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EDITOR'S CHOICE

Two-step induction of *cdsA* promoters leads to upregulation of the glycolipid MPIase at cold temperature

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Glycolipid MPIase, essential for membrane protein integration into the cytoplasmic membrane of *Escherichia coli*, is upregulated at cold temperatures. This upregulation is rapid and sustainable. CdsA, a CDP-diacylglycerol synthase, is a rate-limiting enzyme for MPIase biosynthesis. Upregulation of CdsA is responsible for the increase in the MPIase level at low temperature. Investigation of *cdsA* regulatory regions revealed at least two cold-inducible promoters, a cold-shock promoter that functions transiently and immediately in the cold, and one that is sustainable in the cold. The stability of the *cdsA* transcript was comparable with that of *tufA*, which is not cold-inducible. Thus, *cdsA* is induced through two-step cold-induction to maintain MPIase at a high level rapidly and sustainably in the cold.

Keywords: glycolipid; low temperature; membrane biogenesis; membrane protein integration; MPIase; preprotein translocation

Membrane protein integration into and protein translocation across membranes are vital events for all the living organisms. It is known that these reactions are cold sensitive *per se* [1]. However, how cells overcome the difficulties in protein integration and translocation under cold conditions is totally unknown. In *Escherichia coli*, protein integration and protein translocation proceed with the aid of SecYEG translocase [2–4]. Some membrane proteins of small size or with a transmembrane domain at only the C-terminus are integrated independently of SecYEG. YidC is responsible for the membrane protein integration of these proteins [5]. The expression levels of the SecYEG translocase and YidC do not change under cold conditions [6–8]. We have identified and characterized a glycolipid named MPIase (Membrane Protein Integrase), essential for membrane protein integration and therefore cell growth [9–12]. MPIase is composed of a linear glycan chain comprising 9-11 repeats of the trisaccharide unit of *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-mannosaminuronic acid and 4-acetoamido-4-deoxy-D-fucose, and diacylglycerol through a pyrophosphate linker [10,13]. MPIase directly interacts with membrane proteins and then drives their integration into the cytoplasmic membrane [10,13]. MPIase also significantly stimulates protein translocation by affecting the dimer structure of SecYEG translocase [14,15]. Recently, we found that the expression level of MPIase specifically increases significantly at low temperature (<25 °C) [8]. The increase in the MPIase level is crucial for efficient protein translocation at low temperature [6,8].

CdsA, a CDP-diacylglycerol (CDP-DAG) synthase, biosynthesizes CDP-DAG, using CTP and phosphatidic acid (PA) as substrates [16,17]. CDP-DAG is a precursor of all phospholipids in *E. coli*. In addition to phospholipid biosynthesis, CdsA also biosynthesizes GlcNAc-PP-DAG (Compound I), the

Abbreviations
BPB, bromophenol blue; cat, chloramphenicol acetyltransferase; CDP-DAG, CDP-diacylglycerol; GlcNAc-P, *N*-acetyl-D-glucosamine 1-phosphate; MPIase, Membrane Protein Integrase.

first intermediate for MPIase biosynthesis [11]. GlcNAc-P is substituted by the CMP moiety of CDP-DAG on CdsA, giving Compound I [11]. YnbB is a CdsA paralogue dedicated to MPIase biosynthesis [12]. Both CdsA and YnbB are involved in the increase in the MPIase level at low temperature [8]. When both the *cdsA* and *ynbB* genes on the chromosome are disrupted, the increase in the MPIase level at low temperature is repressed even if CdsA is expressed from a complementary plasmid, in which *cdsA* is placed under the control of the arabinose promoter. Furthermore, the expression level of CdsA is upregulated to the same extent as the increase in the MPIase level in the cold [8,11]. These observations indicate that the increase in the MPIase level is regulated by the CdsA level. It is thought that *cdsA* is the second gene in the *ispU-cdsA-rseP-bamA* operon, all of which are essential for cell growth [18]. However, the promoter responsible for the expression of the *ispU-cdsA-rseP-bamA* operon is not clear. It is reported that when the open reading frame of the *ispU* gene was replaced with the *cat* gene, expression of *cdsA* was disturbed [19], suggesting that there is a region responsible for *cdsA* expression in the open reading frame of *ispU*. Thus, it is totally unknown whether the expression level of CdsA is upregulated at the level of transcription, translation, or stability of mRNA.

In this study, we found that the expression level of MPIase rapidly and sustainably increased after a temperature downshift. The increase in the *cdsA* transcripts, correlated with the increase in the MPIase level, was achieved through two-step cold-induction of promoters with different characteristics.

Materials and methods

Materials

The *E. coli* strains and plasmids used in this study are listed in Table 1. The primers used in this study are listed in Table 2. Antibodies against SecB were raised in rabbits using the purified protein [20]. The anti-MPIase antibodies was raised in rabbits against the purified MPIase chemically cross-linked to keyhole limpet hemocyanin [10]. These antibodies were obtained through commercially available custom services. Rifampicin was from Sigma.

Plasmid construction

Plasmids C1- ~ C6-pCD1-Δ3′, with deletion of the downstream and promoters of *cdsA*, were constructed as follows. A 2.2 kb downstream region of *cdsA* was deleted by PCR using a pair of primers (pUC19 HindIII-3′comp and *cdsA* HindIII-5′) and pCD1 as a template, with a HindIII site following the stop codon of *cdsA*. The PCR product was digested with HindIII, followed by self-ligation, which yielded pCD1-Δ3′. Putative promoters within 2.6 kb upstream of *cdsA* were deleted by PCR using pair primers (pUC19 BglII-5′ and cdsA C1, C2, C3, C4, C5, or C6 BglII-3′comp) and pCD1-Δ3′ as a template. The PCR products were digested with BglII, followed by self-ligation, which yielded plasmids C1- ~ C6-pCD1-Δ3′.

Table 1. *Escherichia coli* strains and plasmids used in this study.

