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Cold Shock Response in *Bacillus subtilis*

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Dedicated to Rudolf K. Thauer in celebration of his 60th birthday

Abstract

Following a rapid decrease in temperature, the physiology of *Bacillus subtilis* cells changes profoundly. Cold shock adaptation has been monitored at the level of membrane composition, adjustment in DNA topology, and change in cytosolic protein synthesis/composition. Some major players in these processes (cold-stress induced proteins and cold acclimatization proteins, CIPs and CAPs) have been identified and mechanisms in cold shock acclimatization begin to emerge; however, important questions regarding their cellular function still need to be answered.

Introduction

Bacillus subtilis has the ability to slip into another skin when times get rough. Upon deprivation of nutrients or slow dehydration, the gram positive bacterium differentiates into a highly resistant spore (Stragier and Losick, 1996). This process takes about 7 h under optimal conditions. Being a common mesophilic soil bacterium, however, *B. subtilis* must be able to respond more rapidly to environmental changes, such as sudden and pronounced changes in temperature or osmolarity (Kempf and Bremer, 1998). *B. subtilis* is mainly found in the surface layers of the soil, where conditions change frequently and transiently. Thus, the physiology of the cells must adjust rapidly for growth and survival under different conditions, and adaptation after a decrease in temperature is discussed in this review.

In general, mesophilic bacteria have to cope with several recognized problems that arise following cold shock: a) membrane fluidity is too low, b) superhelical density of the DNA is too high for opening of the double helix, c) enzyme activities decrease profoundly, but probably to different extents, so protein levels must be adjusted, d) protein folding may be too slow or inefficient, e) ribosomes must be adapted to function properly at low temperatures, and f) secondary structures in RNA affect initiation of translation (Jones and Inouye, 1994; Graumann and Marahiel, 1996; Panoff *et al.,* 1998). Upon a sudden decrease in temperature, *B. subtilis* performs a series of cellular adaptations, which have been monitored in all cellular fractions.

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Membrane Adaptation

At 37°C, branched and straight-chain membrane lipids are fully saturated in *B. subtilis* to ensure the integrity of the membrane (Kaneda, 1991; Grau and de Mendoza, 1993). At lower temperatures, membrane fluidity must increase in order to avoid transtition from a liquid crystalline into a gel-like phase state of the lipid bilayer. To achieve a decrease in phospholipid membrane melting temperature, the ratio of anteiso-to iso-branched fatty acids in B. subtilis is dramatically increased (Klein et al. 1999). In *E. coli*, however, unsaturated fatty acids (UFAs) are synthesized in greater quantity at lower temperatures by a constitutive cytosolic enzyme, ACP synthase II, which is more active at lower temperatures (Cronan and Rock, 1996). In contrast, in *B. subtilis* the synthesis of a membrane desaturase that oxidizes phospholipids in the membrane is induced following cold shock (Figure 1; Aguilar *et al.,* 1998). About 1 h after cold shock, transcription of the desaturase gene (*des*) is transiently induced, reaching 10 to 15-fold higher levels after 4 h. However, deletion of the *des* gene does not cause a detectable phenotype after cold shock (Aguilar *et al.,* 1998). Recent data throw light on this behaviour, by showing that the anteiso-branched fatty acids and not UFAs to be the major fraction after temperature down shift in *B. subtilis*. In a defined minimal medium it has been shown that cold shock adaptation of *B. subtilis* depends on the presence of isoleucine (Ile) or precursors of anteiso-branched chain fatty acids, and that the branching pattern of membrane fatty acids (FA) switches from iso-focused (*B. subtilis* membranes contain a high proportion of branched chain FA, for review see, Kaneda, 1991) to anteiso-dominated after rapid cooling from 37°C to 15°C (Klein *et al.,* 1999). Thus, an Ile-dependent change in the FA-branching profile appears to be the main mechanism for cold shock adaptation of the membrane in *B. subtilis*.

