Cold Shock Response in *Bacillus subtilis*

Peter L. Graumann,¹ and Mohamed A. Marahiel²  

¹Biological Laboratories, Harvard University, Cambridge, MA 02138, USA  
²Biochemie, Fachbereich Chemie, Hans-Meerwein-Straße, Philipps-Universität Marburg, 35032 Marburg, Germany

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). Following a rapid decrease in temperature, negative supercoiling 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 UFA to be the major fraction after cold shock (Aguilar et al., 1998). 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 UFA (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.

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 transition 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 UFA to be the major fraction after cold shock (Aguilar et al., 1998). 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*.

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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, CipP: 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.
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

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

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Class</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcription/Translation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CspB</td>
<td>cold shock protein B</td>
<td>CSI</td>
</tr>
<tr>
<td>CspC</td>
<td>cold shock protein C</td>
<td>CSI</td>
</tr>
<tr>
<td>CspD</td>
<td>cold shock protein D</td>
<td>CSI</td>
</tr>
<tr>
<td><strong>Translation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td>ribosomal protein S6</td>
<td>CSI</td>
</tr>
<tr>
<td>L7/L12</td>
<td>ribosomal protein L7/L12</td>
<td>CSI/CAP</td>
</tr>
<tr>
<td>L10</td>
<td>ribosomal protein L10</td>
<td>CAP</td>
</tr>
<tr>
<td>EF-Ts</td>
<td>GTP/GDP exchange factor</td>
<td>CAP</td>
</tr>
<tr>
<td>EF-Tu</td>
<td>elongation factor</td>
<td>CAP</td>
</tr>
<tr>
<td>EF-G</td>
<td>elongation factor</td>
<td>CAP</td>
</tr>
<tr>
<td><strong>Protein Folding</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPIB</td>
<td>peptidyl/prolyl cis/trans isomerase</td>
<td>CSI</td>
</tr>
<tr>
<td>Tig</td>
<td>trigger factor</td>
<td>CAP</td>
</tr>
<tr>
<td><strong>General Metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CysK</td>
<td>cysteine synthase</td>
<td>CSI</td>
</tr>
<tr>
<td>IlvC</td>
<td>ketolacid reductoisomerase</td>
<td>CSI</td>
</tr>
<tr>
<td>GlnA</td>
<td>glutamine synthase</td>
<td>CAP</td>
</tr>
<tr>
<td>LeuC</td>
<td>betaisopropyl malate DH</td>
<td>CAP</td>
</tr>
<tr>
<td>ThrC</td>
<td>threonine synthase</td>
<td>CAP</td>
</tr>
<tr>
<td>AroF</td>
<td>chorismate synthase</td>
<td>CAP</td>
</tr>
<tr>
<td>Gap</td>
<td>glyceraldehyde phosphate DH</td>
<td>CSI</td>
</tr>
<tr>
<td>TIM</td>
<td>triosephosphate isomerase</td>
<td>CSI</td>
</tr>
<tr>
<td>Fba</td>
<td>fructosebisphosphate aldolase</td>
<td>CSI</td>
</tr>
<tr>
<td>GuaB</td>
<td>inosine monophosphate DH</td>
<td>CAP</td>
</tr>
<tr>
<td><strong>Chemotaxis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CheY</td>
<td>TIP</td>
<td></td>
</tr>
<tr>
<td><strong>Carbohydrate uptake</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hpr</td>
<td>USP</td>
<td></td>
</tr>
<tr>
<td><strong>Iron Uptake</strong></td>
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</tr>
<tr>
<td>DHBA</td>
<td>enterochelin synthase</td>
<td>CSI</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Srf4</td>
<td>surfactin synthase 4, thioesterase</td>
<td>CSI</td>
</tr>
<tr>
<td>Csi12P</td>
<td>CSI</td>
<td>phosphorylated at initiation of sporulation</td>
</tr>
<tr>
<td>SpoVG</td>
<td>CSI</td>
<td>sporulation (septum formation)</td>
</tr>
<tr>
<td>Scp1</td>
<td>SCP</td>
<td>SCP</td>
</tr>
<tr>
<td>Scp2</td>
<td>SCP</td>
<td>SCP</td>
</tr>
<tr>
<td>PspB</td>
<td>phage-shock protein B</td>
<td>CIP</td>
</tr>
<tr>
<td>Csi4b</td>
<td>CSI</td>
<td>unknown</td>
</tr>
<tr>
<td>Csi5</td>
<td>CSI</td>
<td>unknown</td>
</tr>
<tr>
<td>Csi8</td>
<td>RisB or AtpE</td>
<td>CSI</td>
</tr>
<tr>
<td>Csi15</td>
<td>CSI</td>
<td>unknown</td>
</tr>
</tbody>
</table>

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
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 pool 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 cannot rotate freely, and have to exist either in a cis or a trans configuration. Isomerization of these two states is therefore an important rate limiting step in the folding of many protein (Göthel and Marahiel, 1999). This reaction is catalyzed 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 vice 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 elucidated. CSPs share a common fold, a five stranded β-barrel, first described 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 antiparallel 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 length 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 nucleotides 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 susceptibility 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 substoichiometric 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 Wolfe, 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 cannot be overproduced from additional copies of cspB on a plasmid (Graumann et al., 1997). Tight control of CSP levels may be important, because moderate 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 Csp 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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