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Cold Shock Stress-Induced Proteins in *Bacillus subtilis*

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Bacteria respond to a decrease in temperature with the induction of proteins that are classified as cold-induced proteins (CIPs). Using two-dimensional gel electrophoresis, we analyzed the cold shock response in *Bacillus subtilis*. After a shift from 37 to 15°C, the synthesis of a majority of proteins was repressed; in contrast, 37 proteins were synthesized at rates higher than preshift rates. One hour after cold shock, the induction of CIPs decreased, and after 2 h, general protein synthesis resumed. The identified main CIPs were excised from two-dimensional gels and were subjected to microsequencing. Three small acidic proteins that showed the highest relative induction after cold shock were highly homologous and belonged to a protein family of which one member, the major cold shock protein, CspB, has previously been characterized. Two-dimensional gel analyses of a *cspB* null mutant revealed that CspB affects the level of induction of several CIPs. Other identified CIPs function at various levels of cellular physiology, such as chemotaxis (CheY), sugar uptake (Hpr), translation (ribosomal proteins S6 and L7/L12), protein folding (PPiB), and general metabolism (CysK, IlvC, Gap, and triosephosphate isomerase).

Bacteria must adapt to continuous changes in the environment, such as changes in the availability of nutrients or oxygen. Invariably, the response to drastic chemical and physical changes in the surroundings involves the induction of sets of specific proteins. This has been shown for various environmental stresses, including an increased salt concentration, a rise in temperature (heat shock), phage infection, or ethanol treatment (1a, 12, 17, 56). The functions and regulation of some stress-induced proteins, particularly the well-characterized heat shock proteins that act as molecular chaperones (61), have been elucidated.

However, little is known about the functions of proteins induced after a decrease in temperature. A cold shock response has been found in *Escherichia coli* (25), and the induction of proteins in response to cold shock was monitored in *Bacillus* (30, 58, 60), *Listeria* (41), and *Rhizobium* (10) species. In *E. coli*, the number of proteins synthesized decreases drastically after a shift from 37 to 10°C, and cellular growth arrests. After 2 h, only 28 proteins are detectably produced, 14 at rates higher than preshift rates. Three hours after a cold shock, the number of proteins increases again, until after 4 h, normal protein synthesis and growth are resumed. Cold-induced proteins (CIPs) include mainly transcriptional and translational proteins, components of the pyruvate dehydrogenase complex, and CspA, the only protein that is not present at 37°C. Most CIPs are synthesized at a 2- to 10-fold higher level compared with preshift levels, in contrast to CspA, which is induced about 200-fold (25). Other proteins that are continually synthesized after a cold shock have been identified as ribosomal proteins L7, L12, S1, S6B, and S6A, trigger factor, elongation factor Tu (EF-Tu), EF-Ts, EF-G, and the β subunit of RNA polymerase (23). It has been shown that the initiation of translation is a limiting factor for the growth of bacteria at low temperatures (8), which is probably reflected in the preferential synthesis of proteins involved in translation in *E. coli* after cold shock. Interestingly, heat shock proteins are repressed by cold shock, and vice versa (53).

Homologs of the major cold shock protein CspA in *E. coli* have been identified in many eubacteria, including *Bacillus subtilis* (CspB) (59) and other bacilli (51), Streptomycetes (CS7.0) (2), *Listeria monocytogenes* (CspL) (11), *Salmonella typhimurium* (CspS) (28), and *Arthrobacter globiformus* (Csp1) (3). Moreover, *cspA* homologs have been observed in several other gram-positive and gram-negative bacteria (42). This finding indicates that CspA homologs may be present in most eubacteria. Furthermore, in *E. coli*, five homologs to *cspA* (*cspB* to *-F*) have been cloned (29). Among this family of cold shock proteins, an induction after cold shock was observed only for CspA and CspB. As CspA was shown to enhance the transcription of the *hns* and *gyrA* genes, it was suggested that CspA may act as the general transcriptional activator of the cold shock regulon in *E. coli* (24, 27).

The structures of CspB in *B. subtilis* (46, 49) and CspA in *E. coli* (37, 45) have been elucidated by nuclear magnetic resonance and X-ray analysis, revealing very similar β-barrel folds. Mutational analysis of CspB in *B. subtilis* has identified basic and aromatic residues on a three-stranded β-sheet surface that are important for binding to single-stranded DNA (ssDNA) (50). CspA and CspB show a high degree of homology (>45%) to a nucleic acid binding domain of eukaryotic Y-box proteins (16), which is called the cold shock domain. The cold shock domain proteins represent the evolutionarily most conserved protein family so far discovered (16).

In this report, we show that *B. subtilis* has a cold shock response analogous to but distinct from that of *E. coli*. Through microsequencing, we identified 16 major CIPs of *B. subtilis* and detected a family of homologous cold shock proteins that seems to be most important for an adaptation to low temperatures. The function of one of these proteins, CspB, was analyzed in a *cspB* null mutant strain.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* XL-1 Blue {*recA1 endA1 gyrA96 thi hsrR17 supE44 relA1* λ⁻ [F′ *proAB lacI*^{q} *lacZ*DM15 Tn*10* (Tet^r)]} was used for

transformation and propagation of plasmids and was grown in rich medium (59). Strains of *B. subtilis* used in this work were JH642 (wild type; *trpC2 pheA1*) (59), KS3 (*trpC2 pheA1* Δ*cspB cat*) (this study), GW1 (*trpC2 pheA cspB*::*cat*) (59), GW3 (*trpC2 pheA1* P_{cspB}::pTKlac$P_{cspB474}$) (59).

