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Expression Analysis of the *csp*-like Genes from *Corynebacterium glutamicum* Encoding Homologs of the *Escherichia coli* Major Cold-Shock Protein CspA

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Abstract Three *csp*-like genes were identified in the *Corynebacterium glutamicum* genome and designated *cspA*, *cspB*, and *cspA2*. The genes *cspA* and *cspA2* encode proteins, comprising of 67 amino acid residues, respectively. They share 83% identity with each other. Identity of those proteins with *Escherichia coli* Csp proteins was near 50%. The *cspB* gene encodes a protein composed of 127 amino acids, which has 40% and 35% sequence identity with CspA and CspA2, respectively, especially at its N-terminal region. Analysis of the gene expression profiles was done using transcriptional *cat* fusion, which identified not only active expression of the three genes at the physiological growth temperature of 30°C but also growth phase-dependent expression with the highest activity at late log phase. The promoters of *cspA* and *cspA2* were more active than that of *cspB*. The expression of the two genes increased by 30% after a temperature downshift to 15°C, and such stimulation was more evident in the late growth phase. In addition, the *cspA* gene appeared to show DNA-binding activity *in vivo*, and the activity increased at lower temperatures. Interestingly, the presence of *cspA* in multicopy hindered the growth of the host *C. glutamicum* cells at 20°C, but not at 30°C. Altogether, these data suggest that *cspA*, *cspB*, and *cspA2* perform functions related to cold shock as well as normal cellular physiology. Moreover, CspA and its ortholog CspA2 may perform additional functions as a transcriptional regulator.

Keywords: *Corynebacterium glutamicum*, *csp*, cold shock

Corynebacterium glutamicum is a Gram-positive organism and has played a major role in the industrial production of amino acids. Because of its economic importance, the organism has been the target of research to improve amino acid production by genetic engineering, which mainly involves the amplification and deregulation of the biosynthetic genes and enzymes [for reviews see 4, 11, 12, 21, 32, 40]. These days, new approaches, such as improving the stress response mechanism of the organism, are being introduced in the area of strain construction [17, 19]. Understanding the stress response mechanism in the target organism will be a prerequisite for such an approach.

Bacteria respond to various stresses with specific adaptive reactions, thereby ensuring that they survive and continue their growth under new environmental conditions. One example is a cold-shock response. Once *E. coli* cells in an exponential growth phase at 37°C are transferred to 15°C, there is a lag growth period. The cells express a group of high-level cold-shock proteins. Protein CspA is a major cold-shock protein and is one of the nine homologous proteins (CspA to CspI) of *E. coli*, constituting the CspA family [for review, see 28]. As the proteins in the CspA family show a high sequence similarity to each other, they are functionally redundant. For example, all three *csp* genes, *cspB*, *cspC*, and *cspD*, could be deleted to get a lethal phenotype in *B. subtilis* [9], whereas four (*cspA*, *cspB*, *cspE*, and *cspG*) out of the nine *csp* genes would have to be inactivated before obtaining a cold-sensitive phenotype in *E. coli* [43].

Upon cold shock, the *csp* genes promote adaptations of the cells to the new environmental conditions, by functioning at the transcriptional, posttranscriptional, and translational levels. In *E. coli*, CspA proteins have been thought to be like RNA chaperones, which bind to mRNAs and prevent the formation of secondary structures that might prohibit their translation at low temperatures [13]. It has been found that CspA can stimulate the translation of its own messenger [2]. CspA proteins also bind RNA and single-stranded DNA with low affinity and specificity [13]. CspB, CspC, and CspE proteins can bind preferentially to specific RNA and single-stranded DNA sequences [30].

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Those bindings lead to energy-independent melting and/or the destabilization of nucleic acid secondary structures that are formed by a cold shock, to facilitate transcription and translation at low temperatures. CspA proteins of *E. coli* have been shown to stimulate the transcription of two cold-shock genes, *hns* and *gyrA*, and have been suggested to be the general activator of the cold-shock regulon [2, 14, 22].

