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Identification and Transcriptional Control of *Caulobacter crescentus* Genes Encoding Proteins Containing a Cold Shock Domain

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The cold shock proteins are small peptides that share a conserved domain, called the cold shock domain (CSD), that is important for nucleic acid binding. The *Caulobacter crescentus* genome has four *csp* genes that encode proteins containing CSDs. Three of these (*cspA*, *cspB*, and *cspC*) encode peptides of about 7 kDa and are very similar to the cold shock proteins of other bacteria. Analysis by reverse transcription-PCR of the fourth gene (*cspD*), which was previously annotated as encoding a 7-kDa protein, revealed that the mRNA is larger and probably encodes a putative 21-kDa protein, containing two CSDs. A search in protein sequences databases revealed that this new domain arrangement has thus far only been found among deduced peptides of α-proteobacteria. Expression of each *Caulobacter csp* gene was studied both in response to cold shock and to growth phase, and we have found that only *cspA* and *cspB* are induced by cold shock, whereas *cspC* and *cspD* are induced at stationary phase, with different induction rates. The transcription start sites were determined for each gene, and a deletion mapping of the *cspD* promoter region defined a sequence required for maximal levels of expression, indicating that regulation of this gene occurs at the transcriptional level. Deletion of *cspA*, but not *cspD*, caused a reduction in viability when cells were incubated at 10°C for prolonged times, suggesting that *cspA* is important for adaptation to a low temperature.

Bacterial cells face many challenges in the outward environment, being exposed to chemical and physical factors that may considerably affect their growth. Temperature is one of the most critical parameters for bacterial growth, and cells must adapt themselves fairly quickly to sudden temperature changes. Whereas a high temperature causes severe damage to the cells mainly because of protein denaturation, a low temperature may render cells nonviable because of alterations in nucleic acids and membrane lipids.

The response to a low temperature involves a change in the cell membrane lipid composition, with an increase in the proportion of unsaturated fatty acids, to keep the fluidity of the membrane at a low temperature (40). The ribosomes also adapt themselves to translate cold-specific mRNAs by incorporation of ribosomal factors that change their functional properties (24). The structure and topology of the chromosomal DNA is also affected, and its adaptation to the cold involves the induction of proteins that are nucleoid associated, such as H-NS (7).

One of the major difficulties bacteria face during a temperature downshift is the stabilization of secondary structures of nucleic acids, particularly mRNA, which prevents them from being efficiently translated. Upon cold shock, bacteria express a well-defined set of proteins to adapt the cell to the new temperature condition. The first protein described as a major protein induced upon decrease in temperature was CspA from *Escherichia coli* (17), an RNA chaperone which helps to destabilize the secondary structures of the RNA (23). The proposed role for CspA was also to increase mRNA translation and to render mRNA more susceptible to RNase degradation (6, 23). Eight other proteins homologous to CspA were identified in *E. coli* (CspB to CspI), and it was shown that CspA, CspE, and CspC, but not CspB, also act as transcription antiterminators in vitro (3), and CspD has been shown to inhibit DNA replication (56).

Homologues of cold shock proteins have not been found in archaeal genomes, and among the eubacteria they are present in most proteobacteria but not in spirochetes or cyanobacteria, the latter presenting a family of small cold-inducible proteins with RNA-binding domains similar to those found in eukaryotic proteins (36). These small proteins possess a conserved domain called the cold shock domain (CSD) that is composed of two nucleic acid-binding motifs, RNP1 and RNP2, that are crucial for the binding to single-stranded DNA and RNA (38). The CSD is also found in eukaryotic proteins, where it mediates RNA binding and interacts with other RNA-binding domains (20). The role of some of these eukaryotic proteins has been determined, showing that they are involved in coupling transcription of specific mRNAs with their translation and, in some cases, acting as transcription factors (5, 49).

Bacterial genomes contain usually many copies of *csp* genes, but there is a large variation in their patterns of expression. In *E. coli*, only four of the nine cold shock proteins are induced upon cold shock (CspA, CspB, CspG, and CspI) (47, 53), and one (CspD) is induced during stationary phase and upon nutritional starvation (51). *Bacillus subtilis* has three CspA-like proteins, which are all induced at a low temperature (18), and two of them (CspB and CspC) are also induced at stationary phase (21, 25). *Lactobacillus plantarum* has three *csp* genes, but only one (*cspL*) is highly induced at temperature downshift and stationary phase, whereas *cspP* and *cspC* are constitutively expressed (8).

The caulobacters are ubiquitous bacteria found in humid soils and in practically every aquatic environment (34). The

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distinct cell cycle of this bacterium, presenting a sessile phase and an obligatory motile phase, confers a good mode of dispersion through water in search for better nutritional conditions, and may also be important for adaptation to challenging situations, such as extreme environments. These bacteria were reported in frozen soil and bodies of water (1, 10), suggesting that it must be well adapted to live in low temperature. Analysis of the genome content of *Caulobacter* showed that it possesses four genes encoding putative small cold shock proteins (32), but their patterns of expression have not yet been determined. We have investigated here the regulation of the *csp* genes in response to cold shock and growth phase and determined a regulatory sequence important for expression of *cspD* that encodes a peptide presenting a novel CSD arrangement in bacteria. Two strains, one carrying a deletion of the cold-induced *cspA* gene and the other of the stationary-phase-induced *cspD* gene, were generated, and analyses of the phenotype indicate that *cspA*, but not *cspD*, is involved in adaptation to low temperature.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and genetic procedures. *Caulobacter crescentus* was grown at 30°C in peptone-yeast extract medium or minimal M2-glucose medium (9) supplemented with kanamycin (5 μg/ml), tetracycline (1 μg/ml), or nalidixic acid (25 μg/ml) as necessary. *Escherichia coli* strain DH5α (Invitrogen) was used in the cloning procedures. *E. coli* was grown at 37°C in Luria-Bertani medium supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), or tetracycline (12.5 μg/ml) as necessary. Plasmid pRK*lacZ*290 (14) was introduced into *Caulobacter* sp. by conjugation with *E. coli* strain S17-1 (39).

Sequence analyses. Protein sequence analysis was performed with the Protean program, which is included in the Lasergene DNA analysis package DNASTAR (DNASTAR, Inc., Madison, Wis.). The multiple alignments were performed with CLUSTALX (44), and searches in the sequence databases were performed with the BLAST algorithm (2).