Strains and plasmids	Relevant genotype and description	Reference
EK413	MC4100 *ara*$^{+}$	[12]
PS259	*phoAΔPvuII lacΔX74 galE galK rpsL pcnB80 zad1*::Tn*10*	[38]
KS22	EK413 Δ*cdsA*::*cat*	[11]
KS23	EK413 Δ*ynbB* Δ*cdsA*::*cat*	[11]
YS23	EK413 Δ*ynbB* Δ*cdsA*::*cat pcnB80 zad1*::Tn*10*	This study
pAra-CdsA	*cdsA* was cloned into pKQ2 under control of arabinose promoter, spectinomycin-resistant	[11]
pCD1	5.8 kb fragment containing 2.6 kb upstream and 2.2 kb downstream of *cdsA* was cloned into BamHI/HindIII site of pUC19 in opposite direction to *lac* promoter, ampicillin-resistant	[11]
pCD1-Δ3′	Derivative of pCD1 with 2.2 kb downstream of the *cdsA* gene deletion	This study
C1-pCD1-Δ3′	Derivative of pCD1-Δ3′ with upstream of P1 promoter deletion	This study
C2-pCD1-Δ3′	Derivative of pCD1-Δ3′ with upstream of P2 promoter deletion	This study
C3-pCD1-Δ3′	Derivative of pCD1-Δ3′ with promoter P3, P4, and P5 deletion	This study
C4-pCD1-Δ3′	Derivative of pCD1-Δ3′ with promoter P4 and P5 deletion	This study
C5-pCD1-Δ3′	Derivative of pCD1-Δ3′ with upstream of promoter P4 deletion′	This study
C6-pCD1-Δ3′	Derivative of pCD1-Δ3′ with promoter P5 deletion	This study
C3-pCD2-Δ3′	Open reading frame of *cdsA* in C3-pCD1-Δ3′ was replaced with that of *cat*	This study
C4-pCD2-Δ3′	Open reading frame of *cdsA* in C4-pCD1-Δ3′ was replaced with that of *cat*	This study

Table 2. Oligonucleotides used in this study.

Name	Sequence (5′→3′)	Purpose
F1	TTGCTGAAGTATCGCCTGAT	RT-PCR
F2	CAAGACTTTGAAGGGGCG	RT-PCR
F3	GGGGAGCATCGCATTA G	RT-PCR
F4	AGTTGGCTTTATAAGGTCAG	RT-PCR
F5	CCGGCTACAGAGAGTCG	RT-PCR
F6	GCGATGGAGGCGTTCG	RT-PCR
F7	GGCGTGAAGCCGCTCG	RT-PCR
F8	TCTGGGCTCGACCGGCTCGA	RT-PCR
F9	CAGTACGTAACGTGCGTCG	RT-PCR
R1	TTAAAGCGTCCTGAATACCA	RT-PCR
tufA-5′	ACGTACAAAACCGCACGTTA	RT-PCR
tufA-3′comp	TTAGCCCAGAACTTTAGCAA	RT-PCR
cdsA HindIII-5′	AAAAAAGCTTTTAAAGCGTCCTGAATACC	Construction of pCD1-Δ3′
pUC19 HindIII-3′comp	AAAAAAGCTTGGCGTAATCATGGTCATAG	Construction of pCD1-Δ3′
pUC19 BglII-5′	AAAAAGATCTCCGGGTACCGAGCTCGTGGTG	Construction of C1- ~C6-pCD1-Δ3′
C1-BglII-3′comp	AAAAAGATCTCAAGACTTTGAAGGGGCGTT	Construction of C1-pCD1-Δ3′
C2-BglII-3′comp	AAAAAGATCTGGGGAGCATCGCATTAGTAA	Construction of C2-pCD1-Δ3′
C3-BglII-3′comp	AAAA AGATCTAGTTGGCTTTATAAGGTCAG	Construction of C3-pCD1-Δ3′
C4-BglII-3′comp	AAAAAGATCTCCGGCTACAGAGAGTCGCGC	Construction of C4-pCD1-Δ3′
C5-BglII-3′comp	AAAAAGATCTGGCGTGAAGCCGCTCGATTT	Construction of C5-pCD1-Δ3′
C6-BglII-3′comp	AAAAAGATCTTCTGGGCTCGACCGGCTCGA	Construction of C6-pCD1-Δ3′
pCD1 up	AAAAGCAGATATCAGGCGATACTTCAGCAT ATGCGACCCCCATCAGGCTG	Construction of C3- and C4-pCD2-Δ3′
cat-5′ NdeI	ACTTAAGGGTTTTCTACATATGGAGAAA AAAATCACTGG	Construction of C3- and C4-pCD2-Δ3′
cat-3′ comp HindIII	TGAAGCTTACGTTGGTTCATTAGGCGCC GCCCTGCCACTCATCGCAGTA	Construction of C3- and C4-pCD2-Δ3′
cat-5′	ATCACTGGATATACCACCGT	RT-PCR
cat-3′ comp	CTGAATCGCCAGCGGCATCA	RT-PCR

Plasmids C3- and C4-pCD2-Δ3′, in which the open reading frame of *cdsA* has been replaced with that of *cat*, were constructed as follows. The open reading frame of *cdsA* was deleted by PCR using a pair of primers (pCD1 up and pUC19 HindIII-3′comp) and C3- or C4-pCD1-Δ3′ as a template, with an NdeI site at the initiation codon and a HindIII site followed by a stop codon respectively. The open reading frame of *cat* with an NdeI site at the initiation codon and a HindIII site following the stop codon, respectively, was PCR-amplified. The *cat* gene fragment, digested with NdeI and HindIII, was then ligated with the PCR fragment with the *cdsA* deletion, and then digested with NdeI and HindIII, yielding plasmids C3- and C4-pCD2-Δ3′.

RT-PCR analysis

Total RNA was prepared by immediately extracting the cell culture with phenol/chloroform (1 : 1), followed by ethanol precipitation. The precipitate was then treated with RNase-free DNase (Promega, Madison, WI, USA) at 0.1 u/μL at 37 °C for 30 min. After incubation at 65 °C for 10 min to inactivate DNase, an aliquot (50 ng) was subjected to RT-PCR, using a BcaBEST RNA PCR system (Takara, Kusatsu, Shiga, Japan). After the R1 primer (Fig. 2A and Table 2) had been annealed to RNA, the cDNA was synthesized by BcaBEST polymerase at 65 °C for 30 min. The cDNA was then amplified by the addition a primer pair of R1 and one of primers F1-F9 (Fig. 2A and Table 2), and Taq polymerase with 30 cycles of 94 °C-30 s/50 °C-30 s/72 °C-60 s–300 s. An aliquot of the RT-PCR products was then analyzed by 1.5% agarose gel electrophoresis. The primer pair of *tufA*-5′ and *tufA*-3′comp (Table 2) was also used to detect the *tufA* transcript as a control. The primer *tufA*-3′comp was used to synthesize the cDNA.