Chromosome Adjustment

After a temperature decrease, negative supercoiling is increased in the DNA of *B. subtilis* (Grau *et al.,* 1994) and a variety of procaryotes, including hyperthermophilic archaea (Lopez-Garcia and Forterre, 1997). It is thought that underwinding of supercoiled DNA facilitates unwinding of the DNA duplex by helicases during replication and by RNA polymerase at lower temperatures. Artificial inhibition of gyrase activity (which introduces neg. supercoil) prevents the cold shock-induced decrease in linking number in *B. subtilis* (Grau *et al.,* 1994). In *E. coli*, both subunits of gyrase are cold stress-induced proteins (CIPs) (Jones and Inouye, 1994), so increased synthesis of this enzyme appears to account for the change in DNA topology. Inactivation of DNA gyrase also prevents cold dependent accumulation of UFAs (Grau *et al.,* 1994; see above), so transcriptional activation of *des* may depend on the topological state of the DNA. Possibly, the *des* promoter is only active when a certain threshold of negative supercoiling is reached.

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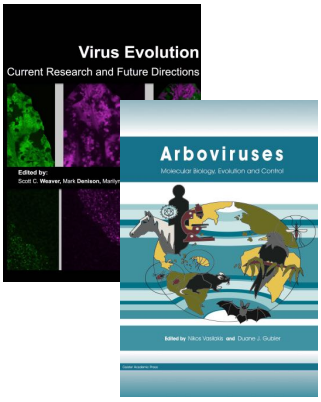

