JOURNAL OF BACTERIOLOGY, Oct. 1999, p. 6449–6455 Vol. 181, No. 20
0021-9193/99/$04.00+0

Pathogenic *Yersinia* Species Carry a Novel, Cold-Inducible Major Cold Shock Protein Tandem Gene Duplication Producing both Bicistronic and Monocistronic mRNA

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Received 10 March 1999/Accepted 15 July 1999

Inverse PCR was used to amplify major cold shock protein (MCSP) gene families from a diverse range of bacteria, including the psychrotolerant *Yersinia enterocolitica*, which was found to have two almost identical MCSP coding regions (*cspA1* and *cspA2*) located approximately 300 bp apart. This tandem gene duplication was also found in *Y. pestis*, *Y. pseudotuberculosis*, and *Y. ruckeri* but not in other bacteria. Analysis of the transcriptional regulation of this MCSP gene in *Y. enterocolitica*, performed by using both reverse transcriptase-PCR and Northern blot assays, showed there to be two cold-inducible mRNA templates arising from this locus: a monocistronic template of approximately 450 bp (*cspA1*) and a bicistronic template of approximately 900 bp (*cspA1/A2*). The former may be due to a secondary structure between *cspA1* and *cspA2* causing either 3′ degradation protection of *cspA1* or, more probably, partial termination after *cspA1*. Primer extension experiments identified a putative transcriptional start site (+1) which is flanked by a cold-box motif and promoter elements (−10 and −35) similar to those found in *Escherichia coli* cold-inducible MCSP genes. At 30°C, the level of both mRNA molecules was negligible; however, upon a temperature downshift to 10°C, transcription of the bicistronic mRNA was both substantial (300-fold increase) and immediate, with transcription of the monocistronic mRNA being approximately 10-fold less (30-fold increase) and significantly slower. The ratio of bicistronic to monocistronic mRNA changed with time after cold shock and was higher when cells were shocked to a lower temperature. High-resolution, two-dimensional protein gel electrophoresis showed that synthesis of the corresponding proteins, both CspA1 and CspA2, was apparent after only 10 min of cold shock from 30°C to 10°C. The data demonstrate an extraordinary capacity of the psychrotolerant *Y. enterocolitica* to produce major cold shock proteins upon cold shock.

The genus *Yersinia* contains 11 species of gram-negative facultative rods, including three pathogens of humans, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, and *Yersinia pestis*, and the salmonid fish pathogen, *Yersinia ruckeri* (20). *Y. enterocolitica* causes gastroenteritis in humans and animals through its ingestion in contaminated food or water (34). It is the ability of *Y. enterocolitica* to grow at temperatures as low as −5°C (2, 35) that has mainly resulted in cases of bacterial septicemia from the transfusion of stored refrigerated blood products (31). On the other hand, *Y. enterocolitica* can grow at temperatures as high as 42°C (34).

The molecular basis of cold tolerance and the roles played by cold-inducible proteins are still poorly understood. The major focus of recent work has been on mesophilic bacteria such as *Escherichia coli* and *Bacillus subtilis* (reviewed in references 15 and 42). Initial work showed that when an exponential-phase culture of *E. coli* is shifted from 37 to 10°C, a novel set of at least 13 proteins is induced (24). Identification of a number of these cold shock proteins has revealed several polypeptides that are involved in transcription and translation. This has led to the suggestion that the cold shock response is an adaptive mechanism facilitating protein synthesis at low temperature (25) and, as reported recently, at early exponential-phase growth (4). In contrast to the relatively minor level of induction (2- to 10-fold) observed for most cold shock proteins during a temperature downshift in *E. coli*, the induction of a novel protein (initially designated as F10.6) was found to be considerably higher (24). This 70-amino-acid polypeptide was shown to be induced 200-fold following a shift from 37 to 10°C (24) and was subsequently termed the major cold shock protein (MCSP) or CspA (10). MCSPs are extremely widespread in eubacteria (reference 8 and this study) and belong to the most conserved group of nucleic acid-binding proteins yet defined in nature: the cold shock domain (CSD) proteins (40). They are characterized by the ability to preferentially bind to single-stranded nucleic acid sequences containing an ATTGG /CCAAT motif (16, 28, 36, 37, 40). In both prokaryotic and eukaryotic organisms, this ability has been shown to be due to two RNA-binding motifs, RNP-1 (KGFGF) and a partial RNP-2 (VFVH) (14, 40). Although a number of roles have been proposed for MCSPs, their most likely function is as molecular chaperones involved with the unfolding of mRNA secondary structures formed at low temperature (17, 22, 23).