The cat reporter assay

Cell pellets, suspended in 10 mM Tris-HCl (pH 7.5)/10% glycerol, were disrupted by sonication at 95% pulse intensity for 30s and the debris were removed by centrifugation at 16 000 ***g*** for 5 min at 4 °C. The supernatant was further centrifuged at 160 000 ***g*** for 60 min at 4 °C to remove the membrane fraction. The supernatant was used as the cytosol fraction. The cytosol fraction was added to 100 mM Tri-HCl (pH 7.5), 0.08 mM 5,5′-dithio-bis (2-nitrobenzoicacid),

Table 3. Growth of the strains used in this study.

	Chromosome		Plasmid		Growth[a]			
					(+) arabinose		(−) arabinose	
	cdsA	*ynbB*	pAra-CdsA	pCD1 derivatives	37 °C	20 °C	37 °C	20 °C
EK413	+	+	−	−	++	++	++	++
KS23	Δ	Δ	+	−	++	++	−	−
YS23	Δ	Δ	+	−	++	++	−	−
YS23	Δ	Δ	+	pCD1	+	+	+	+
YS23	Δ	Δ	+	Δ3′	+	+	+	+
YS23	Δ	Δ	+	C1-Δ3′	++	++	−	−
YS23	Δ	Δ	+	C2-Δ3′	++	++	+	−
YS23	Δ	Δ	+	C3-Δ3′	++	++	+	−
YS23	Δ	Δ	+	C4-Δ3′	++	++	++	++
YS23	Δ	Δ	+	C5-Δ3′	++	++	++	++
YS23	Δ	Δ	+	C6-Δ3′	++	++	++	++

[a]Each strain was steaked onto LB plates, followed by incubation at 37 °C for 18 h and at 20 °C for 72 h with or without 0.2% arabinose. '++' and '+' denote large and small colonies, respectively, while '−' denotes no colony formation.

0.2 mM acetyl-CoA, 0.005% (w/v) chloramphenicol, and then incubated for 10 min at 25 °C. After incubation, the absorbance at 412 nm was measured.

Rifampicin chase experiments

Growing cells cultivated at 37 °C were shifted to 20 °C. After cultivation had been continued for 1 h, 150 μg/mL rifampicin was added to inhibit the *de novo* synthesis of RNA [21]. After specified times, total RNA was extracted and analyzed as described above.

Other methods

The SDS/PAGE was carried out using 1.5% acrylamide, 0.27% *N,N'*-bismethyleneacrylamide [22,23]. Immunoblotting was carried out as described [8]. The bands on agarose gel were quantified by means of a CS analyzer (ATTO). Proteins were quantified using BSA as a standard [24].

Results

The level of the *cdsA* transcript, which is rate-limiting for MPIase expression, is stably upregulated at low temperature

In the previous study, we demonstrated that either *cdsA* or *ynbB* is involved in the increase in the MPIase level at low temperature [8]. The increase in the MPIase was significantly repressed in the *cdsA*/*ynbB* knockout (KS23), even if CdsA was expressed from a complementary plasmid, in which *cdsA* is under the control of the arabinose promoter (pAra-CdsA). KS23/pAra-CdsA was able to grow when arabinose is added to the media at both 37 °C and 20 °C (Table 3). The time course of the increase in the MPIase level after a temperature downshift from 37 °C to 20 °C was examined. Wild-type (EK413) cells were grown at 37 °C until they reached the mid-log phase (OD at 600 nm, ~ 0.8), and then were transferred to 20 °C. The MPIase level at each time was determined by quantitative immunoblotting using anti-MPIase antisera. The MPIase level had already reached the maximum level at 10 min, and was sustained at least until 90 min (Fig. 1A). Consistent with the previous study, an increase in the MPIase level at 20 °C was hardly observed in KS23/pAra-CdsA (Fig. 1A). These results indicate that the increase in the MPIase level is rapid and sustained for a long time at low temperature.

We examined whether the *cdsA* transcript is increased at low temperature by means of RT-PCR, since CdsA is a rate-limiting enzyme for MPIase biosynthesis [11]. The transcript containing the open reading frame of *cdsA* was amplified by means of RT-PCR using a pair of F1/R1 primers (see Fig. 2A). Wild-type cells (EK413) were grown at 37 °C until they reached the early log phase (OD at 600 nm, 0.3–0.5), and then were shifted to 20 °C, and cultivation was continued for another 3 h. We prepared total RNA by immediate phenol/chloroform extraction of cell cultures. We could not observe a difference in the levels of the *cdsA* transcripts between cultures at 37 °C and 20 °C, when total RNA was prepared by means of a commercially available RNA purification kit, in which a step of collecting cells by centrifugation is included. If the temperature of the centrifuge was lower than 37 °C, the *cdsA* transcript increased during the centrifugation (see Fig. 1C). We observed an ~3-