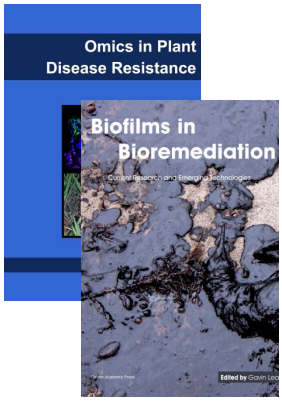

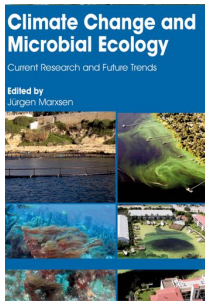

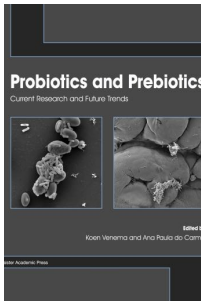

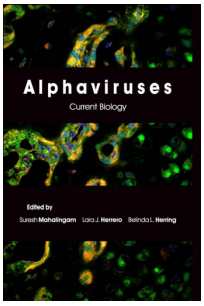

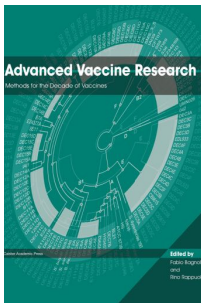

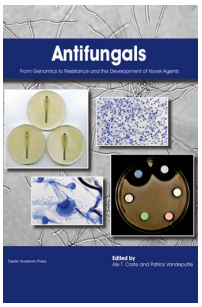

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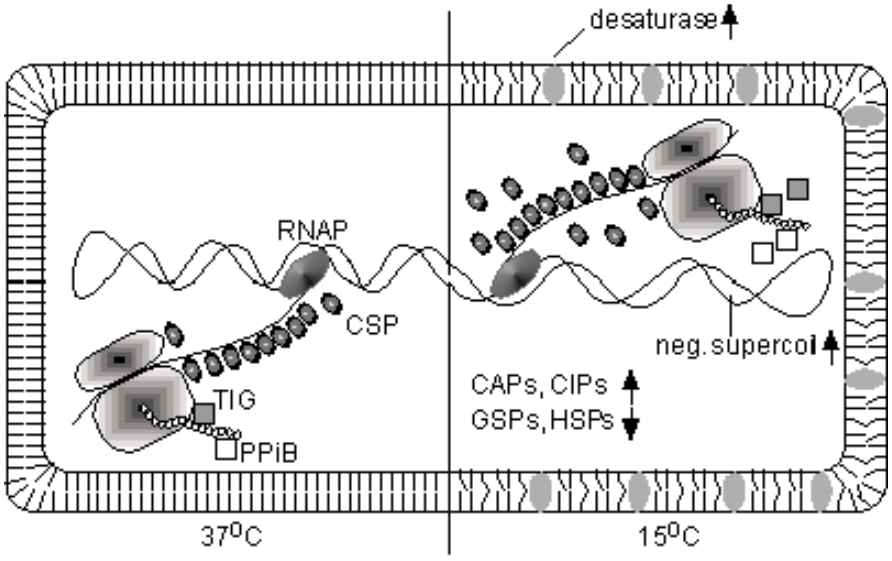
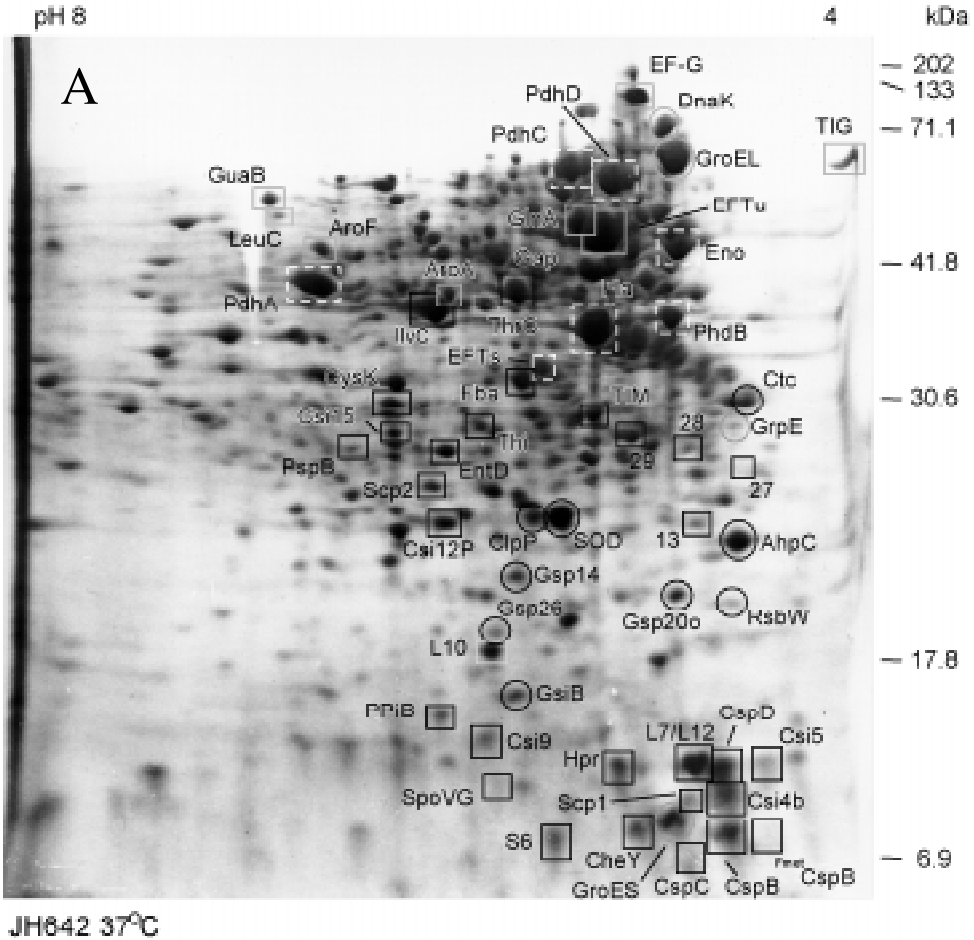


Figure 1. Temperature Adaptation
Model for adaptations following temperature shift down from 37°C to 15°C in *B. subtilis* and other mesophilic bacteria. For details, see text.

Our unpublished results show that the nucleoid in *B. subtilis* is more condensed after cold shock than during exponential growth at 37°C (P. Graumann, unpublished), similar to nucleoids during stationary phase (Koch, 1996). This change in nucleoid structure may be due to a decrease in the cellular transcription/translation capacity. It has been proposed that decondensation of the nucleoid in growing cells is achieved by the coupling of synthesis of membrane proteins and secreted proteins to their incorporation into or transport across membranes. Thus the sites of active DNA transcription are pulled to the membranes (Woldringh *et al.,* 1995).



