Requirement for RNA Helicase CsdA for Growth of *Yersinia pseudotuberculosis* IP32953 at Low Temperatures

Eveliina Palonen, Miia Lindström, Panu Somervuo, Per Johansson, Johanna Björkroth, and Hannu Korkeala

Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland

The expression of *csdA*, encoding an RNA helicase, was induced at 3°C in *Yersinia pseudotuberculosis*. The role of CsdA in *Y. pseudotuberculosis* under cold conditions was confirmed by impaired growth of insertional *csdA* mutants at 3°C. The results suggest that CsdA is crucial for *Y. pseudotuberculosis* survival in the chilled food chain.

Yersinia pseudotuberculosis is an enteropathogen able to grow at low temperatures (25). Recently, infections have been linked to contaminated fresh produce stored at low temperatures (14, 23, 30). Chilled storage gives *Y. pseudotuberculosis* a competitive advantage over many bacteria (8), but only a few studies on cold tolerance mechanisms of *Y. pseudotuberculosis* have been published (24, 25).

The *deaD* encoding a member of the highly conserved DEAD-box (asp-glu-ala-asp) protein family (18) was previously demonstrated to complement a mutation in *rpsB* encoding ribosomal protein S2 in *Escherichia coli* (31). Moreover, DeaD stabilizes mRNAs without ribosomes in this bacterium (12). DeaD was later renamed CsdA (cold shock DEAD-box protein), because it was found to be induced in *E. coli* under cold conditions (15). At low temperatures, CsdA facilitates translation initiation (15, 19) and contributes to the degradation of mRNAs in *E. coli* (17, 28, 33). It is also required for the biogenesis of 50S ribosomal subunits at low temperatures (7, 27) or at 37°C (27) during the early exponential growth phase. Furthermore, at low temperatures CsdA is needed in *E. coli* for the synthesis of stationary-phase sigma factor RpoS (29). The role of CsdA in enteropathogenic *Yersinia* is unknown.

Expression of *csdA* at 3°C relative to its expression at 28°C in *Y. pseudotuberculosis* IP32953 (gratefully received from Elisabeth Carniel, Institut Pasteur, Paris, France) was investigated using quantitative real-time reverse transcription-PCR as described previously (24). The relative expression level of *csdA* in the early logarithmic growth phase at 3°C was 9.4-fold higher ($P < 0.001$; Student's *t* test) than the expression level at 28°C, suggesting that elevated *csdA* transcript levels are needed for *Y. pseudotuberculosis* growth at low temperature. To further investigate the role of CsdA at low temperatures, we constructed three insertional mutations as reported earlier (24). A search for domains of *csdA* (YPTB0486) was performed with the InterProScan tool (6, 11). The Pfam domains were PF00270 (DEAD/DEAH-box helicase; residues 32 to 196), PF00271 (helicase-conserved C-terminal domain; residues 266 to 342), PF03880 (DbpA RNA binding domain; residues 503 to 573), and PF12343 (cold shock protein DEAD-box A; residues 591 to 664). The TargeTron (Sigma-Aldrich Co., St. Louis, MO)-based mutations were targeted to the DEAD/DEAH-box helicase domain (PF00270) between bases 483 and 484 (*csdA*483–484::Ltr Kan^r mutant; referred to as *csdA*483 here) or to the DbpA RNA binding domain (PF03880) between bases 1548 and 1549 (*csdA*1548–1549::Ltr Kan^r mutant; referred to as *csdA*1548 here) or 1578 and 1579 (*csdA*1578–1579::Ltr Kan^r mutant; referred to as *csdA*1578 here). The primers are listed in Table 1. Single-intron

insertion in the mutant genomes was confirmed by Southern blotting (24) (Fig. 1). Growth experiments were performed with the wild-type strain and the mutants at 3°C and 28°C as described previously (24). The values of optical density at 600 nm (OD₆₀₀) for the wild-type and mutant strains correlated with the numbers of viable bacteria.