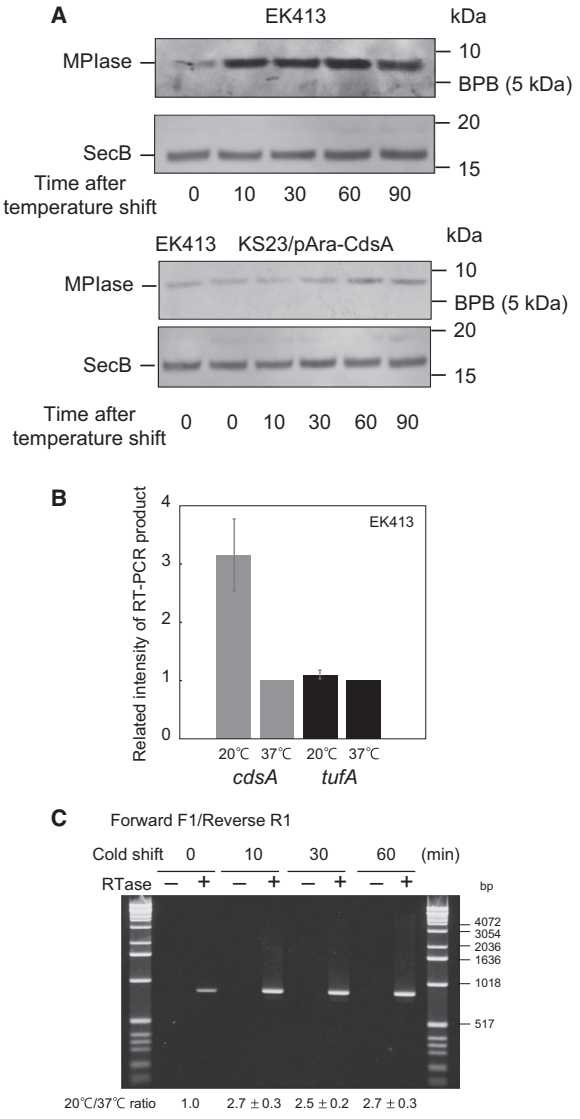

Fig. 1. The *cdsA* transcript and MPIase immediately increase at low temperature. (A) Overnight cultures of EK413 and KS23/pAra-CdsA were 1:100 diluted in LB medium and then cultivated at 37 °C. When cells reached the mid-log phase, they were shifted to 20 °C. At the specified times, total cellular proteins were precipitated with TCA, followed by SDS-PAGE/immunoblotting using anti-MPIase and anti-SecB antisera. (B) EK413 cells were cultivated at 37 °C and then transferred to 20 °C, and cultivation was continued for another 3 h. Total RNA was prepared by phenol/chloroform extraction. The open reading frames of the *cdsA* transcript and the *tufA* transcript were amplified by means of RT-PCR. Three replicates were examined, and the average values are given with SD. (C) EK413 cells were cultivated as described in A, followed by RT-PCR using the indicated pairs of primers. As specified, reverse transcriptase (RT) was omitted (-). Size markers were analyzed in the left and right lanes, and the size of each band in bp is indicated at the right. The 20 °C/37 °C ratio of each sample, determined by three independent experiments, is indicated at the bottom of the gel.

fold increase in the level of RT-PCR product of *cdsA* at 20 °C (Fig. 1B). On the other hand, the level of the RT-PCR product of *tufA*, encoding EF-Tu, remained unchanged in the cold (Fig. 1B). Consistent with these observations, it is reported that the mRNA level of *tufA* does not change in the cold [25]. We next examined the time course of the increase in the *cdsA* transcript after a temperature downshift from 37 °C to 20 °C to confirm that the increase in the *cdsA* transcript is rapid and sustainable. The increase in the level of RT-PCR product reached the maximum level within 10 min. After 10 min, the level of mRNA remained the same until 60 min (Fig. 1C) and 3 h (Fig. 1B), consistent with the increase in the MPIase level in the cold (Fig. 1A). These results indicate that the increase in the level of the *cdsA* transcript, and therefore of MPIase, is rapid and sustainable in the cold.

The *dxr-ispU-cdsA* transcripts are transiently induced, but the *ispU-cdsA* transcript is sustainably induced in the cold

The *cdsA* gene is the second one in the *ispU-cdsA-rseP-bamA* operon, while the promoter(s) responsible for CdsA expression are totally unknown. There are some putative promoters, which can be assigned as a σ^{70} binding site, upstream of *cdsA*. They are referred to as P1–P5, as shown in Fig. 2A. To identify the promoters for CdsA, we performed RT-PCR analysis. We designed forward primers, referred to as F2–F9, immediately downstream of the putative promoters. At 37 °C, the RT-PCR products of F2/R1 to F6/R1 could be detected, while those of F7/R1 to F9/R1 could not (Fig. 2B). On the other hand, the RT-PCR products of F7/R1 and F8/R1 could be detected at 20 °C, in addition to those of F2/R1 to F6/R1 (Fig. 2B). These results suggest that the *dxr-ispU-cdsA* transcript is induced at 20 °C. We examined the time course of the increase in the *dxr-ispU-cdsA* transcript at low temperature. At 0 min, the RT-PCR product of F8/R1 was undetectable (Fig. 2C). The level was significantly increased at 10 min, and then it was gradually attenuated (Fig. 2C). Since the increased amount of the *cdsA* transcript, amplified by F1/R1, did not change until the cultivation time of 60 min (Fig. 1C), we assumed that there is a promoter responsible for the prolonged increase in the *cdsA* transcript downstream of P5. To examine this assumption, time course experiments involving F2/R1, F4/R1, and F6/R1 were performed. The F6/R1 RT-PCR product was increased at 10 min and then it was gradually attenuated (Fig. 2D), indicating that P4 acts as a promoter, however, P4 is not a promoter for a cold-sustainable promoter. On the other hand, the

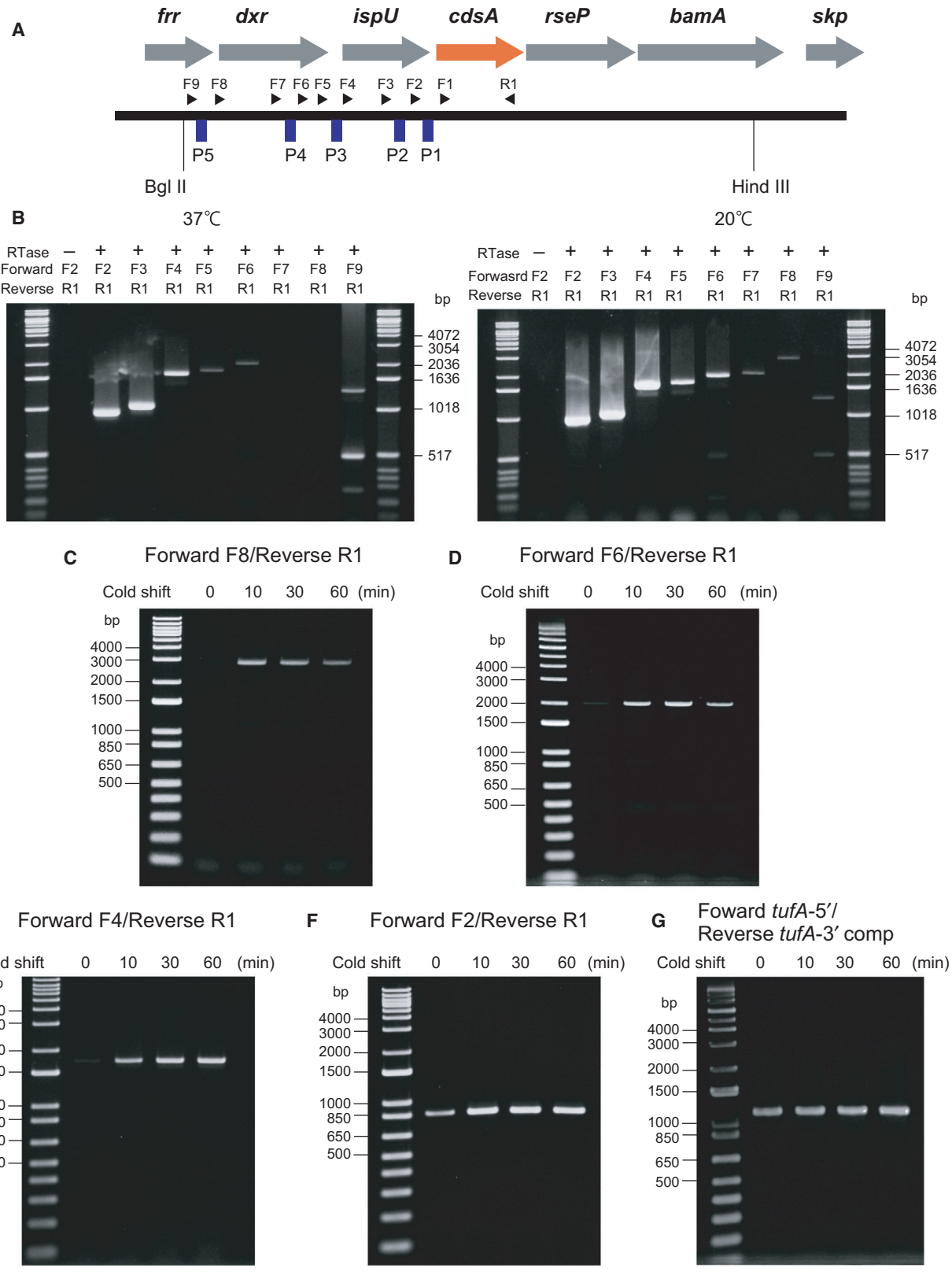
A
frr
dxr
ispU
cdsA
rseP
bamA
skp
F9 F8 F7 F6 F5 F4 F3 F2 F1 R1
P5 P4 P3 P2 P1
Bgl II
Hind III
B
37℃
20℃
RTase
Forward
Reverse
bp
4072
3054
2036
1636
1018
517
C
Forward F8/Reverse R1
D
Forward F6/Reverse R1
Cold shift 0 10 30 60 (min)
4000
3000
2000
1500
1000
850
650
500
E
Forward F4/Reverse R1
F
Forward F2/Reverse R1
G
Foward tufA-5′/
Reverse tufA-3′ comp