E. coli, *B. subtilis*, *Bacillus cereus*, and *Pseudomonas fragi* have all been shown to have families of MCSP homologues, ranging from three in *B. subtilis* to nine in *E. coli* (16, 28, 29, 42). These proteins are all around 70 amino acids. Comparison of the four cold-inducible MCSPs of *E. coli*, CspA, CspB, CspG, and CspI, shows these proteins to have considerable homology (30, 39, 42). Moreover, alignment of the 5′ untrans-

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lated mRNA pertaining to these four MCSPs shows this homology to extend beyond each coding region, with the leading 160 bp of *cspB* and *cspG* having 70% or greater identity (30, 39). These four MCSPs of *E. coli* are differentially regulated at low temperature (7, 39).

To our knowledge, no information on the cold shock response of the psychrotolerant bacterium *Y. enterocolitica* is available. In this study, we show that *Y. enterocolitica* has an MCSP gene duplication that is induced by cold shock to give both monocistronic and bicistronic mRNAs. Furthermore, we demonstrate that both MCSPs are translated and that transcription and translation upon cold shock are extremely rapid.

MATERIALS AND METHODS

Inverse PCR amplification of multiple MCSP gene sequences from gram-positive and gram-negative bacteria. Genomic DNA was purified (1) from a wide range of both gram-positive and gram-negative bacteria, including the psychrotolerant *Bacillus weihenstephanensis* (WSBC10201) and the mesophilic *B. cereus* (WSBC10028), *Enterococcus faecalis* (NCTC 775), *E. coli* (W3110), *Klebsiella pneumoniae* (NCTC 9633), *Listeria monocytogenes* (ATCC 23074), *Micrococcus luteus* (NCTC 2665), *Proteus vulgaris* (NCTC 4175), *Pseudomonas aeruginosa* (PAO1), *Salmonella typhimurium* (LT2), *Staphylococcus aureus* (RN4220), and *Y. enterocolitica* (NCTC 10460). Approximately 1 μg of each DNA was dissolved in 50 μl of water, which was then subdivided into six 8-μl aliquots. Five aliquots were cut individually with the restriction enzymes *Alu*I, *Hha*I, *Hpa*II, *Mbo*I, and *Rsa*I (Roche Diagnostics GmbH, Mannheim, Germany), while the sixth aliquot of uncut DNA acted as a control. After heat inactivation of the restriction enzyme, the cut DNAs were self-ligated (1 U of T4 DNA ligase; Promega) for 4 h at 16°C.

Using a compilation of MCSP DNA sequences gained from more than 30 species of bacteria (8), three degenerate oligonucleotide primers were designed (two pairs) with which to perform inverse PCR. The sequences of these primers are as follows: CSPIF1, 5′ [AG][AG]I GA[CT] GTI TTC GT[AT] CA[CT] TT[CT] I[GC]I GC 3′; CSPIF2, 5′ GGI T[AT]C AAA [AT]CI [CT]T[AG] CAI GAA GG[CT] CA 3′; and CSPIR1, 5′ [GC][GC][AT] [GT]I[AG] AT[AG] AAI CC[AG] AAI CCT TTI TC 3′ (bracketed nucleotides show the degeneracies used, and I represents inosine). PCR was performed with a Techne Progene automated thermocycler with 0.2-ml thin-walled PCR tubes. Reactions were carried out in 50-μl volumes containing 5 μl of 10× PCR buffer (supplied with *Taq* DNA polymerase; Eurogentec), 2 mM $MgCl_2$, 100 pmol of each oligonucleotide primer, 0.2 mM each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 1 U of *Taq* DNA polymerase (Eurogentec), and 1 μl of a ligation mixture or uncut DNA. PCR conditions were as follows: 2 min at 95°C, followed by 35 cycles at 95°C for 15 s, 50°C for 2 min, and 72°C for 3 min, with a final extension at 72°C for 5 min. Amplified products were analyzed on a 1.5% agarose gel (NuSieve; FMC BioProducts). Reactions containing PCR products were then run on a low-melting-point agarose gel (SeaPlaque GTG; FMC BioProducts), and DNA fragments were liquid nitrogen band extracted by using a freeze-thaw procedure (32). Direct sequencing of PCR products was performed with an ABI 373A sequencer (Perkin-Elmer Applied Biosystems) with CSPIR1 and either CSPIF1 or CSPIF2 (depending on which oligonucleotide was used to perform PCR) as sequencing primers. DNA sequences gained from the latter procedure were then used to design specific PCR primers to amplify complete MCSP genes, including their missing central regions.

Preparation of RNA from *Y. enterocolitica*. A 350-ml culture of *Y. enterocolitica* was grown at 30°C to an optical density at 600 nm of 0.5. This was then cold shocked to 10°C in an ice bath. Ten-milliliter samples were taken before (control) and shortly after cold shock (2 min) and then at 10, 20, 30, 45, 60, 90, and 120 min after shock. The cells were centrifuged, and the pellet was frozen in liquid N_2. Total RNA was isolated from the pellets with guanidine-phenol buffer as described before (18).

