter as *clpP*, but rather from at least three different promoters (M.Osteras, A.Stotz, S.Schmid Nuoffer and U.Jenal, manuscript in preparation).

The activity of the ClpXP protease must be under tight temporal control during the *C.crescentus* cell cycle. A simple control mechanism would be to restrict the presence

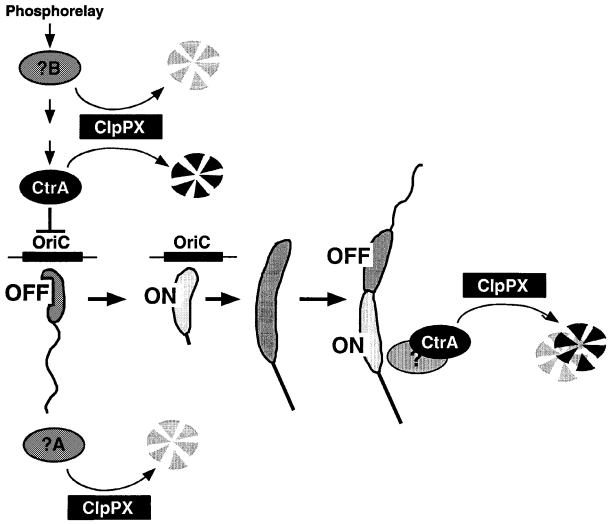

Fig. 9. Model for *C.crescentus* cell-cycle control by the ClpXP protease. ClpXP is required for degradation of CtrA during the G_1-to-S transition and presumably also for removal of CtrA from the ST compartment of the late PD cell (G_2). The phosphorylated form of CtrA blocks replication in the SW cell and in the SW compartment of the late PD cell (OFF) (Quon *et al*., 1998). Degradation and dephosphorylation of CtrA result in the onset of DNA replication in the ST cell and in the ST compartment of the late PD cell (ON) (Domian *et al*., 1997). Since CtrA alone cannot account for the essential nature of ClpXP we postulate one or several additional substrates (marked with '?') for the protease that have to be degraded to allow cells to proceed through the cell cycle. The observed cell-cycle arrest of the *clp* mutants suggests that stabilization of the additional substrate(s) result in a replication block similar to the one in *C.crescentus* cells expressing a stable and constitutive CtrA protein. The additional ClpXP substrates could fall into two groups: (i) proteins that do not affect activity of CtrA and upon stabilization lead to a CtrA-independent cell-cycle arrest; and (ii) proteins that affect CtrA activity [i.e. components of the phosphorelay that results in phosphorylation of the CtrA protein (Wu *et al*., 1998)] and upon stabilization would lead to increased concentrations of phosphorylated CtrA protein. Increased levels of phosphorylated CtrA combined with an increased stability of CtrA would then account for the G_1 cell-cycle arrest observed in clp mutants.

of the ATPase to the corresponding time window or compartment. We have found that both ClpP and ClpX levels are not under cell-cycle control in *C.crescentus* arguing against such a mechanism. However, we cannot exclude the possibility that ClpP and ClpX are separated physically inside the same cell interacting only during specific stages of the cell cycle. Alternatively, ClpP and/or ClpX activity itself could be modulated via the interaction with a regulatory protein. Such a mechanism has been shown for the control of the Lon protease activity in *E.coli* by the bacteriophage T4 protein PinA (Hilliard *et al*., 1998a,b). Finally, the accessibility of CtrA could determine its temporal and spatial degradation pattern. Precedents for such a mechanism is the interaction of ClpX with the Mu transposase, MuA (Levchenko *et al*., 1997b), the degradation of the *E.coli* heat-shock sigma factor, RpoH, by the FtsH protease (Tomoyasu *et al*., 1995), and the degradation of the *E.coli* stationary-phase sigma factor, RpoS, by ClpXP (Zhou and Gottesman, 1998). Interestingly, turnover of RpoS in *E.coli* seems to be controlled by a response-regulator protein, RssB (also called SprE and MviA) (Muffler *et al*., 1996; Pratt and Silhavy, 1996; Zhou and Gottesman, 1998). One of the major tasks in the future will be to determine by which of the above mechanisms the activity of the ClpXP protease is regulated during the *C.crescentus* cell cycle.