Fig. 2. The *dxr-ispU-cdsA* transcripts are transiently induced, but the *ispU-cdsA* transcript is sustainably induced in the cold. (A) The region around *cdsA*. Blue squares indicate the putative promoters, which were assigned as the σ^{70} binding site. Triangles indicate the positions of the primers with the direction. (B) EK413 cells were cultivated at 37 °C (left) and then transferred to 20 °C (right), and cultivation was continued for another 1 h. Total RNA was prepared by phenol/chloroform extraction, followed by RT-PCR. After cDNA had been synthesized using R1 as a primer, PCR was performed using the indicated pair of primers. As specified, reverse transcriptase (RTase) was omitted (-). Size markers were analyzed, and the size of each band in bp is indicated at the right. (C-G) EK413 cells were cultivated as described in Fig. 1A, and then total RNA was prepared at the specified times, followed by RT-PCR. After cDNA had been synthesized using R1 (C, D, and F) or *tufA*-3′comp (G) as a primer, PCR was performed using the F8 (C), F6 (D), F4 (E), F1 (F), and *tufA*-5′ (G) primers. In (B), (C) and (D), 15 μL of each RT-PCR product (50 μL) was applied, while 5 μL was applied in (E) and (F) to analyze the levels quantitatively.

increased levels of the F2/R1 and F4/R1 RT-PCR products did not change during the time course (Figs. 2E and F). These results indicate that both the *dxr-ispU-cdsA* transcript and the *ispU-cdsA* transcript are induced at low temperature, and that the increase in the *ispU-cdsA* transcript is sustainable while that in the *dxr-ispU-cdsA* transcript is transient. Thus, promoter P5 or promoters P5/P4 are cold shock promoter(s), while promoter P3 is a cold-sustainable one, suggesting that the increase in the *cdsA* transcript in the cold is achieved through two-step cold-induction.

The promoter for the *ispU-cdsA-rseP-bamA* operon (P3) is essential for sustainable upregulation of the *cdsA* transcript, and therefore MPIase in the cold

Icho *et al.* [16] reported that a 5.8 kb DNA fragment which contains 2.6 kb upstream and 2.2 kb downstream of *cdsA* (Figs 2A and 3A) directed overproduction of CdsA at 30 °C but not 42 °C, indicating that this fragment contains the region responsible for increase in the CdsA level at low temperature, and therefore that of MPIase. In the previous study, we constructed pCD1, in which the same 5.8 kb DNA fragment containing *cdsA* was cloned into the BamHI/HindIII sites of pUC19 in the opposite direction to the *lac* promoter [11]. Plasmid pCD1 was introduced into YS23 (Δ*pcnB* Δ*cdsA* Δ*ynbB*)/pAra-CdsA. The copy number of the ColE1 type plasmids is maintained at a very low level in the *pcnB* knockout strain [26]. Therefore, the notorious effects of multicopy plasmids can be excluded in YS23. While YS23/pAra-CdsA was not able to grow in the absence of arabinose because of CdsA depletion, YS23/pAra-CdsA/pCD1 was able to grow in the absence of arabinose at both 37 °C and 20 °C (Table 3), indicating that pCD1 has the ability to complement the growth defect of CdsA-depleted strains. Plasmid pCD1 has also the ability to increase the MPIase level at 20 °C as well as EK413 (Fig. 3B), indicating that the 5.8 kb DNA fragment contains the region responsible for the increase in MPIase at low temperature. Next, to examine the involvement of the downstream of *cdsA* in the upregulation of MPIase at low temperature, we constructed plasmid pCD1-Δ3′, in which the downstream region of *cdsA* was deleted (Fig. 3A). Plasmid pCD1-Δ3′ was able to complement CdsA-depletion (Table 3) and increased the MPIase level at low temperature (Fig. 3B), indicating that the downstream of *cdsA* is not necessary for the increase in MPIase in the cold.

To examine whether the putative promoters are involved in the increase in MPIase at low temperature, these promoters were deleted from pCD1-Δ3′, yielding plasmids C1~C6-pCD1-Δ3′ (Fig. 3A). When these plasmids were introduced into YS23/pAra-CdsA, the C4~C6-pCD1-Δ3′ transformants were able to grow in the absence of arabinose at both 37 °C and 20 °C (Table 3). On the other hand, the C2~C3-pCD1-Δ3′ transformants were able to grow at 37 °C, however, the growth was quite slow, and they were cold sensitive (Table 3). The C1-pCD1-Δ3′ transformant was not able to grow at 37 °C and 20 °C (Table 3). These results indicate that promoter P3 is sufficient for *cdsA* expression including cold induction but that promoter P2 is not sufficient for cold induction of *cdsA*. Promoter P1 does not act or is very weak.

