JOURNAL OF BACTERIOLOGY, Nov. 2010, p. 5991–6000 Vol. 192, No. 22
0021-9193/10/$12.00 doi:10.1128/JB.00440-10

SpdR, a Response Regulator Required for Stationary-Phase Induction of *Caulobacter crescentus cspD*▽

Carolina A. P. T. da Silva, Heloise Balhesteros, Ricardo R. Mazzon, and Marilis V. Marques*

Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 1374, 05508-000 São Paulo, SP, Brazil

Received 16 April 2010/Accepted 30 August 2010

The cold shock protein (CSP) family includes small polypeptides that are induced upon temperature downshift and stationary phase. The genome of the alphaproteobacterium *Caulobacter crescentus* encodes four CSPs, with two being induced by cold shock and two at the onset of stationary phase. In order to identify the environmental signals and cell factors that are involved in *cspD* expression at stationary phase, we have analyzed *cspD* transcription during growth under several nutrient conditions. The results showed that expression of *cspD* was affected by the medium composition and was inversely proportional to the growth rate. The maximum levels of expression were decreased in a *spoT* mutant, indicating that ppGpp may be involved in the signalization for carbon starvation induction of *cspD*. A Tn*5* mutant library was screened for mutants with reduced *cspD* expression, and 10 clones that showed at least a 50% reduction in expression were identified. Among these, a strain with a transposon insertion into a response regulator of a two-component system showed no induction of *cspD* at stationary phase. This protein (SpdR) was able to acquire a phosphate group from its cognate histidine kinase, and gel mobility shift assay and DNase I footprinting experiments showed that it binds to an inverted repeat sequence of the *cspD* regulatory region. A mutated SpdR with a substitution of the conserved aspartyl residue that is the probable phosphorylation site is unable to bind to the *cspD* regulatory region and to complement the *spdR* mutant phenotype.

Bacterial stationary phase is characterized by growth arrest as a result of various external causes, such as nutrient starvation, accumulation of toxic compounds, environmental stresses, etc. The mechanisms utilized by the bacterial cell to cope with these phenomena vary accordingly, but in most cases there is a decrease in ribosome activity leading to a severe reduction of protein synthesis. As the cells proceed into longer periods of growth arrest, a reorganization of cell metabolism ensures that the cells will retain viability, maintaining a minimum of essential cell functions (39).

The gene regulation of this passage from exponential growth into stationary phase responds primarily to the nutrient status of the cell. In enterobacteria, the main orchestrator of this shift in metabolism is the sigma factor σ^S, which substitutes for the vegetative sigma factor σ^{70} in binding to core RNA polymerase and initiating transcription of a different set of genes (19, 33). The σ^S-dependent genes include those involved in the stress response as well as metabolic functions, such as uptake and metabolism of amino acids, sugars, and iron and indole production (28). However, no sigma factor with functions equivalent to σ^S had been identified so far in *Alphaproteobacteria*.

Another important player is the second messenger guanosine-bis-3′,5′-diphosphate (ppGpp), which is involved in the response to translational stress induced by nutrient starvation (41). In *Escherichia coli* the levels of this molecule are regulated by two independent enzymes, the ppGpp synthetase RelA and the bifunctional enzyme SpoT, which comprises both ppGpp synthetase and hydrolase activities (20, 51). Other bacterial groups, including the *Alphaproteobacteria*, contain only one RelA-SpoT homolog, which has both ppGpp synthetase and hydrolase activities (37). It has been demonstrated that ppGpp is important for alternative sigma factor competition for binding the RNA polymerase core during cellular stress (25), causing a reduction in transcription of stable RNAs in favor of amino acid biosynthetic operons and catabolism genes (48).

Among the bacterial proteins that are induced at stationary phase are those containing a cold shock domain (CSD), a protein domain comprising two regions, RNP-1 and RNP-2, that are required for single-strand nucleic acid binding (29, 43). The cold shock protein family includes small polypeptides (7 kDa) that are induced upon temperature downshift and stationary phase (15, 24). Bacterial cells usually have multiple copies of *csp* genes, and different subsets of these have distinct patterns of expression, being induced by one or more of these stimuli: cold shock, stationary phase, high osmolarity, metal, H_2O_2, and antibiotics (8, 15, 16, 26, 42, 52). The main proposed role of these proteins is as RNA chaperones maintaining the nucleic acids free from secondary structure at low temperatures, as described for *E. coli* CspA (24, 26). In addition, some members of this family were proposed to have a role in transcription antitermination of cold-induced genes (*E. coli* CspA, CspC, and CspE), activation of transcription, translation initiation (*Bacillus subtilis* CspB), and inhibition of DNA replication (*E. coli* CspD) (2, 17, 34, 40, 49).

Caulobacter crescentus is a free-living aquatic alphaproteobacterium adapted to low-nutrient conditions, and it has a remarkable cell cycle with an obligatory differentiation step that converts the motile swarmer cell into a sessile stalked cell (23, 46). Its oligotrophic metabolism is ensured by an extensive

* Corresponding author. Mailing address: Av. Prof. Lineu Prestes, 1374, 05508-000 São Paulo, SP, Brazil. Phone: 55-11-3091-7299. Fax: 55-11-3091-7354. E-mail: mvmarque@usp.br.

▽ Published ahead of print on 10 September 2010.

biosynthetic capability, along with a broad range of pathways for utilization of alternative nutrient sources (9, 21, 38). When the population reaches stationary phase, a large percentage of the cells die, and the ones that survive undergo a morphological change, becoming elongated and having a helicoidal morphology. Cells at this stage are adapted to even more restrictive nutrient conditions and have increased resistance to several stresses (50).

C. crescentus NA1000 has four genes containing cold shock domains; these are *cspA* and *cspB*, which are induced by low temperature, and *cspC* and *cspD*, which are induced only at stationary phase (31). While the cold-induced CspA and CspB have a single CSD, the two stationary phase-induced genes encode proteins containing two CSDs with molecular masses of 18.3 kDa (CspC) and 21.5 kDa (CspD), a domain arrangement so far found only in *Alphaproteobacteria* (31). Despite the fact that both proteins have two CSDs, their primary structures are very different, with little amino acid sequence similarity outside the RNP regions, suggesting that they may have overlapping but not identical functions.

Phenotypic analyses of *cspC* and *cspD* null mutants, as well as the deletion of both genes, showed that cell viability is severely decreased in the *cspC* and *cspCD* mutants upon entry into stationary phase (3). The *cspC* cells show aberrant morphology at stationary phase, and this phenotype is more severe in the double mutant, suggesting that *cspD* can at least in part compensate for the lack of *cspC* (3). This also results from the fact that both *cspC* and *cspD* are induced upon entry into stationary phase. The regulatory regions of the two genes have some degree of similarity, and a region upstream of the promoter was shown to be required for maximum levels of expression of *cspD* (31).

In this work we unveiled the regulatory signals that trigger *cspD* expression and identified several genes important for its induction at stationary phase; among these signals is a response regulator (SpdR) that belongs to a two-component system with a histidine kinase (SpdS). This is the first report of a two-component system involved in stationary-phase gene regulation in *C. crescentus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids are described in Table 1. *Caulobacter crescentus* strains were grown in PYE medium or minimal M2 medium (12) at 30°C with shaking. When necessary, antibiotics were added at the following concentrations: kanamycin, 5 μg/ml; tetracycline, 1 μg/ml; chloramphenicol, 1 μg/ml; or nalidixic acid, 20 μg/ml. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium. When necessary, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; or tetracycline, 12.5 μg/ml. Plasmids were introduced into *C. crescentus* by conjugation with *E. coli* strain S17-1.

Complementation and expression vectors. Plasmid pCA38 was constructed by inserting a 3.8-kb ApaI fragment from cosmid 1G4 containing the *spoT* gene into vector pUJ142, generating a complementing vector that expresses *spoT* from its original promoter.