On the other hand, expression of *cspA* is regulated by the antagonistic transcriptional control of the activator Fis and the repressor H-NS under nonstress conditions.

E. coli has nine cold-shock-inducible proteins, but only CspA, CspB, CspG, and CspI are cold-inducible [38, 45, 46]. CspC and CspE are constitutively expressed even at 37°C, indicating that the genes also perform normal physiological functions [29, 31]. The *cspD* gene is induced by nutrient starvation, and at the stationary phase [44]. However, their functions in the cell are still unclear. Proteins related to the CspA family have been identified in many bacteria including *Bacillus subtilis* [9, 41], *Lactococcus lactis* [3], *Listeria monocytogenes* [6], *Pseudomonas fragi* [25], and *Streptomyces clavuligerus* [1].

In this study, three ORFs that seem to be related to *cspA* were identified from *C. glutamicum*. Their expression profiles were analyzed by constructing a transcriptional fusion of the *csp* promoters with the *cat* gene in order to elucidate their expression. Based on the results, we suggest that the role of the ORFs is related to cold shock.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains, plasmids, and primers are listed in Table 1. *E. coli* and *C. glutamicum* cells were grown at 37°C in Luria-Bertani (LB, 30) and at 30°C in MB [7], respectively. The minimal media for *E. coli* and *C. glutamicum* were M9 [33] and MCGC [15, 37]. Glucose was added at a concentration of 1%. Ampicillin, chloramphenicol, and kanamycin were added to the final concentrations of 50, 25, and 25 mg/l, respectively.

DNA Technology and Plasmid Construction

Standard molecular cloning, transformation, and electrophoresis protocols were utilized [23, 33]. Plasmids were introduced into *C. glutamicum* cells by electroporation [7], and the preparation of plasmids in *C. glutamicum* cells were

Table 1. Bacterial strains and plasmids used in this study.

Strains, plasmids, and primers	Relevant genotypes or phenotypes	Sources or references
Escherichia coli		
E. coli DH5αF'	F⁻Φ80d*lacZ*ΔM15, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*(rk−, mk+), *supE44*, *relA1*, *deoR*, Δ(*lacZYA-argF*)U169, λ⁻	Bethesda Research Laboratories
E. coli DH5αF'-145	*E. coli* DH5αF' containing plasmid pSL145	[20]
Corynebacterium glutamicum		
AS019E12	Restriction-deficient variant of AS019	[7]
HL1174	*C. glutamicum* AS019E12 carrying pSL430	This study
Plasmids		
pMT1	Shuttle vector; Ap^r, Km^r	[7]
pSK1Cat	*E. coli-C. glutamicum* shuttle promoter-probe vector carrying a promoterless *cat* gene, Km^r	[27]
pSK1CatP_{tac}	pSK1Cat carrying P_{tac}	[27]
pSK1CatP_{180}	pSK1Cat carrying P_{180}	[27]
pSL145	P_{aceB}-*lacZYA*, Tc^r	[20]
pSL430	pMT1 carrying *cspA*	This study
pSL410	pSK1CAT with 378 bp insert carrying P_{cspA} region, P_{cspA}-CAT	This study
pSL411	pSK1CAT with 650 bp insert carrying P_{cspB} region, P_{cspB}-CAT	This study
pSL412	pSK1CAT with 863 bp insert carrying P_{cspA2} region, P_{cspA2}-CAT	This study
Primers		
cspA-forward	5'-ATCGGGGGTAGTCAAG-3'	This study
cspA-reverse	5'-AGGATGTGGGAGGGTT-3'	This study
cspAP-forward	5'-ACCGGATCCCAGCTCTTCACCAAAG-3'	This study
cspAP-reverse	5'-TGTGTCGACCTCTGGGTTGAACCAC-3'	This study
cspBP-forward	5'-ACCGGATCCCTTAGTGGAGTGACG-3'	This study
cspBP-reverse	5'-TGTGTCGACCTTCACTGTTCCGAC-3'	This study
cspA2P-forward	5'-ACCGGATCCTCCGACCTGATCAGAG-3'	This study
cspA2P-reverse	5'-TGTGTCGACTTCGCCGTTGAACCAT-3'	This study

[r]Superscripts indicate resistance. Ap, ampicillin; Km, kanamycin; Tc, tetracycline.

conducted as described previously [47]. Chromosomal DNA from *C. glutamicum* AS019E12 was prepared as described earlier [35]. All restriction enzymes and DNA modifying enzymes were purchased from the Takara Shuzo Co. (Shiga, Japan) and New England Biolabs (Beverly, U.S.A.), and used according to the manufacturer's recommendations.