The expression level of MPIase in these transformants was examined. In the C6~C4- pCD1 transformants, the MPIase level was increased as well as in the pCD1-Δ3′ transformant (Fig. 3B). On the other hand, in the C2~C3-pCD1-Δ3′ transformants, the increase in the MPIase was significantly repressed (Fig. 3B), consistent with the observation that promoter P3 is responsible for the sustainable induction in the cold. To further confirm that promoter P3 is activated in the cold, time course analysis was performed (Fig. 3C). When YS23/C3-pCD1-Δ3′ was transferred to 20 °C, the MPIase level was increased after 30 min, indicating that promoter P3 is a cold-inducible one that acts after the immediate cold-shock response. On the other hand, the MPIase level of YS23/pCD1-Δ3′ increased immediately after the cold exposure. These results again confirmed that promoter P5 or P5/P4 is a

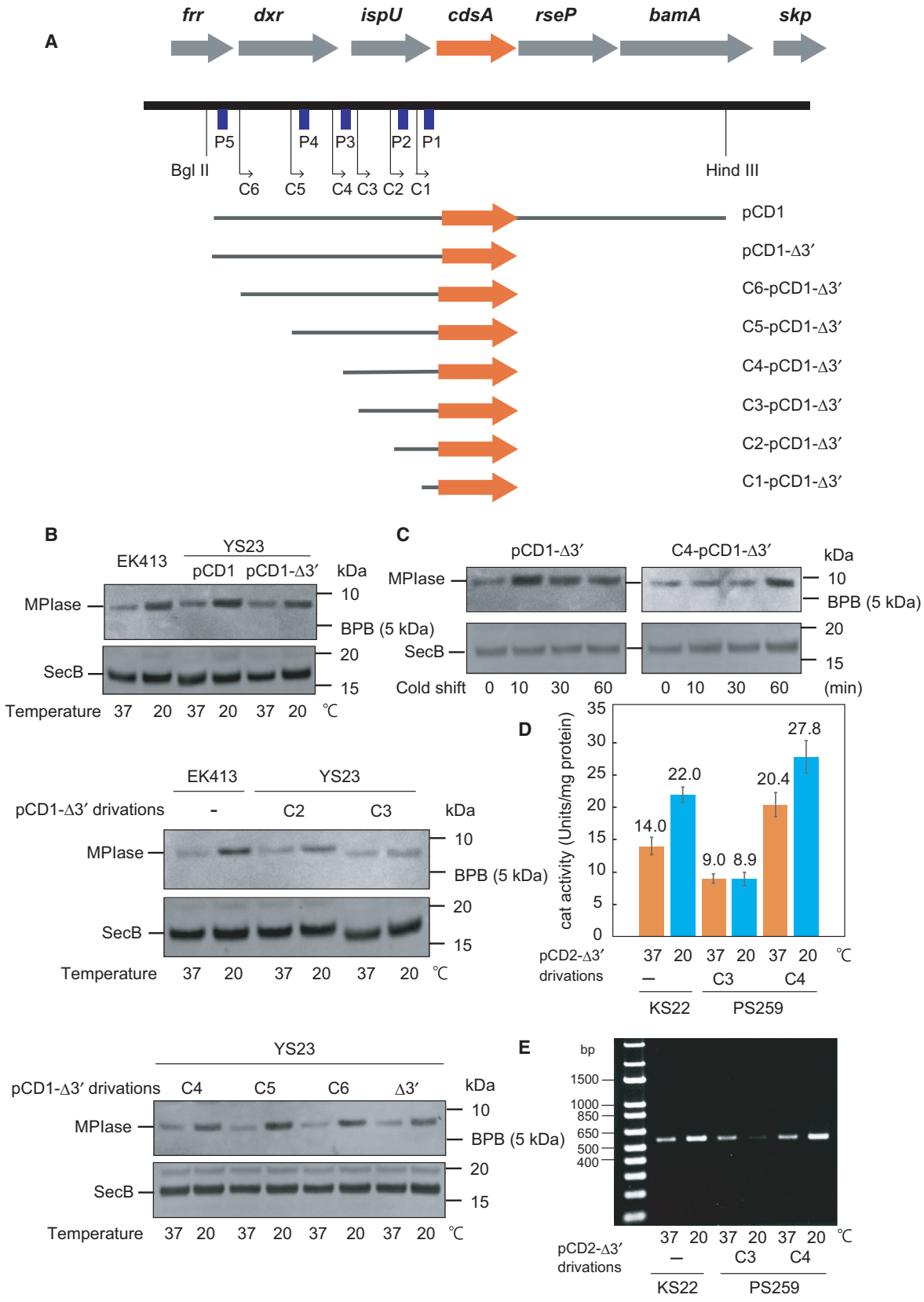
A
frr
dxr
ispU
cdsA
rseP
bamA
skp
P5
P4
P3
P2
P1
Bgl II
Hind III
C6
C5
C4 C3 C2 C1
pCD1
pCD1-Δ3′
C6-pCD1-Δ3′
C5-pCD1-Δ3′
C4-pCD1-Δ3′
C3-pCD1-Δ3′
C2-pCD1-Δ3′
C1-pCD1-Δ3′
B
EK413
YS23
pCD1
pCD1-Δ3′
kDa
MPIase
SecB
10
BPB (5 kDa)
20
15
Temperature 37 20 37 20 37 20 ℃
C
pCD1-Δ3′
C4-pCD1-Δ3′
kDa
MPIase
SecB
10
BPB (5 kDa)
20
15
Cold shift 0 10 30 60 0 10 30 60 (min)
EK413
YS23
pCD1-Δ3′ drivations
-
C2
C3
kDa
MPIase
SecB
10
BPB (5 kDa)
20
15
Temperature 37 20 37 20 37 20 ℃
D
cat activity (Units/mg protein)
35
30
25
20
15
10
5
0
14.0
22.0
9.0
8.9
20.4
27.8
pCD2-Δ3′ drivations
37 20 37 20 37 20 ℃
—
C3
C4
KS22
PS259
YS23
pCD1-Δ3′ drivations
C4
C5
C6
Δ3′
kDa
MPIase
SecB
10
BPB (5 kDa)
20
15
Temperature 37 20 37 20 37 20 37 20 ℃
E
bp
1500
1000
850
650
500
400
pCD2-Δ3′ drivations
37 20 37 20 37 20 ℃
—
C3
C4
KS22
PS259