Transcript analysis by RT-PCR. Analysis of the *cspD* mRNA was carried out by nonquantitative reverse transcription-PCR (RT-PCR) with total RNA from either mid-log- or stationary-phase cells treated with DNase I (amplification grade; Invitrogen) to eliminate any trace of DNA. The primers used were RT-2A and RT-2B (Table 1). Reactions were performed by using the SuperScript One-Step RT-PCR kit (Invitrogen) as recommended by the supplier. The RT-PCR conditions were as follows: 30 min at 55°C and 2 min at 94°C, followed by 35 cycles of 60 s at 94°C, 60 s at 48°C, and 60 s at 72°C, with a final cycle of 7 min at 72°C. Control reactions with only the *Taq* DNA polymerase were carried out to assure that no amplification was due to the presence of DNA in the samples.

Primer extension analysis. Oligonucleotides CSPA-PE, CSPB-PE, CSPC-PE, and CSPD-PE (Table 1), which hybridize to the beginning of the coding region of each gene were labeled with [γ-^{32}P]ATP and used for primer extension experiments. The primers were hybridized to 50 μg of total RNA isolated from log-phase cells grown at either 30 or 10°C for 1 h and 2 h or from cells at stationary phase and then extended with the SuperScript II reverse transcriptase (Invitrogen) as recommended by the supplier. The DNA sequencing ladder was obtained by cycle sequencing with the same primer and, as the template, a plasmid containing the cloned region of each gene by using the Thermosequenase cycle sequencing kit (USB).

Cloning of the promoter regions and gene expression analysis. The regions containing the *csp* genes were amplified from the *C. crescentus* chromosome by PCR with the various primers (see Table 1) as follows: *cspB*, CSPB-A and CSPB-B; *cspD*, CSPD-A and CSPD-B; *cspA*, CSPA-A and CSPA-B; and *cspC*, CSPC-F and CSPC-G.

PCRs were carried out with 1 μg of *C. crescentus* NA1000 chromosomal DNA, 50 pmol of each set of oligonucleotides (described above), 0.2 mM concentrations of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Invitrogen), and 1× PCR buffer (supplied with the enzyme). The PCR conditions were 5 min 94°C, followed by 40 cycles of 90 s at 94°C, 1 min at 50°C, and 1 min at 72°C, with a final cycle of 7 min at 72°C. The amplified fragments were cloned into the TOPO vector from TOPO TA cloning kit for sequencing (Invitrogen) and then confirmed by DNA sequencing.

DNA fragments of the *cspD* regulatory region were either obtained by restriction digestion (as shown in Fig. 6) or by PCR as described above with the primer pair CSPD-A–CSPD-B, CSPD-C–CSPD-B, or CSPD-D–CSPD-B, and the sequence was confirmed by DNA sequencing. DNA fragments of the promoter regions of the *cspA*, *cspB*, and *cspC* genes were obtained by restriction digestion (*cspA*, BamHI/NcoI; *cspB*, EcoRI/SacI; *cspC*, BamHI/EcoRI). All of the fragments obtained were cloned into pRK*lacZ*290 (14) and introduced into *C. crescentus* NA1000 by conjugation. Promoter activities during cold shock and stationary phase were determined by measuring the β-galactosidase activity by the method of Miller (29).

TABLE 1. Primers used in this study

Primer	Sequence (5′-3′)[a]
CSPA-A	CGAAACGGCTCGAGCGATG
CSPA-B	ATAGGATCCGTCGTTCTCAGAACCATC
CSPA-C	AAACTGCAGCTTCACGGTTCCAGTCGC
CSPA-D	TATCTGCAGCAAGCGGCCTAAAGCCCG
CSPA-E	AAAGGGCCCTCGCTGGTGTAGGCCTCGG
CSPA-PE	ATGTTCCTTGTAGGCATAGGTG
CSPB-A	ATAGGATCCGATCGCTCACCGCGAGAC
CSPB-B	GAAAAGCTTCAAGGCCTCACGCTGCGC
CSPB-PE	GAACCACTTTACGGTGCCG
CSPC-F	CGGGGTTTTTTGCGGTGCAATATCGC
CSPC-G	TTAGGATCCTGGCCGCCGTCCTCGGG
CSPC-PE	CCGTTCGCCATATTATCCTCA
CSPD-A	ATTGGATCCATATAACGGCTATGTTCC
CSPD-B	ATAGAATTCTGGTGACGATCTCGACC
CSPD-C	ATGGATCCACTGCCATCTTCGGC
CSPD-D	CCAGGATCCTGGCTTGCCCAATCAGCCC
CSPD-E	AAACTGCAGATTCGTCCCGAGATGATTCC
CSPD-F	AAAGGATCCTGGTGCGGTTCGCACGCGG
CSPD-G	AAAGAATTCGCTGCTCTACAGGGGTTCG
CSPD-PE	AAAATCGTAACCAGACATCCC
RT-2A	TGGTTACGATTTTGAGGACG
RT-2B	GTTGAACCATTTCACCTTGG

[a] Underlined nucleotides indicate restriction sites incorporated into oligonucleotides.

Deletion of *cspA* and *cspD* and viability tests. To delete the coding region of *cspA*, two fragments containing the region upstream and downstream of the gene, were amplified by PCR with primers CSPA-B/CSPA-C and CSPA-D/CSPA-E, respectively (Table 1), and ligated in tandem to the suicide vector pNPTS138. This 1.0-kb ApaI/BamHI fragment contains the flanking regions of the gene without the deleted region. The same was done for the *cspD* coding region, with the primer pairs CSPD-D–CSPD-E and CSPD-F–CSPD-G, generating a 1.6-kb PstI/EcoRI fragment. The pNTPS138 vectors were then introduced into *C. crescentus* NA1000 by conjugation with *E. coli* S17-1, and the genes were deleted by allelic exchange after double recombination. The deletions were confirmed by PCR amplification with primers flanking each gene and by Southern blots.

Determination of survival at low temperature was performed as follows. Cells were grown at 30°C up to mid-log phase and then transferred to 10°C, with agitation. Samples of each culture were taken at different time points, and viability tests were carried out by determination of the number of CFU. The relative survival was calculated as the number of CFU of the mutant strains at each time point divided by the number of CFU of strain NA1000 at the same points, considering that the absorbance at 600 nm for all cultures were identical.