The completely abolished growth of *csdA*483 and the severely impaired growth of *csdA*1548 and *csdA*1578 at 3°C (Fig. 2A) indicate that CsdA, particularly its DEAD-box helicase domain, is indispensable for the growth of *Y. pseudotuberculosis* IP32953 at low temperatures. At the optimal growth temperature of 28°C, mutations had no effect on growth of *Y. pseudotuberculosis* (Fig. 2B), which is in line with findings for *E. coli* in which CsdA was found to be dispensable at 37°C (1, 7, 15, 32). The insertion of the group II intron into the *Y. pseudotuberculosis* *csdA* in both sense (*csdA*1548) and antisense (*csdA*483 and *csdA*1578) orientations hindered growth; thus, the observed growth defect was considered to be due to lack of functional CsdA and not to polar effects resulting from expression of the strong kanamycin promoter in the intron. CsdA was previously reported to be essential for optimal growth at 25°C or below in mesophilic *E. coli*, indicating an important function of CsdA at low temperatures (1, 7, 15, 32). In *Bacillus cereus*, transposon insertion into the 5' untranslated region of *cshA* (BC0259) encoding a CsdA homolog resulted in impaired growth at 10°C (5), while deletion of the entire BC0259 abolished growth at 10°C (26).

During construction of the mutant, the virulence plasmid (pYV) was lost from *csdA*483, and despite several attempts, pYV could not be retained in *csdA*483. However, the other mutants, *csdA*1548 and *csdA*1578, kept their pYV. Frequent loss of the pYV in *Y. enterocolitica* has been previously reported (4). However, the loss of this plasmid is not expected to have distorted the results, since pYV-negative strains generally grow faster than pYV-positive strains and the difference is pronounced at low temperatures (9).

An important role of CsdA at low temperatures was further confirmed by successful complementation of the *csdA* mutation.

Received 20 October 2011 Accepted 30 November 2011

Published ahead of print 9 December 2011

Address correspondence to Eveliina Palonen, eveliina.palonen@helsinki.fi.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.07278-11

TABLE 1 Primers used in the study

Primer	Sequence (5'→3')
<i>csdA</i> -RT-qPCR-left	GTGATGTTGGCGAGATGGAG
<i>csdA</i> -RT-qPCR-right	ATCGTTGAGTGGGAAGCAAA
16S-RT-qPCR-left	GCTCGTGTGTGAAATGTTGG
16S-RT-qPCR-right	TATGTGGTCCGCTGGCTCT
<i>csdA</i> 483-484-IBS	AAAAAAGCTTATAATTATCCTTACATGCCAGCATGTGCGCCCAGATAGGGTG
<i>csdA</i> 483-484-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCAGCATTTTAACTTACCTTTCTTTGT
<i>csdA</i> 483-484-EBS2	TGAACGCAAGTTTCTAATTTTCGATTGTCATGTCGATAGAGGAAAGTGTCT
EBS universal	CGAAATTAGAACTTGCGTTTCAGTAAAC
T7	TAATACGACTCACTATAGGG
<i>csdA</i> 483-484-flank-left	TTGTTGTGGTACCCAGGT
<i>csdA</i> 483-484-flank-right	AGAGAACAACGCGGTCTGAT
<i>csdA</i> 1548-1549-IBS	AAAAAAGCTTATAATTATCCTTAGTTGACGTTTCGCGTGCAGCCCAGATAGGGTG
<i>csdA</i> 1548-1549-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTTCGTTGCCATACTTACCTTTCTTTGT
<i>csdA</i> 1548-1549-EBS2	TGAACGCAAGTTTCTAATTTTCGATTTCAACTCGATAGAGGAAAGTGTCT
<i>csdA</i> 1578-1579-IBS	AAAAAAGCTTATAATTATCCTTAGCTGCCGATATCGTGCAGCCCAGATAGGGTG
<i>csdA</i> 1578-1579-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTTCGATATCACTTACCTTTCTTTGT
<i>csdA</i> 1578-1579-EBS2	TGAACGCAAGTTTCTAATTTTCGATTGTCAGCTCGATAGAGGAAAGTGTCT
<i>csdA</i> -flank-left	TTCTGCTGATAGCCGTGATG
<i>csdA</i> -flank-right	CCGATATAACGGCTGCTGAT
<i>Ninv</i> -left (20)	TAAGGGTACTATCGCGGCGGA
<i>Ninv</i> -right (20)	CGTGAAATTAACCGTCACACT
<i>KvirF</i> -left (16)	TCGTGGCAGCTATGCTGTTT
<i>KvirF</i> -right (16)	ATACGTCGCTCGCTTATCCA
Intron-left	TGGCAATGATAGCGAAACAA
Intron-right	GGTACCGCCTTGTTACATT
<i>csdA</i> NotI	GGCGCGCGGCGCAAAAATCGGACAGAGGGCAA
<i>csdA</i> XhoI	GGCCGCTCGAGTTATGCATCACCAGGAGCGA
<i>csdA</i> 1	GCTTGAAACCTCTTTTGCTGA
<i>csdA</i> 3	GAAAGATGGCCGTCTGGATA
<i>csdA</i> 5	ACGATTGAACTGCCAAAAGG
RP	TTTCACACAGGAAACAGCTATGAC