The plasmid pSL430 was constructed as follows. The region carrying the *cspA* structural gene and its promoter was amplified from the *C. glutamicum* chromosome using primers *cspA*-forward and *cspA*-reverse. The amplified fragment was cloned into the SmaI-digested pMT1 vector. The pSL410 plasmid, which harbors the chloramphenicol acetyltransferase (CAT, EC 2.3.1.28) gene, fused to the downstream region of the *cspA* promoter, was constructed as follows. The *cspA* promoter region was amplified from the chromosome using primers *cspAP*-forward and *cspAP*-reverse, digested with BamHI and SalI. It was then inserted into the pSK1Cat vector, previously digested with the same enzymes. Plasmids pSL411 and pSL412 were constructed by the same scheme, except with different primers. Primers *cspBP*-forward and *cspBP*-reverse and primers *cspA2P*-forward and *cspA2P*-reverse were utilized for the construction of pSL411 and pSL412, respectively.

Cold-Shock Experiment

C. glutamicum cells carrying the appropriate clones were grown in MCGC liquid medium at 30°C. During the appropriate growth phase, the growth temperature was shifted down to 15°C. Culture samples were taken before and 5 h after the temperature shift [18]. Crude extracts were prepared [19] and the chloramphenicol acetyltransferase (CAT) activity was measured as described below.

Biochemical Analysis

The CAT assay was performed as described previously [34]. The chemical reaction was initiated by mixing 1–20 μl of crude extract with 1 ml of reaction mixture (100 mM Tris·HCl pH 7.8, 1 mM DTNB, 0.1 mM acetyl-CoA, and 0.25 mM chloramphenicol). CAT activity was monitored by measuring the optical density at 412 nm, for 5 min. The enzymatic activities of β-galactosidase were determined as described previously [26].

RESULTS

Analysis of the *csp*-like Genes of *C. glutamicum*

In the genome of *C. glutamicum*, a total of three ORFs were identified, which may encode the proteins of the CspA family. Cgl0174 and Cgl0308 were found to encode the proteins composed of 67 amino acid residues, respectively, but Cgl0820 encoded the protein composed of 127 amino acids. The protein product of Cgl0174 showed an 83% identity with that of Cgl0308 (Fig. 1). The protein products of Cgl0174 and Cgl0308 shared a 35% and a 40% sequence identity with the protein encoded by Cgl0820, respectively, especially at its N-terminal region.

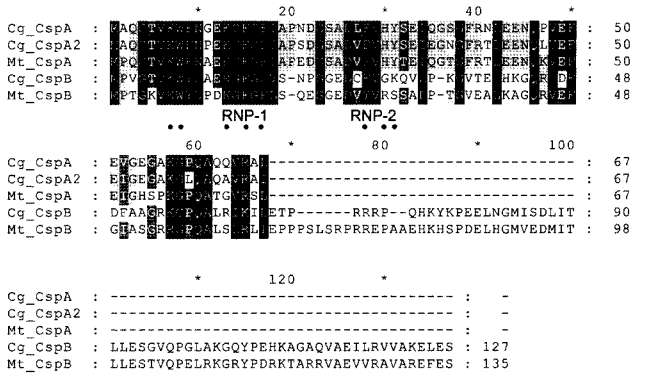

Fig. 1. Multiple amino acid sequence alignment of the CspA, CspB, and CspA2 from *C. glutamicum* with CspA and CspB proteins from *M. tuberculosis*.