The entire *spdR* coding region was amplified by PCR using primers REG-1 and REG-2 (Table 1) and cloned into vector pUJ142, generating plasmid pCA39, which contains the *spdR* gene expressed by the *xylX* promoter. All the PCRs were carried out with the high-fidelity Pfx enzyme (Invitrogen), using a cloned *spdR* coding region as the template. A modified *spdR* coding region was generated by sequential PCR amplification utilizing two complementary mutagenic primers (MutDA1 and MutDA2 [Table 1]) combined with external primers (REG-1 and REG-2) to amplify fragments containing the replacement of the aspartyl codon in position 64 by an alanine codon. The mutant fragment was sequenced to ensure that the correct mutation was introduced and that no additional mutations were generated during amplification. This fragment containing the *spdR*(D64A) gene was cloned in frame into pUJ142, generating plasmid pCA40, and into the expression vector pPROEX-HTa, generating plasmid pCA401.

Construction of *C. crescentus* Δ*spoT* and Δ*spoT* Δ*spdR* mutant strains. A *spoT* deletion mutant strain was generated by allelic exchange. A fragment of 700 bp located upstream and a fragment of 950 bp located downstream of the *spoT* gene (32) were amplified by PCR with the primers RELA-1/REL-A2 and RELA-3/RELA-4, respectively (Table 1). The two fragments RELA-1/RELA-2 and RELA-3/RELA-4 were cloned sequentially into the suicide vector pNTS138, and it was introduced into *C. crescentus* by conjugation with *E. coli* S17-1. The clones were grown in PYE medium containing 3% sucrose, and those that lost resistance to kanamycin were selected for screening by PCR with primers RELA-4 and RELA-5 (Table 1). One of the clones containing the deletion of *spoT* obtained after two recombination events was confirmed as the *spoT* mutant strain SP0200.

A double Δ*spoT* Δ*spdR* mutant strain was constructed using SP0200 as the background strain and generating a Δ*spdR* deletion as described above. Briefly, the flanking regions of *spdR* were amplified by PCR using primer pairs RR1/RR2 and RR3/RR4 (Table 1), generating amplicons of 950 bp and 970 bp, respectively. The two regions were sequentially cloned into pNPTS138 and introduced in *C. crescentus* SP0200 to generate a deletion of *spdR* by double recombination. The resulting Δ*spoT* Δ*spdR* strain was named SP0210.

Analysis of *cspD* expression. *cspD* expression was determined by β-galactosidase activity assays of *C. crescentus* strains NA1000, SP0200, SP0210, and SP0247 harboring plasmid pEL4, which were grown in PYE for 48 h at 30°C with agitation. Strains NA1000 and SP0200 containing plasmid pEL4 were grown in different compositions of M2 medium as follows: M2G (containing 0.2% glucose), M2X (glucose was replaced by 0.3% xylose), M2P (glucose was replaced by 0.2% peptone), or M2GP (containing both glucose and peptone). The cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.1 in the same respective medium, and when the culture was at mid-log phase the expression of the *cspD* promoter was analyzed at different times of growth by determining β-galactosidase activity as described previously (36). For the assays of glucose and ammonium starvation, cultures of NA1000(pEL4) or SP0200(pEL4) were grown in M2G to mid-log phase. The cells were centrifuged and resuspended in M2 medium containing 0.02% glucose or depleted of NH_4Cl. The expression of *cspD* was analyzed every hour for 5 h and after 24 h by measuring β-galactosidase activity at each time point.

Screening of a transposon mutant library. A library of 7,500 *C. crescentus* mutants containing an insertion of the Tn*5* transposon (reference 22 and this work) was transferred into PYE plates containing kanamycin and incubated at 30°C for 48 h. *E. coli* S17-1 containing the plasmid pEL4 was spread over PYE plates and incubated for 2 h at 37°C, and the colonies of the library were transferred on top of the *E. coli* S17-1 lawn. After growth for 12 h at 30°C, the conjugants were transferred into PYE plates containing kanamycin, tetracycline, and nalidixic acid and incubated at 30°C. The mutants were grown in 150 μl PYE medium in 96-well plates for 2 days, and 4 μl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (20 mg/ml) was added to the cultures. This experiment used NA1000(pEL4) as a positive control and NA1000(pEL5) (31) as a negative control for the intensity of the blue color developed. The colonies that had a color similar to or lighter than that of the negative control were selected for β-galactosidase assays. Clones in which β-galactosidase activity was less than 50% of that of NA1000(pEL5) were selected for identification of the disrupted gene.

Identification of Tn*5* insertion sites. The Tn*5* insertion sites were identified by reverse PCR using primers R3 and L7 as described previously (5). The PCR conditions were as follows: 5 min 95°C; 30 cycles of 1 min at 95°C, 1 min at 42°C or 52°C, and 1 min at 72°C; and a final cycle of 7 min at 72°C. The amplified bands were used for automatic DNA sequencing; the primers used for the sequencing were the same used in the reverse PCR. The identification of the insertion site was determined by comparison to the *C. crescentus* NA1000 genome at the GenBank database.

Immunoblot analysis. The polyclonal anti-CspD serum was previously obtained by immunizing New Zealand rabbits (3). Cultures were grown at 30°C in PYE medium for 48 h, and total protein extracts were obtained by sonication followed by centrifugation to remove cell debris. Equal amounts of proteins were separated by electrophoresis in a 15% polyacrylamide-SDS gel and then transferred to nitrocellulose membranes. The membranes were incubated for 16 h with 1:50 anti-CspD antiserum diluted in TBSTT (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.03% Tween 20, 0.02% Triton X-100) and then with anti-rabbit–alkaline phosphatase conjugate (Sigma) at a dilution of 1:5,000 in TBS containing 5% milk. The bands were developed using 0.5 mg/ml nitroblue tetrazolium

TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this work

Strain, plasmid, or oligonucleotide	Description	Reference or source
Caulobacter crescentus strains		
NA1000	Synchronizable derivative of CB15	13
SP0200	NA1000 Δ*spoT*	This work
SP0247	NA1000 *spdR*::Tn*5*	This work
SP0210	NA1000 Δ*spoT* Δ*spdR*	This work
Δ*spdR* mutant	CB15N ΔCC0247::Tet^r	47
Δ*spdS* mutant	CB15N ΔCC0248::Tet^r	47
E. coli strains		
DH5α	*supE44 lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA11 gyrA96 thi-1 relA1*	18
S17-1	294::RP4-2(Tc::Mu)(Km::Tn*7*)	45
Plasmids		
pEL4	pRK*lacZ*290 with complete *cspD* regulatory region	31
pEL5	pRK*lacZ*290 with truncated *cspD* regulatory region	31
pCA38	pUJ142 containing the *spoT* gene	This work
pCA39	pUJ142 containing the *spdR* gene	This work
pCA40	pUJ142 containing the *spdR*(D64A) gene	This work
pCA401	pPROEX-HTa containing the *spdR*(D64A) gene	This work
pPROEX-HTa	Expression vector (Amp^r)	Invitrogen
pGEM-T Easy	Cloning vector (Amp^r)	Promega
pNPTS138	Suicide vector containing *oriT sacB* (Kan^r)	D. Alley
pSUP2021	Plasmid containing Tn*5* transposon	45
pUJ142	Xylose-inducible promoter (Chlor^r)	34
pRK*lacZ*290	Transcription fusion vector *lacZ*, replicon IncP1, *oriT* (Tet^r)	14
Trx-His CC0247	Expression vector with cloned coding region of *spdR*	47
Trx-His CC0248	Expression vector with cloned kinase domain of *spdS*	47
Cosmid1G4	pLAFR5 containing nucleotides 1710345–1739376 of the *C. crescentus* genome	C. Stephens
Oligonucleotides		
RELA-1	GGGGCCCGCGTATCTGAACG	This work
RELA-2	GGGATCCGCTTCGGTCACAGCGGACG	This work
RELA-3	GGGATCCCAAGCACCTGACCAACATC	This work
RELA-4	CCCTGCGGCGCGGAATTCGTCG	This work
RELA-5	GAGGCCTTGTTGGAAGCCGCC	This work
CSP2-A	ATTGGATCCATATAACGGCTATGTTCC	This work
CAROL-1	AAGCTTCAAAATCGTAACCAGACATCCC	This work
REG-1	CGAATTCATGGCGGATATCGGAGAACT	This work
REG-2	CTTCGAAGCGAGGGAGCAACTTAAAGC	This work
RR-1	CGGGCCCCCAACTCCAATCTGCTGTGG	This work
RR-2	TGGATCCTCCGCCATAAAAGTCAGCGC	This work
RR-3	CGGATCCAGAAGCGTTTAAGTTGCTCCCTC	This work
RR-4	AGAATTCGTTTGAATTATAATCGGGAGGA	This work
MutDA1	CATGCTGTTCTGGCCATGCGGCTGGAG	This work
MutDA2	CTCCAGCCGCATGGCCAGAACAGCATG	This work
R3	ATGTGACCTCCTAACATGGT	4
L7	CCATCTCATCAGAGGGTAGT	4

(NBT) and 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate (BCIP) in alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 5 mM $MgCl_2$, 100 mM NaCl).