Northern blot analysis of cold-induced *Y. enterocolitica* MCSP mRNA. Northern blotting, with 20 μg of total RNA, was carried out as described previously (27) with the following changes. The RNA was blotted with a vacuum blotter at 70 mbar for 1 h. The membrane was then air dried at 37°C for 20 min before it was cross-linked with 0.3 J/cm^2. Immediately after cross-linking, the membrane was prehybridized. The blot was washed at 30°C, if not indicated otherwise, as follows: two times for 5 min each in 2× SSPE (1× SSPE is 0.15 M NaCl, 100 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 20 min at 55°C in 0.2× SSPE, 20 min in blocking buffer, followed by 1 h of conjugation with anti-digoxigenin (DIG) AP (Roche Diagnostics), two times for 5 min each in blocking buffer, two times for 5 min each in 1× phosphate-buffered saline (plus 0.5% sodium dodecyl sulfate), two times for 5 min each in assay buffer, and finally transferred to the substrate solution (250 μM CDP-Star; Tropix) for 5 min at room temperature. The blot was exposed to Curix HC1.000G film (Agfa Gevart, Köln, Germany) for 3 to 15 min. Hybridization solution was made with DIG-labelled oligonucleotide (YeA1-DIG, 5′ GCC ACA ATA CTG TTT TGC CAC AAT ATG T 3′) or DIG-labelled PCR products amplified with the primers Melmack (5′ GCT GCT GGC ACG TAG TTA 3′) and Alex (5′ ACT GGG ACT GAG ACC GG 3′) in accordance with the Boehringer Mannheim manual.

Primer extension. Primer extension was conducted as described previously (26) with the following minor changes: 5 μg of total RNA (shock at 10°C for 10 min) was incubated with 4 μl of the primer YeA1R2(+1) (2 pmol; 5′ GCC ACA ATA CTG TTT TGC CAC 3′) and 2.6 μl of H_2O for 1 min at 94°C and then 10 min at 50°C. All subsequent steps were conducted at 50°C. A sequencing reaction for a ladder was carried out with different plasmids and primers in accordance with the manual of the Sequenase V2.0 DNA sequencing kit (Amersham Pharmacia Biotech, Freiburg, Germany).

RT-PCR of cold-induced *Y. enterocolitica* bicistronic *cspA1/A2* mRNA. Reverse transcriptase (RT)-PCR was carried out with the primer YeRTR (5′ GGC TAT CAC CTT CAT CGC 3′) for MCSP mRNA as described for primer extension but without using labelled dATP. PCR was conducted by using the primers YeRTF (5′ CAT CGG TTT GGA CAC CAG AC 3′) and YeRTR, with the following parameters: 95°C for 10 s, 50°C for 15 s, and 72°C for 20 s for 20 cycles.

Southern blot analysis of *Y. enterocolitica* DNA. Ten micrograms of DNA was cut with the restriction enzymes *Ssp*I, *Eco*RV, *Eco*RI. Fragments were run on a 1.2% Tris-acetate-EDTA agarose gel and blotted on a nylon membrane (Hybond-N^+; Amersham Pharmacia Biotech). Hybridization and washing were carried out as described for Northern blotting above.

Prediction of secondary mRNA structure. The secondary structure of the bicistronic *cspA1/A2* mRNA from *Y. enterocolitica* was obtained by using the folding software program MFOLD (38, 43, 43a).

Two-dimensional polyacrylamide gel electrophoresis. For the preparation of protein samples, shocked cells were centrifuged and the pellets were resuspended in solubilization buffer as described previously (12). Cell lysis was performed by a single passage through a French press (SLM Aminco Inc., Rochester, N.Y.) cell at 20,000 lb/in^2. The cell extract was then centrifuged for 45 min (15,400 × *g* at 4°C). Two-dimensional gel electrophoresis of the supernatant was performed as described before (11-13a) by using immobilized pH gradient (IPG) recipes (pH 4 to 7 and pH 5 to 6) described previously (33). For analytical purposes, samples of approximately 70 μg, for microseparation ca. 700 μg, of protein per gel were used. Proteins were resolved by isoelectric focusing with Pharmacia's DryStrip kit. The protein solution was applied at the anodic side of the IPG gel strips. The sample was run into the gel at low voltages (gradient pH 4 to 7, 150, 300, and 600 V, each for 3 h; gradient pH 5 to 6, 150, 300, and 600 V, each for 5 h). Isoelectric focusing was done for 12 h at 3,500 V (gradient pH 4 to 7) and for 12 h at 1,500 V and 12 h at 3,500 V (gradient pH 5 to 6). The IPG gel strips either were used immediately for the second-dimension run or were stored at −80°C. The IPG gel strips were equilibrated two times as described previously (12). In the second dimension, self-casted sodium dodecyl sulfate-pore gradient gels on plastic backing (12) with a gel size of 190 by 250 by 0.5 mm^3 were run for 0.75 h at 300 V and 4.5 h at 600 V on a Multiphor electrophoresis unit (Pharmacia). The gels were stained with silver (3), or the proteins were blotted onto polyvinylidene difluoride membranes (Millipore) with Tris-borate transfer buffer (12) and stained with Coomassie brilliant blue R-250 (Serva). Selected protein spots either were directly applied to an automated protein sequencer to obtain N-terminal amino acid sequences or were digested with trypsin and the mass of the peptide fragments was analyzed with an automated mass spectrometer (matrix-assisted laser desorption ionization [MALDI]; Toplab, Martinsried, Germany).

