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Induction of Cold Shock Proteins in *Bacillus subtilis*

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Abstract. The cold shock response in the Gram-positive soil bacterium *Bacillus subtilis* is described. Cells were exposed to sudden decreases in temperature from their optimal growth temperature of 37°C. The *B. subtilis* cells were cold shocked at 25°C, 20°C, 15°C, and 10°C. A total of 53 polypeptides were induced at the various cold shock temperatures and were revealed by two-dimensional gel electrophoresis. General stress proteins were identified by a comparative analysis with the heat shock response of *B. subtilis*. Some unique, prominent cold shock proteins such as the 115 kDa, 97 kDa, and 21 kDa polypeptides were microsequenced. Sequence comparison demonstrated that the 115-kDa protein had homology to the TCA cycle enzyme, aconitase.

Prokaryotic and eukaryotic organisms respond to environmental temperature changes by the induction of a set of globally regulated genes. The response of microorganisms to elevated temperatures (termed the heat shock response) has been extensively studied [4, 6, 7, 14, 16–18]. In the Gram-negative *Escherichia coli* and the Gram-positive *Bacillus subtilis* at least 17 and 66 proteins respectively are known to constitute the heat shock response [1, 11, 17, 18]. It has also been shown that *E. coli* synthesizes a minimum of 14 proteins when subjected to low temperatures [12, 13]. Several of these cold shock proteins were identified and were shown to participate in maintaining the translational and transcriptional capacity of the cell [12, 13, 15]. One of the cold shock proteins, CspA (CS7.4), was uniquely synthesized only at low temperatures and has been shown to function as a possible transcriptional enhancer for cold shock genes [9, 15].

Little is known about the cold shock response of the soil bacterium *B. subtilis*, which has been otherwise studied in detail for several developmental responses such as sporulation, competence, and heat shock [25]. Willimsky and coworkers isolated CspB from *B. subtilis*, a protein homologous to CspA [28]. Studies with mutants in which the intact copy of the *cspB* gene in the *B. subtilis* chromosome was replaced with a *cat* interrupted copy showed that the protein product of this gene has a role in cell viability at low temperatures [28]. However, CspB is not the only protein functioning in cell protection at these low temperatures, since preincubation of cells at low temperatures prior to cold shock compensated for the absence of the CspB protein [28].

The study in this manuscript utilizes a two-dimensional gel analysis system to resolve the extent of the cold shock response of *B. subtilis* and peptide microsequencing techniques to begin to identify uniquely synthesized cold shock proteins.

Materials and Methods

Strains used. Bacillus subtilis strain YB886 (*trpC2, metB5, xin-*) was used in all experiments.

Growth of cells and protein labeling. The procedures for growing and labeling cells for the analysis of the heat shock response have been previously described [17]. A similar procedure was developed for the growth and labeling of cells under cold shock conditions. Briefly, cells were grown in minimal media supplemented with the appropriate amino acids at 37°C in an incubator shaker. The cells were harvested at the mid-exponential phase of growth and resuspended in minimal media that contained tryptophan at 20 μg/ml but with a decreased methionine concentration to 0.02 μg/ml. The cells were then equilibrated in the shaker incubator for 10 min prior to being cold shocked at 25°C, 20°C, 15°C, and 10°C for 30 min. The control was maintained at 37°C. Cells were labeled with [35]S-methionine for 30 min after shifts to these temperatures as previously described [12, 17].

Sample preparation and two-dimensional gel electrophoresis. The procedures used in sample preparation and one- and two-dimensional gel electrophoresis have been previously described [17, 19–21].

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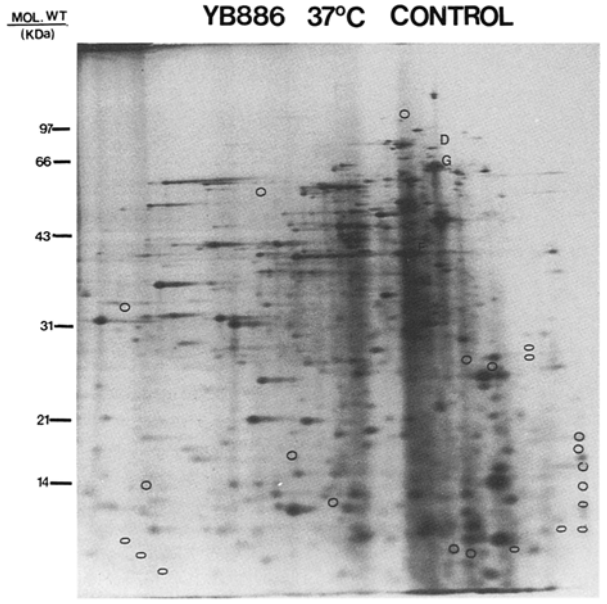