Expression of the SpdR and SpdS proteins and phosphotransfer assays. Plasmids Trx-His CC0247 and Trx-His CC0248, with the response regulator SpdR and the histidine kinase domain of SpdS fused with polyhistidine tails (47), were used for overexpression of the corresponding proteins. Plasmid pCA401 was used for overexpression of the mutated SpdR(D64A) protein. The proteins were expressed in *E. coli* DH5α at 37°C by induction with 300 μM IPTG (isopropyl-β-D-thiogalactopyranoside), and the purification was performed using Ni affinity chromatography (Qiagen).

The phosphotransfer assay was carried out as described previously (47). Briefly, 5 μM purified His-SpdS was autophosphorylated in storage buffer containing 5μCi [γ-^{32}P]ATP at 30°C for 15 min, and then 5 μM purified SpdR was added to the reaction mixture. The phosphotransfer assay was carried out with a reaction mixture containing the histidine kinase alone as a control, and at different times after addition of SpdR (30 s, 2 min, 10 min, and 30 min), the reaction was stopped by the addition of 3.5 μl of 4× sample buffer (500 mM Tris-Cl [pH 6.8], 8% SDS, 40% glycerol, 400 mM β-mercaptoethanol). The samples were separated by 10% SDS PAGE, and the gel was dried and exposed to X-rays film.

Electrophoretic mobility shift assays (EMSA). The promoter region upstream of the *cspD* gene was amplified by PCR using primers CSP2-A and CAROL-1 (Table 1). All the following steps were carried out as previously described (10). The probes were end labeled with 20 μCi [γ-^{32}P]ATP using T4 polynucleotide kinase (Invitrogen), and the DNA binding reaction was carried out in 30 μl containing 0.05, 0.1, 0.25, 0.5, and 1 μM purified proteins. Purified His-SpdR or His-SpdR(D64A) proteins were previously incubated with SpdS in the presence of 0.5 mM ATP under conditions described previously (47). In competition assays, a 30-fold excess of unlabeled probe was used to challenge the labeled probe, either of the same fragment (specific competitor) or of a fragment corresponding to the coding region of gene CCNA01712 (nonspecific competitor). After incubation at 30°C for 30 min, the samples were loaded onto a native 5% polyacrylamide gel and electrophoresed in 0.5× Tris-borate (TB) buffer for 2 h at 40 mA. Radioactive species were detected by autoradiography.

DNase I footprinting. DNase I footprinting assays were performed as described previously (10). The same primers used for amplification of the DNA

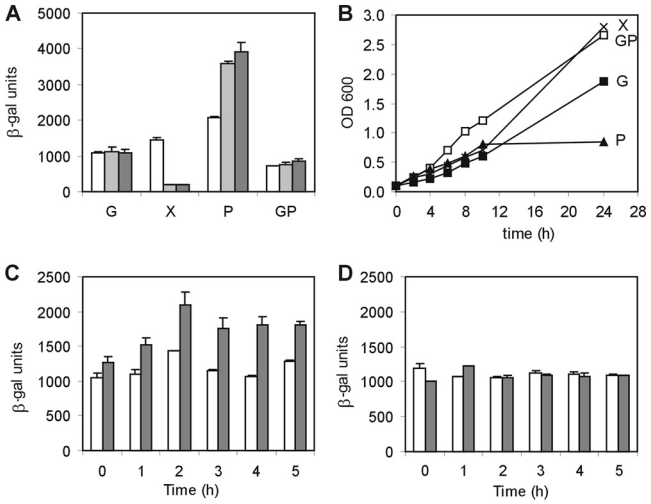

FIG. 1. Expression of *cspD* in *C. crescentus* NA1000. (A) β-Galactosidase activity assay with wild-type strain NA1000 harboring plasmid pEL4. The cells were grown in different media: G, M2G (containing 0.2% glucose); X, M2X (glucose was replaced by 0.3% xylose); P, M2P (glucose was replaced by 0.2% peptone); GP, M2GP (containing both glucose and peptone). Cultures were incubated at 30°C, and the expression of *cspD* was determined at mid-exponential phase (white bars), after 24 h (light gray bars), and after 48 h (dark gray bars). (B) Growth curves of *C. crescentus* NA1000 harboring pEL4 in different media. (C) Expression of *cspD* in NA1000(pEL4) was determined by β-galactosidase activity assays at mid-exponential phase in M2 medium containing 0.2% glucose (white bars) and at several time points after washing and inoculation in M2 medium containing 0.02% glucose (gray bars). (D) β-Galactosidase activity was determined at mid-exponential phase in M2 medium containing NH_4Cl (white bars) and at several time points after washing and inoculation in M2 medium without NH_4Cl (gray bars). Error bars indicate standard deviations.

fragments for EMSA were used for PCR amplification of the *cspD* promoter region. The reactions and the conditions were the same as used in the EMSA, except that in this case the probe had a single ^{32}P-labeled end. The probe was incubated with increasing amounts of purified His-SpdR protein (0.1, 0.25, and 0.5 μM). The DNA was digested with 0.05 U of RQ1 RNase-free DNase I (Promega) for 1 min. Reaction mixtures were run on a 6% polyacrylamide-urea sequencing gel alongside sequencing ladders of the fragments generated with the same respective primers.

RESULTS

Expression of *cspD* under different nutrient conditions. The *cspD* gene is induced upon entry into stationary phase (31), and previous observations from our group showed that its expression was affected by the medium composition. In order to determine more precisely which nutrient could be affecting expression, we used a transcriptional *cspD-lacZ* fusion to evaluate *cspD* expression in media containing different carbon sources. Cultures grown in each medium were freshly diluted in the same medium, and β-galactosidase activity was determined in mid-log phase and early stationary phase (24 h and 48 h). As observed in Fig. 1A, the gene is highly induced in media containing peptone, similar to what was observed in PYE (31), but not in media containing sugars (the gene is not induced in M2G and is repressed in M2X). Moreover, in a medium containing both glucose and peptone, there is a decrease in the maximum levels of expression, suggesting that the presence of glucose could be inhibiting expression. When we analyze the growth of the cultures in each medium, we can observe that growth rates are higher in media containing sugars than in those containing only peptone (Fig. 1B). In fact, an inverse correlation appears to exist between the growth rate and the levels of *cspD* transcription, and this could be independent of the carbon source utilized.

A further evaluation of the role of nutrient availability in *cspD* expression was carried out to determine whether the gene is induced in response to carbon and nitrogen starvation. The cultures were grown in minimal M2 medium to mid-log phase and then washed and resuspended either in the same medium or in M2 with a low glucose concentration (0.02%) or lacking ammonium chloride. As depicted in Fig. 1C and D, *cspD* was induced by low glucose but not by ammonia depletion, even though growth was arrested in both cases (not shown). These data indicate that *cspD* gene regulation responds to the levels of the carbon source, being induced at low concentrations, and that expression varies depending on the carbon source available and the growth rate.

In order to separate the effects of carbon source and growth rate, we then analyzed the expression of *cspD* in a *spoT* null mutant strain. In *C. crescentus*, as in other bacteria, SpoT is the only enzyme responsible for ppGpp synthesis and degradation (32), so we constructed a mutant strain containing a *spoT* deletion (SP0200) which has the expected phenotype of relaxed growth upon nutrient starvation (compare growth in M2-peptone in Fig. 1B and 2B). A *spoT* strain carrying a plasmid containing the *cspD-lacZ* construct was then grown in the same media as the wild-type NA1000 strain (Fig. 1), and expression was measured by β-galactosidase assay. As depicted in Fig. 2A, the induction of *cspD* at stationary phase in medium containing peptone is no longer observed. This can be confirmed by immunoblot analysis of CspD in the *spoT* mutant, where very low levels of protein are observed after 48 h of growth in PYE (Fig. 3D). When we analyzed the growth curves in each medium, we can see that the growth rates are high in all media (Fig. 2B), which again correlates inversely to the levels of *cspD* expression. The induction of *cspD* in M2-peptone medium was restored when the *spoT* gene was added back to strain SP0200 (Fig. 2C). Moreover, the induction observed in the shift to low-glucose medium does not occur in the *spoT* strain (Fig, 2D), indicating that it is dependent on SpoT-mediated signaling.