Nucleotide sequence accession numbers. Complete sequences of MCSPs were deposited in the GenBank database and include *E. coli cspB-cspF* (accession no. AF003590) and *cspH* (accession no. AF003591), *M. luteus cspA* (accession no. AF019905), *P. aeruginosa cspA* (accession no. U82822), *S. aureus cspB* and *cspC* (accession no. AF003592 and AF003593), *S. typhimurium cspH* (accession no. AF006035), and *Y. enterocolitica cspA1/A2* (accession no. U82821).

RESULTS

A *cspA* gene duplication in *Y. enterocolitica*. Inverse PCR of bacterial genomic DNA, performed with the degenerate MCSP oligonucleotide pairs CSPIR1-CSPIF1 and CSPIR1-CSPIF2, resulted in the amplification of at least one MCSP sequence from all bacteria tested (see Materials and Methods for the list of strains). In the majority of cases, control PCRs containing uncut genomic DNA did not give amplified products. Exceptions to this were *E. coli* and *Y. enterocolitica*, which were found to give products of between 400 and 500 bp. Sequencing of the PCR products from *E. coli* and *Y. enterocolitica* confirmed these DNAs to each contain two MCSP sequences, *E. coli* having divergent MCSP coding regions (corresponding to *cspB-cspF* and *cspG-cspH*, respectively) and *Y. enterocolitica* having a tandem repeat (Fig. 1).

Comparison of the two MCSP coding regions from *Y. enterocolitica*, designated *cspA1* and *cspA2*, shows these se-

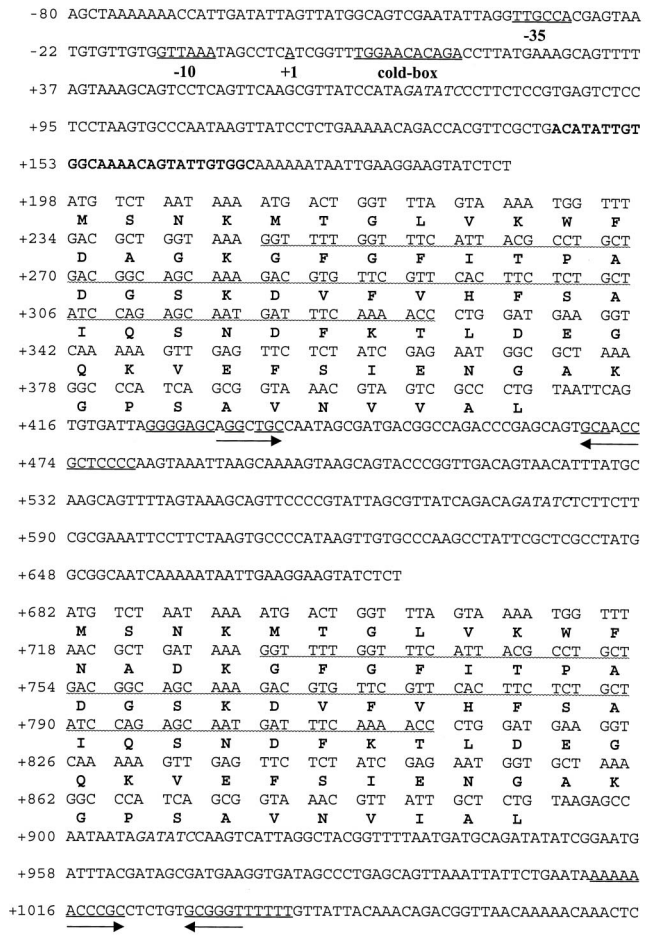

FIG. 1. Nucleotide sequence of *cspA1/cspA2* from *Y. enterocolitica*. *cspA1* (upper protein sequence) and *cspA2* (lower protein sequence) differ only in the 13th and 15th amino acids and in the third amino acid of the C terminus. Putative transcriptional start site (+1), putative promoter regions (−10, −35), and the cold-box are indicated. Sequence in boldface type shows the position of the A1 probe (YeA1-Dig). The underlined sequence indicated by upper facing arrows shows the putative transcriptional termination structure of the monocistronic mRNA. The underlined sequence indicated by lower facing arrows shows the transcriptional termination structure of the polycistronic mRNA. The sequence underlined by a wavy line in *cspA1* can fold in an antiparallel direction to the same region of *cspA2*, forming an extensive secondary structure. *Eco*RV restriction sites, which cut at positions +73, +579, and +909, are indicated by italics.

quences to be almost identical (96.7%), with their deduced protein sequences only differing in amino acids 13 and 15 (CspA1, DAG; CspA2, NAD) and a single amino acid at their C termini (CspA1, VVAL; CspA2, VIAL). Furthermore, there is a large degree of homology between the 5′ untranslated regions of these two genes, with the first 175 bp upstream of *cspA1* and the corresponding 154 bp upstream of *cspA2* showing approximately 70% identity. Primer extension experiments identified the 5′ end of the *cspA1/A2* mRNA as lying 197 bp upstream of the first coding region (*cspA1*, ATG) (data not shown). This is probably the transcriptional start site +1 because it is in the same position as that in *E. coli* and is flanked by a cold-box motif and promoter elements (−10 and −35) similar to those found in *E. coli* MCSP genes (7, 10, 21).