csdA and its putative native promoter, as predicted by BROM (softberry, Inc., Mount Kisco, NY), were amplified by PCR (Table 1) and ligated to the high-copy-number plasmid pBluescript II KS+ (here called pBluescript) (Stratagene, La Jolla, CA), resulting in pBluescript-*csdA*. The complemented mutants showed enhanced growth at 3°C compared to the vector-only controls, although the phenotype of the wild-type strain was not fully restored (Fig. 2C to E). However, as the complemented strains continued to grow throughout the experiment, loss of the complementation plasmid is very unlikely. Introduction of pBluescript-*csdA* into the wild-type strain did not influence growth (Fig. 2F).

To investigate the possible role of *csdA* in freeze-thaw tolerance, duplicate aliquots of overnight cultures of the IP32953 wild-type strain and the mutants were frozen to −20°C. Cryovials were

thawed and refrozen 15 times at 24-h intervals by incubation for 10 min in a 25°C water bath and freezing again to −20°C. The numbers of CFU per milliliter were determined before and upon freezing at 72-h intervals. The average numbers of viable cells of the wild-type, *csdA*1548, and *csdA*1578 strains decreased from 10⁸ to 10² and those of the *csdA*483 decreased from 10⁸ to 10¹ ($P > 0.05$; Student's *t* test). Thus, mutations in *csdA* did not affect the freeze-thaw tolerance of *Y. pseudotuberculosis*. This is in line with a previous study showing that a mutation in an RNA helicase gene prevented growth of *Listeria monocytogenes* at low temperatures but had no effect on freeze-thaw tolerance (2).

The maximum and minimum growth temperatures of the IP32953 wild-type and mutant strains were determined using a Gradiplate W10 temperature gradient incubator (BCDE Group, Helsinki, Finland) under aerobic conditions as described previously (10). Strains IP32953, *csdA*1548, and *csdA*1578 grew throughout the temperature gradient (1.7 to 6.3°C), indicating that the minimum growth temperatures of these strains are lower than 1.7°C. The minimum growth temperature of *csdA*483 was 5.6°C and was thus significantly higher ($P < 0.05$, Student's *t* test) than that of the wild-type strain. The maximum growth temperatures of IP32953, *csdA*483, *csdA*1548, and *csdA*1578 were 43.0°C, 43.7°C, 43.2°C, and 43.2°C, respectively, indicating no significant differences between strains.

Apparently, the main function of *csdA* in *Y. pseudotuberculosis* occurs at a low temperature. In *E. coli*, the main role of CsdA at a

FIG 1 Southern blots to demonstrate single insertions. Single bands of approximately 11.5 kb in size indicate single-intron insertion in mutants *csdA*483, *csdA*1548, and *csdA*1578. Linear vector pACD4K-C served as a positive control. Lanes: M, molecular weight marker; 1, pACD4K-C; 2, wild type; 3, *csdA*483; 4, *csdA*1548; 5, *csdA*1578.