Stress induction and radioactive labeling of cultures. Cultures of *B. subtilis* were grown to mid-log phase (optical density at 600 nm of 0.5) in M9 medium (34) supplemented with tryptophan and phenylalanine (each at 0.01% [wt/vol]) and yeast extract (0.01% [wt/vol]). Aliquots of 4 ml were transferred into preincubated flasks containing NaCl to a final concentration of 1 M (salt shock), with shaking at 37°C, 15°C (cold shock), 48°C (heat shock), or at 37°C. [^{35}S]methionine (20 μCi; Hartmann Analytics, Braunschweig, Germany) was added at 5, 30, 60, 90, 120, and 150 min after cold shock (labeling was stopped after 30 min), at 10 min after heat shock (labeling for 10 min), at 10 min after salt shock (labeling for 10 min), and at 37°C in parallel to the stress induction (labeling for 10 min). Aliquots were put on ice, 1/10 volume of stop solution (0.1 M Tris-HCl [pH 7.5], 0.01 M methionine, 1 mg of chloramphenicol per ml) was added, and the samples were centrifuged at 4°C and 12,000 rpm for 10 min. Cells were washed in wash buffer (0.1 M Tris-HCl [pH 7.5], 0.1 mg of chloramphenicol per ml), centrifuged as before, and frozen or directly resuspended in breakage buffer (10 mM Tris-HCl [pH 7.4], 1 mg of $MgCl_2$ per ml, 50 μg of lysozyme [U.S. Biochemical] per ml, 50 μg of DNase I [Amersham] per ml, 50 μg of RNase A [Sigma] per ml). Cells were disrupted with a Branson model B15 Sonifier (twice for 30 s each time). After pelleting of unbroken cells and insoluble material (4°C, 12,000 rpm, 15 min), the incorporation of radioactivity in the supernatant was measured (5 μl was applied to Whatman 2MM paper, dried, washed with ice-cold 10% trichloroacetic acid for 10 min, washed with ice-cold 5% trichloroacetic acid for 10 min, washed twice with ethanol for 10 min, and finally dried) in a liquid scintillation counter (Packard CA1900). The protein concentration in the supernatant fluid was determined by the Bradford test (5). The cellular extracts were dried in a Speed Vacumeter (Uniequip) and resuspended in a suitable volume of buffer E (9.6 mg of dithioerythritol [Biomol] per ml, 38.5 mg of 3-[3-cholamidopropyl-dimethyl-ammonio]-1-propanesulfonate [CHAPS; Sigma] per ml, 0.25 mg of phenylmethylsulfonyl fluoride [Sigma] per ml, 0.52 g of urea per ml, 50 μl of Servalyt pH 3-10 per ml), resulting in 7×10^5 cpm in 10 μl (for analytical two-dimensional [2D] gels) or 600 μg of unlabeled protein in 20 μl (preparative 2D gels).

Analytical and preparative 2D PAGE. Two dimensional polyacrylamide gel electrophoresis (PAGE) was performed as described previously (56), using a Bio-Rad ProteanIIxI system, with the following specifications: Servalyt pH 3-10 and 5-7 were used for the first-dimension electrofocusing (3.3-mm diameter), which was run for 15 h at 400V and then for 1 h at 600 V; 1 mm sodium dodecyl sulfate-polyacrylamide gels (13.5% 30%:08% acrylamide/*N*-bisacrylamide [wt/vol]) were run in the second dimension. Analytical gels were fixed for 30 min (50% methanol, 10% acetic acid), rinsed in water for 45 min, and dried on Whatman 2MM paper. Kodak X-Omat films were exposed for 5 days at room temperature. Preparative gels were fixed as described above, stained with Coomassie brilliant blue R-250 (U.S. Biochemical), and destained (20% ethanol, 10% acetic acid).

Microsequencing. For N-terminal microsequencing, the spots from several (8 to 12) Coomassie blue-stained 2D gels were cut out. The gel pieces were concentrated as described previously (43), but using 5% polyacrylamide as the spacer and a 15% polyacrylamide gel underneath. The resulting protein spot was blotted onto a polyvinylidene difluoride membrane (Immobilon P), using a carbonate buffer system (10 mM $NaHCO_3$, 3 mM Na_2CO_3). After staining by amido black (0.1% in 40% methanol–10% acetic acid), the spot was excised from the membrane and installed into the blot cartridge of a model 473A protein sequencer (Applied Biosystems). A faster modification of the NORM standard cycle was used for sequencing as specified in the Applied Biosystems manual for the microcartridge. To effect deformylation of proteins, the polyvinylidene difluoride membrane in the blot cartridge was wetted with 50 ml of 1 N methanolic HCl, and the cartridge was mounted into the holder and kept for 2 h at 45°C. Afterwards it was flushed for 5 min with nitrogen, and the sequencing was started with the begin cycle. Because D and E residues were esterified by this procedure, the positions of the corresponding phenylthiohydantoins in the chromatogram were determined by using CNBr fragments of myoglobin esterified with 1 N methanolic HCl as described above.

Construction of a *cspB* deletion mutant. To create the total deletion of the *cspB* gene (Fig. 1), chromosomal DNA was prepared from a 1.5-ml overnight culture of JH642 grown in 2× yeast-tryptone (YT) rich medium, using Qiagen tip columns as instructed by the manufacturer. The upstream and downstream regions flanking the *cspB* gene were amplified by PCR (1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C for 5 cycles and 1 min at 94°C, 1 min at 60°C, and 1.5 min at 72°C for 30 cycles, using a Perkin-Elmer model 480 DNA thermal cycler). Primers csp5Bam (5′-CGGGATCCCGTGCAGACGGTGAACATGCAG-3′), csp5Eco (5′-CGGAATTCCGCCTCCTAAAGCGATCATAACG-3′), csp3Eco (5′-CGGAATTCCGTCCCGCTTCATGAAGATTCC-3′), and csp3Hind (5′-CCCAAGCTTGGGCTGGGGCTGACAATGGTTG-3′) were used. The amplified 0.5-kb 5′ and 0.8-kb 3′ fragments were cut with corresponding endonucleases (*Bam*HI and *Eco*RI for the 5′ fragment and *Eco*RI and *Hin*dIII for the 3′ fragment; U.S. Biochemical) and sequentially inserted into pBluescriptII SK$^-$ (Stratagene) cut with the same endonucleases. A 1.3-kb *Eco*RI fragment from plasmid pZD327 (62), containing the chloramphenicol acetyltransferase gene (*cat*), was inserted into the *Eco*RI site (between the 5′ and 3′ fragments). Five micrograms of the resulting plasmid, pPCR3-cat-PCR4, was linearized with *Sca*I, and competent cells of *B. subtilis* JH642 were transformed as described previously (26). Cells containing a *cat* marker were isolated on 2× YT agar plates containing 5 μg of chloramphenicol per ml. The *cspB* knockout (Fig. 1) in the resulting strain, KS3, was verified both by Southern blot analysis of *Eco*RI-digested chromosomal DNA of KS3 with a PCR fragment containing the *cspB* gene (51) and by Western blot (immunoblot) analysis (59).