Conserved and functionally similar amino acids are marked with black and shaded boxes, respectively. Important residues involved in the nucleic acid binding site are indicated with filled dots. RNA-binding motifs RNP-1 and RNP-2 are indicated. Abbreviations: Cg_CspA, CspA2 of *C. glutamicum* (YP 224470); Cg_CspA2, CspA of *C. glutamicum* (YP 224607); Cg_CspB, CspB of *C. glutamicum* (NP 600049); Mt_CspA, CspA of *M. tuberculosis* H37Rv (NP 218165); Mt_CspB, CspB of *M. tuberculosis* H37Rv (CAA17677).

Three cold-shock genes, *cspA*, *cspB*, and *cspC*, are found in the genome of *M. tuberculosis*. The proteins encoded by Cgl0174 and Cgl0308 showed an approximately 77% identity with CspA of *M. tuberculosis* (Fig. 1), and the protein encoded by Cgl0820 showed a 53% identity with CspB of *M. tuberculosis*. Based on these findings, ORFs Cgl0308, Cgl0174, and Cgl0820 were designated *cspA*, *cspA2*, and *cspB*, respectively.

On the other hand, the CspA and CspA2 of *C. glutamicum* showed an approximately 53% sequence identity with the CspA of *E. coli*. Their identity with the other Csp proteins of *E. coli* was near 50%. On the contrary, the CspB of *C. glutamicum* had low identity (10% to 20%) with the Csp proteins from *E. coli* (data not shown).

Scrutiny of the CspA and CspA2 proteins identified the two RNA-binding motifs of RNP-1 (KGFGFI) and RNP-2 (FVHY), which are highly conserved among the Csp proteins (Fig. 1). In addition, the aromatic and basic amino acid residues, which are important for binding single-stranded DNA in *E. coli* CspA and *B. subtilis* CspB, were also found to be conserved in *C. glutamicum* CspA and CspA2 (Fig. 1). The presence of those conserved motifs and residues in *C. glutamicum* Csp proteins suggest that CspA and its homolog CspA2 are probably nucleic acid-binding proteins, as demonstrated for other members of the Csp family (see below).

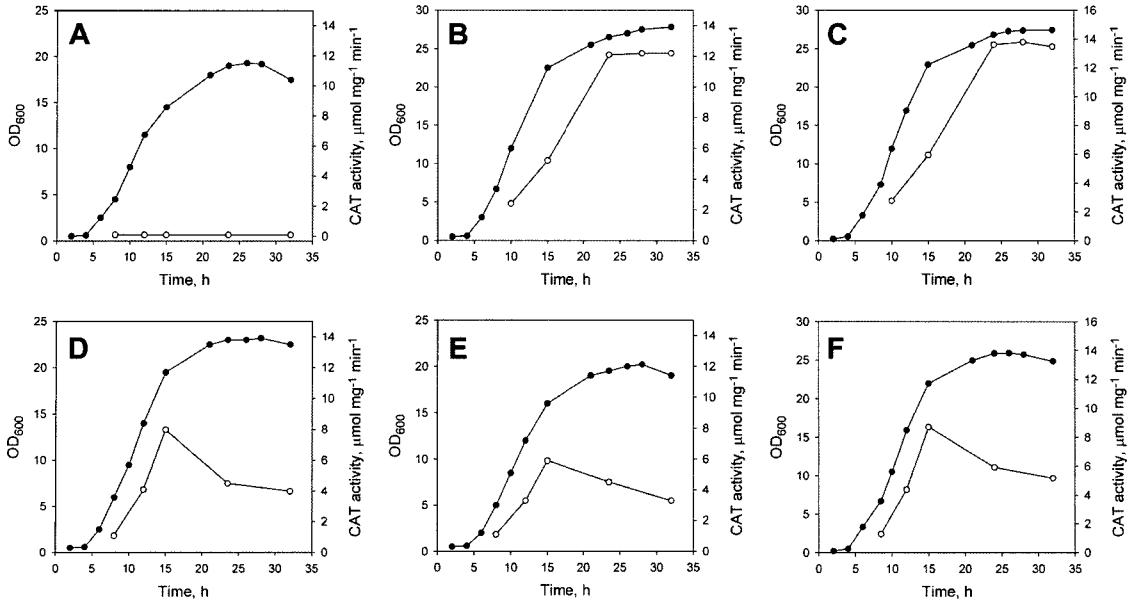

Fig. 2. Expression of the *cspA*, *cspB*, and *cspA2* genes during the growth of the *C. glutamicum* strains at 30°C on MCGC minimal medium containing 1% glucose as a sole carbon source.