Materials and methods

Bacterial strains, plasmids, media, enzymes and chemicals

The strains and plasmids used in this study are shown in Table I. *Escherichia coli* strains were grown at 37°C in LB (Sambrook *et al*., 1989), supplemented with ampicillin (100 μg/ml), carbenicillin (100 μg/ml), kanamycin (30 μg/ml), tetracycline (10 μg/ml), chloramphenicol (30 μg/ml), spectinomycin (50 μg/ml) or streptomycin (30 μg/ml) as necessary. *Caulobacter crescentus* strains were grown in either PYE complex medium or M2 minimal glucose (Ely, 1991) and M2 minimal xylose medium (0.2% xylose), respectively, supplemented with nalidixic acid (20 μg/ml), kanamycin (20 μg/ml), tetracycline (2 μg/ml), where indicated.

A horseradish peroxidase (HRP) conjugate of goat anti-rabbit IgG was purchased from Gibco-BRL (Gaithersburg, MD), Renaissance Western Blot chemiluminescence reagent from DuPont NEN (Boston, MA), restriction enzymes from New England Biolabs (Beverley, MA), resin for purification of His-tagged proteins from Novagen (Madison, WI), kits for the isolation and purification of plasmid DNA from Qiagen (Hilden, Germany), the 'QuickChange' kit for site-directed mutagenesis from Stratagene (La Jolla, CA), and YO-PRO-1 DNA stain for flow cytometry was from Molecular Probes (Eugene, OR).

Overexpression and purification of ClpP and ClpX His-tagged fusion proteins and generation of antibodies

A fragment of the *clpP* gene encoding amino acids 1–205 was generated by PCR and cloned into pET-21a resulting in construct pETP. A fragment of the *clpX* gene encoding amino acids 1–417 was generated by PCR and cloned into pET-21a resulting in construct pETX. These constructs contained protein fusions of either *clpP* or *clpX* with an N-terminal T7 epitope tag and a C-terminal His tag. DNA sequence analysis was performed to verify the in-frame nature of the fusions.

To induce expression of the *clp* fusion genes in *E.coli*, BL21(DE3) was transformed with pETP and pETX. Cultures of the obtained strains were grown to an OD_{600} of 1.0 and then induced by adding IPTG to a final concentration of 1 mM. Three h after induction the cells were harvested by centrifugation and lyzed by sonication. The overexpressed fusion proteins were found in inclusion bodies and were concentrated and washed by consecutive centrifugation steps. Resolubilization of inclusion bodies was performed in buffer containing 6 M urea and affinity purification on Sephadex nickel columns was carried out as described earlier (Jenal *et al*., 1994). The purified fractions were separated on a preparative SDS–polyacrylamide gel and the bands corresponding to the ClpP and ClpX fusion proteins were cut out. The polyacrylamide was crushed, mixed with Freund's adjuvant and used to immunize rabbits. Immunization and sampling of the sera was performed by Eurogentec (Seraing, Belgium).

Construction of chromosomal clpP and clpX interposon mutations

To construct chromosomal *clpP* and *clpX* mutants both genes were independently disrupted by insertion of an omega cassette (Prentki and Krisch, 1984) and the chromosomal wild-type genes were exchanged by the mutated copies via homologous recombination. A 2.7 kb *Eco*RI–*Bam*HI fragment containing *clpP* and its flanking regions was cloned into the vector pNPT228 resulting in construct pUJ112. A *Sma*I fragment containing the omega cassette was then cloned into the blunt-ended single *Xho*I site in *clpP* resulting in construct pUJ115. An *Afl*II–*Spe*I fragment containing the *clp* region with the interposon was then subcloned from pUJ115 into pNPTS138, to produce pUJ116. pNPTS138 contains two selection markers for the subsequent homologous recombination step: *nptI*, conferring kanamycin resistance; *sacB*, conferring sucrose sensitivity. To isolate a disrupted *clpX* copy a 2.2kb *Apa*I–*Eco*RV fragment containing *clpX* and flanking regions was cloned into pNPTS138 resulting in construct pTF20. A *Sac*I–*Xmn*I internal *clpX* fragment was then exchanged by the *Sma*I fragment containing the interposon resulting in construct pTF21. pUJ116 and pTF21 were integrated into the chromosome of *C.crescentus* wild-type strain NA1000 via conjugation and homologous recombination. The correct loci of integration into the chromosome were verified by Southern blot analysis. The resulting strains (LS2037, NA1000::pUJ116; UJ127, NA1000::pTF21) contained a wild-type and a disrupted copy of either *clpP* or *clpX*. To select for the second recombination event cultures of LS2037 and UJ127 were grown on PYE overnight and aliquots were then spread on PYE plates containing 3% sucrose. Colonies that were able to grow on sucrose were then tested for loss of the plasmid encoded kanamycin resistance and for the presence of the spectinomycin/streptomycin resistance from the interposon. Sucrose-resistant clones that retained kanamycin resistance had not lost the plasmid by homologous recombination and presumably had acquired sucrose resistance through the mutational inactivation of the *sacB* gene. Kanamycin-sensitive, spectinomycin-sensitive colonies had lost the plasmid and recombination left a wild-type *clp* locus in the chromosome of these strains. Mutant strains with a disrupted chromosomal *clpP* or *clpX* copy were identified by their sensitivity to kanamycin and resistance to spectinomycin and streptomycin.