Fig. 1. Two-dimensional equilibrium gel protein profile of *Bacillus subtilis* YB886 grown at 37°C. The autoradiograms were made from whole cell extracts of cells labeled in the mid-exponential phase of growth. Equal amounts of radioactivity were loaded on each gel. The basic end of the gels is to the left, and the acidic proteins are on the right hand side. Open circles indicate the position of the cold shock-induced proteins.

Protein microsequencing. Proteins resolved by one-dimensional gel electrophoresis were transferred to a polyvinylinedifluoride (PVDF) membrane according to a BioRad Laboratories (Richmond, California) transfer protocol. CAPS (3-[cyclo-hexylamino]-1-propanesulfonic acid) transfer buffer containing 20% methanol (pH 11) was used in a BioRad transblotter to transfer the proteins to the membranes according to the manufacturer's instructions. The transfer conditions were at 90V for 1 h. The membranes were then stained with Coomassie brilliant blue R-250 in 50% methanol solution for 5 min and then destained with a 50% methanol and 10% acetic acid solution to obtain a complete protein pattern. Membranes were air dried, and the correct protein band was excised and sequenced by Edmann degradation with an automated gas phase sequenator.

Results

Bacillus subtilis cells were shifted in temperature from 37°C to 15°C, and the protein response was analyzed by both a one- and two-dimensional equilibrium gel electrophoresis system. For demonstration of the existence of a significant cold shock response, at least 13 proteins could be visualized at the cold shock temperature of 15°C with a one-dimensional gel electrophoresis system (not shown). A two-dimensional gel analysis system has greater resolution capabilities to reveal all cold shock-induced proteins, and this system was used in the following studies. A cold shock to 15°C served as a standard for examining cold shock in *B. subtilis*. In Figs. 1 and 2 are shown the polypeptide profiles from cells grown at 37°C (Fig. 1) and those shifted and labeled at 15°C (Fig. 2). At least 24 cold shock-related polypeptides could be seen by two-dimensional gel electrophoresis of cells shocked and labeled at the temperature of 15°C (Fig. 2). The polypeptides matched the molecular weights of those seen in one-dimensional assays; however, several additional low-molecular-weight proteins could be detected by the two-dimensional gel electrophoresis.

It was also important to determine whether progressive decrease in temperature would result in the specific appearance of proteins at various temperatures. Consequently, the cells were grown at 37°C and then were shifted to either 25°C, 20°C, or 10°C to determine the induction of proteins over the whole lower growth range of *B. subtilis*. At the more moderate cold shock temperature of 25°C, the basic response of 24 polypeptides (as seen at 15°C) was evident with several additional proteins (numbers 25–30) also induced (Fig. 3). Some of these must represent temperature-specific proteins since they do not constitute a part of the core cold shock proteins induced under all cold shock temperatures. Cold shock proteins found in common with the representative 15°C gel were also induced at the cold shock temperatures of 20°C (Fig. 4) and 10°C (Fig. 5). Additional temperature-specific proteins were observed under cold shock at 20°C (cold shock proteins numbered 31–53) (Fig. 4), and several from this group also appeared at a cold shock of 10°C (Fig. 5). One could presume that a common set of proteins would be induced at different cold shock temperatures, with specific proteins appearing at different temperatures, as needed by the cell at those conditions.

A comparative gel showing the heat shock response of *B. subtilis* indicated that several of these induced cold shock proteins were also induced under heat shock conditions (Fig. 6). Examples of such general stress proteins are cold shock proteins numbered 15, 16, 18, 20, 48, and 49, which were induced under both temperature stresses. Prominent heat shock proteins such as DnaK and GroEL, which are elevated under heat shock conditions (marked as D and G in the figures), were not visibly induced under cold shock conditions.