The expressions of the *csp* genes were monitored by CAT activity, as described in Materials and Methods. Cell extracts were prepared from *C. glutamicum* AS019E12 harboring pSK1Cat (Panel A), pSK1P$_{tac}$-*cat* (Panel B), pSK1P$_{180}$-*cat* (Panel C), pSK1P$_{cspA}$-*cat* (Panel D), pSK1P$_{cspB}$-*cat* (Panel E), or pSK1P$_{cspA2}$-*cat* (Panel F). Closed and open circles represent the cell growth and CAT activities, respectively. One representative data point is shown out of several repetitive experiments.

Expression of the *C. glutamicum csp* Genes

In order to analyze the expression profiles for the *csp* genes from *C. glutamicum*, the promoter region of the *cspA*, *cspA2*, and *cspB* genes were amplified using PCR. The amplified 0.3 to 0.8 kb DNA fragments were introduced into the promoter probe vector pSK1Cat, which carries a promoterless *cat* gene as the reporter. After introducing the recombinant plasmids into the *C. glutamicum* cells, the transformed cells were grown on MCGC minimal medium at 30°C to analyze the expression from the *csp* promoters by monitoring the chloramphenicol acetyltransferase (CAT) activity.

As shown in Fig. 2 (Panel A), no CAT activity was detected in the cells carrying the empty vector pSK1Cat. The cells carrying the *tac* promoter (P$_{tac}$) fused to the front of the *cat* gene (pSK1P$_{tac}$-*cat*) had a maximum CAT activity of 12 μmol/mg/min, and their expression profile almost coincided with the growth pattern. Similarly, the cells carrying the P$_{180}$ promoter (pSK1P$_{180}$-*cat*), a strong promoter previously isolated from *C. glutamicum*, represented the highest CAT activity of 14 μmol/mg/min (Fig. 2, Panel C). The expression profile of the P$_{180}$ promoter was almost identical to that of the P$_{tac}$ promoter. However, the CAT activity of the cells carrying the *cspA* promoter (P$_{cspA}$-*cat*) was the highest (8 μmol/mg/min) at the late exponential phase, and decreased subsequently (Fig. 2, Panel D). The expression and activity profiles exerted by the *cspA2* promoter were almost identical to those of the *cspA* promoter (Fig. 2, Panel F). Although the expression profile of the *cspB* promoter was similar to those of the *cspA* and *cspA2* promoters, the highest CAT activity shown by the *cspB* promoter was relatively low (6 μmol/mg/min) (Fig. 2, Panel E). These results indicate that (1) all three *csp* promoters of *C. glutamicum* are considerably active at 30°C, (2) the three genes carry independent promoters, and (3) the expression from the promoters are regulated according to the cellular growth phase by an unknown mechanism.

In order to analyze the functionality of the *csp* promoters in *E. coli*, the above-mentioned clones were introduced into *E. coli* DH5αF' cells and the corresponding CAT activity was assayed. Unlike the expression patterns observed in *C. glutamicum*, the *csp* promoters showed CAT activity profiles that agreed with the growth patterns (data not shown). The highest CAT activities were 2 μmol/mg/min by the *cspA* promoter and 1.5 μmol/mg/min by the *cspB* promoter, which were considerably lower when compared with those of *C. glutamicum*. These results indicate that the regulatory mechanism governing the expression of the *C. glutamicum csp* genes may differ from that of *E. coli*.