Generation of conditional clpP and clpX mutants

To generate conditional *clpP* and *clpX* mutants, strains were constructed that contained a single functional copy of either *clpP* or *clpX* under the control of the inducible xylose promoter (Meisenzahl *et al*., 1997). Primers ClpP5′RI (5′-AAA GAA TTC ATG ATG TAC GAT CCG GTT) and ClpP3′Sp (5′-AAA ACT AGT CCG GCG ACA CCG AGA TGC) were used to amplify by PCR the entire *clpP* gene including the downstream transcriptional terminator signals (M.Osteras, A.Stotz, S.Schmid Nuoffer and U.Jenal, manuscript in preparation). Similarly, the *clpX* gene was amplified using primers ClpX5′RI (5′-AAA GAA TTC ATG ACG AAA GCC GCG AGC) and ClpX3′Sp (5′-AAA ACT AGT GCT TCG AAA GCA CGC GCT). Both products were then cloned into the *Eco*RI–*Spe*I sites of plasmid pUJ83, resulting in constructs pUJ165 (*clpP*) and pUJ166 (*clpX*), respectively. This resulted in a translational fusion between the first six codons of the *xylX* gene and the complete *clpP* or *clpX* coding regions. A *Spe*I–*Hin*dIII fragment was then subcloned from pUJ165 (P_{xylX}::clpP) and from pUJ166 (P_{xylX}::clpX) into the vector pPHU281 (Hübner *et al*., 1993) resulting in constructs pUJ175 (*clpP*) and pUJ176 (*clpX*). pUJ175 and pUJ176 were introduced into the chromosomal *xylX* locus strains LS2037 and UJ127, respectively, by conjugational transfer and subsequent homologous recombination. Insertion at the correct loci of plasmids pUJ175 and pUJ176 was verified by Southern blot analysis. The resulting strains had a total of three *clpP* or *clpX* copies: strain UJ218 contained a functional *clpP* copy fused to P_{xylX}, a *clpP* wild-type gene and a *clpP* gene disrupted by an omega cassette in the *clp* locus. Strain UJ220 contained a functional *clpX* copy fused to P_{xylX}, a *clpX* wild-type gene and a *clpX* gene disrupted by an omega cassette in the *clp* locus. To isolate strains that carried a single functional *clpP* or *clpX* gene in their *xylX* locus, sucrose selection was carried out with strains UJ218 and UJ220. Cultures of both strains were grown overnight on PYE complex medium and then spread onto plates containing 3% sucrose and 0.1% xylose or glucose. Sucrose-resistant colonies were then tested for their antibiotic resistance profile as described above. Colonies that were kanamycin sensitive, spectinomycin and