RESULTS

Sequence analysis of open reading frames (ORFs) containing the CSD. There are four genes encoding small cold shock proteins similar to *E. coli* CspA in the genome of *Caulobacter* (32). The genes were arbitrarily named as follows: *cspA* (CC2903), *cspB* (CC0665), *cspC* (CC2623), and *cspD* (CC1387). Three of the peptides (CspA, CspB, and CspC) showed a higher degree of similarity to each other and to *E. coli* CspA and possess one CSD, which harbors the nucleic acid-binding motifs RNP1 and RNP2 (Fig. 1A). These proteins

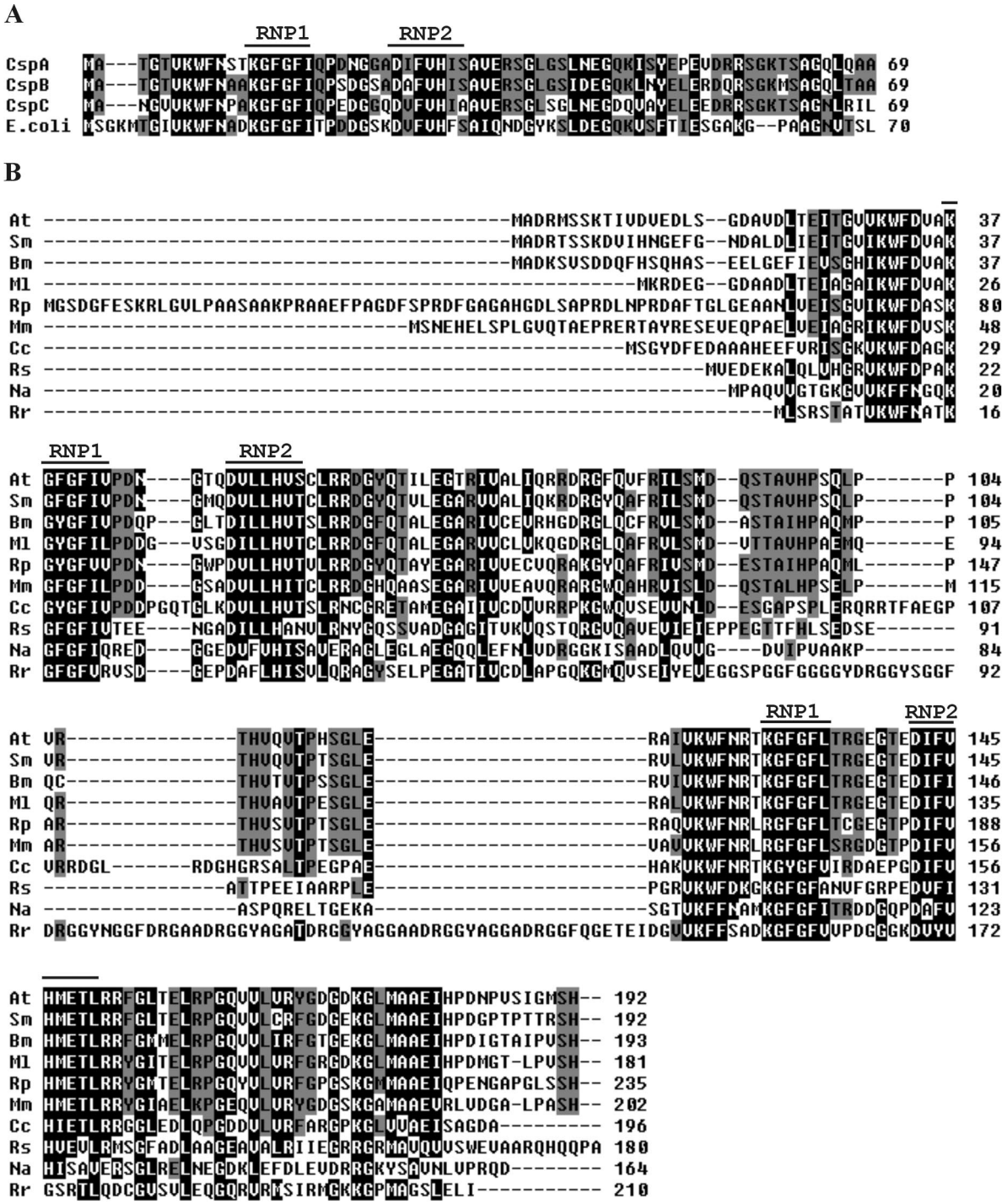


FIG. 1. (A) Amino acid sequence comparison of the three *C. crescentus* genes containing one CSD with *E. coli cspA* (GenBank no. AAB18533). (B) Amino acid sequence comparison of the proposed new annotation of *cspD* from *C. crescentus* (Cc) with other predicted α-*Proteobacteria* peptides showing two CSDs. Above the sequences are indicated the two RNA-binding motifs (RNP1 and RNP2). Residues shaded in black indicate conserved residues present in at least 90% of the proteins; those shaded in dark gray are present in at least 60% of the proteins. The GenBank no. and abbreviations are as follows: *Agrobacterium tumefaciens* (At; AAK87573), *Sinorhizobium meliloti* (Sm; CAC46297), *Brucella melitensis* (Bm; AAL51912), *Mesorhizobium loti* (Ml; BAB47810), *Rhodopseudomonas palustris* (Rp; ZP_00012063), *Magnetospirillum magnetotacticum* (Mm; ZP_00049605), *Rhodobacter sphaeroides* (Rs; ZP_00006193), *Novosphingobium aromaticivorans* (Na; ZP_00093526), and *Rhodospirillum rubrum* (Rr; ZP_00013462).