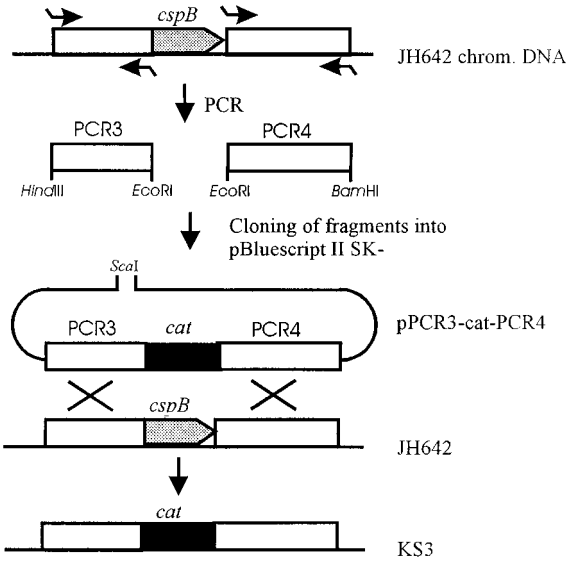

FIG. 1. Construction of the *B. subtilis cspB* null mutant strain KS3 by a double-crossover recombination event. The boxes indicate the upstream and downstream regions of the *cspB* gene, which is indicated by a filled arrow. The black box indicates *cat*, which mediates chloramphenicol resistance. The locations of restriction sites introduced by PCR (*Hin*dIII, *Eco*RI, and *Bam*HI) and used for linearization of the integration plasmid pPCR3-cat-PCR4 (*Sca*I) are shown. Horizontal arrows indicate the PCR primers. The figure is not drawn to scale.

RESULTS

Analysis of the cold shock response of *B. subtilis*. The minimal growth temperature for *B. subtilis* was determined to be between 8 and 9°C in rich medium and between 12 and 13°C in minimal medium (MM). A sudden decrease from 37 to below 13°C in MM (or to below 10°C in rich medium) resulted in the lysis of cells, indicating that the adaptation to low temperatures after a cold shock is vital for *B. subtilis*. After a cold shock to 13°C or higher, the growth of *B. subtilis* continued with a reduced doubling time of over 24 h but no apparent growth lag. This is in contrast to an arrest of growth for 4 h reported for *E. coli* after cold shock (25). A drop from 37 to 15°C during mid-log phase was chosen as standard cold shock in our assays, as this is close to the maximum shift of 37 to 13°C in MM.

On autoradiograms of 2D gels from cells labeled at 37°C, the synthesis of approximately 250 discrete intracellular proteins was detectable (Fig. 2a). During the first 0.5 h after cold shock, the synthesis of a majority of cytosolic proteins decreased, whereas 23 proteins were expressed at a higher level than at 37°C (Fig. 2b; Table 1). Eighteen identified CIPs were excised from preparative 2D gels of extracts from cells that had been subjected to 3 h of cold shock (15°C) and were microsequenced (Table 2). The identities of CIPs were determined by a data search of the EBI database (Hengston, England), using the programs FASTA and Blitz.

The L7/L12 protein is not visible on autoradiograms, as it contains no methionine. It was identified as a CIP by compar-