Sequence analysis of specific cold shock polypeptides. Prominent and distinct cold shock proteins, as visual-

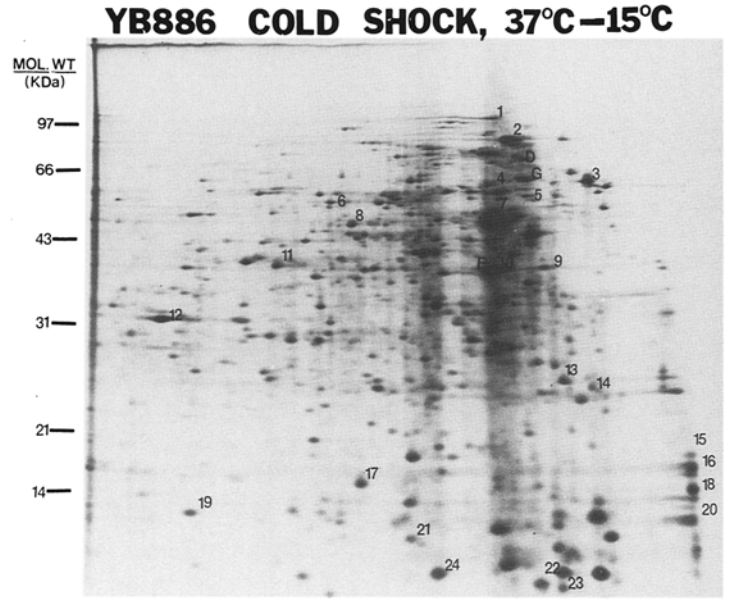

Fig. 2. Protein profile of *Bacillus subtilis* cells grown at 37°C, then shifted to 15°C, as described in Materials and Methods. The cold shock of 15°C was selected as the standard of cold shock within the *Bacillus subtilis* growth range. Proteins induced, as compared to the control gel, were assigned a number. Circles were not utilized for all 53 induced cold shock proteins to reduce cluttering the gels. Letter abbreviations on gels are as follows: D, DnaK; G, GroEL; F, flagellin; E, enolase. Relative positions of these proteins have been determined from previous research in our laboratory [17].

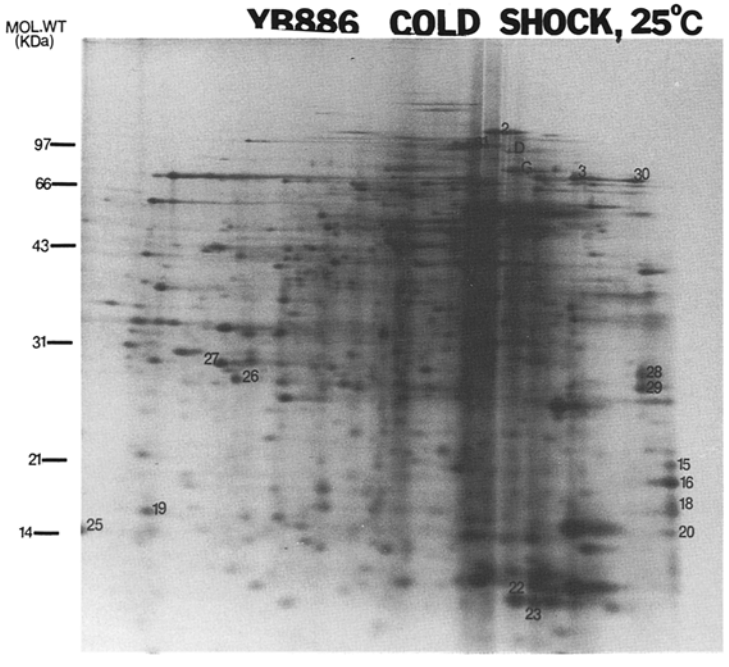

Fig. 3. Protein profile of *Bacillus subtilis* cells shocked at 25°C.

ized by the one-dimensional gel analysis, were isolated from PVDF membranes and were microsequenced, as described in Methods. Polypeptide sequences were obtained for the 115kDa, 97kDa, and 21kDa unique cold shock proteins that were obvious after shifts from 37°C to both 15°C and 10°C (Table 1). A comparative sequence analysis was obtained from the international protein sequence database. From this analysis, the N-terminal sequence of the 115-kDa polypeptide was homologous to citrate hydrolase, commonly referred to as aconitase. The peptide sequence had 84% homologous identity to the amino