Cytosolic Response

In the cytosol, protein synthesis changes markedly after cold shock (Lottering and Streips, 1995; Graumann *et al.,* 1996; Figure 1). While synthesis of the majority of proteins decreases, a subset of CIPs is transiently induced, with a peak at about 1 h after temperature decrease from 37°C to 15°C. Thereafter, synthesis of most proteins resumes and induction of CIPs declines, such that 2 h after cold shock a new steady state of protein synthesis is reached (Graumann *et al.,* 1996). This pattern of synthesis is different from that at 37°C. Therefore, through a transient response, the cells adapt to the lower temperature with an adjustment of protein composition.

Although some proteins are induced in response to cold shock and salt stress or heat shock (SCP or TIP, see Table 1), generally, the synthesis of CSPs decreases following heat shock, and that of heat shock and general stress proteins (HSPs and GSPs) after cold shock (Graumann *et al.,* 1996; see Figure 2). Likewise, heat shock and cold shock response are mutually exclusive in *E. coli* (Jones and Inouye, 1994).

Although the change in the pattern of protein synthesis is profound, the overall variation in protein composition is only moderate, because overall protein synthesis is much lower - only about 15-20% - after transition from 37°C to 15°C. 3 h after cold shock, the concentration of most CIPs is at least < 2 fold higher, and that of others, *e.g.* GSPs