Taken together, these results suggest that *cspD* is induced when cells enter a slow-growth period in response to low nutrient levels, particularly carbon sources. The reduced expression of *cspD* in the *spoT* strain does not allow us to define whether its transcription is directly activated by the second messenger ppGpp or whether it is responding to another signal transduction network affected in this mutant.

Identification of genes important for *cspD* expression. With the purpose of identifying the players in the regulatory systems involved in *cspD* expression, a library of 7,500 transposon mutants was screened in a search for mutants with reduced *cspD* expression at stationary phase. The plasmid containing the *cspD-lacZ* fusion was introduced into the mutant strains by conjugation, and the cultures were grown for 48 h in PYE medium. Expression was evaluated by adding X-Gal to the culture medium, and clones that showed a blue color lighter than that of the positive control were selected for further

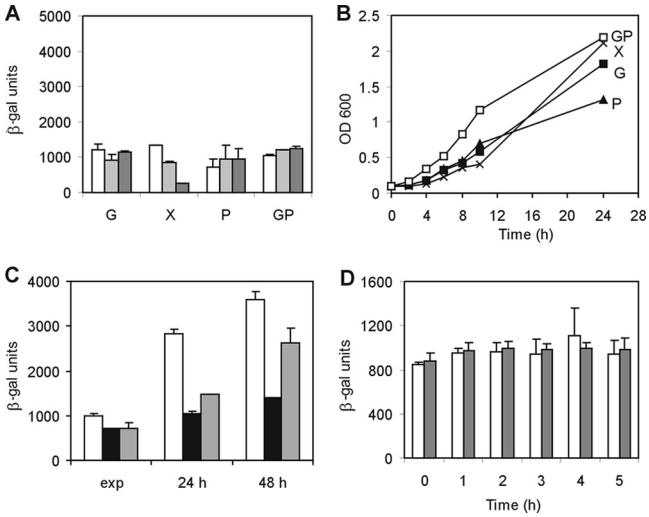

FIG. 2. Expression of *cspD* in the SP0200 strain. (A) β-Galactosidase activity assay was carried out with strain SP0200 harboring pEL4. The cells were grown in different media: G, M2G (containing 0.2% glucose); X, M2X (glucose was replaced by 0.3% xylose); P, M2P (glucose was replaced by 0.2% peptone); GP, M2GP (containing both glucose and peptone). Cultures were incubated at 30°C, and the expression of *cspD* was determined at mid-exponential phase (white bars), after 24 h (light gray bars), and after 48 h (dark gray bars). (B) Growth curves of SP0200(pEL4) in different media. (C) Complementation of the *spoT* mutation on *cspD* expression. Expression of *cspD* was determined by β-galactosidase activity assays of *C. crescentus* strains NA1000 (white bars), SP0200 (black bars), and SP0200(pCA38) (gray bars) harboring pEL4. Assays were performed at mid-exponential phase (exp) and stationary phase (24 h and 48 h). (D) Expression of *cspD* in SP0200(pEL4) was determined by β-galactosidase activity assays at mid-exponential phase in M2 medium containing 0.2% glucose (white bars) and at several time points after washing and inoculation in M2 medium containing 0.02% glucose (gray bars). Error bars indicate standard deviations.

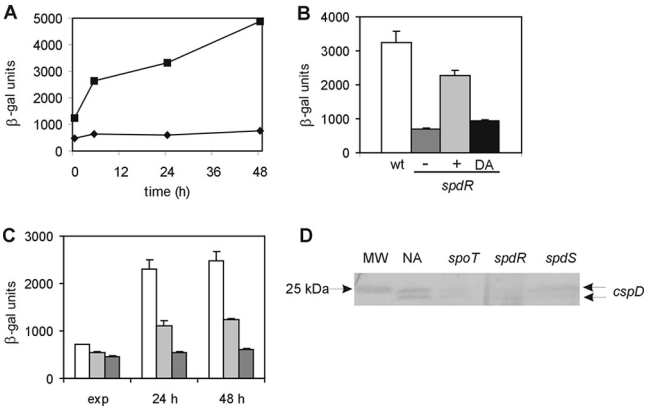

FIG. 3. Expression of *cspD* in *spoT* and *spdR* mutant strains. (A) β-Galactosidase activity assay of *cspD* expression in strains NA1000 (squares) and SP0247 (diamonds) carrying vector pEL4 in PYE medium after different times of growth. (B) Complementation of the *spdR* mutation on *cspD* expression. β-Galactosidase activity assay was carried out with *C. crescentus* strains NA1000 (white bars), SP0247 (dark gray bars), SP0247(pCA39) (carrying the wild-type [wt] *spdR* gene) (light gray bars), and SP0247(pCA40) [carrying the mutated *spdR*(D64A) gene] (black bars) harboring pEL4. Assays were performed at stationary phase (48 h). (C) β-Galactosidase activity assay of *cspD* expression in strains NA1000 (white bars), SP0200 (light gray bars), and SP0210 (dark gray bars) carrying vector pEL4 in PYE medium at exponential phase (exp) and stationary phase (24 h and 48 h). (D) Immunoblot analysis using a polyclonal anti-CspD antiserum of total protein extracts obtained from 48-h cultures of the NA1000, *spoT*, *spdR*, and *spdS* strains grown for 48 h. NA, NA1000 extract; *spoT*, SP0200 extract; *spdR*, Δ*spdR* mutant strain (47) extract; *spdS*, Δ*spdS* mutant strain (47) extract.

analysis. Expression of *cspD* in these selected clones was then quantitatively determined by β-galactosidase activity assays. Ten clones that showed a reduction of at least 50% in the levels of expression were subject to identification of the disrupted gene and are shown in Table 2.

Four of the genes encode proteins involved in transport across the membranes that belong to different systems, i.e., an ABC transporter (CCNA_02670), a permease (CCNA_00254), a TonB-dependent receptor (CCNA_02106), and a putative export system (CCNA_01611). Two independent insertions on neighboring genes (CCNA_02186 and CCNA_02188) also caused a decrease in *cspD* expression. CCNA_02186 encodes the small subunit of acetolactate synthase, an enzyme involved in the biosynthesis of leucine, isoleucine, and valine, and the product of CCNA_02188 contains a thioesterase superfamily domain. A gene encoding a glucokinase (CCNA_03269) is also important for *cspD* expression. A strain with a mutation in this gene is still able to grow in M2 containing glucose, but at a lower rate than the wild-type strain, and it shows a similar growth rate in PYE (data not shown). This indicates that this enzyme is probably not the main glucokinase of *C. crescentus* (a paralog is CCNA_02133), but it confers a better fitness to cells growing with glucose as the sole carbon source. A mutant with a mutation in a gene encoding an acylamino acid-releasing enzyme (CCNA_03839), which is involved in peptide hydrolysis, was also identified in our screen. When analyzing them altogether, we can identify a common feature to these mutations, which is that they disrupted genes that are probably important for nutrient uptake or carbon metabolism. This is in accordance with the fact that *cspD* is induced by glucose starvation, indicating that an imbalance in the carbon metabolism might interfere with the signals that are important for *cspD* induction.