Interestingly, the coding regions of the *Y. enterocolitica* *cspA1/A2* mRNA can fold and hybridize to each other, resulting in an extensive secondary structure. This secondary structure begins with the 17th codon in *cspA1* (GGU), which can bind in an antisense direction to the 44th codon of *cspA2* (ACC) and vice versa (Fig. 1).

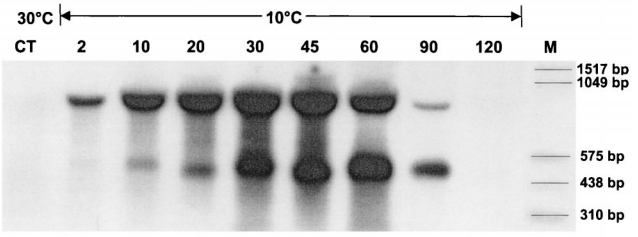

FIG. 2. Northern blots of *Y. enterocolitica* cold shocked to 10°C. The signals were gained by a probe binding to the 5′ untranslated region of *cspA1* (YeA1-Dig). Time after cold shock is given in minutes. The upper signal is approximately 900 bp and represents the bicistronic mRNA. The lower signal is approximately 450 bp and represents the monocistronic mRNA. CT, control.

Transcription of *Y. enterocolitica* MCSP gene duplication. Northern blot analysis of *Y. enterocolitica* mRNA was conducted at both 30°C and after cold shock to 10°C, hybridizing with the oligonucleotide A1 probe specific to the 5′ untranslated region of *cspA1*. This analysis showed that two cold-inducible mRNA templates are produced from this MCSP sequence (Fig. 2): a monocistronic template of approximately 450 bp (*cspA1*) and a bicistronic template of approximately 900 bp (*cspA1/A2*). At 30°C, the level of the bicistronic mRNA was low, with the level of monocistronic mRNA almost negligible. However, upon a temperature downshift to 10°C, transcription was both substantial and immediate.

To confirm that the bicistronic transcript contained the complete coding regions of both *cspA1* and *cspA2*, RT-PCR was performed with mRNA taken at 30°C and at 10°C after 30 min by using an oligonucleotide back primer downstream of the *cspA2* coding region. After 20 PCR cycles, an intense product of approximately 900 bp was obtained from the cold-shocked sample, with virtually no product obtained from the control sample (data not shown). The former product was sequenced and contained both *cspA1* and *cspA2* gene sequences (Fig. 1).

To ensure that the A1 probe used in the Northern blots specifically hybridized to *cspA1/A2*, a Southern blot analysis of *Y. enterocolitica* genomic DNA was conducted. This procedure confirmed that the A1 probe hybridized to only one region of this bacterium's DNA (data not shown). Furthermore, by including an *Eco*RV digest of this DNA (a restriction enzyme known to cut before *cspA1*, between *cspA1/A2* and after *cspA2* [see Fig. 1], to give 456-bp and 330-bp DNA fragments, respectively), it can be confirmed that both signals visible on the Northern blots are mRNAs flanked by *cspA1* and not a second MCSP gene sequence.

Comparison of mono- and bicistronic mRNA synthesis. To compare the levels of monocistronic and bicistronic mRNA transcribed from this MCSP gene sequence before and after cold shock, quantitative Northern blot analysis was performed. mRNA was isolated at 30°C and compared to a dilution range of mRNA taken at 10°C after 30 min. This showed that at this time point the levels of bicistronic and monocistronic mRNA were induced 300- and 30-fold, respectively (Fig. 3). However, at 30°C the level of monocistronic mRNA was approximately fourfold higher than that of the bicistronic mRNA. When cold shocked to 10°C, a high level of bicistronic mRNA was observed at 30 min, whereas a high level of monocistronic mRNA was not recorded until 60 min, after which time both transcripts diminished. Neither transcript was visible 120 min after the initial temperature downshift (Fig. 2).