Table I. Strains and plasmids

Strain	Genotype	Reference, source
E.coli		
BL21(DE3)	F$^-$ *lon ompT* r_B^- m_B^- λDE3	Novagen
DH10B	F$^-$*mcrA* Δ(*mrr hsdRMS mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX*74 *end*A1 *rec*A1 *deo*R Δ(*ara, leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L	Gibco-BRL
S17-1	M294::RP4-2 (Tc::Mu) (Km::Tn*7*)	Simon *et al.* (1983)
C.crescentus		
NA1000	*syn*-1000, synchronizable derivative of *C.crescentus* wild-type strain	Evinger and Agabian (1977)
LS2037	NA1000*clpP*::pUJ116	this work
UJ32	LS2037/pUJ158	this work
UJ36	NA1000*clpP*::Ω/pUJ158	this work
UJ127	NA1000*clpX*::pTF21	this work
UJ159	UJ127/pUJ170	this work
UJ199	NA1000*clpP*::Ω*xylX*::pUJ174	this work
UJ200	NA1000*clpX*::Ω*xylX*::pUJ175	this work
UJ218	LS2037*xylX*::pUJ174	this work
UJ220	UJ127*xylX*::pUJ175	this work
UJ222	NA1000*clpX*::Ω/pUJ170	this work
UJ223	NA1000*clpP*::Ω*xylX*::pUJ167/pUJ158	this work
UJ224	NA1000*clpX*::Ω*xylX*::pUJ168/pUJ170	this work
UJ270	NA1000*clpP*::Ω*xylX*::pUJ167	this work
UJ271	NA1000*clpX*::Ω*xylX*::pUJ168	this work

Plasmid	Description	Reference, source
pBBR1MCS-Gent	pBBR1-based Gent^R broad host range vector	Kovach *et al.* (1994)
pBGS18T	Kan^R pUC18 derivative with oriT	D.Alley
pBluescript SK (+)	Amp^R cloning vector	Stratagene
pET-21a	Amp^R vector for protein overexpression	Novagen
pGML11	RK2-based Gent^R broad host range vector	D.Alley
pJS14	pBBR1-based Chlor^R broad host range vector	J.Skerker
pLITMUS28	Amp^R cloning vector	New England Biolabs
pMR10	RK2-based Kan^R broad host range vector	R.Roberts and C.Moor
pMR20	RK2-based Tet^R broad host range vector	R.Roberts and C.Moor
pNPT228	Kan^R derivative of pLITMUS28 with oriT	D.Alley
pNPT228XNE	*xylX* locus in pNPT228	U.Jenal, unpublished
pNPTS138	Kan^R derivative of pLITMUS28 with *sacB* and oriT	D.Alley
pPHU281	Tet^R derivative of pUC18 with oriT	Hübner *et al.* (1993)
pTF3	*Pst*I–*Mlu*I fragment containing *clpP* in pMR20	this work
pTF20	*Apa*I–*Eco*RV fragment containing *clpX* and flanking regions in pNPTS138	this work
pTF21	Omega-cassette inserted in *Sac*I–*Xmn*I *clpX* fragment of pTF20	this work
pTF33	*Pst*I–*Mlu*I fragment containing *clpPS107A* in pMR20	this work
pUJ83	*Nru*I–*Eco*RI fragment from xylose locus in pLITMUS28	this work
pUJ112	*Eco*RI–*Bam*HI fragment containing *clpP* and flanking regions in pNPT228	this work
pUJ115	Omega-cassette inserted in *Xho*I site of *clpP* of pUJ112	this work
pUJ116	*clpP*::Ω and flanking regions from pUJ115 in pNPTS138	this work
pUJ145	*Eco*RI–*Hin*dIII fragment contining the entire *clp* locus in pMR20	this work
pUJ148	*Eco*RI–*Bam*HI fragment containing *clpP* and flanking regions in pMR20	this work
pUJ158	*Pst*I–*Mlu*I fragment containing *clpP* and flanking regions in pJS14	this work
pUJ165	*clpP* in-frame fusion to *xylX* in pUJ83	this work
pUJ166	*clpX* in-frame fusion to *xylX* in pUJ83	this work
pUJ167	*clpP* in-frame fusion to *xylX* in pNPT228XNE	this work
pUJ168	*clpX* in-frame fusion to *xylX* in pNPT228XNE	this work
pUJ170	*Bam*HI–*Xho*I fragment containing *clpX* in pMR20	this work
pUJ174	*xylX–clpP* fusion from pUJ165 in pPHU281	this work
pUJ175	*xylX–clpX* fusion from pUJ166 in pPHU281	this work

streptomycin resistant, as well as tetracycline resistant were then assayed for their growth dependence on xylose by streaking onto PYE plates that either contained 0.1% xylose or 0.1% glucose. Xylose-dependent expression of ClpP in strain UJ199 and of ClpX in strain UJ200 was assayed by immunoblot analysis (see below).