Expression of *cspD* at stationary phase was particularly low in one of the mutants, which was disrupted in a gene encoding a putative response regulator (CCNA_00247). This gene was then named *spdR*, for *s*tationary-*p*hase *cspD r*egulator, based on evidence obtained in this work (see below). *cspD* expression was determined at the exponential and early stationary phases (24 h and 48 h) in both wild-type strain NA1000 and the *spdR* mutant strain SP0247 (Fig. 3A). Expression was lower in the mutant even at exponential phase (time zero), and failed to increase when cells reached stationary phase, keeping a basal level of transcription. The expression of *cspD* is partially restored in the *spdR* strain containing the *spdR* gene in *trans* under the control of a xylose promoter (Fig. 3B). It should be noticed that in the expression experiments no xylose was added to the medium due to the catabolite repression effect in the presence of xylose (Fig. 1A), so *spdR* expression was due to the natural leakage of the *xylX* promoter. In order to evaluate the contributions of SpdR and SpoT to *cspD* regulation, an *spdR spoT* double mutant strain was constructed. As can be observed in Fig. 3C, expression of *cspD* in the double mutant

TABLE 2. Expression of *cspD* in mutant strains of *C. crescentus*

Strain[a]	Disrupted ORF[b]	Putative ORF product[c]	β-Galactosidase activity (Miller units)[d]
NA1000(pEL4)	Wild type		3,698.7 ± 33.5
NA1000(pEL5)	Wild type		740.1 ± 21.0
23/11C	CCNA_00247	Response regulator	623.0 ± 28.5
22/7H	CCNA_00254	RarD family membrane permease	1,980.3 ± 52.8
5/3	CCNA_01611	Transporter, drug/metabolite exporter family	2,121.7 ± 125.9
1/6G	CCNA_02106	TonB-dependent outer membrane receptor	1,977.8 ± 120.0
5/4E	CCNA_02186	Acetolactate synthase 3 regulatory subunit	1,740.0 ± 37.7
24/5E	CCNA_02188	Cytosolic protein	1,998.6 ± 47.9
4/2C	CCNA_02603	Phosphatidylserine decarboxylase	1,903.8 ± 5.6
2/6F	CCNA_02670	ABC transporter ATP binding protein	1,022.3 ± 22.5
46/2A	CCNA_03269	Glucokinase	1,863.6 ± 149.2
30/3C	CCNA_03839	Acylamino acid-releasing enzyme	2,102.6 ± 4.8

[a] The promoter constructs used are shown in parentheses. All mutant strains contained pEL4.
[b] Numbers indicate the open reading frame (ORF) designation for *C. crescentus* NA1000 as deposited in GenBank.
[c] Obtained from the sequence databases.
[d] Values are averages and standard deviations of data obtained in three experiments. Data refer to measurements at 24-h time point.

shows a further decrease, suggesting that the two regulators have additive roles in *cspD* regulation. While *cspD* expression in the *spoT* mutant still increases slightly in stationary phase, the *spdR* mutant (Fig. 3A) and the double mutant (Fig. 3C) do not show this induction, suggesting that SpdR is the major contributor to the stationary-phase induction of *cspD*. The *spdR* gene is probably cotranscribed with a gene encoding a membrane-spanning histidine kinase (CCNA_00248), which was here named *spdS*. Immunoblot analysis of CspD was carried out in *spdR* and *spdS* deletion strains at stationary phase (48 h of growth) (Fig. 3D). The results showed that there is very little CspD protein in the *spdR* and *spdS* mutant strains, although the decrease in protein amount was less striking in the *spdS* strain.

Roles of SpdR and SpdS in activation of *cspD* transcription. The SpdR protein has a putative conserved aspartyl residue (D64) that could receive a phosphoryl group from a cognate histidine kinase. In order to establish whether SpdS is able to autophosphorylate and to transfer its phosphate to SpdR, a phosphorylation assay was carried out (Fig. 4). Incubation of SpdS with [^{32}P]ATP led to incorporation of the gamma phosphate by SpdS in an autophosphorylation reaction that is typical for this sort of enzyme (47). Addition of purified SpdR to the phosphorylated SpdS caused a very efficient transfer of the labeled phosphate, with detection of labeled SpdR after 0.5 min. These results indicate that SpdS is the cognate histidine kinase of SpdR, and they probably work together regulating genes in response to signals sensed outside the cytoplasmic membrane.

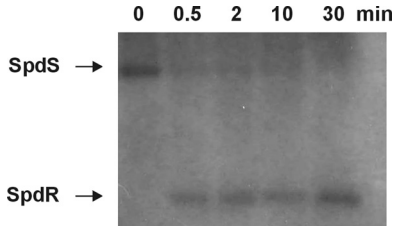

FIG. 4. Phosphate transfer between SpdS and SpdR. The purified kinase domain of SpdS was previously autophosphorylated with [^{32}P]ATP and then was mixed with purified His-SpdR. The reaction was stopped at several time points, and phosphotransfer to SpdR was evaluated by SDS-PAGE. Lane 1, autophosphorylated SpdS (time zero); lanes 2 to 5, products of the phosphotransfer reaction after 0.5, 2, 10, and 30 min, respectively.

The levels of β-galactosidase activity driven by the complete *cspD* promoter in the *spdR* mutant are similar to those obtained with a promoter fusion that lacks the upstream activation region (31) (Table 2), suggesting that SpdR could bind directly to this region and activate transcription of *cspD*. Binding of purified His-SpdR protein was assessed by electrophoretic gel mobility shift assay (EMSA), using as the probe a fragment containing the complete *cspD* regulatory region. As shown in Fig. 5A, the probe was shifted in the presence of from 50 nM up to 1 μM His-SpdR that was previously phosphorylated *in vitro*, and a more pronounced retardation was observed with the increase in protein concentration. This result suggests that the SpdR protein is able to oligomerize when binding to the probe. As a control, purified His-SpdS at the same concentrations was not able to shift the probe. The specificity of SpdR binding was demonstrated in a competition assay, using His-SpdR that was previously phosphorylated *in vitro* (Fig. 5B). The shifted band was competed out by excess unlabeled DNA probe (specific competitor) but not by an unlabeled nonspecific DNA. Therefore, EMSA experiments confirmed *in vitro* specific binding of the phosphorylated SpdR regulator to the *cspD* regulatory region.

In order to establish the importance of phosphorylation for SpdR binding, the same experiment was carried out using His-SpdR(D64A), which contains a substitution of the aspartyl residue at position 54 for alanine. This is the probable phosphorylated residue of SpdR, as part of a conserved region in most response regulators. As can be observed in Fig. 5A, in this case the protein was not able to shift the probe even when previously subjected to a phosphotransfer reaction, indicating that phosphorylation at this site is required for SpdR binding. The importance of phosphorylation for SpdR activity *in vivo* was assessed by complementation studies using an expression plasmid carrying the mutated SpdRD64A version of the SpdR-coding region

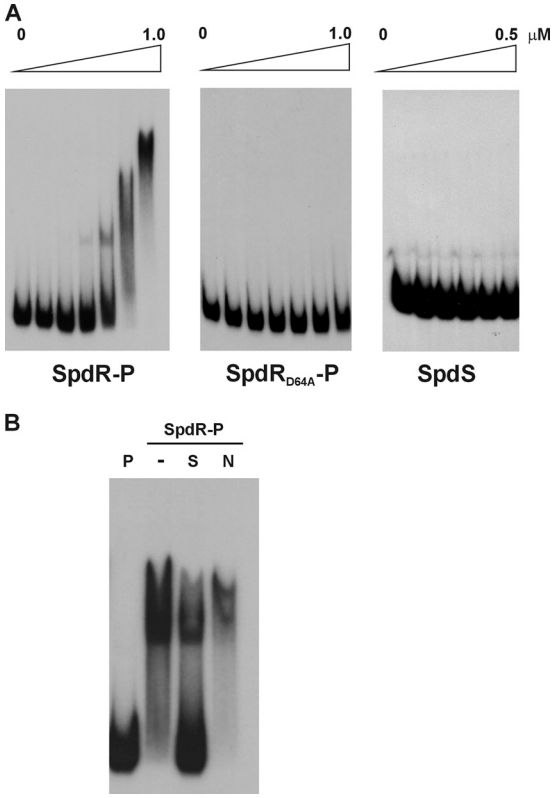

FIG. 5. SpdR binding to the regulatory region of *cspD*. (A) A DNA fragment corresponding to the regulatory region of *cspD* was ^{32}P labeled and incubated or not with increasing concentrations (0.025, 0.05, 0.1, 0.25, 0.5, and 1 μM) of purified protein as indicated. His-SpdR and the mutated version His-SpdR(D64A) were previously incubated in phosphotransfer reactions with SpdS. As a negative control, autophosphorylated SpdS was used alone. (B) Competition assays were carried out with 0.5 μM purified His-SpdR that was previously phosphorylated *in vitro* by SpdS. P, labeled probe; −, probe incubated with His-SpdR; S, probe incubated with His-SpdR in the presence of a 30-fold excess of unlabeled specific probe; N, probe incubated with His-SpdR in the presence of a 30-fold excess of nonspecific DNA competitor.