Effects of Cold Shock on the Expression of the *csp* Genes

To assess the function of the *csp* genes in regard to a cold shock, we introduced each clone, such as pSK1P$_{tac}$-*cat*, pSK1P$_{180}$-*cat*, pSK1P$_{cspA}$-*cat*, pSK1P$_{cspB}$-*cat*, or pSK1P$_{cspA2}$-

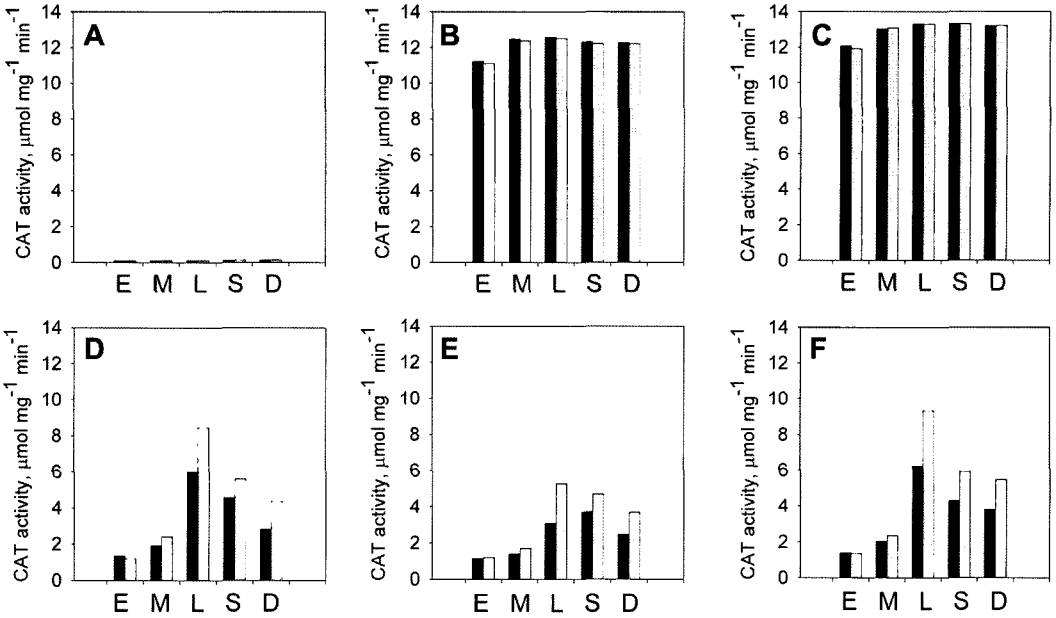

Fig. 3. Effects of cold shock on the expression of the *cspA*, *cspB*, and *cspA2* genes.
C. glutamicum AS019E12 harboring plasmid pSK1Cat (Panel A), pSK1P$_{tac}$-*cat* (Panel B), pSK1P$_{180}$-*cat* (Panel C), pSK1P$_{cspA}$-*cat* (Panel D), pSK1P$_{cspB}$-*cat* (Panel E), or pSK1P$_{cspA2}$-*cat* (Panel F) were grown in MCGC liquid medium at 30°C to the appropriate growth phase. After the growth temperature was shifted down to 15°C, the cells were grown for an additional 5 h. Then, cell extracts from each culture were prepared and the CAT activity was measured. The filled and shaded bars represent the before temperature shift and 5 h after temperature shift, respectively. One representative data point is shown out of several repetitive experiments. Abbreviations: E, early-log phase; M, mid-log phase; L, late-log phase; S, stationary phase; and D, death phase.

cat into *C. glutamicum*. Next, we analyzed the CAT activity at various growth phases after a temperature downshift from 30°C to 15°C. As shown in Fig. 3, the CAT activities, which were measured 5 h after temperature downshift, in the *C. glutamicum* cells carrying pSK1P$_{tac}$-*cat* or pSK1P$_{180}$-*cat* were not affected by either the growth phase or the cold shock (Fig. 3, Panels B and C), but the CAT activities in the cells carrying pSK1P$_{cspA}$-*cat* or pSK1P$_{cspA2}$-*cat* increased to 30% after cold shock (Fig. 3, Panels D and F). In addition, the magnitude of the increase in the CAT activity induced by the cold shock was the largest in the late-exponential growth phase. The *cspB* promoter also showed a similar pattern of increment in the CAT activity upon cold shock, although the overall activity was at a 50% level as compared with those of *cspA* and *cspA2* (Fig. 3, Panel E).

