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Ribosome-binding site interference caused by Shine–Dalgarno-like nucleotide sequences in *Escherichia coli* cells

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Two-cistronic expression plasmids are useful for high-level expression of heterologous genes in *Escherichia coli* cells by preventing the inhibition of translational initiation. In the process of constructing a two-cistronic expression plasmid pCbSTCR-4 containing the fragments of the porcine cytochrome b_5 (Psb5) and NADPH-cytochrome P450 reductase (PsCPR) genes as the first and second cistrons, respectively, the presence of a specific region in the first cistron that lowered the accumulation level of the *PsCPR* was suggested [Kimura, S., *et al.* (2005) *J. Biochem.* 137, 523–533]. In this study, a disturbing nucleotide sequence similar to a Shine–Dalgarno (SD) sequence (SD-like sequence), AGGAG, was identified at the 5′-upstream region near the SD sequence for the second cistron. Silent mutations in the SD-like sequence that lowered the similarity to a typical SD sequence increased the accumulation level of PsCPR. SD-like sequences introduced into mono-cistronic expression plasmids for the *Psb5* and *PsCPR* genes also decreased the accumulation level of these proteins. The SD-like sequence also decreased the accumulation level of the insoluble PsCPR protein. This type of ribosome-binding site interference is useful not only for precise control of protein accumulation but also for increasing the soluble form of recombinant proteins in *E. coli* cells.

Keywords: Gene expression/mRNA/ribosome-binding site/Shine–Dalgarno sequence/two-cistronic expression.

Abbreviations: CBB, Coomassie Brilliant Blue; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; Psb5, solubilized domain of porcine cytochrome b_5; PsCPR, solubilized domain of porcine NADPH-cytochrome P450 reductase; RBS, ribosome-binding site; SD, Shine–Dalgarno; SD1, SD sequence for the first cistron; SD2, SD sequence for the second cistron.

Heterologous gene expression is a necessary technique for the identification, characterization and *in vitro* studies of proteins. *Escherichia coli* is widely used as a host organism for the production of recombinant proteins, because of its fast growth, simple culture, uncomplicated nutritional and sterility requirements, and extensive characterization. The expression levels of heterologous genes in *E. coli* cells are affected by a number of factors, at both the transcriptional and translational levels (*1*). Various plasmid vectors for the over-expression of heterologous genes in *E. coli* cells have been constructed to contain a high-copy number origin of replication, strong promoters such as *tac* and *T7* promoters (*2*, *3*) and a ribosome-binding site (RBS), which includes the Shine–Dalgarno (SD) sequence and is complementarily recognized by the 3′-end region of the 16S rRNA in the 30S subunit of the ribosome, the anti-SD sequence, in *E. coli* (*4*). Although the maximum known length of the SD:anti-SD duplex is 12 or 13 nucleotides (*5*), the SD sequence in most *E. coli* genes is shorter. Despite the widespread use of heterologous gene expression, the target genes are not always highly expressed. One reason for the low level of gene expression with a conventional high-level expression plasmid is the formation of unfavourable intramolecular double-stranded structures of mRNA at the RBS (*6*). We had previously elucidated that the expression level of the wild-type solubilized domain of the porcine NADPH-cytochrome P450 reductase (PsCPR) gene (*PsCPR* gene) in *E. coli* cells, using expression plasmid pCW^{+}_{ori} (*7*,*8*), is markedly inhibited by the formation of a highly stable intramolecular double-strand structure of mRNA at the RBS (*9*). Recently, Kudla *et al.* clearly showed that the stability of mRNA folding near the ribosomal binding site, not codon bias, played a predominant role in shaping expression levels of individual genes (*10*). Therefore, a universal expression plasmid that enables high-level heterologous gene expression independent of the target genes, especially one that overcomes the formation of intramolecular secondary structure of mRNA, is required.