Comparison of the two mRNA transcripts shows that the

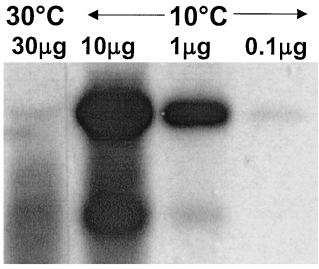

FIG. 3. Northern blot of a dilution series of cold-shocked *Y. enterocolitica* mRNA (10°C, 30 min) detected by using the A1-probe. The relative amounts of bi- and monocistronic mRNA from *cspA1/A2* were determined with the software Image Master 1D Elite (Amersham Pharmacia Biotech). The bicistronic mRNA shows a 300-fold increase; the monocistronic one shows a 30-fold increase.

ratio of bicistronic and monocistronic mRNA increases with decreasing temperature (Fig. 4). At 0 and 5°C, bicistronic mRNA is present at a high level, with relatively little monocistronic mRNA appearing. In contrast, at 15°C, similar levels of bi- and monocistronic mRNA are present, while at 20°C, the level of monocistronic mRNA seems to be higher.

Cold-induced synthesis of *Y. enterocolitica* CspA1 and CspA2. Protein extracts gained from cultures of *Y. enterocolitica* that were grown at 30°C and then cold shocked at 10°C for increasing periods of time were analyzed by two-dimensional gel electrophoresis. Initial studies using conventional gel gradients between pH 4 and 7 identified MCSPs around 7 kDa (Fig. 5A). However, it was not possible to determine how many separate MCSPs this spot contained. To increase resolution, protein extracts were focused in a narrow immobilized pH gradient between pH 5 and 6, over 18 cm, which to our knowledge has not been used before. As can be seen in Fig. 5B, the protein spot of interest actually consists of at least three proteins (marked 1, 2, and 3 on Fig. 5B). The N-terminal sequences of each of these spots was determined. Since it was unclear which of the spots 2 or 3 contains CspA2 (or another unknown MCSP), a peptide mass fingerprint (MALDI) was executed with each of these spots. The data are summarized in Table 1. The mixture of Csps in spot 2 is most probably due to carryover.

MCSP tandem gene duplications in other species of *Yersinia*. To determine whether the MCSP gene duplication found in *Y. enterocolitica* was present in other species of *Yersinia*, three additional members of this genera were investigated. *Y. pseudotuberculosis* (biotype III; environmental isolate from H. Wolf-Watz, University of Umea, Umea, Sweden) and *Y. ruckeri* (NCIMB 1316) were both probed by PCR, using the oligonucleotide primers CSPIR1 and CSPIF2, and in both cases gave a 450-bp product (similar in size to that gained from *Y. enterocolitica*). Sequencing of these DNA fragments (data not shown) confirmed the flanking regions of each product to be MCSP sequences homologous to *Y. enterocolitica cspA1/A2*.

Analysis of the *Y. pestis* database (Pathogen Sequencing Group, Sanger Centre, Cambridge, United Kingdom) (37a) confirmed an MCSP gene duplication to also be present in this *Yersinia* species. Due to a number of unknown bases being present in the latter sequence, this entire region of DNA was PCR amplified and resequenced (*Y. pestis* DNA was a gift from R. Titball, Defence Evaluation Research Agency, Porton Down, United Kingdom). Like those of *Y. enterocolitica*, the sequences of CspA1 and CspA2 of *Y. pestis* show only minor changes. Furthermore, both of these proteins are 94% identical to their comparative homologues in *Y. enterocolitica*. Alignment of the DNA sequences of both of these MCSP gene duplications, *Y. enterocolitica cspA1/A2* with *Y. pestis cspA1/A2*, shows that there is considerable homology (85% identity) throughout these entire sequences (approximately 950 bp), including the 5′ untranslated region (data not shown).

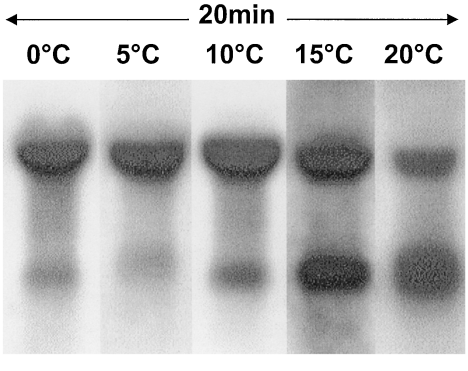

FIG. 4. Northern blots of cold-shocked *Y. enterocolitica* mRNA obtained at different temperatures with the A1 probe. Each cold shock lasted for 20 min. Changes in the proportion of bicistronic and monocistronic mRNA are most obvious at 15 and 20°C.

DISCUSSION

Only *Yersinia* species carry a tandem *cspA* gene duplication. Recently, it has been shown that *Lactococcus lactis* has two sets of MCSP genes, *cspA-cspB* and *cspC-cspD*, both being separated by approximately 300 bp. Comparison of these two MCSP loci showed them to be homologous, with 79% identity over 800 bp (41). Similarly, in this study we amplified two MCSP loci from *E. coli* that are located at 35 and 22 min on the chromosome. Each of these loci again contains two MCSP genes, *cspB-cspF* and *cspG-cspH*, that are separated by approximately 300 bp and show high levels of homology (more than 70% identity over 750 bp). We found no evidence to suggest that *S. typhimurium* has the *cspBF-cspGH* duplication found in *E. coli*, although subsequent work has demonstrated these sequences to be present in *Shigella* species (data not shown). The high levels of homology shown between these genes (loci) give strong evidence that they have arisen by sequence duplication.