These results indicate that the expression of the *csp* genes depends not only on the growth phase of the cells, but also on cold-shock stress. When the experiments were performed using *E. coli* as the host cell, the stimulatory effects of cold shock were also observed, although the magnitude was much smaller (data not shown).

In Vivo DNA-binding Activity of the Csp Proteins

In our previous report, we observed that the CspA protein affects the expression of the *aceB* gene, probably by interacting with the promoter region of the gene [16, 20]. The *aceB* gene encodes one of the enzymes catalyzing the glyoxylate bypass in *C. glutamicum*. Although the physiological significance of such an interaction has not yet been addressed, we assumed that the interaction may be valuable as a tool for an indepth study of the Csp function. Based on this notion, we tested the effects of cold shock on DNA binding of the Csp proteins to the promoter of the *aceB* gene. In this experiment, the plasmid pSL430, a *cspA* clone, was introduced into an *E. coli* DH5αF'-145

Table 2. *In vivo* DNA-binding activity of CspA[a].

Strain	Growth temperature	Plasmids	Phenotype	β-Galactosidase activity, mU[b]	% Activity
E. coli DH5αF'-145	37°C	pMT1	-	15±1.2	100
		pSL430	*cspA* clone	6.6±0.5	44
	20°C	pMT1	-	8.4±0.9	100
		pSL430	*cspA* clone	1.1±0.2	13

[a]After *E. coli* DH5αF' cells were grown in LB, β-galactosidase activities were measured as described previously [20].
[b]One unit of activity was defined as the amount of enzyme that hydrolyzed 1 mole of ONPG in 1 min at 30°C.

cell carrying a reporter plasmid that has *lacZYA* fused to the downstream of the *aceB* promoter. Then, we monitored the effect of the CspA2 protein on the *aceB* promoter by analyzing the β-galactosidase activity of the cell. Compared with the *E. coli* DH5αF'-1 cells carrying an empty vector, the cells harboring plasmid pSL430 represented a 56% reduction in β-galactosidase activity (Table 2). Interestingly, when the experiment was performed at 20°C rather than 37°C, the magnitude of reduction in β-galactosidase activity was greater at 87%, suggesting a stronger interaction of the CspA protein with the *aceB* promoter region. The results suggest that the CspA protein may possess DNA-binding activity and also exert a regulatory effect at the level of transcription. That is, the binding of the CspA2 protein(s) to the promoter region of the *aceB* gene might have interfered with the binding of the RNA polymerase to the region, resulting in a reduced expression of *lacZ*. Even though the reason that the CspA proteins bind on the *aceB* promoter region is not yet clarified, it is still interesting to note that the CspA protein may have the potential to interact with the promoter region of a gene, and such an interaction is stimulated by a low temperature.

Knowing that the expression of the *csp* gene is stimulated by a low temperature, we analyzed the effect of *cspA* expression in *C. glutamicum* in regard to growth at a low temperature. As shown in Fig. 4, *C. glutamicum* cells carrying the *cspA* clone in multicopy showed slower growth rate at 20°C in the MCGC minimal medium. The growth inhibitory effect of the *cspA* gene was not observed when cells were cultivated at 30°C (Fig. 4). For the 20°C cells, the excess amount of CspA might have interfered