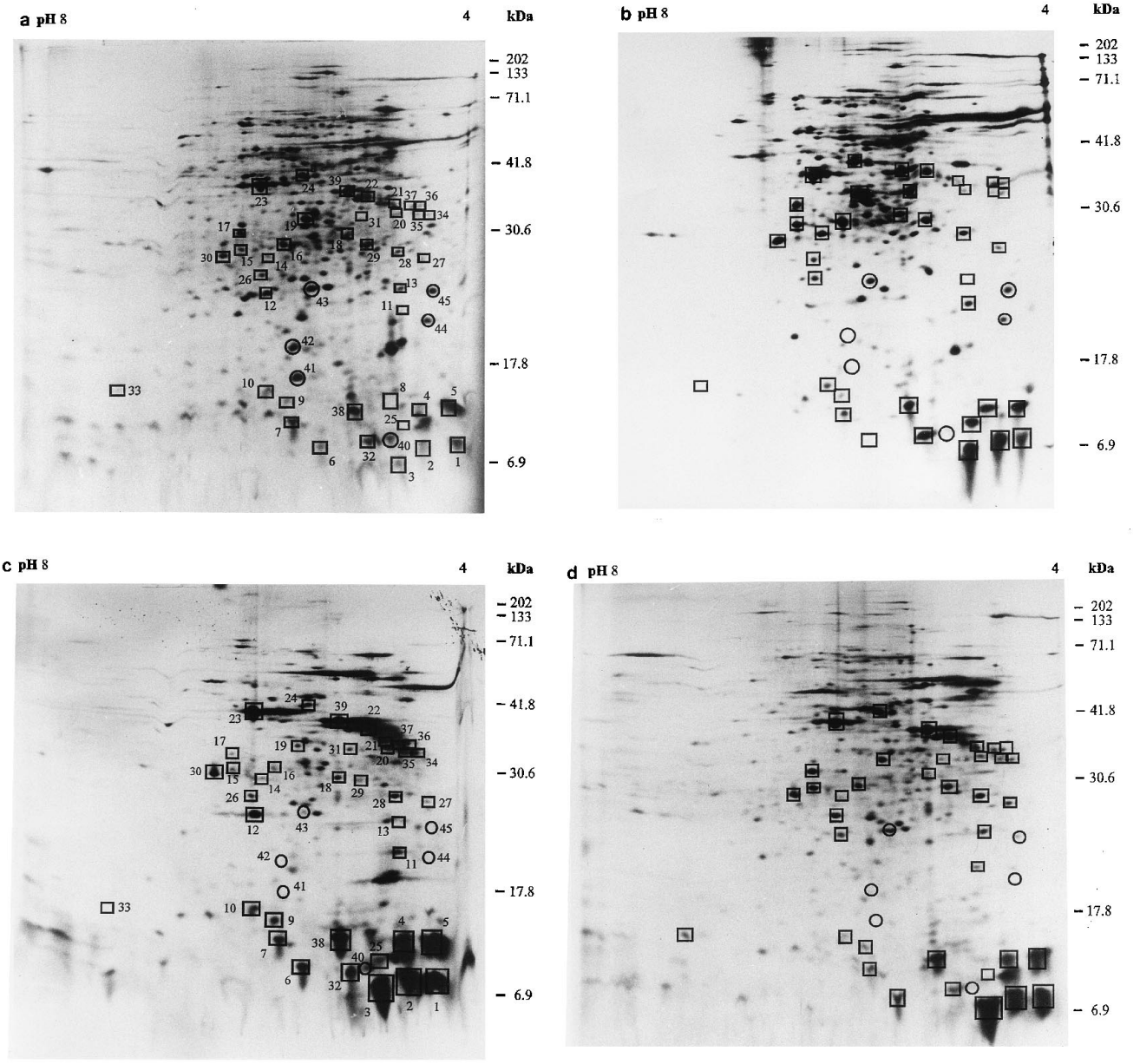

FIG. 2. Autoradiograms from 13.5% second-dimension gels containing cytosolic extracts from [^{35}S]methionine-labeled cells of *B. subtilis* JH642 grown at 37°C (labeled for 10 min) (a) and shifted from 37 to 15°C and labeled for 5 to 30 min (b), 30 to 60 min (c), and 90 to 120 min (d). Protein spots whose synthesis increased relative to preshift levels or was not affected after cold shock are boxed; proteins whose synthesis decreased are circled. The corresponding proteins are listed in Table 1. The position of protein L7/L12 (spot 8), which does not contain methionine, is indicated in panel a. Kaleidoscope Prestained Standards (Bio-Rad) were used as markers.

ison of cold shock Coomassie blue-stained gels with gels containing extracts from cells growing at 37°C (not shown). Spot 9 (Fig. 2) revealed two sequences which could be identified as RisB (involved in the last step of riboflavin synthesis) and AtpE, the E subunit of ATPase (Table 2). As it could not be determined whether RisB or AtpE or both proteins are CIPs, spot 9 is referred to as Csi9. Interestingly, the CspB sequence was detected in two spots (1 and 2 [Table 2; Fig. 2]), which migrated at similar sizes but with different pI values. The CspB sequence from spot 1 could be identified only after a deformylation step, which shows that CspB exists as a formylated as well as deformylated protein under cold shock conditions in *B. subtilis.*

At 30 to 60 min after the shift to 15°C, protein synthesis decreased further to about 75 detectable proteins (Fig. 2c). With the exception of Csi13, Scp2, Scp3, and Tip2 (not induced) and Csi16, Csi19, CysK, and Scp5 (repressed), all CIPs were induced relative to levels at 37°C (Table 1). Moreover, CspB, CspC, CspD, PspB, Csi5, Csi12P, and IlvC were further induced in relation to the first half hour after cold shock, such that predominantly cold shock proteins were synthesized. One hour after cold shock (60 to 90 min), an increase in the total number of proteins synthesized was apparent, including many proteins whose synthesis had previously been repressed (not shown). Conversely, the expression of CspB, CspC, CspD, CheY, Hpr, Csi9, Csi18, Csi12P, SpoVG, and IlvC decreased

TABLE 1. Proteins affected by cold shock identified by 2D PAGE and shown in Fig. 2

Spot	Protein[a]	Size (kDa)	Induction after:					
			Cold shock				Salt stress, 10–20 min	Heat shock, 10–20 min
			0–30 min[b]	30–60 min	60–90 min	90–120 min		
	Csps/Csis							
1	^{Fmet}CspB	7.4	>	>>	>>	>>	<	−
2	CspB	7.4	>	>>	>>	>>	<	<
3	CspC	7.2	>	>>	>>	>>	<	−
4	CspD	7.4	>	>>	>>	>	+	<
5	Csi5	10.0	>	>>	>>	>	<	<
6	S6	11.1	+	>	>	>	+	+
7	SpoVG	10.8	+	>	+	+	<	+
8	L7/L12	12.4	—[c]					
9	Csi9	12.0	+	>	+	+	−	+
10	PPiB	13.5	>	>	>	+	−	+
11	Csi11	20.0	>	>	>	>	−	−
12	Csi12P	22.0	>	>>	>	+	<	+
13	Csi13	23.0	−	−	−	>	−	−
14	Csi14	27.5	>	+	+	+	−	−
15	Csi15	28.5	>	+	+	+	+	<
16	Csi16	28.5	>	<	+	+	−	<
17	CysK	32.8	>	<	+	>	−	<
18	TIM	32.0	>	+	<	<	<	−
19	Csi19	32.5	>	<	+	+	+	<
20	Csi20	33.5	+	>	>	+	<	+
21	Csi21	34.5	+	>	>	+	<	+
22	Csi22	35.5	>	>	>	>	<	+
23	IlvC	37.5	>	>>	>	+	<	<
24	Gap	35.7	>	+	+	+	+	+
	Scps							
25	Scp1	8.5	>	>	>	−	>	−
26	Scp2	26.0	+	+	>	>	>	<
27	Scp3	27.0	>	>	>	>	>	+
28	Scp4	28.5	>	>	>	>	>	<
29	Scp5	29.5	>	<	+	+	>	−
30	PspB	26.0	>	>>	>>	+	>	+
31	Scp7	33.5	>	>	>	>	>	−
	Tips							
32	CheY	13.3	>	>	+	+	+	>
33	Tip2	14.0	−	−	−	>	−	>
34	Tip3	33.5	+	>	>	>	−	>
35	Tip4	33.5	+	>	>	>	−	>
36	Tip5	34.5	+	>	>	>	−	>
37	Tip6	34.5	+	>	>	>	−	>
	Universally induced protein							
38	Hpr	9.2	+	>	>	>	>	>
	Selected proteins repressed or not affected by cold shock							
39	Flagellin	37.5	+	+	+	+	<	+
40	GroES	10.2	−	<	<	−	+	>
41	GsiB	14.5	−	−	−	−	>	>
42	Gsp26	15.5	−	−	−	−	>	>
43	ClpP	21.5	+	<	<	<	>	>
44	RsbW	19.5	<	−	−	−	>	>
45	Gsp22	20.5	<	−	−	<	>	>