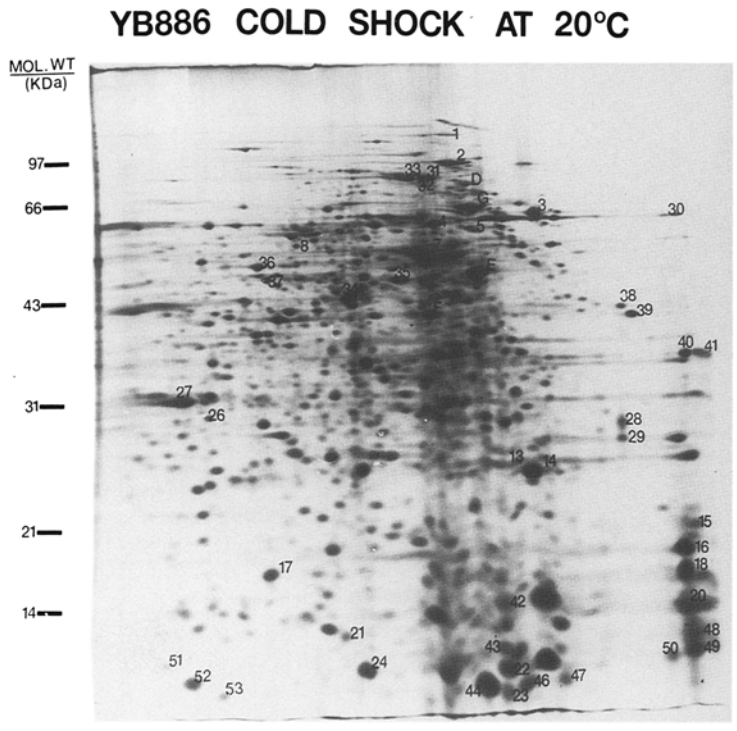

Fig. 4. Protein profile of *Bacillus subtilis* cells shocked at 20°C.

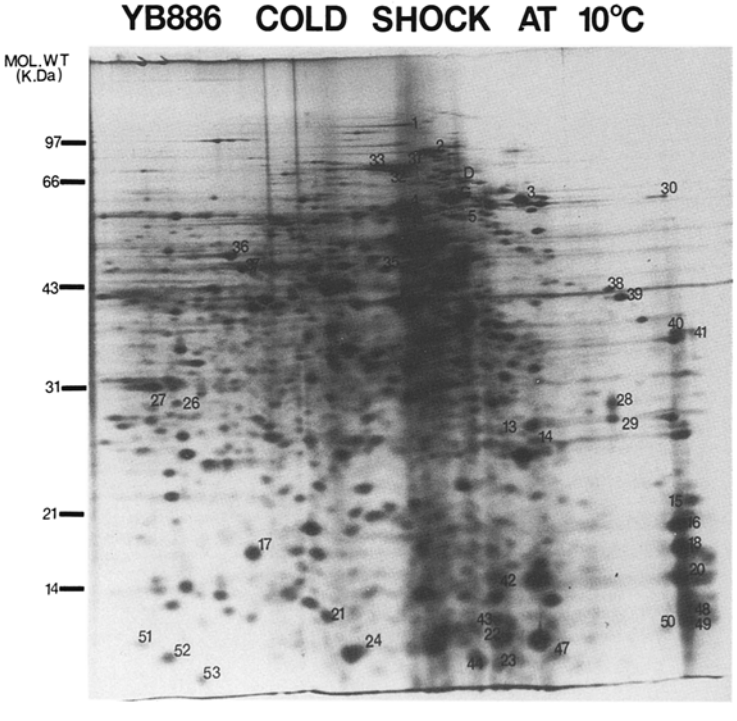

Fig. 5. Protein profile of *Bacillus subtilis* cells shocked at 10°C.

terminal aconitase sequence of *B. subtilis*. Failure to achieve a 100% identity was due to ambiguous sequence information and missing sequence information (marked with an X in Table 1). The sequences of the other two polypeptides (97 and 21 kDa) could not be matched to any sequence information in the database and, as expected, had no similarity to CspA or CspB [28].