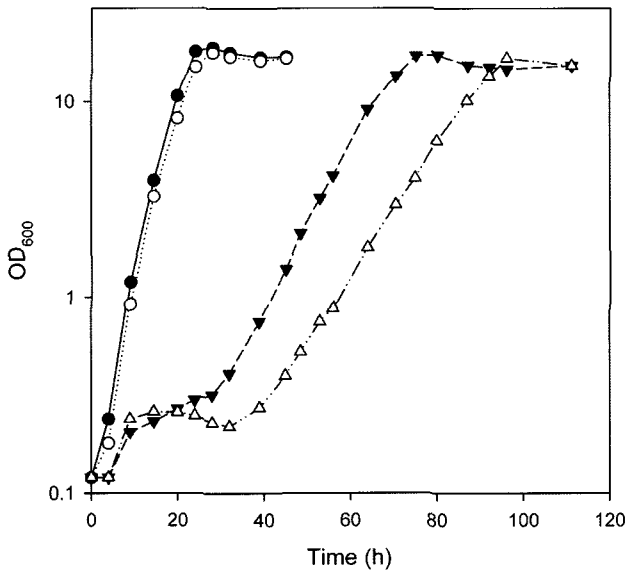

Fig. 4. Growth of *C. glutamicum* cells carrying the *cspA* clone. Cells were cultivated in MCGC minimal medium either at 30°C (circles) or 20°C (triangles). Symbols: ● and ▼, *C. glutamicum*/pMT1; ○ and △, *C. glutamicum*/pSL430.

with cellular physiology by the nonspecific binding of the proteins to DNA. This may support the notion that *cspA* and *cspA2* play regulatory roles by binding to DNA.

DISCUSSION

Csp proteins are found in multiple copies, and with varying homologies within a given bacterial species [45]. In this report, three *csp*-like genes have been found in *C. glutamicum*. This is similar to *M. tuberculosis*, which possesses 3 *csp*-like genes and is closely related to *C. glutamicum*. The function of the *csp* genes in those two organisms has never been studied. Therefore, this is the first study addressing the function of the *csp* genes of *C. glutamicum*. Although the genetic organization of the *csp* genes of *C. glutamicum* show a difference from that of *E. coli*, the Csp proteins from various organisms possess several features in common. The CspA and CspA2 proteins of *C. glutamicum* are homologous to Y-box proteins of several bacteria and eukaryotes, which are known to bind single-stranded nucleic acids [42]. The Y-box has a sequence feature of ATTGG (or CCAAT in the opposite strand) and is known to be present in the promoter region of certain genes. The binding of the CspA protein may stabilize the open complex formed by the RNA polymerase [24]. Scrutiny of the *aceB* promoter region of *C. glutamicum* also identified several related sequences. The finding that the expression of the *aceB* gene is repressed in the presence of CspA proteins may indicate that the proteins could bind to the promoter region of the gene, which then interferes with the RNA polymerase to interact with the promoter rather than to stabilize the open complex. Because the expression of the *csp* genes in *E. coli* was considerably lower when compared with that of *C. glutamicum*, and the *csp* genes were only marginally induced in *E. coli* by cold shock, the increased repression of *aceB* by the CspA protein in *E. coli* at lower temperature might have been caused by stronger binding of the protein to DNA rather than by increased expression of the *cspA* gene.

Recently, it has been proven that the *E. coli* CspA is not restricted to cold-shocked cells [2]. CspA is one of the most abundant proteins in cells coming out of the stationary phase and during the early exponential growth at 37°C. In the early exponential phase of growth at 37°C, CspA represents up to 1% of the total soluble proteins. The level of cold-shock induction of the *cspA* expression is growth cycle-dependent, being about 30-fold when the cells are cold shocked during the mid-late exponential growth, and no more than 3-fold in cells shocked during very early exponential growth [10]. The expression pattern of *C. glutamicum cspA*, *cspA2*, and *cspB* at optimal growth temperature was found to be similar to those of *E. coli*

[45], *B. subtilis* [8], and *Lactobacillus plantarum* [5] *csp* genes. The *C. glutamicum* genes also showed growth phase-dependent induction. Our data suggest that *C. glutamicum* Csp proteins might play a role at optimal temperature as well as for cold-shock adaptation, possibly acting as alternative translation initiation factors and/or as RNA chaperones, as suggested in *E. coli* [39].

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