[a] PPiB, peptidylprolyl *cis/trans* isomerase; CysK, cysteine synthase; PspB, phage shock protein of *Bacillus* species; IlvC, ketol-acid reductoisomerase; Gap, glyceraldehyde dehydrogenase.
[b] −, no synthesis detectable. Synthesis relative to preshift: >, enhanced; <, reduced; +, no change.
[c] L7/L12 does not contain methionine and was identified as a CIP on Coomassie blue-stained gels.

relative to the level in the previous period, whereas Csi11 was further induced and Scp2 was newly induced. At 90 to 120 min after cold shock, the expression of about 150 proteins could be detected (Fig. 2d). This continued without noticeable fluctuations during 150 to 180 min and 180 to 210 min (not shown). These data reveal that 2 h after a cold shock, cells exhibited a new pattern of protein synthesis, which then remained constant for 1 h. During this last period in the cold shock response (90 to 120 min), the synthesis of CspD, CheY, PPiB, Csi5, Csi11, Csi12P, Csi20, Csi21, IlvC, and PspB further decreased or was undetectable in the case of Scp1. In contrast, Csi13 and Tip2 were expressed at a higher level than during preceding periods of the cold shock. The synthesis of all other CIPs remained constant. This finding suggests that some CIPs (like Scp1) may be important for adaptation after a cold shock, whereas the majority are needed for continued growth at low temperatures.

TABLE 2. Proteins identified from preparative 2D gels

Spot	Sequence[a]	Protein	Reference(s) or source
1	MLEGKVKWFNSEKGFGF ::::::::::::::::: MLEGKVKWFNSEKGFGF	CspB*	59
2a	MLEGKVKWFNSEKGFGFIEVEGQDDVFVHFS ::::::::::::::::::::::::::::::: MLEGKVKWFNSEKGFGFIEVEGQDDVFVHFS	CspB	59
2b	AIVKATDQSFSAET :::::::::::::: MAIVKATDQSFSAET	Thioredoxin	9
3	MEQGTVKWFNAEKGFGFIERENGDDVFV ::::::::::::::::::::::::::: MEQGTVKWFNAEKGFGFIERENGDDVFV	CspC	57
4	MQNGKVKWFNNEKGFGFIEVEGGDDVFVHFTAIEGDGYKS	CspD	This study
6	MRKYEVMYIIRPNIDEESKKAVI ::::::::::::::::::::::: MRKYEVMYIIRPNIDEESKKAVI	S6	19
7	MEVTDVRLRRV ::::::::::: MEVTDVRLRRV	SpoVG	20
8	ALNIEEIIASVKEATVLELNDLVKAIEEEF ::::::::::::::::::::::::::::::: ALNIEEIIASVKEATVLELNDLVKAIEEEF	L7/L12	21
9a	MNIIQGNLVGTGLKIGIVVG :::::::::::::::::::: MNIIQGNLVGTGLKIGIVVG	RisB	31
9b	MKTVKVNIVTPDGPVYDA :::::::::::::::::: MKTVKVNIVTPDGPVYDA	AtpE	13
10	MKTGYFLLEDGNKIEFELYPEAAPGT ::::::::::::::::::::::::::: MKTGYFLLEDGNKIEFELYPEAAPGT	PPiB	18
12	MLFFVDTANIDEIRE ::::::::::::::: MLFFVDTANIDEIRE	Csp9P/OrfU	35, 52
15	MNFSLEGRNIVVMGVANKRQI	Unknown/Csi15	This study
17	VRVANSITELIGN ::::::::::::: MVRVANSITELIGN	CysK	38
18	MRKPIIAGNWKMNKTLGEAVSFVEEVK ::::::::::::::::::::::::::: MRKPIIAGNWKMNKTLGEAVSFVEEVK	TIM	36
23	GKTVAVIGYGSQGHA ::::::::::::::: MVKVYYNGDIKENVLAGKTVAVIGYGSQGHA	IlvC	54
24	AVKVGINGFGRIGRNVFRAALNNPEVEVVA ::::::::::::::::::::::::::::::: AVKVGINGFGRIGRNVFRAALNNPEVEVVA	Gap	55
30	SIIGRFKDIMXANINALLEKAE .:..:: ::. :::::::::::: MGIFSRFADIVNANINALLEKAE	PspB PspA (*E. coli*)	This study 7
32	AHRILIVDDAAF :::::::::::: MAHRILIVDDAAF	CheY	4
38	AQKTFKVTADSGIHARPATVLVQTAS ::::::::::::::::::::::::::: MAQKTFKVTADSGIHARPATVLVQTAS	Hpr	36
40	MLKPLGDRVVIELVESEEKT :::::::::::::::::::: MLKPLGDRVVIELVESEEKT	GroES	48

[a] Upper sequences were determined by microsequencing; lower sequences are from proteins that have been described previously. Colons mark identical amino acid residues; single points mark conservative exchanges.

TABLE 3. Global alignment of CspB-homologous proteins in *B. subtilis*

Protein	Sequence[a]	% Identity/homology to CspB
	RNP-1 RNP-2	
CspD	MQNGKVKWFNNE**KGFGFIEV**EGGDD**VFVH**FTAIEGDGYKS	78/85
CspB	MLEGKVKWFNSE**KGFGFIEV**EGQDD**VFVH**FSAIQGEGFKTLEEGQAVSFEIVEGNRGPQAANVTKEA	
CspC	MEQGTVKWFNAE**KGFGFIER**ENGDD**VFVH**FSAIQSDGFKSLDEGQKVSFDVEQGARGAQAANVQK-A	71/79

[a] Sequences of CspB and CspC were deduced from the corresponding genes; the sequence of CspD was deduced from microsequencing. The CspB protein was chosen as a reference for comparison. Colons mark identical positions; single points mark conservative exchanges. Underlined sequences have been determined by microsequencing; RNP motifs are in boldface.