As an approach to prevent the formation of an unfavourable intramolecular double-stranded structure of mRNA at the RBS, a bacterial polycistronic gene expression system is used, as described by Schoner *et al.* (*11*–*13*). Many genes in bacteria are expressed using polycistronic mRNAs, in which each cistron has a RBS upstream of the initiation codon in the intercistronic region. When the termination codon of the first cistron is positioned near the SD sequence of the next cistron, translation proceeds sequentially through the cistrons (*14*). Since the ribosome prevents the

formation of an intramolecular double-stranded structure at the RBS for the second cistron, the second cistron can be translated without the formation of secondary structure at the RBS. Therefore, it is considered that a more universal high-level expression plasmid, in which gene expression is independent of the nucleotide sequences of heterologous genes, can be constructed by aligning a highly translatable gene as the first cistron and the target gene as the second cistron in order to co-translate both cistrons without complete ribosome dissociation. We constructed the two-cistronic expression plasmid pCbSTCR for co-expression of the *PsCPR* gene as the second cistron with the first cistron through prevention of the inhibition of translational initiation caused by the intramolecular local secondary structure at the RBS in mRNA (*15*). The first cistron in pCbSTCR contains the solubilized domain (Ala^{7}–Lys^{93}) of the porcine cytochrome b_5 (Psb5) gene (*Psb5* gene), which has additional nucleotides, GCTAAGGAGGTC**TAA** containing a SD sequence for the second cistron (SD2) (underlined) upstream of the TAA (bold) encoding the termination codon of the first cistron, at the 3′-terminal end. For convenience, plasmid pCbSTCR is hereafter designated pCTCR-93 in this paper, according to the number of the Psb5-derived last amino acid residue (Lys^{93}).

Schoner reported that the length of the first cistron should be kept to a minimum to avoid unnecessary expenditure of energy and precursors for the synthesis of the peptide it encodes (*13*). Therefore, we systematically reduced the length of the first cistron in pCTCR-93 to improve the accumulation level of PsCPR (*15*). Unexpectedly, we found that a shorter length of the first cistron did not always increase the accumulation level of the second cistronic gene product, although the deletion of the nucleotides encoding Ser^{22}–Gly^{45} in Psb5 largely increased the accumulation level of PsCPR (*15*), suggesting the presence of specific nucleotide(s) that lowered the accumulation level of the second cistronic gene product in the deleted region.

In this study, we describe the identification of a SD-like sequence that lowered the second cistronic gene product of PsCPR, and demonstrate that the accumulation level of the heterologous gene product can be changed by modulation of the SD-like sequence at the near-upstream region of the SD sequence not only in a two-cistronic expression system but also in a mono-cistronic expression system. In addition, we demonstrate that the modulation of protein accumulation using a SD-like sequence is useful for synthesizing heterologous protein in *E. coli* cells in the soluble form.

Materials and methods

Materials

Plasmid pCW^{+}_{ori} (*7*, *8*) was kindly provided by Dr F. W. Dahlquist of the University of Oregon. This plasmid contains two tandem *tac* promoters and an RBS, which is highly complementary to the 3′-end region of the 16S rRNA in the 30S subunit of the *E. coli* ribosome. *Escherichia coli* strain BL21 [F^{-} ompT $hsdS_B$ ($r_B^- m_B^-$) *gal dcm*] was from Novagen. The enzymes for recombinant DNA technology were from TaKaRa, Toyobo, New England BioLabs and Nippon Gene. The oligonucleotides used were synthesized by Sigma-Aldrich.