Fig. 3. Promoter P3 is essential for sustainable upregulation of MPIase in the cold. (A) The structures of plasmid pCD1 and its derivatives are indicated. Blue squares show the putative promoters, which were assigned as the σ^{70} binding site. Arrows indicate the 5′ ends of the constructs. (B) An overnight culture of the indicated cells was 1:100 diluted in LB medium without arabinose. When the cells reached the mid-log phase, they were shifted to 20 °C, and cultivation was continued for another 2 h. The MPIase level was determined by immunoblotting. SecB was analyzed as a loading control. BPB ran to 5 kDa in this gel system. (C) YS23/pCD1-Δ3′ (left) and YS23/C4-pCD1-Δ3′ (right) cells, grown at 37 °C to the mid-log phase, were shifted to 20 °C. An aliquot was withdrawn at the specified time, followed by determination of the MPIase by immunoblotting. SecB was analyzed as a loading control. (D) The indicated cells were cultivated in LB medium at 37 °C (orange) and then transferred to 20 °C (blue), and cultivation was continued for another 2 h. For KS22/pAra-CdsA, 0.2% arabinose was added. The cytosol fraction was prepared and then the cat activity was measured as described under "Materials and methods". The experiments were performed three times and the average values are given together with SD. (E) The indicated cells were cultivated as described in Fig. 2C. Total RNA was prepared by phenol/chloroform extraction. The open reading frame of the *cat* transcript were amplified by means of RT-PCR using a primer pair of *cat*-5′/*cat*-3′ comp.

cold-shock one, and that promoter P3 is a cold-sustainable one.

We also performed a cat reporter assay to reveal that promoter P3 is responsible for the increase in the *cdsA* transcript. The open reading frame of *cdsA* in C4-pCD1-Δ3′ was replaced with that of *cat*, yielding plasmid C4-pCD2-Δ3′. Plasmid C4-pCD2-Δ3′ was introduced into a *pcnB*-deficient strain (PS259). The cat activity in the cells cultivated at 37 °C and 20 °C was measured. The cat activity was increased by ~ 1.5 fold at 20 °C compared to that at 37 °C (Fig. 3D). The cat activity in KS22 (*cdsA*::*cat*)/pAra-CdsA, in which open reading frame of chromosomal *cdsA* is replaced with that of *cat,* was also increased by ~ 1.5 fold. The higher activity in the C4-pCD2-Δ3′ transformant may reflect the dimerization of the plasmid, since PS259 is *recA*$^{+}$. These results indicate that C4-pCD2-Δ3′ reflects the chromosomal response to the cold. When promoter P3 was deleted, the increase in the cat activity was abolished (Fig. 3D, C3). The increases in the cat activities coincided with the increases in the cat transcripts (Fig. 3E). We observed an increase in the level of the RT-PCR product of *cat* in the C4-pCD2-D3′ transformant as well as in KS22/pAra-CdsA (Fig. 3E). On the other hand, an increase in the *cat* transcript was not observed at 20 °C in the P3-pCD2-D3′ transformant (Fig. 3E). These results indicate that promoter P3 is activated upon cold and that is necessary for the sustainable increase in the *cdsA* transcript, and therefore in the MPIase level.

Stabilization of the *cdsA* transcript is not responsible for the increase in its transcript at low temperature

Stabilization of mRNA is a major strategy for accumulation of mRNA encoding cold shock proteins in the cold [27,28]. It is known that the 5′-end of the stem loop structure is responsible for the stabilization of mRNA encoding cold shock proteins like Csp family proteins [29]. We found a sequence capable of forming a stable stem-loop structure proximal to the 5′ end of *cdsA* (Fig. 4A). To examine whether the stem loop structure stabilizes the *cdsA* transcript at low temperature, we compared the half-lives of the RT-PCR products amplified with F1/R1 (Fig. 4B) and F2/R1 (Fig. 4C), by means of rifampicin chase experiments. The half-life of the *cdsA* transcript amplified using the F1/R1 primer increased ~ 3-fold at 20 °C (7.8 min) as compared with at 37 °C (2.5 min) (Fig. 4B). The half-life of the transcript amplified with the F2/R1 primers also increased ~ 3-fold at 20 °C as with F1/R1 (Fig. 4C). These half-lives are comparable with that of the *tufA* transcript (the 20 °C/37 °C ratio is ~ 3.5) (Fig. 4D). These results indicate that the stabilization of mRNA does not explain the increase in the *cdsA* transcript.

Discussion

In this article, we searched for the promoter regions of *cdsA* necessary for the cold-induction of MPIase. Contrary with the prediction, the upstream region of the *ispU-cdsA-rseP-bamA* operon, including *dxr*, is also involved in the control of expression of this operon. The transcript starting from promoter P5 was immediately induced upon cold exposure, but then was gradually attenuated similarly to the cold-shock response [29,30]. On the other hand, the level of the shorter transcript containing *cdsA* remained high throughout the cultivation in the cold. Promoter P3 for the operon was responsible for induction in the cold after the cold-shock response to keep the MPIase level high throughout the cold cultivation. Thus, the transcript containing *cdsA* is kept high through two-step induction in the cold. At an early stage after the temperature downshift, promoter P5 acts, and then promoter P3 begins to act so that the *cdsA* expression and therefore the MPIase expression is kept high in the cold. While *ynbB*, a paralogue of *cdsA* [12], is also involved