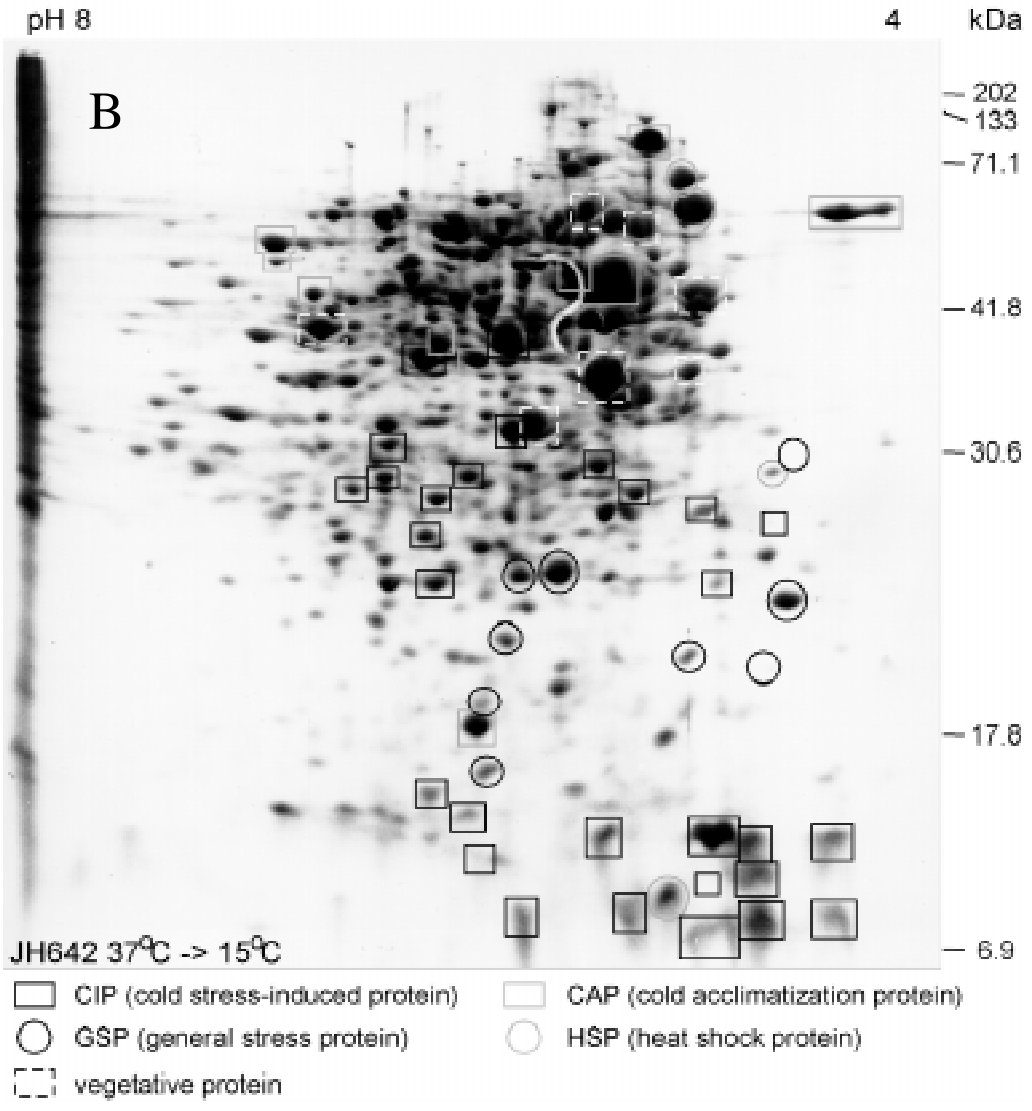


Figure 2. Stress Induction of Proteins.
Coomassie stained second dimension SDS-PAGE 2D gels with 700 μg of soluble proteins from *B. subtilis* cells A) grown at 37°C during mid-exponential phase, and B) 3 h after cold shock to 15°C. HSPs are induced after heat shock, and function as protein chaperones; GSPs are induced after ethanol, salt and other stress (SOD: superoxide dismutase, AhpC: katalase, ClpP: protease, other GSPs have no known function; Bernhardt *et al.*, 1997); vegetative proteins are not stress inducible, except for TCA enzymes after glucose starvation (Fla: flagellin, PdhA-D: subunits of pyruvate dehydrogenase, Eno: enolase; Schmid *et al.*, 1997). Identification of protein spots (see Table 1 for CIPs/CAPs) from (Graumann *et al.*, 1996), (Antelmann *et al.*, 1997), Swiss-Prot accession numbers: Csi4B - P81094, Csi5 - P81095, Scp1 - P81099, Scp2 - P81100. Note that although some proteins are cold stress induced according to pulse-labelling experiments, their levels do not change visibly in Coomassie stained gels.

Table 1. Identified Cold Shock Stress Induced Proteins (CIPs) and CAPs in *Bacillus subtilis*

Protein	Name	Class	Function
Transcription/Translation			
CspB	cold shock protein B	CSI	initiation of translation, RNA-chaperone (?)
CspC	cold shock protein C	CSI	initiation of translation, RNA-chaperone (?)
CspD	cold shock protein D	CSI	initiation of translation, RNA-chaperone (?)
Translation			
S6	ribosomal protein S6	CSI	translation, folding of 16S RNA?
L7/L12	ribosomal protein L7/L12	CSI/CAP	translation, interaction with EFTu/aa-tRNA
L10	ribosomal protein L10	CAP	translation, interaction with EFTu/aa-tRNA
EFTs	GTP/GDP exchange factor	CAP	elongation of translation
EFTu	elongation factor	CAP	elongation of translation
EF-G	elongation factor	CAP	elongation of translation
Protein Folding			
PPiB	peptidylprolyl *cis/trans* isomerase	CSI	protein chaperone
Tig	trigger factor	CAP	protein chaperone (associated with ribosome)
General Metabolism			
CysK	cysteine synthase	CSI	amino acid synthesis
IlvC	ketolacid reductoisomerase	CSI	amino acid synthesis (Val, Ile)
GlnA	glutamine synthase	CAP	amino acid synthesis (Gln)
LeuC	betaisopropyl malate DH	CAP	amino acid synthesis (Leu)
ThrC	threonine synthase	CAP	amino acid synthesis (Thr)
AroF	chorismate synthase	CAP	amino acid synthesis
Gap	gyceraldehyde phosphate DH	CSI	glycolysis
TIM	triosephosphate isomerase	CSI	glycolysis
Fba	fructosebisphosphate aldolase	CSI	glycolysis
GuaB	inosine monophosphate DH	CAP	nucleotide synthesis
Chemotaxis			
CheY		TIP	regulation of flagellar rotation
Carbohydrate uptake			
Hpr		USP	PEP:phosphotransferase system
Iron Uptake			
DHBA	enterochelin synthase	CSI	synthesis of enterochelin (Fe-chelator)
Other			
Srf4	surfactin synthase 4, thioesterase	CSI	surfactin (fatty acid ?) synthesis
Csi12P		CSI	phosphorylated at initiation of sporulation
SpoVG		CSI	sporulation (septum formation)
Scp1		SCP	kinase-domain ?
Scp2		SCP	homology to Te-resistence from *Alcaligenes*
PspB	phage-shock protein B	CIP	membrane-associated (?) stress protein
Csi4b			CSI unknown
Csi5			CSI unknown
Csi9	RisB or AtpE		CSI riboflavin synthase or E- ATPase
Csi15			CSI unknown