Construction of plasmids

Plasmids pCTCR-44, -43, -42, -41, -40, -39, -38, -33 and -27, in which the 3′-terminal region of the *Psb5* gene in pCbSTCR-3 (*15*) was systematically deleted, were constructed using polymerase chain reaction (PCR) as follows. The oligonucleotide primers used are tabulated in Table 1. For the construction of pCTCR-39, DNA fragments containing the shortened *Psb5* gene were amplified from pCbSTCR-3 (*15*), which is hereafter renamed pCTCR-45, using forward primer 5′-CCGGATCCATCGATGCTTAGG-3′ (primer 1) containing a *Bam*HI site (underlined) and two reverse primers: 3′-GCTAAACTGGTTTAAA*CGATTCCTCCAGAT*-5′ (primer 2) and 3′-*CGATTCCTCCAGAT*TGTATACTAACTCTG G-5′ (primer 3), in which italics and underlining indicate the common nucleotide sequence and an *Nde*I site, respectively. The resultant fragment was inserted into pCTCR-45 using the *Bam*HI and *Nde*I sites. Plasmids pCTCR-33 and -27 were constructed by the same method as that for the construction of pCTCR-39 except for the use of reverse primers 4 and 5, instead of primer 2, respectively. Plasmids pCTCR-44, -43, -42, -41, -40 and -38 were also constructed similarly using primers 6–11 instead of primer 2, respectively, and using pCPb5 (*15*, *16*) as a template instead of pCTCR-45.

Plasmids pCTCR42-GAG, -GGA, -GAA and -AAA, which have silent mutations in the region encoding $Leu^{40}Glu^{41}Glu^{42}$ in the plasmid pCTCR-42, were constructed as follows. For the construction of pCTCR42-GAG, DNA fragments were amplified from pCTCR-42 using primer-1 containing a *Bam*HI site and two reverse primers, primer-3 containing an *Nde*I site and 3′-GGTTTAAAAACC**TT**CT C*CGATTCCTCCAGA*-5′ (primer 12), which has a mutagenic nucleotide (bold) and a nucleotide sequence (italic) in common with primer 3. The resultant fragment was inserted into pCTCR-45 using the *Bam*HI and *Nde*I sites. Plasmids pCTCR42-GGA, -GAA and -AAA were constructed by the same method as that for the construction of pCTCR42-GAG except for the use of mutagenic reverse primers 13–15, which have mutagenic nucleotides, instead of primer 12, respectively.

Plasmids pCTCR-41-GA, -AG and -AA, which have silent mutations in the region encoding $Leu^{40}Glu^{41}$ in pCTCR-41, were constructed as follows. For the construction of pCTCR-41-GA, DNA fragments were amplified from pCTCR-41 using primer-1 containing a *Bam*HI site and two reverse primers, 3′-ACTGGTTTAAAAACC **TT***CGATTCCTCCAGA*-5′ (primer 16), which has a mutagenic nucleotide (bold) and a nucleotide sequence (italic) in common with primer 3. The resultant fragment was inserted into pCTCR-45 using the *Bam*HI and *Nde*I sites. Plasmids pCTCR41-AG and pCTCR41-AA were constructed by the same method as that for the construction of pCTCR41-GG except for the use of reverse primers 17 and 18 containing mutagenic nucleotides, instead of primer 16, respectively.

Plasmids pCTCR-93-GAG, -GGA, -GAA and -AAA, which have silent mutations in the region encoding $Leu^{40}Glu^{41}Glu^{42}$ in pCTCR-93, were constructed as follows. For construction of pCTCR-93-GAG, two DNA fragments of the partial *Psb5* gene were obtained from pCTCR-93 using distinct primer pairs of primers 19 and 3, and primer 1 and mutagenic primer 20. The two purified PCR fragments were mixed and re-amplified using primers 1 and 3. The resulting secondary PCR product was inserted into pCTCR-21 using the *Bam*HI and *Nde*I sites. Plasmids pCTCR-93-GGA, -GAA and -AAA were constructed by the same method as that for the construction of pCTCR-93-GAG except for the use of primers 21–23 containing mutagenic nucleotides, instead of primer 20, respectively.