(Fig. 3B). The results showed that the mutated protein failed to complement the phenotype of *cspD* activation, confirming that phosphorylation of SpdR in the D64 residue is required *in vivo* for activation of *cspD* transcription.

The *cspD* regulatory region was further analyzed by DNase I footprinting assay to determine the sequence of SpdR binding (Fig. 6). Probes consisting of the *cspD* promoter region were ^{32}P labeled at the 5′ end of either strand, incubated with increasing amounts of His-SpdR protein, and digested with DNase I. A region corresponding to positions −89 to −66 with respect to the most upstream transcriptional start site of the *cspD* gene was protected by the SpdR protein in both assays (Fig. 6A). Within this region there is an inverted repeat sequence separated by five nucleotides that could be the motif recognized by SpdR (Fig. 6B). This region had been previously identified as necessary for maximum levels of *cspD* transcription (31), and our results showed that this is achieved by binding of SpdR and activation of transcription.

DISCUSSION

In this work we have studied the regulation of the *cspD* gene, establishing the signals that trigger its induction at early stationary phase and uncovering part of the regulatory network that modulates its expression. We have observed that the induction of *cspD* responds to carbon, but not nitrogen, starvation and that it is dependent on the type of carbon source present in the medium. The gene is highly induced in media containing amino acids (peptone) but not in media containing sugars (glucose or xylose), and when cells were grown in xylose the expression of *cspD* was further repressed at later times of stationary phase. Also, the presence of glucose in the medium containing peptone causes a decrease in the maximum levels of expression compared to those with peptone alone. This effect might be related to catabolite repression, although not much is known about how this is achieved in *Caulobacter*. Meisenzahl and coworkers reported that repression of lactose utilization of *C. crescentus* on complex medium can be achieved equally by glucose and xylose, indicating that both sugars may be used as primary carbon sources (35). Moreover, dibutyryl cyclic AMP was shown to stimulate the expression of inducible catabolic enzymes, although the intracellular concentration of cyclic AMP does not vary with carbon source (44).

These results must be taken carefully, since the growth rate of *C. crescentus* is much higher in media containing sugars than in those containing only amino acids (Fig. 1B). This could indicate that gene induction is somehow responding to the growth rate instead of the carbon source available. This effect was observed earlier in *E. coli*, where the expression of *cspD* also varied in response to different carbon sources, and the results indicated that the *cspD* expression is inversely dependent on the growth rate and independent of the sigma factor σ^S (52). In fact, circuits modeling gene expression predicted that even the concentration of a protein product of an unregulated gene can exhibit growth rate dependence (27). It is intriguing, however, how the growth rate at exponential phase may interfere with the expression of the gene at the onset of stationary phase. This is more so when we consider that *cspD* expression is not increased even at later times during stationary phase when cultures have a high growth rate, despite the fact that nutrients should have run out at this point.

When *cspD* is overproduced in *E. coli* it causes a lethal effect, and this has been attributed to the fact that it may impair DNA replication, probably by binding to single-stranded DNA (ssDNA) (53). However, increased expression of *cspD* in *C. crescentus* does not appear to have the same drastic effect (data not shown), suggesting that it may play a distinct role in the cell physiology and may be regulated differently. Another interesting fact is that *C. crescentus* CspD appears as two bands in SDS-PAGE, one of them migrating more slowly than predicted by its molecular weight (MW) (3). This indicates that two isoforms of CspD exist in *C. crescentus*, with one of them likely being subject to posttranslational modification, and this may have an effect upon its function in the cell.

A possible link between the growth rate and *cspD* expression would be the second messenger ppGpp. In *E. coli*, ppGpp is produced in response to most stresses and nutrient limitations besides amino acid starvation, and cells grown in minimal

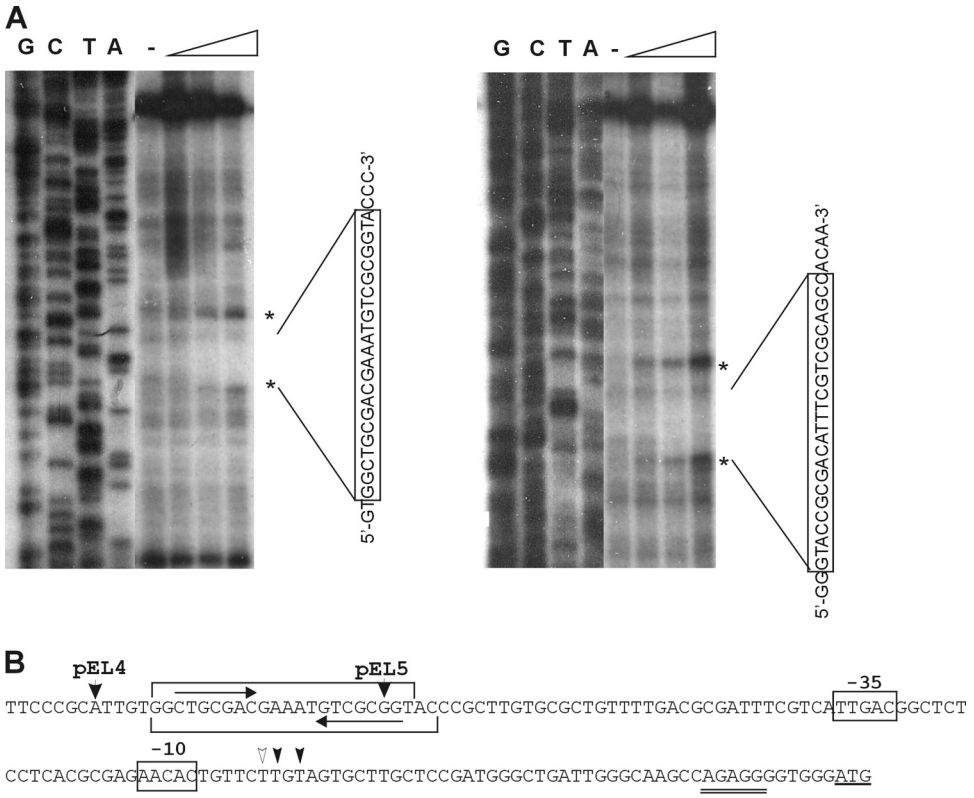

FIG. 6. (A) DNase I footprinting assays of SpdR in the *cspD* regulatory region. Probes containing the regulatory region of *cspD* were end labeled and incubated in the presence or absence of increasing concentrations of purified His-SpdR (0.1, 0.25, and 0.5 μM, respectively). The reaction mixtures were treated with DNase I, and reactions were run in a urea-polyacrylamide gel along with a DNA sequencing reaction with the same labeled primer. A minus sign indicates no protein. The protected regions are indicated with the respective sequences, and asterisks mark hypersensitive sites. (B) DNA sequence of the regulatory region of *cspD*. The −35 and −10 promoter regions are indicated above the sequence. Black arrowheads indicate the transcription start sites, and a white arrowhead indicates the stationary-phase start site of *cspD*. The start codon is underlined, and the ribosome binding site is double underlined. The SpdR-protected region in each strand is indicated by brackets. An inverted repeat within the SpdR binding site is indicated by arrows. The 5′ ends of the transcriptional fusions pEL4 and pEL5 are indicated.

medium accumulate more ppGpp than cells grown in rich medium at stationary phase (7, 52). In *C. crescentus*, deprivation of glucose as the energy source blocks the transition of the stalked cell to the swarmer cell and prevents the beginning of DNA replication. ppGpp was shown not to be necessary for blocking polar morphogenesis, but it is required to inhibit DNA replication, since in the *spoT* mutant there is an imbalance of the levels of CtrA and DnaA that prevents replication initiation (32).