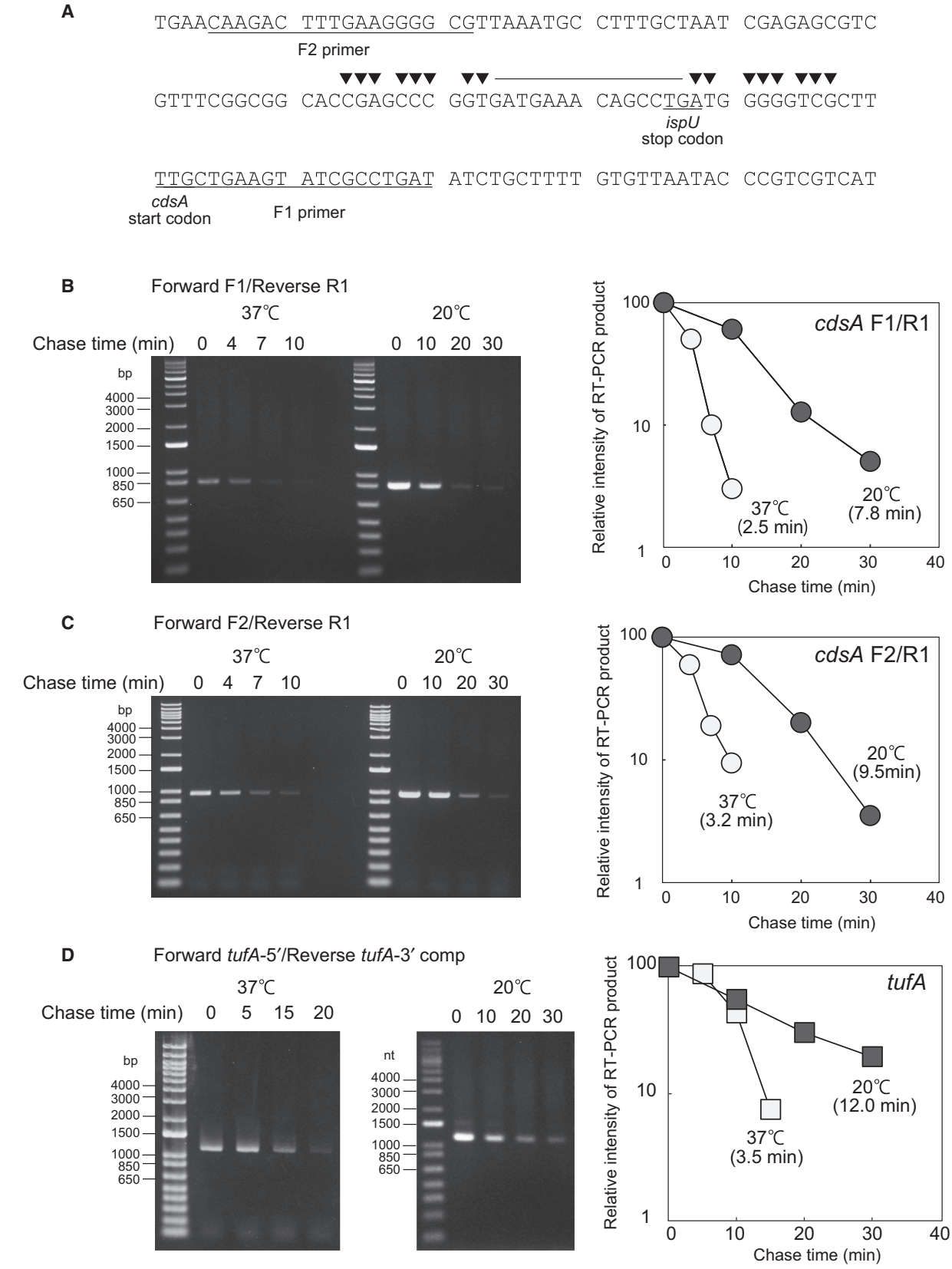
A
TGAACAAGAC TTTGAAGGGG CGTTAAATGC CTTTGCTAAT CGAGAGCGTC
F2 primer
GTTTCGGCGG CACCGAGCCC GGTGATGAAA CAGCCTGATG GGGGTCGCTT
ispU
stop codon
TTGCTGAAGT ATCGCCTGAT ATCTGCTTTT GTGTTAATAC CCGTCGTCAT
cdsA
start codon
F1 primer
B
Forward F1/Reverse R1
37℃
20℃
Chase time (min) 0 4 7 10 0 10 20 30
bp
4000
3000
2000
1500
1000
850
650
Relative intensity of RT-PCR product
cdsA F1/R1
37℃
(2.5 min)
20℃
(7.8 min)
Chase time (min)
C
Forward F2/Reverse R1
37℃
20℃
Chase time (min) 0 4 7 10 0 10 20 30
cdsA F2/R1
20℃
(9.5min)
37℃
(3.2 min)
D
Forward tufA-5′/Reverse tufA-3′ comp
37℃
20℃
Chase time (min) 0 5 15 20 0 10 20 30
nt
tufA
20℃
(12.0 min)
37℃
(3.5 min)

Fig. 4. Stabilization of the *cdsA* transcript is not responsible for its increase in the cold. (A) DNA sequence near the initiation codon of *cdsA*. The positions of primers F1 and F2 are underlined. A sequence capable of forming a stem-loop structure is indicated by a reverse triangle and a line respectively. (B, C, and D) EK413 cells were cultivated in LB medium to the mid-log phase and then treated with rifampicin (150 μg/ml). An aliquot of the culture (500 μL) was withdrawn at the specified times, followed by RNA extraction and RT-PCR using the primer pairs of F1/R1 (B), F2/R1 (C), and *tufA*-5′/*tufA*-3′comp (D). The levels of RT-PCR products were determined and plotted against time after the addition of rifampicin.

in the increase in the MPIase level in the cold, the effect is weak and marginal [8]. Therefore, the effect of *ynbB* is a backup of that of *cdsA*.

Recently, we demonstrated that the increase in the MPIase level in the cold is necessary for the efficient preprotein translocation in the cold [8], presumably because a higher level of MPIase is necessary to keep the SecYEG translocon in the active form in the cold [8,15]. Under cold conditions where the growth rate is slowed down, the rate of phospholipid biosynthesis seems not to be so high. Therefore, it is likely that the upregulation of *cdsA* is necessary only to increase the MPIase level. The unnecessary CDP-DAG synthesized by the overproduced amount of CdsA would be hydrolyzed by Cdh, a CDP-DAG hydrolase [31].

When cells are exposed to cold, a series of cold shock proteins are induced [30,32]. The induction of these proteins is transient and reduced to a new basal level [30,32]. In some cases, the transcript becomes very stable in the cold, forming stem-loop structures [32]. The transcript containing *cdsA* also possesses a region that can form a stem-loop structure near the initiation codon. However, unlike the cold-shock proteins, the half-lives of the transcripts were comparable to that of the cold-uninduced gene. Therefore, the cold induction of *cdsA* can be explained by the cold-inducible promoters discussed above.

Our results indicate that not only *cdsA* but also *dxr* and *ispU* increase at low temperature. Dxr is 1-deoxy-D-xylulose -5-phosphate reductoisomerase [33]. IspU is undecaprenyl diphosphate synthase [34,35]. Both are involved in undecaprenol biosynthesis as lipid carriers for cell wall carbohydrates, peptidoglycan, and enterobacterial common antigen (ECA) [36,37]. It is totally unknown why undecaprenol must be upregulated in the cold. Apart from its lipid carrier function, undecaprenol might be involved in the increase in the membrane fluidity in the cold, since undecaprenol contains unsaturated lipids.

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Author contributions

KS and KN designed and supervised the study; KS and YS performed experiments; KS and KN analyzed data and wrote the manuscript. All authors approved the manuscript.

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