Plasmids pCTT-TCTA, -AGGA, -AGAA, -AAGA and -AAAA, in which mutations were introduced at the near-upstream region of the SD sequence of pCPsCPR-TT (*9*), were constructed as follows. For construction of pCTT-TCTA, DNA fragments were amplified from pCPsCPR-TT using reverse primer 24 and mutagenic forward primer 5′-CCGGATCCATCGATGCTAAGGAGGTCATAT-3′ (primer-25), which has a *Bam*HI site (underlined) and a mutagenic base (bold). The *Bam*HI–*Xho*I fragment of pCPsCPR-TT was replaced with the *Bam*HI–*Xho*I fragment of the resultant fragment to construct plasmid pCTT-TCTA. Plasmids pCTT-AGGA, -AGAA, -AAGA and -AAAA were constructed by the same method as that for the construction of pCTT-TCTA except for the use of reverse primers 26–29 instead of primer 25, respectively.

Table 1. Primers used in this study.

primer	sequence
1	5′-CCGGATCCATCGATGCTTAGG-3′
2	3′-GCTAAACTGGTTTAAA*CGATTCCTCCAGAT*-5′
3	3′-*CGATTCCTCCAGATT*GTATACTAACTCTGG-5′
4	3′-GGACGTGGTGTTTCAC*CGATTCCTCCAGAT*-5′
5	3′-GTTCTCGTGGACCGAT*CGATTCCTCCAGAT*-5′
6	3′-AAACCTCCTCGTAGGA*CGATTCCTCCAGAT*-5′
7	3′-TAAAAACCTCCTCGTA*CGATTCCTCCAGAT*-5′
8	3′-GTTTAAAAACCTCCTC*CGATTCCTCCAGAT*-5′
9	3′-CTGGTTTAAAAACCTC*CGATTCCTCCAGAT*-5′
10	3′-AAACTGGTTTAAAAAC*CGATTCCTCCAGAT*-5′
11	3′-CATGCTAAACTGGTTT*CGATTCCTCCAGAT*-5′
12	3′-GGTTTAAAAACC**T**TCTC*CGATTCCTCCAGA*-5′
13	3′-GGTTTAAAAACCTCCT**T***CGATTCCTCCAGA*-5′
14	3′-CTGGTTTAAAAACCT**T**CT**T***CGATTCCTCCAGA*-5′
15	3′-CTGGTTTAAAAA**T**CT**T**CT**T***CGATTCCTCCAGA*-5′
16	3′-ACTGGTTTAAAAACCT**T***CGATTCCTCCAGA*-5′
17	3′-ACTGGTTTAAAAA**T**CTC*CGATTCCTCCAGA*-5′
18	3′-ACTGGTTTAAAAA**T**CT**T***CGATTCCTCCAGA*-5′
19	5′-CATCCTGGTGGGGAAGAAGTC-3′
20	3′-GTTTAAAAACCT**T**CTCGTAGGACCACCCCT-5′
21	3′-GTTTAAAAACCTCCT**T**GTAGGACCACCCCT-5′
22	3′-GTTTAAAAACCT**T**CT**T**GTAGGACCACCCCT-5′
23	3′-GTTTAAAAA**T**CT**T**CT**T**GTAGGACCACCCCT-5′
24	3′-CTCATTAGGCGGGCTCAGATCTGC-5′
25	5′-CCGGATCCATCGATGCT**A**AGGAGGTCATAT-3′
26	5′-CCGGATCCA**AG**GA**G**GCT**A**AGGAGGTCATAT-3′
27	5′-CCGGATCCA**AG**GA**A**GCT**A**AGGAGGTCATAT-3′
28	5′-CCGGATCCA**AA**GA**G**GCT**A**AGGAGGTCATAT-3′
29	5′-CCGGATCCA**AA**GA**A**GCT**A**AGGAGGTCATAT-3′

Underlines in the sequences of primers 1, 3 and 25–29 represent *Bam*HI, *Nde*I and *Bam*HI sites, respectively. The common sequence among primers 2–18 is shown in italic. Mutagenic nucleotides are shown in bold letters.

Plasmids pCb5-TT, pCb5-AGGA, -AGAA, -AAGA and -AAAA were constructed by replacing the *Nde*I–*Hin*dIII fragments of pCTT-TCTA, -AGGA, -AGAA, -AAGA and -AAAA with the *Nde*I–*Hin*dIII fragment of pCPb5 containing the *Psb5* gene, respectively.