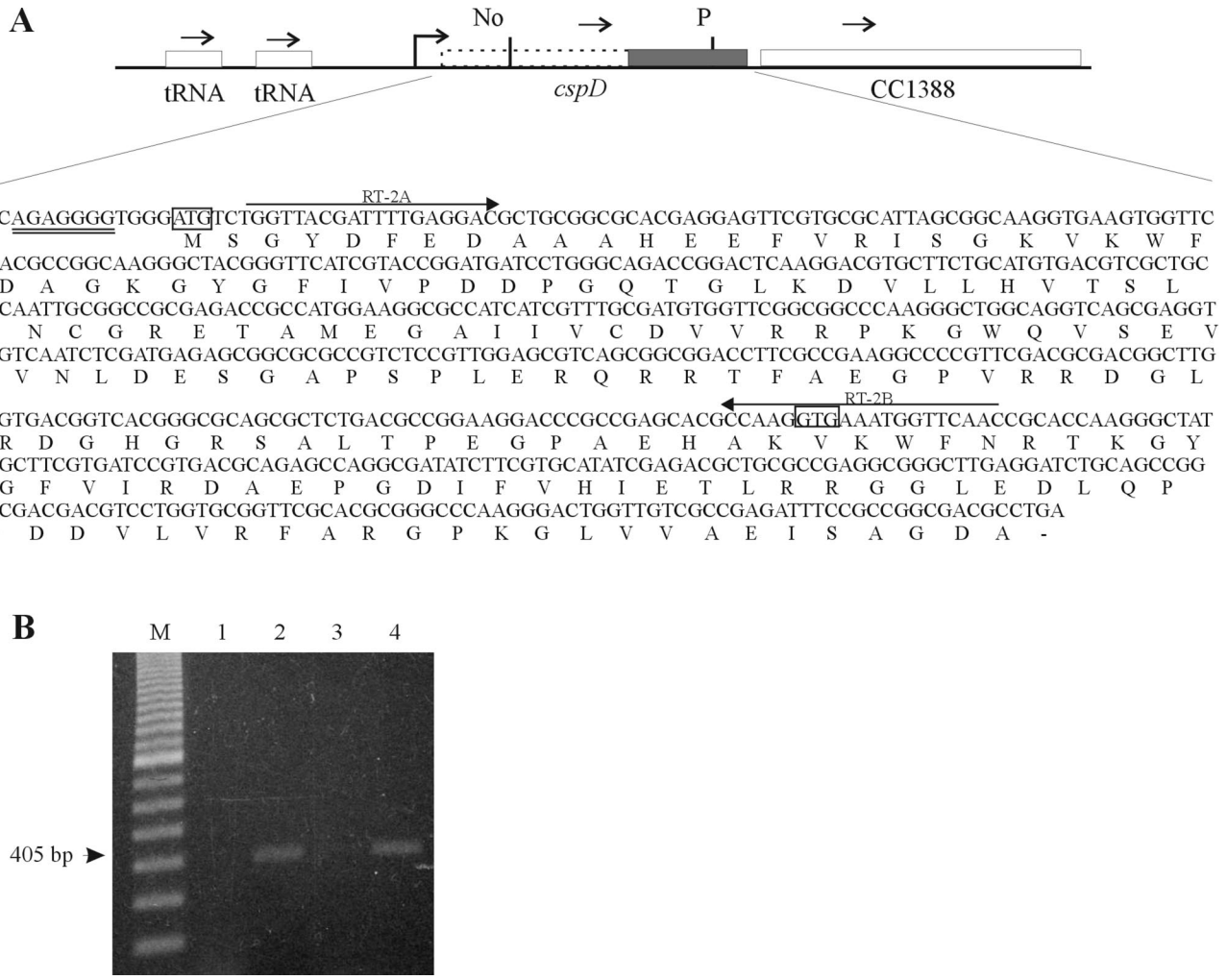


FIG. 2. Determination of the existence of a longer *cspD* transcript. (A) The scheme indicates the region of the *cspD* gene, showing the new proposed coding region (dotted lines), and the originally annotated coding region (dark box). Below is shown the sequence of the proposed *cspD* coding region, indicating the position of the two primers used in the RT-PCR (arrows). The new (ATG) and annotated (GTG) start sites are boxed. Ribosomal binding site is double underlined. (B) RNA was isolated from mid-log phase cells (lanes 1 and 2) and from cells at 24 h after entry into stationary phase (lanes 3 and 4) and treated with DNase I previous to the experiment. RT-PCR was performed with a pair of oligonucleotides—one that hybridizes close to the ATG of the proposed longer *cspD* ORF and one at the beginning of the annotated *cspD* coding region. Control reactions, carried out with *Taq* DNA polymerase but without reverse transcriptase, yielded no amplified bands (lanes 1 and 3), confirming that there is no contamination of DNA in the samples. The expected 405-nt fragment obtained for both samples is indicated by an arrow.

have similar predicted molecular mass of 7 kDa but have different pIs, being CspB and CspC acidic (pI 5.74 and 4.82, respectively) and CspA neutral (pI 7.16).

The original genome annotation of the fourth gene (*cspD*) identified a coding region corresponding to 192 bp, but a more detailed sequence analysis showed that the coding region is probably 588 bp long, coding for a putative protein of 21.5 kDa and a predicted pI of 5.94 (Fig. 2A). In order to investigate whether the *cspD* coding region is larger than what was previously determined, we performed an RT-PCR experiment, with primers that hybridize with the beginning of the proposed longer ORF and the beginning of the annotated *cspD*, respectively (Fig. 2A). A specific product was obtained, indicating that there is an mRNA encompassing the whole predicted ORF and suggesting that *cspD* could encode a 21.5-kDa protein (Fig. 2B). This putative protein contains two CSDs of 70 residues separated by a nonconserved region of 52 residues. A search in protein databases revealed that other proteins with the same domain structure are found in α-*Proteobacteria* but not in other eubacteria (Fig. 1B). Interestingly, we could not find any similar peptide in the two *Rickettsia* species that have their complete genome sequence determined.

These proteins share extensive similarity in their CSDs, but the amino terminus and the region between the two CSDs show high divergence, with the *Caulobacter*, *Rhodopseudomonas*, and *Rhodospirillum* proteins having insertions in these regions. An analysis of the CspD sequence with respect to backbone chain flexibility indicated that the region between residues 87 and 128 is highly flexible. A longer insertion at this same relative position was also seen in a *Rhodospirillum* homolog (Fig. 1B), suggesting that there may be less selective pressure on this interdomain region than on the CSDs.

The next ORF, CC1388, encodes a conserved 184-amino-acid protein, which possesses a domain of unknown function (DUF192) when analyzed by the PFAM program (4). The same genetic organization of *cspD* and CC1388 found in *C. crescentus* was observed in other α-*Proteobacteria*, except for *M. magnetotacticum*, *R. rubrum*, and *N. aromaticivorans*, in which the CC1388 homolog is found elsewhere in the genome.

Expression of *csp* genes in response to cold shock. It was observed for several bacteria that the expression of some homologues of CspA increases with cold shock, whereas other homologues are not induced under this condition. To determine whether the *C. crescentus csp* genes are induced by cold shock, the promoter region of each gene was cloned upstream of a *lacZ* gene in a reporter plasmid, and expression was analyzed by β-galactosidase activity (Fig. 3). It should be noted, however, that with reporter genes the results are only indicative of the time and extent of the induction, and some variation may occur, as reported by Goldenberg et al. for the *cspA* promoter (16). Figure 3B shows that expression of fusions carrying *cspA* or *cspB* promoters was increased by cold shock. On the other hand, expression of fusions carrying *cspC* or *cspD* promoters did not show any increase under the same conditions, indicating that these genes are not cold induced, similarly to *E. coli cspC*, *cspD*, or *cspE* genes (51, 55).

In order to evaluate the temperatures for which the *cspA* and *cspB* gene expression is maximized, we tested the levels of transcription at several temperatures ranging from 0 to 40°C (Fig. 3B). We observed that there is no induction at temperatures lower than 10°C (0 and 5°C) or higher than 20°C (30 and 40°C) and that both genes are induced at 10, 15, and 20°C. The *cspA* gene showed higher levels of induction, and expression was still going up after 4 h at 15 and 20°C, whereas the peak of expression of *cspB* was at 2 h and remained stable after that.