Csi11, Csi12P, Scp1, and Sci7 were synthesized only in response to a cold shock. All other CIPs were present at 37°C. Therefore, the adaptation to a cold environment was mediated mainly by proteins that were synthesized under optimal conditions. In addition, the relative synthesis of several proteins was not affected by a cold shock but remained rather constant throughout the period of the response. Such proteins may represent components vital for metabolism or cell structure, such as flagellin (identified in reference 1a).

Overall, these results suggest that a dynamic response to decreased temperatures exists in *B. subtilis*, with this response being maximal during the second half hour after a cold shock and decreasing until a pattern of expression of cytosolic proteins that is different from that at 37°C is reached.

After a cold shock induced by a shift from 37 to 10°C, no cold shock response was observed in *B. subtilis*; rather, a severely reduced amount of protein synthesis with a pattern comparable to that at 37°C was found (not shown). The lack of a cold shock response may be the reason for the lysis of *B. subtilis* after a shift to 10°C in minimal medium. This finding is in contrast to the results of Lottering and Streips (30), who have monitored proteins induced by a shift to 10°C. A reason for this may be the presence of amino acids in the labeling medium (30). This is in accordance with our observation that *B. subtilis* can survive a cold shock to 10°C without lysis of cells in full medium but not in M9 minimal medium.

Induction of cold shock proteins in response to other stresses. After a shift from 37 to 15°C, a total of 38 proteins were reproducibly expressed at a higher level (Fig. 2). These CIPs can be grouped into three categories. Twenty-one proteins were induced only after a cold shock, not in response to heat shock or salt stress (not shown), and thus represent true cold shock- and cold stress-induced proteins (Csps/Csis). Six proteins were induced in response to cold as well as to heat shock (48°C) and were therefore named temperature-induced proteins (Tips) (Tip1 to -6). An enhanced expression of seven proteins was detected after cold shock and salt stress (1 M NaCl) but not after heat shock. These proteins were named stress and cold shock proteins (Scps) (Scp1 to -7). Of the identified CIPs, CspB, CspC, CspD, S6, PPiB, CysK, Csi12P, triosephosphate isomerase (TIM), IlvC, and Gap (previously identified by Schmidt et al. [47a]) are Csps/Csis. CheY belongs to the group of Tips, and PspB to the group of Scps.

In contrast to the identified Tips and Scps, the expression of several heat shock and general stress proteins (the latter are induced in response to various stresses) was repressed after a cold shock (Fig. 2; Table 1). Representatives for this group, GsiB, Gsp14, Gsp22, RsbW, ClpP (described in references 1a and 56) and GroES (identified in this work and by Antelmann [1]), are circled in Fig. 2 and 3. Conversely, after a heat shock, most CIPs (CspB, CspC, CspD, CysK, Csi5, Csi11, Csi13, Csi15, Csi18, Csi19, and IlvC) and Scps (Scp1, Scp2, Scp4, Scp5, and Scp7) were repressed, whereas in response to salt stress, the synthesis of CspB, CspC, Csi12P, Csi5, Csi9, Csi11, Csi13, Csi14, Csi18, Csi20, Csi21, IlvC, SpoVG, PPiB, and CysK as well as Tip2 to -6 was reduced (not shown). This result reveals that the cold shock response comprises proteins that are also induced during heat shock and general stress responses but is generally antagonistic to the latter responses in *B. subtilis*. Only one protein showed an enhanced expression after all kinds of stresses. Microsequencing of the corresponding spot revealed this protein to be Hpr (previously identified as Gsp17u by Völker et al. [56]), which is involved in the phosphoenolpyruvate:sugar phosphotransferase system (PTS).

Identification of a family of CspB-homologous proteins. Watanabe and Muto have identified an open reading frame (*cspC*) in chromosomal DNA of *B. subtilis* with strong homology to the *cspB* gene (57). In our study, the predicted *cspC* gene product has been revealed to be a cold shock-induced protein (Fig. 2). The calculated size (7.2 kDa) and pI (4.53) of the *cspC* product match the data derived from 2D gel analysis (Table 1; Fig. 2). Furthermore, a third homologous protein sequence, which has not been described previously, was identified as a Csp in 2D gels (Fig. 2) and was therefore named CspD. The alignment of the amino acid sequences of CspB, CspC (deduced from amino acid sequences), and CspD (determined from microsequencing [Table 1]) revealed identities of 71% (CspB and CspC) and 78% (CspB and CspD) between all proteins (Table 3). RNP-1 and RNP-2 motifs, which are essential for binding of CspB to ssDNA (50), are almost 100% conserved between all proteins, with the exception of one change in the eighth position of RNP-1 in CspC. This result shows that a family of CspB-homologous proteins whose members are cold inducible exists in *B. subtilis*. All three proteins have similar sizes (7 to 9 kDa) and a very acidic pI (CspB, 4.3; CspC, 4.5; CspD, 4.3). It is evident that all three CspB homologs show the most pronounced relative induction after cold shock of all CIPs and are synthesized in a greater amount than any other protein at 15°C (Fig. 2). The protein family is thus likely to be most important for the adaptation of *B. subtilis* to low temperatures.

Phenotype of a *cspB* null mutant. Previously, the disruption of the *cspB* gene was shown to increase the freezing sensitivity of *B. subtilis* (59). To determine to what extent the major cold shock protein CspB contributes to the cold shock response, we performed 2D PAGE analysis with strain KS3, in which the entire *cspB* gene has been replaced by *cat* (Fig. 1). At 37°C, few differences were visible between KS3 and the wild type (not shown). After cold shock, the relative induction of several CIPs (CspC, CspD, Csi5, Csi11, Csi19, IlvC, PspB, Scp1, Scp3, Scp4, Scp7, and Tip3 to -6 [Fig. 3a]) was detected in KS3. However, several CIPs (CheY, PPiB, CysK, Csi14, Csi15, Csi16, and Scp5) were not expressed at a higher level after a cold shock than at 37°C (Fig. 3a). In contrast, one protein (Csp46 [Fig. 3a]) was reproducibly overexpressed in KS3 but was undetect-