The entire nucleotide sequences of the manipulated parts of the mutant plasmids were confirmed with ABI PRISM 310 or 3130 Genetic Analyzers.

Expression and analysis of protein accumulation

The heterologous genes in the expression plasmids were expressed in *E. coli* strain BL21. The cells containing expression plasmids were cultivated in a 2 × YT medium containing 50 μg ml^{-1} ampicillin with shaking at 37°C overnight, and then inoculated into the LB medium containing 50 μg ml^{-1} ampicillin at a ratio of 1/200 (v/v). Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM for the induction of gene expression after shaking at 37°C for 1.5 h. Protein accumulation in the cells was analysed by SDS–PAGE using 7.5% (w/v) and 15% (w/v) polyacrylamide gels of whole cell extracts for PsCPR and Psb5 as previously described, respectively (*9*,*15*). Proteins on the gels were stained with Coomassie Brilliant Blue (CBB) R-250. For distribution analysis of synthesized PsCPR, cells were harvested at relevant incubation times after induction with IPTG and were lysed on ice by sonication with a model 450 sonifier (Branson Ultrasonic). The lysate was subjected to centrifugation at 18,000 × *g* for 20 min. The supernatants were used as the intracellular soluble fractions of *E. coli* cells. The pellets were resuspended in 10 mM Tris–HCl containing 1 mM EDTA (pH 7.2) and used as the insoluble fractions of *E. coli* cells. The proteins in these fractions were analysed by SDS–PAGE as described above.

Results

Identification of the region that disturbed the accumulation of PsCPR

To roughly identify the region that disturbed the accumulation of PsCPR protein (second cistronic product of pCTCR-45), mutant plasmids pCTCR-39, -33 and -27 were constructed and the accumulation of PsCPR protein in *E. coli* cells was analysed (Fig. 1A). In pCTCR-39, -33, -27 and -21, the nucleotides in pCTCR-45 encoding Leu^{40}–Gly^{45}, Tyr^{34}–Gly^{45}, Ile^{28}–Gly^{45} and Ser^{22}–Gly^{45} of the *Psb5* gene were deleted, respectively. Here, plasmid pCbSTCR-4 (*15*) was renamed pCTCR-21. The band present in whole cell extracts of *E. coli* BL21/pCTCR-39 cells at the position of PsCPR on the gel was obviously thicker than that from BL21/pCTCR-45 after IPTG induction, while those of BL21/pCTCR-33, -27 and -21 were similar to that of BL21/pCTCR-39. These results suggest that the nucleotides encoding Leu^{40}–Gly^{45} contain a region that disturbs the expression level of the *PsCPR* gene.

Then the nucleotides encoding Leu^{40}–Gly^{45} were deleted from the 3′-side by three nucleotides to analyse the disturbing nucleotides in more detail (Fig. 1B). The bands of PsCPR from *E. coli* BL21/pCTCR-44, -43 and -42 cells were only slightly thinner or almost the same as that from BL21/pCTCR-45, while the thickness of the bands from BL21/pCTCR-41, -40 and -39 gradually increased in this order. Those from BL21/pCTCR-39 and -38 were almost the same. The accumulation level of PsCPR gradually increased with the deletion of nucleotides encoding Leu^{40}Glu^{41}Glu^{42}. The deletion of these nucleotides eliminated the SD-like sequence AGGAG in pCTCR-42, suggesting that this SD-like sequence disturbs the accumulation of the second cistronic product.