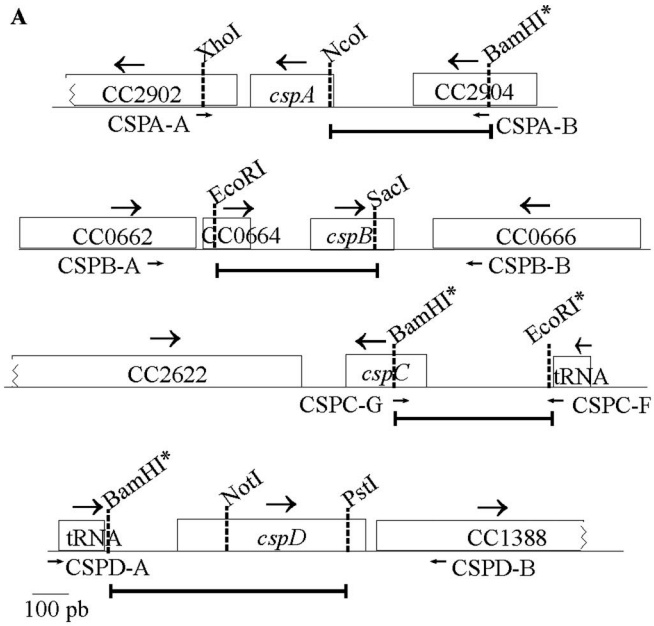
These results prompted us to determine whether the genes are induced at stationary phase, as with the *cspD* gene in *E. coli* (51). There is a great increase in enzyme activity at the onset of stationary phase when cells carry promoter fusions of *cspB* (3.8-fold), *cspC* (5.3-fold), and *cspD* (6.5-fold) (Fig. 4) and a very small increase (1.2-fold) with the promoter fusion of *cspA*. Once at stationary phase, the expression of these genes did not significantly change even 24 h after the onset of this phase, with the exception of the *cspD*/*lacZ* fusion, which showed a small increase after this time.

Analysis of transcription start sites. The transcription start sites of the *csp* genes were determined by primer extension analyses (Fig. 5). All genes showed multiple start sites, and the *cspA* and *cspB* transcripts were greatly induced after 2 h at 10°C (Fig. 5A and B), whereas *cspC* and *cspD* were induced at stationary phase (Fig. 5C and D). Despite the fact that the *cspB*/*lacZ* fusion showed an increased β-galactosidase activity at stationary phase (Fig. 4), we could not detect an increase in the *cspB* transcript, suggesting that the results may be due to some interference of the fusion to *lacZ*. Transcripts from *cspA* and *cspB* were detected from RNA of cells growing at 30°C at longer exposure times (not shown). If we consider the major transcripts of each gene, the results showed that the 5′-untranslated regions of the cold-induced *cspA* and *cspB* genes are longer (127 and 143 nucleotides [nt], respectively) than those of the genes that are not cold induced (*cspC* [64 nt] and *cspD* [45 nt]). Long 5′-untranslated regions are found in several cold-induced genes and have a role in regulation of gene expression (11, 12, 16, 47, 48). The −35 and −10 sequences of the major transcripts share low similarity among the promoters, but there are AT-rich regions upstream of the *cspB* −35 region, similar to what was reported for *E. coli cspA* (16, 30).

For the *cspD* gene, the −35 (TTGACGG) and −10 (GCG AGAAC) regions follow the consensus proposed for promoter regions of *Caulobacter* housekeeping genes (27). Two start sites were observed when RNA isolated from exponential-phase cells was used, with the downstream signal being the more intense (Fig. 5D), and three signals were observed when stationary-phase RNA was used—two corresponding to the log-phase RNA and a new one located upstream. All three bands were more intense in stationary phase than in the log phase, indicating that the induction observed at this growth phase is due to an increase in mRNA level whose transcription initiates from the same promoter.

The regulatory region of *cspD* was further analyzed by cloning several promoter fragments containing progressive deletions each in front of a *lacZ* reporter gene (Fig. 6). Deletion analysis showed that there is no promoter activity downstream of the NotI site (pEL1), which is 225 bp upstream of the annotated start codon. Fragments comprising the region from the upstream tRNA gene to the PstI site drive the maximal values of β-galactosidase activity in the log phase and also a great induction (~5.5-fold) in the stationary phase. These levels of expression are observed for all constructs that contain the region downstream of position −98 (pEL2, pEL3, and pEL4). The activity of pEL5 is much lower than that of pEL4, although the promoter region is present in this construction, which suggests that the region between positions −98 and −73 is necessary for maximal *cspD* expression in both log and stationary phases. The sequence found in this region comprises two imperfect direct repeats (Fig. 5D), and a similar sequence was also found upstream of the *cspC* gene (Fig. 5C), whose promoter fusion showed similar levels of β-galactosidase activity during stationary phase. These results indicate that this region may be a regulatory site involved in the maximal levels of expression of *cspD*, but it is not involved in the growth-phase-specific induction.