CSI, cold stress induced protein (transient induction only after cold shock)
CAP, cold acclimatization protein (higher amount at cold temperatures)
SCP, salt and cold stress induced protein
TIP, temperature (cold and heat shock-) induced protein
USP, universal stress protein
DH, dehydrogenase

and many vegetative proteins, is lower (Figure 2). On the other hand, the amount of some proteins that are not strongly cold induced is also higher: probably through a slight but steady increase in synthesis, proteins like EFTs, EFTu and GlnA are present in a higher concentration 3 h after cold shock (Figure 2). These proteins, as opposed to CIPs, are termed cold acclimatisation proteins (CAPs, Table 1).

Through reverse genetics, the identity of several members of the cold shock stimulon has been determined (Graumann *et al.,* 1996; Figure 2 and Table 1). The function of some CIPs is intriguing: like in *E. coli*, several CIPs/CAPs are associated with translation, and may adapt the ribosome to function at lower temperature. PPiB and TigBs in *B. subtilis* are folding catalysts that act as prolyl-isomerases (see below), and CspB-D may be RNA chaperones (see below). Several CIPs are involved in intermediary metabolism (Table 1), and may simply be needed at a higher level because they may be more inefficient enzymes at low temperatures than others in the corresponding biochemical pathway. CheY is phosphorylated by CheA, and regulates the direction of

fagellar rotation; HPr is involved in the PTS system. Both proteins may be diffusion-limited factors in chemotaxis and sugar uptake, respectively, and therefore be induced to compensate for lower diffusion after cold shock. The function of CIPs such as Srf4 or PspB, however, is still a mystery.

Genes encoding CIPs are not clustered, but are distributed over the *B. subtilis* chromosome. Their induction after cold stress is probably achieved at the post-transcriptional level. A cold shock-like response can be induced upon treatment of *B. subtilis* with a low concentration of a translational inhibitor (Graumann *et al.,* 1997); the same effect has previously been reported for *E. coli* (Van Bogelen and Neidhardt, 1990). In addition, cold shock induction of *cspB* transcription is less than 2-fold, but the synthesis of CspB strongly increases even when the *cspB* gene is under control of a constitutive promoter (Graumann *et al.,* 1997). Induction of CIPs may therefore be achieved by a predominant translation of an existing pools of mRNA, which was also proposed for *E. coli* (Jones and Inouye, 1996). After cold shock, impaired initiation of translation (Jones and Inouye, 1996) may allow only the limited synthesis of CIPS, and resumption of general protein synthesis due to the adaptation of the translational machinery.

Protein Folding at Low Temperature: A Major Role for Prolyl Isomerases?

Peptidyl-prolyl bonds in proteins can not rotate freely, and have to exist either in a *cis* or a *trans* configuration. Isomerisation of these two states is therefore an important rate limiting step in the folding of many protein (Göthel and Marahiel, 1999). This reaction is catalysed by an ubiquitous class of enzymes called prolyl cis/trans isomerases. Two such enzymes are known in *B. subtilis*: PPiB and trigger factor (TigBs). Synthesis of both enzymes increases after cold shock (Figure 1, Graumann *et al.,* 1996), as was also found for *E. coli* Tig protein (Kandror and Goldberg, 1997). Interestingly, an E. coli *tig* mutant was found to be cold sensitive (Kandror and Goldberg, 1997), Whereas, a *B. subtilis tig/ppiB* double mutant was impaired in stress adaptation in minimal medium (Göthel *et al.,* 1998). Thus, prolyl isomerization appears to be a committing step in protein folding at low temperatures, in contrast to heat shock conditions, where general misfolding and aggregation appear to be defective. In agreement with this, the synthesis of GroEL and GroES protein chaperones (HSPs) is reduced after cold shock (Graumann *et al.,* 1996).