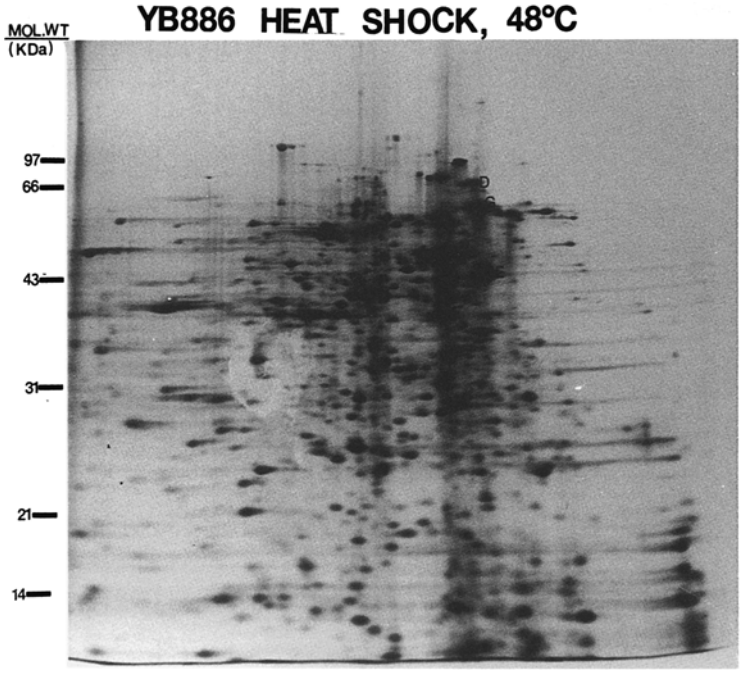

Fig. 6. Protein profile of *Bacillus subtilis* cells heat shocked to 48°C, as described previously [17].

Table 1. Results of polypeptide sequencing and comparisons

	1	2	3	4	5	6	7	8	9	10	11	12	13
115 kDa	A	N	E	Q	X	T	A	A	X	K	V	F	(R)
Aconitase	A	N	E	Q	K	T	A	A	K	D	V	F	Q
	1	2	3	4	5	6	7	8					
97 kDa	A	R	E	F	S	L	E	(K)					
	9	10	11	12	13	14	15	16					
	T	G	N	(I)	(G)	(I)	(M)	(A)					
	1	2	3	4	5	6	7	8					
21 kDa	M	R	T	T/(G)	P/(E)	M	A/(E)	N					
	9	10	11	12	13	14	15	16					
	A	S	T	(I)	X	R	X	G					

Sequences are listed with the amino terminus first. Parentheses indicate weaker identification. A backlash indicates an equal probability of either amino acid residue at this position. An X indicates the absence of sequence information. Protein sequences were obtained from distinct Coomassie-stained bands on PVDF membranes corresponding to induced cold shock proteins as seen on autoradiograms of cold-shocked cells.

Discussion

This study focused on the adaptive synthesis of proteins by the soil bacterium *B. subtilis*, as the organism adjusts to growth at temperatures approaching its lower growth capability limits. From these cold shock experiments it was observed that this bacterium induces a significant set of proteins (designated as the cold shock proteins) at temperatures below its optimal range of growth, as assayed by two-dimensional gel electrophoresis. The overall response has not been previously characterized in *B. subtilis*, except for the identification of a homolog to a uniquely synthesized *E. coli* cold shock protein [22, 28]. The uniquely synthesized *E. coli* cold shock protein (F10.6) has been cloned and sequenced and determined to be a DNA-binding protein showing remarkable sequence similarity to the DNA-binding domain of a family of eukaryotic nucleic acid-binding proteins [9, 15, 26, 29]. A protein of similar size to CS7.4, designated as CspB and possessing 60% amino acid sequence iden-

tity to CspA of *E. coli*, has recently been identified in *B. subtilis* [22, 28]. This protein also has high affinity for specific DNA sequences [10]. *Streptomyces clavulugerus* also contains a protein with a calculated size of 7.0 kDa and 56% amino acid sequence identity to CS7.4 [2]. This protein, designated SC7.0, had an apparent molecular mass of 8 kDa on SDS polyacrylamide gels. In our study, several acidic and basic proteins possessing similar mobilities to CS7.4 on SDS gels were uniquely induced under cold shock conditions, though the CS7.4 analog was not specifically identified. However, a partial identity of this protein could be obtained by Western blotting with antibodies to CS7.4 or from gel purification methods enabling detection by staining procedures.