To construct xylose-dependent *clpP* and *clpX* strains with a kanamycin-resistance instead of a tetracycline-resistance marker the *clpP* and *clpX* PCR products (see above) were cloned into the *Eco*RI–*Spe*I sites of vector pNPT228XNE resulting in constructs pUJ167 and pUJ168, respectively. pUJ167 was introduced into the *xylX* locus of strain UJ36 (NA1000 *clpP*::Ω / pUJ158) resulting in strain UJ223, and pUJ168 was introduced into the *xylX* locus of strain UJ222 (NA1000 *clpX*::Ω / pUJ170) resulting in strain UJ224. The only functional *clpP* copy of UJ36 is located on plasmid pUJ158 and strain UJ222 contains a single functional *clpX* copy on plasmid pUJ170. To remove the plasmid borne *clpP* and *clpX* copies from strains UJ223 and UJ224, respectively, pUJ158 was chased with plasmid pBBR1MCS-Gent and pUJ170 was chased with plasmid pGML11. The resulting strains were then restreaked three times without gentamycin selection and clones that had spontaneously lost pBBR1MCS-Gent (UJ270) or pGML11 (UJ271) were isolated. Xylose-dependent expression of ClpP in strain UJ270 and of ClpX in strain UJ271 was assayed by immunoblot analysis (see below).

Immunoblots

Immunoblots were performed as described previously (Jenal and Shapiro, 1996). Polyclonal sera against ClpP or ClpX were diluted 1:5000 and

1:1000, respectively, before use. Polyclonal serum that specifically recognizes CtrA (Domian *et al*., 1997) was diluted 1:5000 before use. Secondary antibody (goat anti-rabbit IgG, HRP-coupled) was used in a 1:10 000 dilution. Immunoblots were developed using the Renaissance kit from DuPont NEN, following the manufacturer's instructions.

Flow cytometry

Mixed populations of NA1000 wild-type strain, UJ199 and UJ200 mutants were grown in PYE complex medium to an OD_{600} of 0.5. *Caulobacter crescentus* wild-type cells were synchronized as described previously (Stephens and Shapiro, 1993) and pure populations of swarmer cells (G_1) or late PD cells (G_2) were harvested at an OD_{600} of 0.5. Mixed and pure cell populations were washed in 1 volume of staining buffer (10 mM Tris, 1 mM EDTA, 50 mM NaCitrate pH 7.2) and fixed immediately in 70% ethanol at –20°C for 1 h. Fixed cells could be stored up to 1 month at –20°C in ethanol. As an additional control, wild-type cells were grown in PYE to an OD_{600} of 0.3 before rifampicin (15 μg/ml) was added to the culture for 3 h. Cells were then harvested and fixed as described above. Fixed cells were washed in 1 volume staining buffer and incubated in 1 volume staining buffer containing 0.1 mg/ml RNaseA for 30 min at room temperature. After adding 0.5 volumes staining buffer containing 1.5 μM YO-PRO-1 the cells were incubated in the dark for 2 h and immediately analyzed with a FACScan (Beckton Dickinson). If necessary, cells were diluted with staining buffer to an optimal cell number of 10^6–10^7/ml. For each measurement the DNA content of 5×10^4 cells was determined.

Cell-cycle experiments

To analyze cell-cycle-dependent CtrA degradation in the mutant strains UJ199 and UJ200 under non-permissive conditions, cultures of both strains were grown in PYE medium containing 0.1% xylose (PYEX) to an OD_{600} of 0.5–0.8. Cells were then washed three times in PYE medium containing 0.1% glucose (PYEG) and diluted into fresh PYEG at an OD_{600} of 0.05. Cultures were grown in PYEG to an OD_{600} of 0.5–0.6 and samples were taken periodically to monitor the decrease of ClpP and ClpX concentrations by immunoblots. After the cultures had reached an OD_{600} of 0.5–0.6 motility had decreased drastically. Cells were then harvested and synchronized as described previously (Stephens and Shapiro, 1993). Pure populations of swarmer cells were resuspended in either PYEG or PYEX and allowed to proceed through the cell cycle. Progression of the cell cycle was monitored microscopically and samples were collected for immunoblot analysis.

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