FIG 2 (A and B) Growth curves and growth rates (GR; expressed in OD units per hour [31]) of the *Yersinia pseudotuberculosis* IP32953 wild-type strain and *csdA* mutants at 3°C (A) and at 28°C (B). (C to F) Data represent growth of the mutants, complemented mutants, vector-only controls, and the wild-type strain at 3°C. In the graphs in panels A and C to F, measured values are shown at 20-h intervals; in panel B, measured values are shown at 1-h intervals. Error bars indicate minimum and maximum values.

low temperature was proposed to relate to its helicase function (1, 13, 32) and mRNA decay (1, 33). Also, cold-shocked *Y. enterocolitica* cells refused to resume growth until cold shock protein mRNAs were degraded (21, 22). Whether CsdA is responsible for cold shock protein mRNA degradation at low temperatures in *Y. pseudotuberculosis* warrants further investigation.

ACKNOWLEDGMENTS

This work was supported by the Finnish Center of Excellence in Microbial Food Safety Research, Academy of Finland (grants 118602 and 141140), the Doctoral Program of the Faculty of Veterinary Medicine of the University of Helsinki, the Finnish Veterinary Foundation, the Walter Ehrström Foundation, and the Medical Fund of the University of Helsinki.

We thank Erika Pitkänen, Kirsi Ristkari, and Heimo Tasanen for technical assistance.