Analysis of *cspA* and *cspD* mutants. *cspA* is the major cold-induced *csp* gene, and it probably has a role in adaptation of *Caulobacter* to low temperature, whereas the *cspD* gene could be more important in adaptation to stationary phase. In order to verify this hypothesis, two mutant strains were generated in which the *cspA* gene and *cspD* genes were deleted (NA1000 [Δ*cspA*] and NA1000 [Δ*cspD*], respectively). The strains were tested for survival at low temperature (10°C) and at stationary phase. Neither strain showed any defect in survival at stationary phase (results not shown), and when cells were incubated for prolonged times at 10°C, the parental NA1000 strain and the *cspD* mutant did not show any decrease in survival (Fig. 7). On the other hand, the *cspA* mutant showed a decrease in survival after 24 h at 10°C and kept that reduced viability after 48 h. These results suggest that *cspA* is important for *Caulobacter* to withstand low temperature, whereas the role of *cspD* is probably not directly related to cold survival.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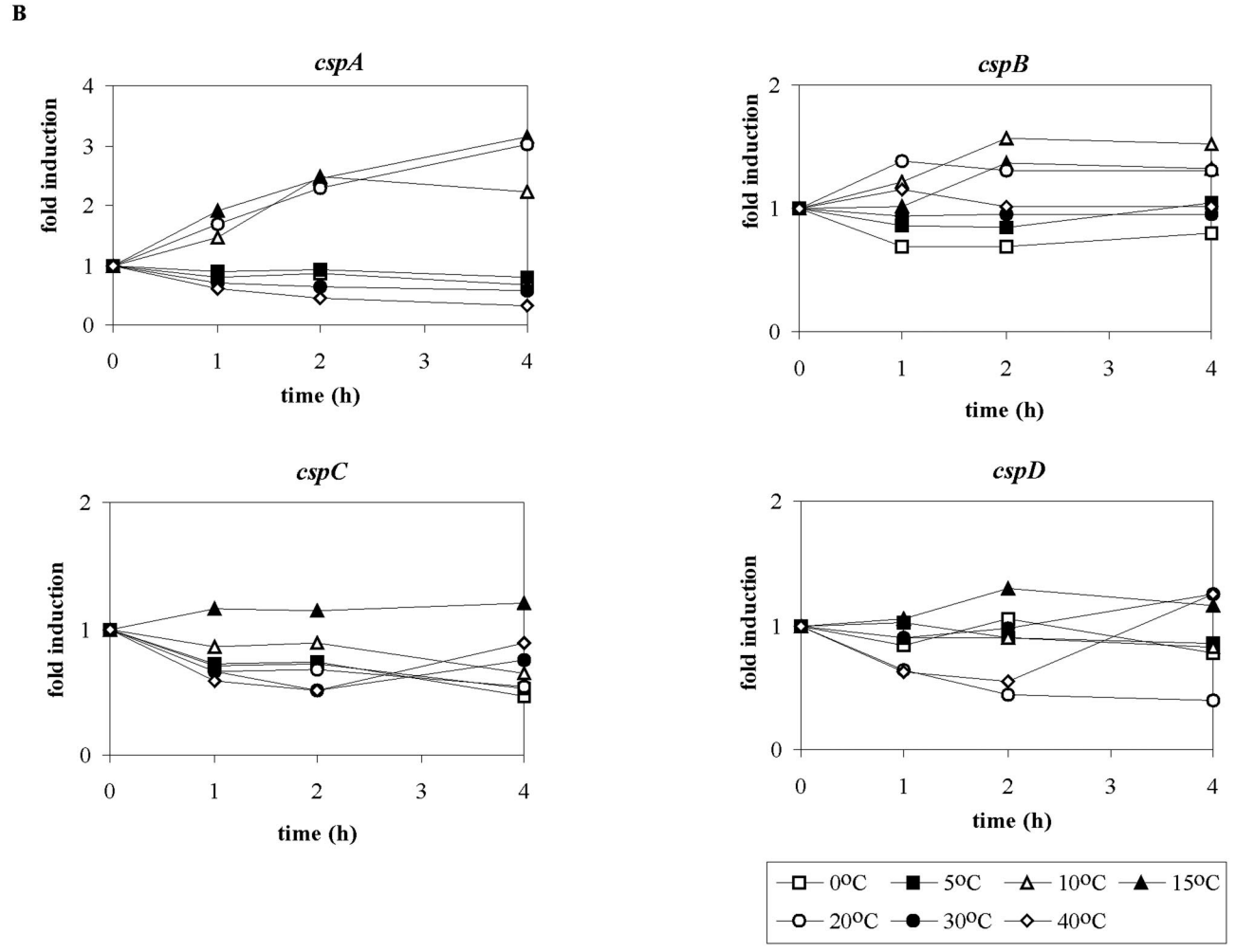
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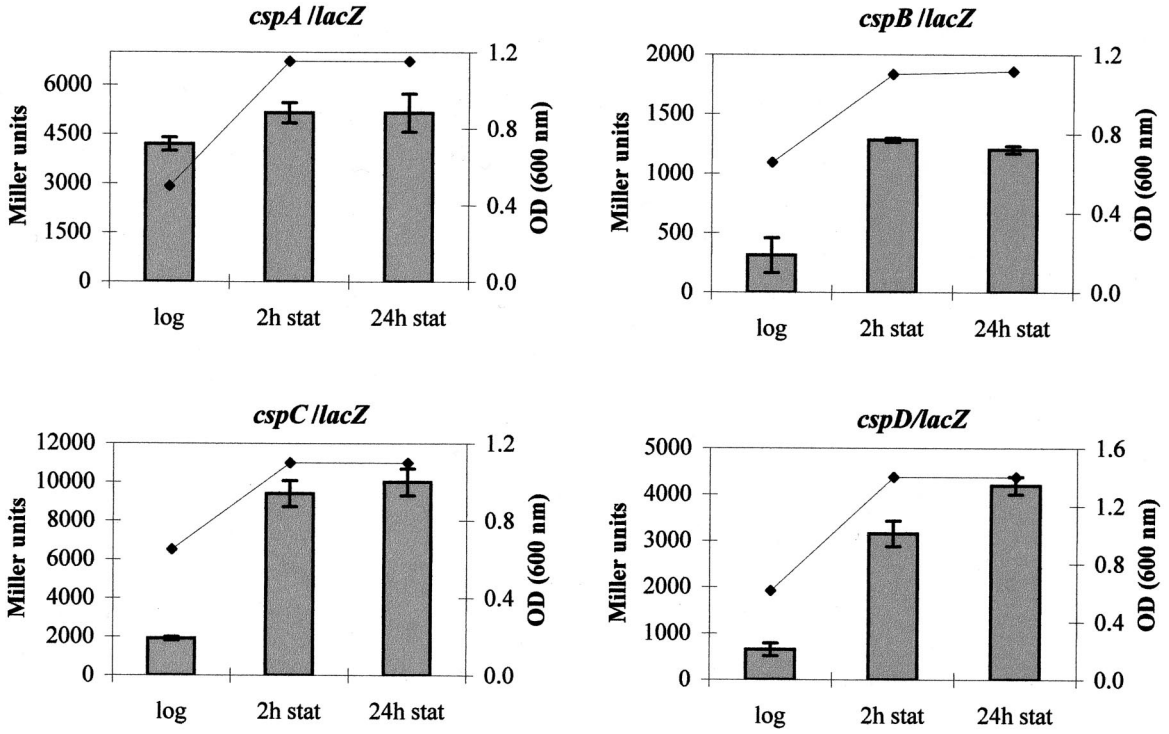

FIG. 4. Growth-phase-dependent expression of the *csp* genes. Expression of the *csp* genes was determined from cells harboring the respective promoter fusions at mid-log phase and at 2 and 24 h after entry into stationary phase. The β-galactosidase activity is expressed in Miller units (29), and growth was monitored by measuring the optical density (OD) at 600 nm.

DISCUSSION

The cold shock response in bacteria involves the activation of several genes important for adjusting the essential cellular processes to the new temperature. The best-studied cold shock genes are those encoding small proteins (7 kDa), which are very conserved among bacteria (33), playing a major role during cold shock adaptation but also important under normal growth conditions. The prototype of this family, the CspA protein from *E. coli*, is composed of five antiparallel β-strands and presents a very efficient folding (31, 35, 37). The finding of eukaryotic proteins sharing sequence similarity with the bacterial cold shock proteins showed that the small cold shock proteins are constituted of a single domain, the CSD, which is conserved from bacteria to humans (20). CSDs have also been determined to be integral components of larger proteins in eukaryotes (22).