Cold Shock Proteins: Important Function Not Only After Cold Stress

Cold shock proteins (CSPs) are the most strongly induced CIPs in *E. coli* and *B. subtilis*, as well as in a variety of other eubacteria. CSPs are small (7-7.5 kDa) proteins, highly conserved between even distantly related bacterial branches, and exist in families of up to 9 members (Graumann and Marahiel, 1998; Yamanaka *et al.,* 1998). There are 3 *csp* genes (B, C and D) in *B. subtilis*, and all three gene products are cold inducible, while only three out of nine *csp* genes in *E. coli* are cold inducible. On the other hand, *E. coli cspD* is induced in response to starvation and stationary phase, but not after cold shock (Yamanaka *et al.,* 1998). In *Lactococcus* sp., four CSPs are cold stress induced, while a fifth gene is constitutively expressed (Wouters *et al.,* 1998). Thus, members of the CSP families can be differentially regulated. Interestingly, CspB and CspC, but not CspD, are also major stationary phase induced proteins in *B. subtilis* (Graumann and Marahiel, 1999), revealing that synthesis of CSPs can be increased in response to different stresses. Interestingly, rapid inactivation of ribosomes occurs after cold shock as well as after entry into stationary phase (Jones and Inouye, 1996; Wada, 1998), and may be a common trigger for CSP induction.

Deletion of any *B. subtilis csp* gene does not result in a detectable phenotype at physiological temperatures. A *cspB* null mutant has been shown to be sensitive to direct freezing from 37°C and a subsequent thawing. This phenotype could be partly restored by a pre-cold shock adaptation at 15°C prior to freezing (Willimsky *et al.,* 1992). However, deletion of *cspB* and *cspC* or *cspD* results in a defect in cold stress acclimatization, and growth at 15 as well as 37°C. A *cspC/cspD* mutant is only defective in growth at low temperatures. These results show that CspB performs the most important function, and is complemented by CspC (mainly at low temperatures) and CspD (since a *cspB/D* mutant shows a stronger defect at 37°C than a *cspB/C* mutant, and vive versa at 15°C). Deletion of all three *csp* copies is only possible when *cspB* is present and induced in *trans*; in the absence of CSP production, *B. subtilis* is unable to grow, even at 37°C, which reveals an essential function of CSPs under optimal growth conditions. Since CspB and CspD are stable proteins *in vivo* (CspC becomes stable under cold shock conditions, Schindler *et al.,* 1999), these results show that an increase in CSP synthesis and concentration is necessary for cold stress adaptation. Moreover, a *cspB/C* double mutant shows cell lysis after entry into stationary phase, indicating that an increase in synthesis of CspB and CspC (see above, Graumann and Marahiel, 1999) is also important for adaptation.

Function of CSPs as RNA Chaperones

Although it is clear that CSPs function at the level of transcription and/or translation, their exact mode of function still needs to be illucidated. CSPs share a common fold, a five stranded β-barrel, first descibed for CspB of *B. subtilis* (Schindelin *et al.,* 1993; Schnuchel *et al.,* 1993). They carry conserved RNP1- and 2 motifs that are essential for binding to ssDNA and RNA (Schröder *et al.,* 1995) on a surface composed of three antiparrallel β-sheet. Although CSPs have a preference for sequences such as ATTGG, they bind rather non specifically and cooperatively to single stranded nucleic acids (Graumann *et al.,* 1997). Affinity to RNA increases with increasing lenght of the substrate, but binding to longer (> 25 nucleotides) molecules requires that the RNA is devoid of secondary structures. CspA from *E. coli* has been shown to possess RNA-chaperone activity in vitro (Jiang *et al.,* 1997), which has led to the model that CSPs bind to nascent mRNA during transcription and prevent the formation of secondary structures that would inhibit initiation of translation. After cold shock, a higher concentration of CSPs may be needed to counterbalance