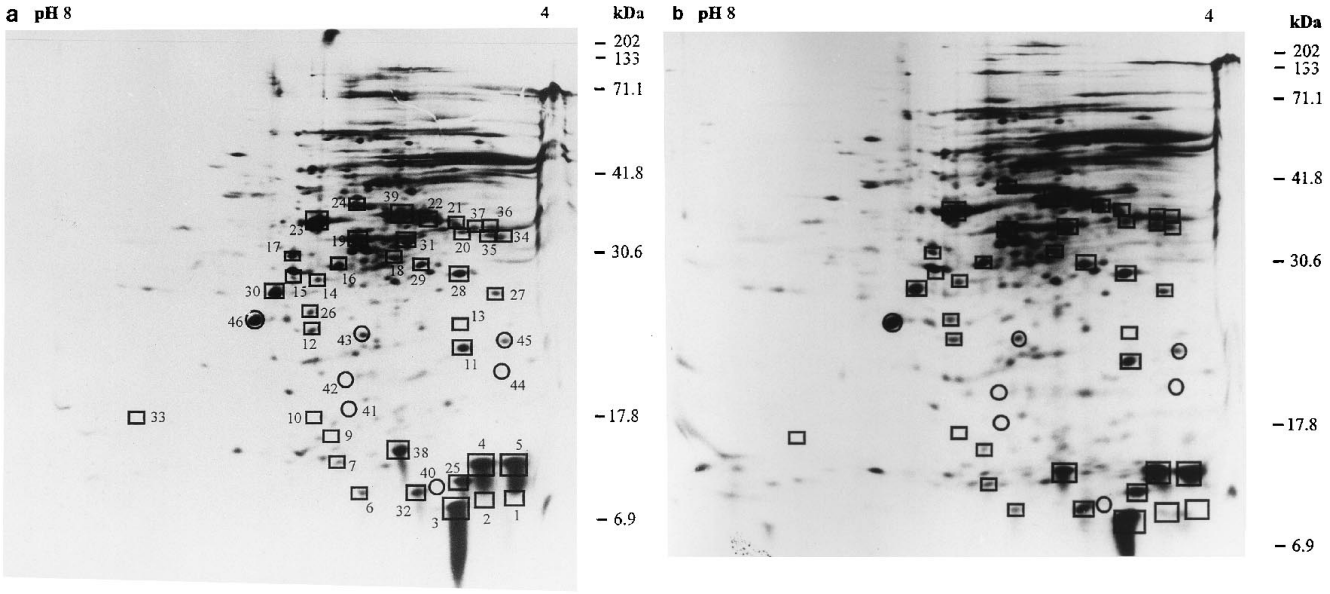

FIG. 3. Autoradiograms from 13.5% second-dimension gels containing cytosolic extracts from [^{35}S]methionine-labeled cells of *B. subtilis* KS3 (*cspB*::*cat*) shifted from 37 to 15°C and labeled for 5 to 30 min (a) and 30 to 60 min (b). Protein spots whose synthesis increased relative to preshift levels or was not affected after cold shock are boxed; proteins whose synthesis decreased are circled. The corresponding proteins are listed in Table 1. Spot 46, whose synthesis increased after a cold shock in KS3, is circled, as it is not a CIP in cells from the wild type. Kaleidoscope Prestained Standards (Bio-Rad) were used as markers.

able after cold shock in wild-type cells. In addition, CspC, CspD, and Csi5 were induced to a higher level in KS3 than in the wild type. In the following half hour after cold shock (Fig. 3b), no noticeable alteration of the pattern of protein synthesis could be detected, in contrast to the wild type, in which general protein synthesis further decreased and the induction of several CIPs was further enhanced. In this period, Csi12P, SpoVG, Csi9, and S6 were not induced and CspC, CspD, Csi5, and Hpr did not show an enhanced synthesis in KS3, in contrast to IlvC, Gap, Csi11, and Csi19, which revealed a higher induction, as in the wild type. Within the third half hour after cold shock, no noticeable difference in the pattern of protein expression compared with the previous periods was observed in KS3 (not shown). During the fourth half hour after cold shock, an increased synthesis of CspC, CspD, Csi12P, CysK, and Csi15 was detectable (not shown), at the time when the cold shock response is completed in the wild type. The responses to heat shock were identical in GW1 (*cspB* interruption [59]), GW3 (*cspB* promoter interruption [59]), and the wild type (data not shown).

From these observations, it is evident that CspB has an influence on the expression of 15 CIPs and thus that a *cspB* deletion results in the loss of the normal cold shock response. The data suggest that CspB is a modulator of the cold shock regulon. However, no difference in the growth after a cold shock to 15°C was observed between the wild-type and *cspB* mutant strains, which shows that the diminished cold shock response in KS3 is sufficient for survival of a cold shock.

DISCUSSION

In this study, we have analyzed the continuous pattern of protein synthesis after cold shock in *B. subtilis* by 2D PAGE and determined the identities of 16 major CIPs by microsequencing. It is evident that protein synthesis is drastically altered after a temperature shift from 37 to 15°C. The synthesis of the majority of cytosolic proteins decreased or was arrested following cold shock, whereas the relative production of 38 proteins increased markedly. The synthesis of CIPs reached a maximum 30 to 60 min after a cold shock and decreased thereafter until a stable pattern of protein synthesis was reached after 120 min (Fig. 2). This pattern of protein synthesis after cold stress is analogous to the heat shock response. Thus, in addition to the existence of CIPs as described by Lottering and Streips (30), we show that a dynamic cold shock response exists in *B. subtilis.*

The experiments also revealed that the responses to cold shock, heat shock, and general stress are related, as several cold shock proteins were also induced by salt stress or heat shock. However, most cold shock proteins were repressed after heat shock and general stress, which demonstrates that the response to cold shock has an antagonistic effect on heat shock and general stress responses, and vice versa. This is in contrast to the heat shock and general stress responses in that a majority of general stress proteins are also induced in response to heat shock (56).