We show here that *C. crescentus* has four predicted peptides that present the CSD; three of them belong to the *E. coli* CspA family of 7-kDa proteins (CspA, CspB, and CspC), and one of them belongs to a novel class of bacterial proteins that possesses two CSDs (CspD). Despite the modular nature of the CSD, which has been clear for some time (20), proteins with this two-domain structure have not yet been described. One possible reason for this could be that we have identified this particular arrangement in predicted proteins only from proteobacteria of the α subdivision (Fig. 1B), whose genome sequences only recently became available.

The role of *Caulobacter* CspD and these novel α-*Proteobacteria* proteins possessing a double CSD structure in the cell is still unknown. A protein with five CSDs in humans, the UNR protein, has been described (22) that was found to bind single-stranded DNA and RNA with high affinity and double-stranded DNA with lower affinity (13); only three of the domains are sufficient to confer the same affinity for RNA as does the full-length protein (45). The interaction of Unr with a second protein, the gene regulator ALL-1, requires two CSDs, suggesting that this double-domain arrangement could be necessary for protein-protein interaction (26). Another interesting observation is that some bacterial cold shock proteins are able to form dimers in vitro (28, 56), although the physiological relevance of this is still unclear. It is tempting to speculate that in some cold shock proteins from α-*Proteobacteria*, dimerization of the CSD was ensured by encoding the two domains within the same polypeptide.

FIG. 3. (A) Schematic representation of the *csp* genes. The DNA fragments cloned to the pRK*lacZ*290 vector in the transcriptional fusions are indicated by bars. The small arrows indicate the primers used to amplify each region from the genome. Some restriction sites are indicated, and the sites inserted by PCR are labeled with an asterisk. (B) Analysis of the cold induction of the *csp* genes. Cells harboring the transcription fusions of each gene were grown at 30°C up to mid-log phase and were then transferred to different temperatures. Expression of each construct was measured by β-galactosidase activity assays (29) at sequential time points, and the results are shown as relative measurements of induction.

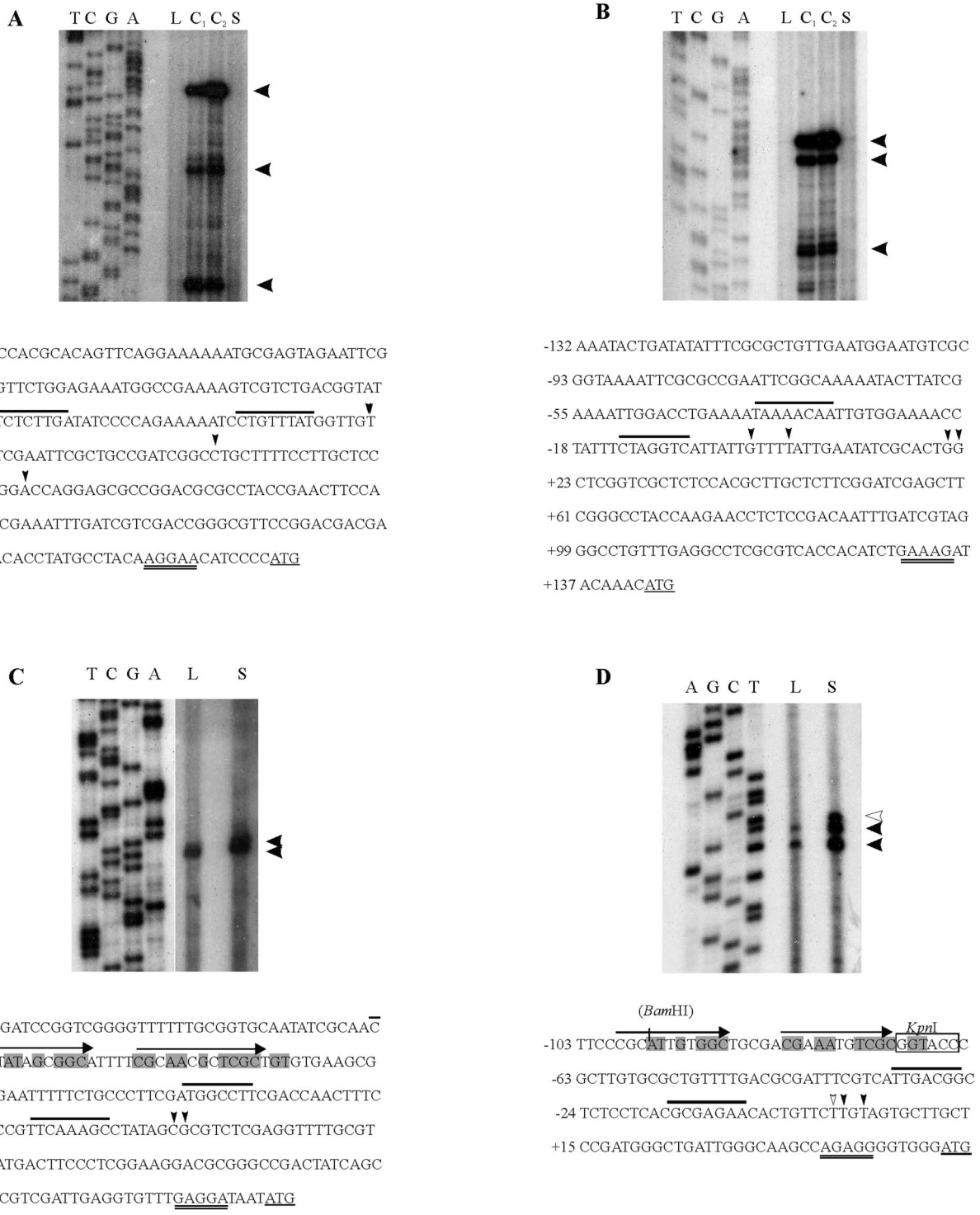

FIG. 5. Determination of the transcription start sites of *csp* genes. Primer extension analysis was carried out with total RNA from exponential-phase cells (L) or stationary-phase cells (S) at 30°C or incubated at 10°C for 1 h (C_1) or 2 h (C_2). The primers were end labeled with ^{32}P and extended with reverse transcriptase to determine the transcription start sites and were also used in DNA sequencing reactions (shown on the left). Below each panel is shown the respective regulatory regions: *cspA* (A), *cspB* (B), *cspC* (C), and *cspD* (D). Black arrowheads indicate the transcription start sites, and a white arrowhead indicates the stationary-phase start site of *cspD*. The most upstream start sites were arbitrarily chosen as position +1, and the −35/−10 sequences are overlined (for clarity, only the most upstream promoters are indicated). The start codons are underlined, and the ribosome-binding sites are double underlined. In panel D, the KpnI restriction site used for the transcription fusion pEL5 (Fig. 6) is boxed, and the position of the BamHI restriction site introduced by PCR in construct pEL4 is shown in parentheses. In panels C and D, the arrows indicate two imperfect direct repeats, and the shaded nucleotides indicate the regulatory sequence of *cspD* that is also found in *cspC*.