In contrast to *Y. enterocolitica*, all of the above MCSP genes of *L. lactis* and *E. coli* are transcribed divergently and monocistronically. In each of the latter bacteria, it is the single MCSP locus that has been duplicated and not tandem genes, as in the case of *Y. enterocolitica*, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. ruckeri*. Furthermore, although families of MCSPs have been identified in a wide range of bacteria (16, 17, 28, 29, 42), *Y. enterocolitica* is the first bacterium in which bicistronic MCSP mRNA has been found. This finding is especially intriguing in this particular bacterium due to its exceptional ability to grow at low temperature.

Differential appearance of mono- and bicistronic *cspA1/A2* mRNA. It has been shown that *cspA* mRNA is constitutively transcribed in *E. coli* but that, at elevated temperatures, this mRNA is rapidly degraded and may not be translated. Following cold shock, *cspA* mRNA is specifically and temporally stabilized (9). These findings could help to explain the immediate and substantial quantities of bicistronic mRNA produced by *Y. enterocolitica* upon cold shock from 30 to 10°C (Fig. 2). A sequence that is characteristic of a conventional transcription termination site can be found downstream of the *cspA2* coding region (Fig. 1). This site, which is approximately 900 bp down-

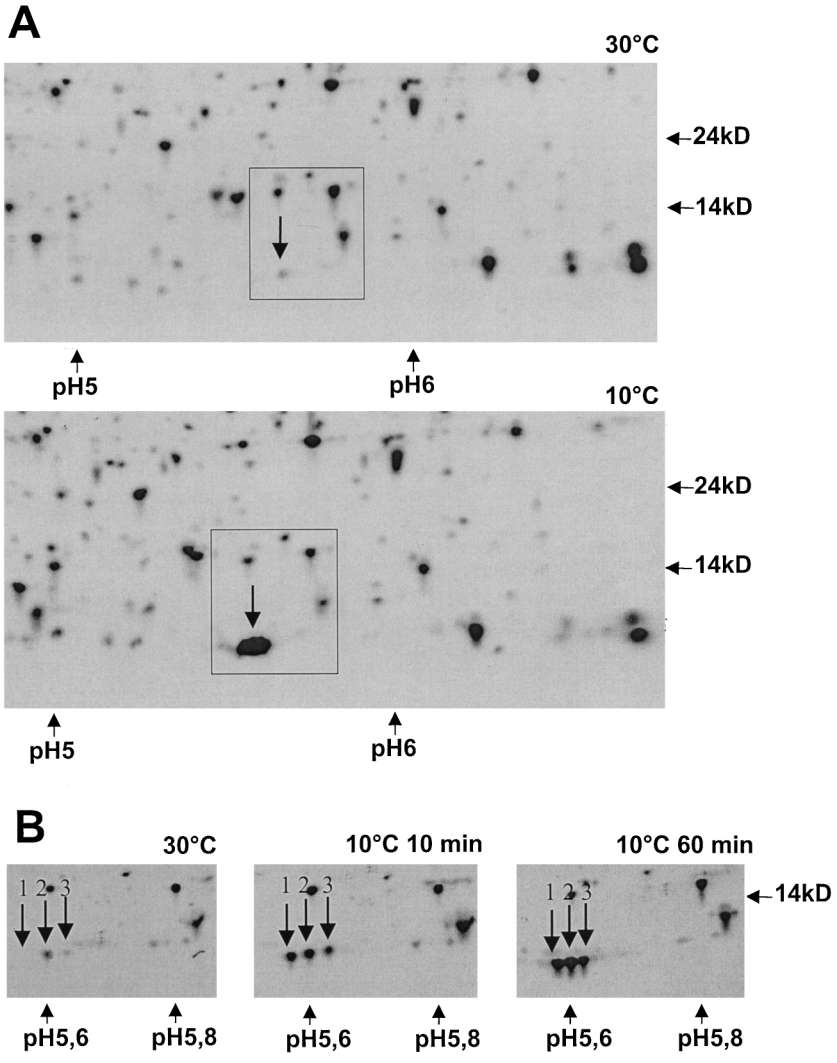

FIG. 5. Two-dimensional gels from *Y. enterocolitica*. Molecular weights and pH values are indicated. MCSPs are indicated by downward arrows. (A) Windows of the broad-range gels showing proteins from pH 4 to 9 at 30°C and after a shock of 60 min to 10°C; (B) windows of the narrow-range gels from pH 5 to 6 of the control (30°C) and shock experiments to 10°C for 10 min and 60 min.

stream of the +1, is most probably where the bicistronic messenger terminates.