REFERENCES

- Awano N, et al. 2007. Complementation analysis of the cold-sensitive phenotype of the *Escherichia coli* *csdA* deletion strain. *J. Bacteriol.* 189: 5808–5815. doi:10.1128/JB.00655-07.
- Azizoglu RO, Kathariou S. 2010. Inactivation of a cold-induced putative RNA helicase gene of *Listeria monocytogenes* is accompanied by failure to grow at low temperatures but does not affect freeze-thaw tolerance. *J. Food Prot.* 73:1474–1479.
- Baranyi J, Roberts TA. 1994. A dynamic approach to predicting bacterial growth in food. *Int. J. Food Microbiol.* 23:277–294.
- Blais BW, Phillippe LM. 1995. Comparative analysis of *yadA* and *ail* polymerase chain reaction methods for virulent *Yersinia enterocolitica*. *Food Control* 6:211–214.
- Broussolle V, et al. 2010. Insertional mutagenesis reveals genes involved in *Bacillus cereus* ATCC 14579 growth at low temperature. *FEMS Microbiol. Lett.* 306:177–183.
- Chain PSG, et al. 2004. Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. *Proc. Natl. Acad. Sci. U. S. A.* 101:13826–13831.
- Charollais J, Dreyfus M, Iost I. 2004. CsdA, a cold-shock RNA helicase from *Escherichia coli*, is involved in the biogenesis of 50S ribosomal subunit. *Nucleic Acids Res.* 32:2751–2759.
- Fredriksson-Ahomaa M, Korkeala H. 2003. Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food, and environmental samples: a methodological problem. *Clin. Microbiol. Rev.* 16:220–229.
- Goverde RLJ, Kusters JG. 1994. Growth rate and physiology of *Yersinia enterocolitica*; influence of temperature and presence of the virulence plasmid. *J. Appl. Bacteriol.* 77:96–104.
- Hinderink K, Lindström M, Korkeala H. 2009. Group I *Clostridium botulinum* strains show significant variation in growth at low and high temperatures. *J. Food Prot.* 72:375–383.
- Hunter S, et al. 2009. InterPro: the integrative protein signature database. *Nucleic Acids Res.* 37:D211–D215.
- Iost I, Dreyfus M. 1994. mRNAs can be stabilized by DEAD-box proteins. *Nature* 372:193–196. doi:10.1038/372193a0.
- Jain C. 2008. The *E. coli* RhlE RNA helicase regulates the function of related RNA helicases during ribosome assembly. *RNA* 14:381–389.
- Jalava K, et al. 2006. An outbreak of gastrointestinal illness and erythema nodosum from grated carrots contaminated with *Yersinia pseudotuberculosis*. *J. Infect. Dis.* 194:1209–1216. doi:10.1086/508191.
- Jones PG, Mitta M, Kim Y, Jiang W, Inouye M. 1996. Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 93:76–80.
- Kaneko S, Ishizaki N, Kokubo Y. 1995. Detection of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from pork using the polymerase chain reaction. *Contrib. Microbiol. Immunol.* 13:153–155.
- Khemici V, Toesca I, Poljak L, Vanzo NF, Carpousis AJ. 2004. The RNase E of *Escherichia coli* has at least two binding sites for DEAD-box RNA helicases: functional replacement of RhlB by RhlE. *Mol. Microbiol.* 54:1422–1430.
- Linder P, et al. 1989. Birth of the D-E-A-D box. *Nature* 337:121–122.
- Lu J, Aoki H, Ganoza MC. 1999. Molecular characterization of a prokaryotic translation factor homologous to the eukaryotic initiation factor eIF4A. *Int. J. Biochem. Cell Biol.* 31:215–229.
- Nakajima H, et al. 1992. Detection and identification of *Yersinia pseudotuberculosis* and pathogenic *Yersinia enterocolitica* by an improved polymerase chain reaction method. *J. Clin. Microbiol.* 30:2484–2486.
- Neuhaus K, et al. 2003. The AGUAAA motif in *cspA1/A2* mRNA is important for adaptation of *Yersinia enterocolitica* to grow at low temperature. *Mol. Microbiol.* 50:1629–1645.
- Neuhaus K, Rapposch S, Francis KP, Scherer S. 2000. Restart of exponential growth of cold-shocked *Yersinia enterocolitica* occurs after down-regulation of *cspA1/A2* mRNA. *J. Bacteriol.* 182:3285–3288.
- Nuorti JP, et al. 2004. A widespread outbreak of *Yersinia pseudotuberculosis* O:3 infection from iceberg lettuce. *J. Infect. Dis.* 189:766–774. doi:10.1086/381766.
- Palonen E, Lindström M, Karttunen R, Somervuo P, Korkeala H. 2011. Expression of signal transduction system encoding genes of *Yersinia pseudotuberculosis* IP32953 at 28°C and 3°C. *PLoS One* 6:e25063. doi:10.1371/journal.pone.0025063.
- Palonen E, Lindström M, Korkeala H. 2010. Adaptation of enteropathogenic *Yersinia* to low growth temperature. *Crit. Rev. Microbiol.* 36:54–67.
- Pandiani F, et al. 2010. Differential involvement of the five RNA helicases in adaptation of *Bacillus cereus* ATCC 14579 to low growth temperatures. *Appl. Environ. Microbiol.* 76:6692–6697.
- Peil L, Virumäe K, Remme J. 2008. Ribosome assembly in *Escherichia coli* strains lacking the RNA helicase DeadD/CsdA or DbpA. *FEBS J.* 275:3772–3782.
- Prud'homme-Généreux A, et al. 2004. Physical and functional interactions among RNase E, polynucleotide phosphorylase and the cold-shock protein, CsdA: evidence for a 'cold shock degradosome'. *Mol. Microbiol.* 54:1409–1421.
- Resch A, Večerek B, Palavra K, Bläsi U. 2010. Requirement of the CsdA DEAD-box helicase for low temperature riboregulation of *rpoS* mRNA. *RNA Biol.* 7:796–802.
- Rimhanen-Finne R, et al. 2009. *Yersinia pseudotuberculosis* causing a large outbreak associated with carrots in Finland, 2006. *Epidemiol. Infect.* 137: 342–347.
- Toone WM, Rudd KE, Friesen JD. 1991. *deaD*, a new *Escherichia coli* gene encoding a presumed ATP-dependent RNA helicase, can suppress a mutation in *rpsB*, the gene encoding ribosomal protein S2. *J. Bacteriol.* 173: 3291–3302.
- Turner AMW, Love CF, Alexander RW, Jones PG. 2007. Mutational analysis of the *Escherichia coli* DEAD box protein CsdA. *J. Bacteriol.* 189: 2769–2776.
- Yamanaka K, Inouye M. 2001. Selective mRNA degradation by polynucleotide phosphorylase in cold shock adaptation in *Escherichia coli*. *J. Bacteriol.* 183:2808–2816.