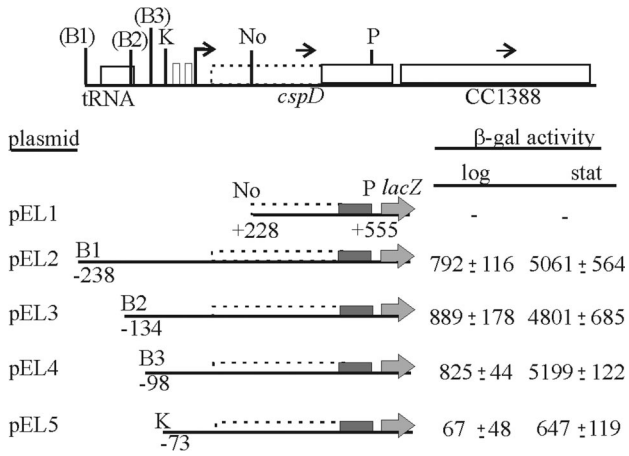

FIG. 6. Deletion mapping of *cspD* promoter region. The scheme of the *cspD* locus is shown above, indicating the previously annotated coding region (solid line), and the proposed extended coding region (dashed line). The bent arrow indicates the transcription start sites determined by primer extension. Restriction sites are indicated as follows: B, BamHI; K, KpnI; No, NotI; P, PstI. The sites in parentheses were introduced by PCR and are not in the original sequence. The plasmids carrying the constructs were introduced into *C. crescentus* NA1000, and promoter activity was measured by β-galactosidase assays both in exponential-phase (log) and stationary-phase cells (stat). The results are in Miller units (29) and are the average of at least three independent assays, with the respective standard deviation.

We showed that the fusions containing the promoter regions of *cspA* or *cspB* are highly induced by a temperature downshift from 10 to 20°C, whereas those containing the *cspC* and *cspD* promoters are not. It has been demonstrated that the expression of *E. coli* CspA is regulated at the transcriptional level during cold shock and that the expression of β-galactosidase under control of its promoter was increased three- to fivefold upon a decrease in temperature (16, 43, 46). The cold inducibility of *E. coli* CspA, as well as *B. subtilis* CspB, is also the result of increased mRNA stability at a low temperature (6, 12, 15, 25). The presence of a sequence called upstream box in the long 5′-untranslated region and a sequence downstream of the initiation codon (called the downstream box) seem to increase translation efficiency in *E. coli* (11, 30, 41, 54). Although *C. crescentus cspA* and *cspB* genes have long 5′-untranslated regions, no sequences similar to the consensus for *E. coli* boxes could be found. The use of transcriptional fusions prevents the regulation at the level of translation, since the reporter gene has its own translation signals; therefore, the increase in expression observed for *Caulobacter cspA* and *cspB* promoter fusions is a result of transcription and/or mRNA stability.

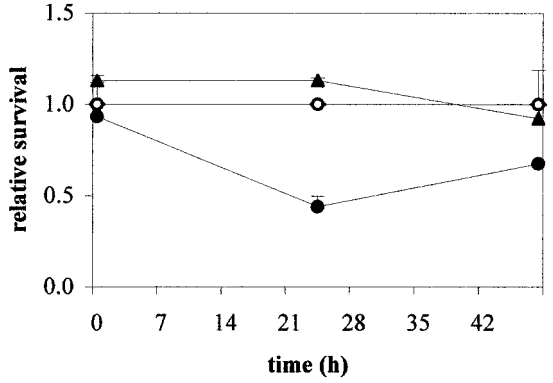

FIG. 7. Viability of the mutant strains at 10°C. Cells of the parental strain NA1000 (○), NA1000 (Δ*cspA*) (●), and NA1000 (Δ*cspD*) (▲) were grown at 30°C up to early log phase and then transferred to 10°C. Aliquots were taken before (0 h) and 24 or 48 h after incubation at 10°C, and serial dilutions were plated to determine the number of CFU. Survival rates were determined relative to NA1000 at each time point.

The promoter fusions of three of the genes (*cspB*, *cspD*, and *cspC*) showed similar degrees of induction of β-galactosidase when cells entered stationary phase, but the promoter fusions of *cspC* and *cspD* genes did not show any increase in expression at a low temperature. Other CspA homologues in *E. coli* were described that are not induced by cold shock (51, 55) and, among them, *cspD* is induced during stationary phase. The *Caulobacter cspD* gene is regulated at the transcriptional level, since the presence of an upstream regulatory region is essential for maximal levels of expression. A sequence similar to this activator sequence is also present in the promoter region of the *cspC* gene, but it is not found in the regulatory region of another stationary-phase-induced gene, *katG* (42). Since this element is not responsible for the growth phase regulation, the stationary-phase induction observed could be a result of both transcriptional regulation and increased mRNA stability. In *B. subtilis*, two of three small cold shock-induced proteins, CspB and CspC, are also induced in the stationary phase and were shown to be essential for adaptation to this phase (20, 25). Since the *Caulobacter cspC* and *cspD* genes are induced in the stationary phase, their role is probably related more specifically to adapting the cell to survive long periods of growth arrest. The environmental signals that trigger the expression of these two genes are still not determined, but they might respond to the nutritional status of the cell, as described for CspA and CspD from *E. coli* (52).

Gene knockout of *Caulobacter cspA* and *cspD* showed that these genes are not essential at 30°C, but the *cspA* strain shows a lower survival rate during prolonged growth at 10°C. The phenotype observed is consistent but not severe, indicating that the lack of a single *csp* gene is not very deleterious to the cell. Cells carrying deletions of individual *E. coli csp* genes or even a triple deletion (Δ*cspA* Δ*cspB* Δ*cspG*) were also shown to be viable, but a combination of four deletions (Δ*cspA* Δ*cspB* Δ*cspG* Δ*cspE*) presented a cell division defect at a low temperature (50). It was shown that when *E. coli* cells carry a double or triple *csp* deletion, there is a compensatory induction of the remaining *csp* homologues (50). In *B. subtilis*, double *cspB*/*cspC* or *cspB*/*cspD* deletions show a reduction in growth rate at both 15 and 37°C and lower viability at stationary phase (19). Although the *Caulobacter cspD* gene is induced at stationary phase, it is not essential for viability at this phase. The knockout of the other two genes, as well as obtaining double mutations, will enable us to determine the respective role of each gene in response to cold shock and stationary-phase survival.

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