The greatest relative increase in synthesis after a cold shock was detected for several small acidic proteins. These were predominantly produced during the second half hour after a temperature drop and continued to be synthesized at a high level thereafter. The protein that was most highly induced after cold shock was the well-characterized major cold shock protein CspB. The protein gave two spots on 2D gels; one was the deformylated protein, and the other was the formylated protein. However, it is not known whether this chemical modification has any influence on the stability of CspB, which has a low thermodynamic stability and exhibits extremely rapid unfolding and refolding reactions (47). Two other spots were identified as close CspB homologs, CspC and CspD. The similarity of these main cold shock proteins (identity of >70%) reveals that a family of small cold shock proteins exists in *B. subtilis*, all members of which receive the greatest cold shock induction of all CIPs. This finding suggests that Csp proteins are likely to be most important for an adaptation to low temperatures.

To assess the function of the major cold shock protein CspB, we have analyzed the cold shock response of a *cspB* deletion from the chromosome (KS3, Δ*cspB cat*). From 2D gels, it can

be observed that a response to cold shock is detectable within the first half hour in KS3. However, in contrast to the wild type, the synthesis of several CIPs does not increase relative to the level at 37°C. During successive periods after cold shock, no change in the pattern of protein synthesis was visible, in contrast to the wild type. Thus, CspB is involved in the regulation of the synthesis of 15 CIPs, whose level of induction is directly and profoundly affected after a cold shock. The lack of an induction of CIPs may be the reason for the cold-sensitive phenotype of GW1 (*cspB*::*cat*) and KS3, whose survival after a shift from 37 to −80°C is 10-fold lower than that of the wild type. As CspC and CspD are still induced after a cold shock in KS3, they may partially compensate for the loss of CspB. This is likely to be the case during a preincubation at 15°C before freezing of the *cspB* mutant, a treatment which markedly increases the number of surviving cells (59).

One possible mechanism of the regulatory function for the CspB protein family may be the stabilization of the RNA polymerase-DNA open complex (RP_o) at low temperatures. As plasmid DNA isolated from *B. subtilis* growing at 20°C is significantly more negatively supercoiled than the same DNA from cells growing at 37°C (14), it is probable that after a cold shock, additional proteins are needed to facilitate the formation of RP_o. A possible function of Csps at RP_o is supported by the finding that the CspB homolog in *E. coli*, CspA, binds to the promoter of the cold-inducible *hns* gene in the presence of RNA polymerase but not in its absence (6). Furthermore, CspB binds to ssDNA with high affinity to ATTG sequences (15) and was shown to form dimers in phosphate buffer (33). A CspB dimer could bind to both strands of unwound DNA and thereby stabilize RP_o. A similar mode of DNA binding has been observed in the case of the eukaryotic transcription factor YB-1 (32), which contains a CspB-homologous nucleic acid binding domain (cold shock domain). If CspC and CspD are also able to form dimers, heterodimers of the three Csps would allow multiple interactions at cold shock promoters. However, as we have found that CspB binds to RNA with a sequence specificity similar to that for ssDNA but with a lower affinity (unpublished results), CspB homologs could also act at the level of translation.

In addition to the Csps, we have identified several other CIPs, including ribosomal proteins S6 and L7/L12, which have also been identified as cold shock proteins in *E. coli*. This finding suggests that the continued synthesis of these proteins is important for many mesophilic bacteria. It is interesting that mutations within the leader region of 16S RNA, a region important for the correct folding of 16S RNA, result in cold-sensitive cells (40). Even though the synthesis of functional ribosomes is reduced after a cold shock, which is observed in any response after growth arrest (22), the continued production of S6 and L7/L12 could be essential for the correct assembly of rRNA at low temperatures.

The PPiB protein in *B. subtilis*, which is induced after cold shock, catalyzes the isomerization of peptidylprolyl bonds and belongs to the family of cyclophilins, which are inhibited by cyclosporin A (18). Our results indicate that PPiB is important for cold adaptation of *B. subtilis*, as could also be the case for other bacterial cyclophilins. It is interesting that trigger factor in *E. coli*, which possesses prolyl isomerase activity, is a CIP (23), which leads to the assumption that isomerase activity is important at low temperatures. The Csi12P protein, which was identified as a main CIP, had previously been identified as a protein phosphorylated by ATP during the sporulation of *B. subtilis* (35). The encoding gene, *orfU*, is cotranscribed with *tsr*, which lies downstream of *spo0F* (52). It remains to be examined whether phosphorylation is involved in the regulation of the cold shock response. The identification of CheY, which regulates the rotation of the flagella (39), as a Tip suggests that chemotaxis is important after a drop in temperature, as it also is after an increase. Furthermore, it may indicate that thermotaxis and chemotaxis are modulated by overlapping systems. In this study, Hpr was shown to be the only protein whose synthesis increased after heat, cold, and general stress. Since Hpr is involved in the PTS (44), the uptake of sugars by the PTS appears to play a vital role under all stress conditions.

After a cold shock, a minimum of 75 distinct spots are detectable (Fig. 2c), implying that proteins that are probably essential for continued metabolism are synthesized. In addition, only four proteins whose synthesis was induced de novo could be detected, showing that most CIPs serve functions that are also important under optimal growth conditions. Because of the increase in the relative synthesis of CysK, IlvC (catalyzing the second step in valine and isoleucine synthesis), TIM, and Gap (Table 1), it appears that glycolysis and the synthesis of amino acids are of considerable importance after a cold shock. These proteins may represent vital enzymes that are rather cold sensitive and whose increased production is necessary to keep up a minimal level of anabolic and catabolic metabolism. Alternatively, TIM and Gap may be important to more efficiently degrade metabolites other than glucose, such as glycerol from the degradation of fatty acids.

It is evident that the cold shock response of *B. subtilis* is analogous to but different in many aspects from the response in *E. coli*. The number of proteins synthesized after a drop in temperature is about three times as high as in *E. coli*, and the response is approximately twice as fast, 2 h compared with 4 h (25). As Lottering and Streips (30) have already noted, this is physiologically sensible, as *B. subtilis*, being a soil bacterium, is constantly being subjected to changes in temperature, unlike the usual situation for *E. coli*. It is also apparent that the CIPs identified in *B. subtilis* have a broad spectrum of functions. Several CIPs are even induced in response to osmotic stress and heat shock. This observation reveals that the cold shock response is a rather complex process, connected with heat shock and general stress response. It remains to be elucidated which detailed function the Csp protein family serves.

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