In the enteric bacterium *E. coli*, an abrupt decrease in the growth temperature of the cells results in the cessation of growth for several hours, as the cells adapt to the new environment [12, 13]. During this period, overall protein synthesis is greatly decreased and the pattern of cold shock protein synthesis is unique [12]. The repressed proteins include also the heat shock proteins. Since the growth of the cell is restricted because of an inhibition of the cellular translation machine, the cold shock response is thought to facilitate the expression of genes involved in translation [3, 12, 13, 27]. Indeed, some of the cold shock proteins identified in *E. coli* participate in the transcription and translation operations of the cell or form part of operons containing genes involved in these processes [12].

The growth of *B. subtilis* was slowed by shifts from the optimal growth temperature to a lower growth temperature, although there was no observable lag period in the growth of the cells as observed in *E. coli*. This probably relates to *Bacillus* being a common soil bacterium, which is relatively well adapted to life under conditions of constant fluctuations in temperature. Consequently, this study could focus on the induction of cold shock proteins upon immediate exposure of the cells to low temperature conditions. This sudden exposure to low temperatures resulted in the induction of as many as 53 total proteins when this organism was shifted to several lower temperatures, with several of these proteins being common to all temperatures, while others were specific to a particular shock temperature. The proteins could be grouped into two broad classes, namely, those proteins that apparently function in cellular metabolism under optimal conditions but are enhanced during cold shock, and those that are uniquely synthesized under the cold shock conditions. Although the identity of most of these proteins is not known, presumably several are involved in enhancing transcription and maintaining the translational capacity of the cell at low temperature conditions [15, 26, 27].

The identity of one of the cold shock proteins of *B. subtilis* from the current study could be obtained through sequence comparisons of the induced polypeptide with those contained in a protein sequence database. The 115-kDa polypeptide, reproducibly induced at increasingly low temperatures, had identity to aconitase, a TCA cycle enzyme encoded for by the *citB* gene that converts citrate to isocitrate via the intermediate, *cis*-aconitate [5]. The purified aconitase protein from *B. subtilis* has a probable monomer molecular weight between 100 kDa and 120 kDa [5]. The promoter region for this gene has consensus sequences for the binding of the RNA polymerase associated with either the major, vegetative sigma-43, or the sporulation sigma factor, sigma-29 [8]. In addition, the *citB* promoter has several regions in common with the promoters of *amyE* and *spoVG*, both of which are activated at the end of the stationary phase of growth [8]. Evidence also implicates aconitase for being controlled by the sporulation cascade, because signals that initiate sporulation such as depletion of carbon, nitrogen, or phosphate also activate the expression of aconitase [5]. Thus, the appearance of aconitase under cold shock conditions suggests the possibility of overlapping regulons with the sporulation event, or common induction and transcriptional signals. This is not an altogether novel idea since several of the adaptational networks of *B. subtilis*, such as competence, chemotaxis, and sporulation, are reactions occurring in the stationary phase of growth [25]. The common induction of aconitase during cold shock and sporulation might possibly be limited to a shared signal transducer which ultimately leads to the expression of the *citB* gene under both conditions. The identification of several additional cold shock proteins that also function in the sporulation event would further support the theory of overlapping adaptational regulons existing in *B. subtilis*.

An initial characterization of the heat shock response of *B. subtilis* by two-dimensional electrophoresis has been recently published [17]. Although the role of most of these heat shock proteins in *B. subtilis* has not been extensively studied, there is initial evidence that the DnaK homolog is necessary for normal cellular morphology [24]. The likely physiological role of GroEL and select other heat shock proteins in *B. subtilis* will likely be elucidated in the near future after the recent isolation and sequencing

of the *groEL* operon [16, 23]. However, the major heat shock proteins, DnaK and GroEL, were not induced by exposure to cold shock, as also has been reported for *E. coli* [12]. Likewise, the roles of the cold shock proteins in *B. subtilis* will be determined from studies with mutants in which the effect of a loss of one or more cold shock proteins can be evaluated regarding the survival of the cell. Analysis of mutants will also assist in determining how components of the cold shock response function in cell adaptation to survival at lower temperatures.

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