Expression of *cspD* in a *spoT* mutant strain indicated that *cspD* induction in peptone-containing medium was lost even after 48 h, a time when the nutrients would probably be completely depleted from the medium. Moreover, the carbon starvation induction of *cspD* was also dependent on the presence of *spoT*. While in *E. coli* ppGpp is required for effective transcription of amino acid biosynthetic genes (51), this does not seem to be the case in *C. crescentus*, since the *spoT* mutant does not display a phenotype of amino acid auxotrophy (32). Expression of the *cspD* ortholog in *E. coli* is very low in the *relA spoT* double mutant, implying that ppGpp is also a positive factor for the expression of *cspD* in this bacterium (52). The results indicated that in *C. crescentus spoT* is required for expression of *cspD*, either via a direct effect of ppGpp on its transcription or indirectly by the effects on the overall cell response to carbon starvation and growth. In a global analysis of *Lactobacillus lactis* gene expression in response to carbon limitation (isoleucine or glucose starvation), the stringent response and growth rate were compared, and the results showed that the stringent response is not the general mechanism controlling growth rate modifications or glucose starvation (11).

In this work we have identified several genes that affect the expression of *cspD* at stationary phase. Most of these genes are involved in nutrient transport and metabolism of carbon compounds, such as amino acids and glucose, in agreement with the fact that *cspD* is induced upon carbon, but not nitrogen, starvation. Among the genes identified, one encoded a response regulator, SpdR, which was necessary for activation of *cspD* transcription at stationary phase. Interestingly, the expression of the activator *spdR* itself was also reported to vary depending on the medium composition (21). An *spdR spoT* double mutant shows even lower levels of *cspD* expression, indicating that the effects of each regulator are additive. We could postulate that SpoT is required for signaling for carbon starvation, while SpdR could be involved in signaling for other physiological changes that occur at the onset of stationary phase.

The identification of such a regulator is very important, since very little is known about the control of stationary-phase gene

expression in *C. crescentus*. Cells at this stage are better adapted to cope with very-low-nutrient conditions and have increased resistance to several stresses (50). Some of the genes involved in the increased resistance to stresses at this phase are regulated by extracytoplasmic function (ECF)-type sigma factors, such as some oxidative stress genes that are regulated by the extracytoplasmic sigma factor SigF (1). Moreover, several small regulatory RNAs are induced at stationary phase and by nutrient starvation, suggesting that the regulatory network controlling gene expression at this phase may be more complex than one relying on a single master regulator (30).

SpdR is part of a two-component system and is probably encoded in the same transcriptional unit with the histidine kinase SpdS. Mutants with mutations in these two genes were previously obtained and showed a wild-type phenotype regarding doubling time in PYE, motility, and presence of polar structures (47). We showed that SpdS can phosphorylate SpdR *in vitro*, in agreement with previous data that histidine kinases display a kinetic preference for their *in vivo* cognate substrate (47). Moreover, the aspartyl residue D64 was shown to be necessary both for SpdR binding to the *cspD* promoter (Fig. 5A) and for *cspD* activation in stationary phase (Fig. 3B). However, CspD protein seems to be present at somewhat higher levels in the *spdS* mutant strain than in an *spdR* strain (Fig. 3D), suggesting that other histidine kinases may relay the signal to SpdR. Domain analysis of SpdS showed that it has five transmembrane regions at the amino terminus and a phosphoacceptor and ATP binding domain at the carboxy terminus, indicating that the signal for its phosphotransfer activity could be the binding of an extracellular molecule. Recently attention has been given to auxiliary factors that interact with histidine kinases to modify their activity (6), raising the possibility that SpdS activity could also be the target for a second level of regulation.

The SpdR protein consists of an amino-terminal response regulator domain and a carboxy-terminal helix-turn-helix domain. SpdR binds to an imperfect inverted repeat positioned relatively far upstream from the −35 region of *cspD* (between positions −87 and −69). This suggests that it may require the formation of dimers or oligomers to allow interaction with the RNA polymerase and activate transcription and that interaction with the carboxyl-terminal domain of the α subunit probably occurs (4). In fact, *in vitro* DNA binding assays indicate the formation of higher-order structures of SpdR upon interaction with DNA (Fig. 5A). Despite the fact that *cspC* shows a pattern of expression similar to that of *cspD*, no SpdR binding sequence can be found upstream of the *cspC* promoter, and DNA binding assays with purified SpdR did not detect any binding to this region (data not shown), confirming that the regulation of these two *csp* genes is independently achieved.

The role of SpdR in activating the expression of *cspD* at the onset of stationary phase suggests that this regulator may be activated by phosphorylation triggered by nutrient starvation, most likely related to the carbon source. The determination of the complete regulon of SpdR and its involvement in stationary-phase adaptation is currently under investigation.

ACKNOWLEDGMENTS

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). During the course of this work, C.A.P.T.D.S., H.B., and R.R.M. were supported by fellowships from FAPESP. M.V.M. was partially supported by CNPq.

We thank Michael Laub for the His-SpdR- and His-SpdS-expressing clones and the *spdR* and *spdS* mutant strains. We also thank Rogério Lourenço for helping with the His-SpdR and His-SpdS purification and Suely Gomes for critical reading of the manuscript.

REFERENCES

1. **Alvarez-Martinez, C. E., R. L. Baldini, and S. L. Gomes.** 2006. A Caulobacter crescentus extracytoplasmic function sigma factor mediating the response to oxidative stress in stationary phase. J. Bacteriol. **188:**1835–1846.
2. **Bae, W., B. Xia, M. Inouye, and K. Severinov.** 2000. *Escherichia coli* CspA-family RNA chaperones are transcription antiterminators. Proc. Natl. Acad. Sci. U. S. A. **97:**7784–7789.
3. **Balhesteros, H., R. R. Mazzon, C. A. da Silva, E. A. Lang, and M. V. Marques.** 2010. CspC and CspD are essential for *Caulobacter crescentus* stationary phase survival. Arch. Microbiol. **192:**747–758.
4. **Browning, D. F., and S. J. Busby.** 2004. The regulation of bacterial transcription initiation. Nat. Rev. Microbiol. **2:**57–65.
5. **Brun, Y. V., and L. Shapiro.** 1992. A temporally controlled sigma-factor is required for polar morphogenesis and normal cell division in Caulobacter. Genes Dev. **6:**2395–2408.
6. **Buelow, D. R., and T. L. Raivio.** 2010. Three (and more) component regulatory systems—auxiliary regulators of bacterial histidine kinases. Mol. Microbiol. **75:**547–566.
7. **Cashel, M., D. R. Gentry, V. J. Hernandez, and D. Vinella.** 1996. The stringent response, p. 1458–1496. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, DC.
8. **Chanda, P. K., R. Mondal, K. Sau, and S. Sau.** 2009. Antibiotics, arsenate and H2O2 induce the promoter of Staphylococcus aureus cspC gene more strongly than cold. J. Basic Microbiol. **49:**205–211.
9. **Chatterjee, D. K., and A. W. Bourquin.** 1987. Metabolism of aromatic compounds by *Caulobacter crescentus*. J. Bacteriol. **169:**1993–1996.
10. **da Silva Neto, J. F., V. S. Braz, V. C. Italiani, and M. V. Marques.** 2009. Fur controls iron homeostasis and oxidative stress defense in the oligotrophic alpha-proteobacterium Caulobacter crescentus. Nucleic Acids Res. **37:**4812–4825.
11. **Dressaire, C., E. Redon, H. Milhem, P. Besse, P. Loubiere, and M. Cocaign-Bousquet.** 2008. Growth rate regulated genes and their wide involvement in the Lactococcus lactis stress responses. BMC Genomics **9:**343.
12. **Ely, B.** 1991. Genetics of *Caulobacter crescentus*. Methods Enzymol. **204:**372–384.
13. **Evinger, M., and N. Agabian.** 1977. Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. J. Bacteriol. **132:**294–301.
14. **Gober, J. W., and L. Shapiro.** 1992. A developmentally regulated *Caulobacter* flagellar promoter is activated by 3′ enhancer and IHF binding elements. Mol. Biol. Cell **3:**913–926.
15. **Graumann, P., T. M. Wendrich, M. H. Weber, K. Schröder, and M. A. Marahiel.** 1997. A family of cold shock proteins in *Bacillus subtilis* is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures. Mol. Microbiol. **25:**741–756.
16. **Graumann, P. L., and M. A. Marahiel.** 1999. Cold shock proteins CspB and CspC are major stationary-phase-induced proteins in *Bacillus subtilis*. Arch. Microbiol. **171:**135–138.
17. **Graumann, P. L., and M. A. Marahiel.** 1998. A superfamily of proteins that contain the cold-shock domain. Trends Biochem. Sci. **23:**286–290.
18. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
19. **Hengge-Aronis, R.** 2002. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. Microbiol. Mol. Biol. Rev. **66:**373–395.
20. **Hogg, T., U. Mechold, H. Malke, M. Cashel, and R. Hilgenfeld.** 2004. Conformational antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p)ppGpp metabolism during the stringent response. Cell **117:**57–68. (Corrected.)
21. **Hottes, A. K., M. Meewan, D. Yang, N. Arana, P. Romero, H. H. McAdams, and C. Stephens.** 2004. Transcriptional profiling of *Caulobacter crescentus* during growth on complex and minimal media. J. Bacteriol. **186:**1448–1461.
22. **Italiani, V. C., and M. V. Marques.** 2003. Isolation and characterization of *Caulobacter* mutants impaired in adaptation to stationary phase. Brazilian J. Microbiol. **34:**85–90.
23. **Jenal, U.** 2000. Signal transduction mechanisms in *Caulobacter crescentus* development and cell cycle control. FEMS Microbiol. Rev. **24:**177–191.
24. **Jiang, W., Y. Hou, and M. Inouye.** 1997. CspA, the major cold-shock protein of *Escherichia coli*, is an RNA chaperone. J. Biol. Chem. **272:**196–202.
25. **Jishage, M., K. Kvint, V. Shingler, and T. Nystrom.** 2002. Regulation of sigma factor competition by the alarmone ppGpp. Genes Dev. **16:**1260–1270.