With shock at temperatures not quite as low, monocistronic mRNA is produced predominantly (Fig. 4); this may be sufficient to overcome the impediment caused by this mild cold shock. The most significant difference between the appearance of these two transcripts at 10°C is that bicistronic mRNA is immediately available for translation at 10°C, whereas mono-

TABLE 1. Summary of results from N-terminal sequencing and MALDI

Spot no.	N-terminal sequence	Peptide masses[a]	Corresponding peptide fragment sequences[a]	MCSP contained in spot
1	SNKMTGLVKWFDAGKG	ND[b]		B, A1
2	SNKMTGLVKWFNADKG	723.3	11-KWFDAGKG-16 (CspB or CspA1)	A1, A2, B
	MSNKMTGLVKWFNADKG	780.3	11-KWFNADKG-16 (CspA2)	
		926.5	61-KGPSAVNVVAL-70 (CspA1)	
		940.5	61-KGPSAVNVIAL-70 (CspA2)	
3	SNKMTGLVKWFNADKG	780.3	11-KWFNADKG-16 (CspA2)	A2
		940.5	61-KGPSAVNVIAL-70 (CspA2)	

[a] Peptide masses and fragments from MALDI are examples showing fragments at the relevant amino acid signatures which are specific for CspA1, CspA2, and CspB (6), respectively.
[b] ND, not determined.

cistronic mRNA does not significantly emerge until 30 min later (Fig. 2).

The ratio of monocistronic to bicistronic mRNA increases with the duration of the cold shock (Fig. 2) and decreasing shock temperature (Fig. 4). This change of the ratio of monocistronic to bicistronic mRNA could be reflective of transcription termination downstream of *cspA1*. Although a classical termination structure does not appear to be present between *cspA1* and *cspA2*, sequence homology which indicates the formation of a large loop structure (Fig. 1) exists; this structure may act as a temperature-dependent second termination site. It cannot be excluded, on the other hand, that this loop structure acts as 3′ degradation protection. Investigations of the *rpsO* operon in *E. coli* showed that a secondary structure (t1) is located between *rspO* and *pnp*, which can act either as a terminator or as 3′ degradation protection. A second terminator (t2) is found downstream of *pnp*. Therefore, *rpsO* is sometimes transcribed monocistronically and sometimes bicistronically together with *pnp* (5, 19). Further investigations of the *cspA1/A2* tandem in *Yersinia* will show whether a similar mechanism occurs in this bacterium.

Both CspA1 and CspA2 are synthesized. In case the bicistronic mRNA indeed contributes to an elevated cold shock response, it should be fully translated; i.e., CspA2 should be present after cold shock. By electrophoresing proteins on two-dimensional gels with an extremely narrow pH range between 5 and 6 (Fig. 5B), it is possible to separate cold-inducible MCSPs. At least three cold-inducible MCSPs could be found, CspA1, CspA2, and CspB (see Results). Surprisingly, CspA1 and CspA2 separate at the narrow-range two-dimensional gel, although their sequences differ by only three amino acids (see Results). We conclude that CspA2 is synthesized and contributes to the cold shock response of *Y. enterocolitica*.

Does *Y. enterocolitica* have a higher translational capacity for MCSP than *E. coli*? Studies in *E. coli* have shown that *cspA* mRNA is maximally induced 30-fold following a temperature downshift from 37 to 15°C and that this peak induction occurs after approximately 60 min at this lower temperature (9). These data correspond well with the induction of monocistronic *cspA1* mRNA in *Y. enterocolitica* (Fig. 2). In contrast, a high level of bicistronic *cspA1/A2* mRNA in *Y. enterocolitica* occurs after only 30 min and is induced at least 300-fold, 10-fold more than the monocistronic transcript (Fig. 2 and Fig. 3) of *Y. enterocolitica* and *E. coli*. If it is true that the basal levels of the monocistronic MCSP mRNAs are similar in both bacteria at non-cold shock temperatures, *Y. enterocolitica* would have a much greater translational capacity than *E. coli* upon cold shock with respect to these single MCSP sequences (loci).

Additionally, the fact that this transcript contains two copies of the coding region, which are both translated, means that *Y. enterocolitica* is able to synthesize this protein more effectively than bacteria that have monocistronic MCSP mRNAs only, such as *E. coli*, assuming that translation levels of these two mRNAs are comparable. Indeed, synthesis of CspA1 and CspA2 is clearly seen only 10 min after cold shock. However, in order to finally conclude that the rapid accumulation of these MCSPs is essential for *Y. enterocolitica*'s superior ability to adapt to low temperature, a knockout mutant of *cspA2* needs to be constructed.

ACKNOWLEDGMENTS

K. Neuhaus and K. P. Francis contributed equally to this work.

The majority of the gene sequences reported in this manuscript were discovered by Kevin P. Francis in the laboratory of Gordon S. A. B. Stewart at the University of Nottingham (ROPA grant 42/CEL 04626). We thank Günther Boguth and Christian Obermaier for support and technical assistance in preparing the two-dimensional gels.

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