increased stability of intramolecular basepairing in mRNA. On the other hand, affinity of CSPs to RNA is rather low (μmolar-range, Graumann *et al.,* 1997), consequently may be allowing the ribosomes to displace CSPs and initiate translation on a linear template. This model has recently received substantial support. Following photocrosslinking of nascent RNA to proteins in active transcription complexes, CspE was found to be a major RNA-bound constituent in *E. coli* (Hanna and Liu, 1998). CspE was heavily crosslinked to a short RNA of about ten nucleotidees in length only when this RNA was associated to the transcription complex. Moreover, *B. subtilis* CSPs were found to be stable proteins *in vivo*, in contrast to their high suscepteability to proteolytic degradation *in vitro* (CspB folds extremely rapidly with a low kinetic barrier towards unfolding, therefore about 1% of all molecules are present in an unfolded state in solution, Schindler *et al.,* 1995). However, addition of a substocheometric amount of nucleic acid ligand strongly protected the CSPs against protease attack (Schindler *et al.,* 1999), which suggests that in the cell, CSPs are predominantly complexed most likely with mRNA.

Intriguingly, coupling of transcription to translation was also shown to be performed by the eukaryotic Y-box proteins, which contain a domain (cold shock domain) that is highly conserved to CSPs (Graumann and Marahiel, 1998; Matsumoto and Wolffe, 1998). Recently, a structural fold similar to CSPs was found in domain(s) within the S1 (Bycroft *et al.,* 1997), and IF1 ribosomal proteins of *E. coli* (Sette *et al.,* 1997) as well as in eukaryotic/archaeal eIF-5A factor (Kim *et al.,* 1998), all of which are involved in initiation of translation. Their common structure, called OB (oligomer-binding)-fold, has therefore been adapted during evolution to perform a variety of tasks in RNA/ribosome interactions.

The *csp* genes in *B. subtilis* appear to be autoregulated. Deletion of one or two genes results in higher synthesis of the remaining CSP(s). On the other hand, CspB can not be overproduced from additional copies of *cspB* on a plasmid (Graumann *et al.,* 1997). Tight control of CSP levels may be important, because moderate induction of *B. subtilis* CspB in *E. coli* at 37°C leads to a strong decrease in growth rate and a change in the pattern of protein synthesis (not seen after induction of a CspB mutant impaired in RNA-binding, Graumann and Marahiel, 1997). The highest affinity of *B. subtilis* CSPs was found for a sequence at the 5' end of their untranslated leader regions (5' UTRs). Possibly, CSPs bind tightly to their 5' UTRs and thereby reduce translation of *csp* mRNA.

Cold Shock Response in Other *Bacilli*

Cold shock from 65°C to 45°C induces a transient decrease in colony forming units in cultures of the thermophilic *Bacillus stearothermophilus* for 3 h, followed by resumption of exponential growth (Wu and Welker, 1991). During the adaptation period, the synthesis of several membrane and cytosolic proteins was induced. The presence of a translational inhibitor was found to increased the length of the adaptation suggesting that a change in protein synthesis is needed for cold stress adaptation. Interestingly, heat shock suppressed the synthesis of CIPs, while cold shock suppressed HSP production. Thus, thermophilic *bacilli* appear to have an analogous cold shock response to that of *B. subtilis*.

On the other end, the psychrophilic bacterium *B. cereus* was shown to contain at least 6 members of the Csps family, one of them was strongly induced after cold shock. CSPs seem to be an ubiquitous class of proteins. They are present in psychrophilic, mesophilic and thermophilic *bacilli* (Schröder *et al.,* 1993, Perl *et al.,* 1998). It will be interesting to find out if they are generally major CIPs in *bacilli*, and if they perform a similar, essential function at low and optimal temperatures. Increased demand for RNA-chaperone activity at lower temperatures may be a recurring theme in many organisms: recently, a small RNA-binding protein (CIRP) was identified as the first cold shock protein in mammalian cells - including humans (Nishiyama *et al.,* 1997).

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