26. **Jones, P. G., R. A. VanBogelen, and F. C. Neidhardt.** 1987. Induction of proteins in response to low temperature in Escherichia coli. J. Bacteriol. **169:**2092–2095.
27. **Klumpp, S., Z. Zhang, and T. Hwa.** 2009. Growth rate-dependent global effects on gene expression in bacteria. Cell **139:**1366–1375.
28. **Lacour, S., and P. Landini.** 2004. SigmaS-dependent gene expression at the onset of stationary phase in Escherichia coli: function of sigmaS-dependent genes and identification of their promoter sequences. J. Bacteriol. **186:**7186–7195.
29. **Landsman, D.** 1992. RNP-1, an RNA-binding motif is conserved in the DNA-binding cold shock domain. Nucleic Acids Res. **20:**2861–2864.
30. **Landt, S. G., E. Abeliuk, P. T. McGrath, J. A. Lesley, H. H. McAdams, and L. Shapiro.** 2008. Small non-coding RNAs in Caulobacter crescentus. Mol. Microbiol. **68:**600–614.
31. **Lang, E. A. S., and M. V. Marques.** 2004. Identification and transcriptional control of *Caulobacter crescentus* genes encoding proteins containing a cold shock domain. J. Bacteriol. **186:**5603–5613.
32. **Lesley, J. A., and L. Shapiro.** 2008. SpoT regulates DnaA stability and initiation of DNA replication in carbon-starved *Caulobacter crescentus*. J. Bacteriol. **190:**6867–6880.
33. **Maeda, H., N. Fujita, and A. Ishihama.** 2000. Competition among seven Escherichia coli sigma subunits: relative binding affinities to the core RNA polymerase. Nucleic Acids Res. **28:**3497–3503.
34. **Mascarenhas, J., M. H. Weber, and P. L. Graumann.** 2001. Specific polar localization of ribosomes in Bacillus subtilis depends on active transcription. EMBO Rep. **2:**685–689.
35. **Meisenzahl, A. C., L. Shapiro, and U. Jenal.** 1997. Isolation and characterization of a xylose-dependent promoter from *Caulobacter crescentus*. J. Bacteriol. **179:**592–600.
36. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
37. **Mittenhuber, G.** 2001. Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). J. Mol. Microbiol. Biotechnol. **3:**585–600.
38. **Nierman, W. C., T. V. Feldblyum, M. T. Laub, I. T. Paulsen, K. E. Nelson, J. A. Eisen, J. F. Heidelberg, M. R. Alley, N. Ohta, J. R. Maddock, I. Potocka, W. C. Nelson, A. Newton, C. Stephens, N. D. Phadke, B. Ely, R. T. DeBoy, R. J. Dodson, A. S. Durkin, M. L. Gwinn, D. H. Haft, J. F. Kolonay, J. Smit, M. B. Craven, H. Khouri, J. Shetty, K. Berry, T. Utterback, K. Tran, A. Wolf, J. Vamathevan, M. Ermolaeva, O. White, S. L. Salzberg, J. C. Venter, L. Shapiro, and C. M. Fraser.** 2001. Complete genome sequence of *Caulobacter crescentus*. Proc. Natl. Acad. Sci. U. S. A. **98:**4136–4141.
39. **Nyström, T.** 2004. Stationary-phase physiology. Annu. Rev. Microbiol. **58:**161–181.
40. **Phadtare, S., J. Alsina, and M. Inouye.** 1999. Cold-shock response and cold-shock proteins. Curr. Opin. Microbiol. **2:**175–180.
41. **Potrykus, K., and M. Cashel.** 2008. (p)ppGpp: still magical? Annu. Rev. Microbiol. **62:**35–51.
42. **Schmid, B., J. Klumpp, E. Raimann, M. J. Loessner, R. Stephan, and T. Tasara.** 2009. Role of cold shock proteins in growth of *Listeria monocytogenes* under cold and osmotic stress conditions. Appl. Environ. Microbiol. **75:**1621–1627.
43. **Schroder, K., P. Graumann, A. Schnuchel, T. A. Holak, and M. A. Marahiel.** 1995. Mutational analysis of the putative nucleic acid-binding surface of the cold-shock domain, CspB, revealed an essential role of aromatic and basic residues in binding of single-stranded DNA containing the Y-box motif. Mol. Microbiol. **16:**699–708.
44. **Shapiro, L., N. Agabian-Keshishian, A. Hirsch, and O. M. Rosen.** 1972. Effect of dibutyryladenosine 3′:5′-cyclic monophosphate on growth and differentiation in Caulobacter crescentus. Proc. Natl. Acad. Sci. U. S. A. **69:**1225–1229.
45. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Biotechnology **1:**784–790.
46. **Skerker, J. M., and M. T. Laub.** 2004. Cell-cycle progression and the generation of asymmetry in *Caulobacter crescentus*. Nat. Rev. Microbiol. **2:**325–337.
47. **Skerker, J. M., M. S. Prasol, B. S. Perchuk, E. G. Biondi, and M. T. Laub.** 2005. Two-component signal transduction pathways regulating growth and cell cycle progression in a bacterium: a system-level analysis. PLoS Biol. **3:**e334.
48. **Traxler, M. F., S. M. Summers, H. T. Nguyen, V. M. Zacharia, G. A. Hightower, J. T. Smith, and T. Conway.** 2008. The global, ppGpp-mediated stringent response to amino acid starvation in Escherichia coli. Mol. Microbiol. **68:**1128–1148.
49. **Weber, M. H., A. V. Volkov, I. Fricke, M. A. Marahiel, and P. L. Graumann.** 2001. Localization of cold shock proteins to cytosolic spaces surrounding nucleoids in Bacillus subtilis depends on active transcription. J. Bacteriol. **183:**6435–6443.
50. **Wortinger, M. A., E. M. Quardokus, and Y. V. Brun.** 1998. Morphological adaptation and inhibition of cell division during stationary phase in *Caulobacter crescentus*. Mol. Microbiol. **29:**963–973.
51. **Xiao, H., M. Kalman, K. Ikehara, S. Zemel, G. Glaser, and M. Cashel.** 1991. Residual guanosine 3′,5′-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. J. Biol. Chem. **266:**5980–5990.
52. **Yamanaka, K., and M. Inouye.** 1997. Growth-phase-dependent expression of *cspD*, encoding a member of the CspA family in *Escherichia coli*. J. Bacteriol. **179:**5126–5130.
53. **Yamanaka, K., W. Zheng, E. Crooke, Y. H. Wang, and M. Inouye.** 2001. CspD, a novel DNA replication inhibitor induced during the stationary phase in *Escherichia coli*. Mol. Microbiol. **39:**1572–1584.