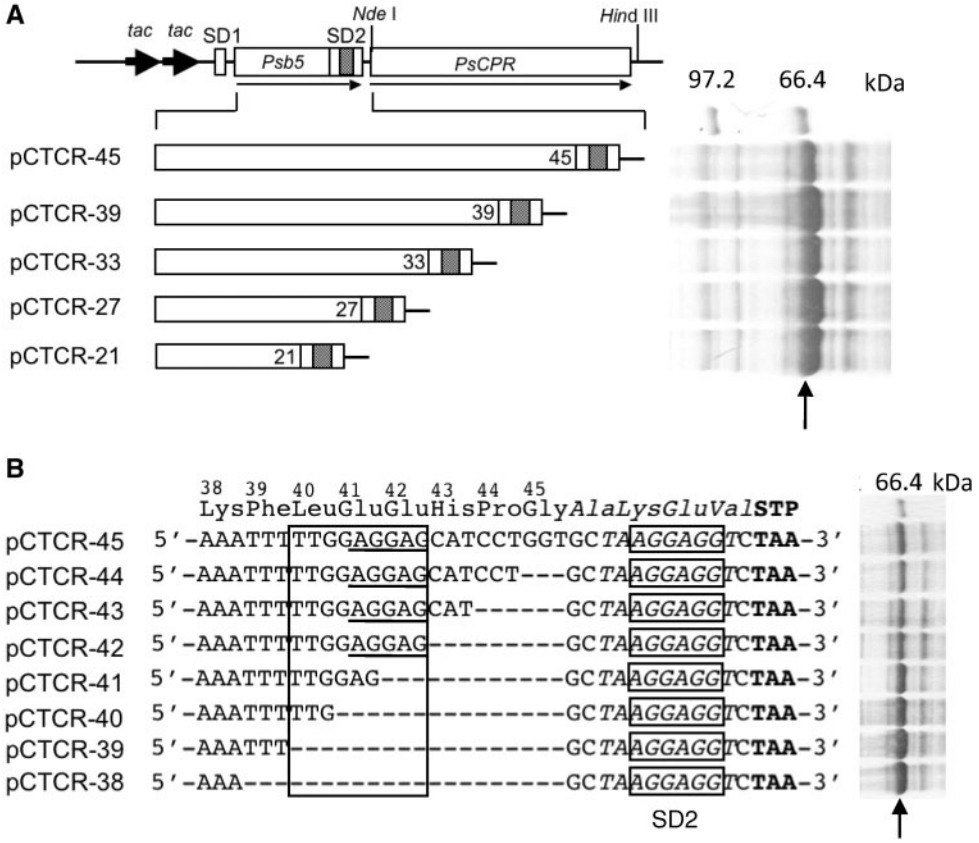

Fig. 1 Construction of plasmids carrying deletion mutations and SDS–PAGE of whole cell extracts of *E. coli* cells containing these plasmids. (A) Schematic representation of two-cistronic mutant plasmids that have systematic deletions of the nucleotides encoding Ser^{22}–Gly^{45} in the *Psb5* gene (left), and the SDS–PAGE gel of the whole cell extracts of *E. coli* BL21 cells containing the plasmids (right). The numbers in schematic bars are Psb5-derived last amino acid numbers. The number of amino acid residues starts from the N-terminal Ser of wild-type Psb5. SD1 (open box) and SD2 (shadowed box) are the SD sequences for the expression of the *Psb5* and *PsCPR* genes, respectively. (B) Partial nucleotide sequences of the pCTCR-45 and its mutant plasmids, in which the triplets encoding Leu^{39}–Gly^{45} were systematically deleted from the 3′-side (left), and a SDS–PAGE gel of the whole cell extracts of *E. coli* BL21 cells containing the plasmids (right). The hyphens (-) indicate the deletions of the nucleotides. The nucleotides encoding Leu^{40}–Glu^{42} are boxed. The underlined AGGAG is complementary to the 3′-terminus of *E. coli* 16S rRNA. STP in bold indicates the position of the nucleotides encoding the termination codon (TAA). The nucleotides that are complementary to the 3′-terminal region of *E. coli* 16S rRNA are shown in italics, and those encoding the hypothetical RBS (SD sequence) are boxed. Additional C-terminal amino acid residues (AlaLysGluVal) are shown in italics also. In both (A) and (B), proteins on the 7.5% (w/v) polyacrylamide gel were stained with CBB R-250. The arrows under the gels indicate the position of the bands of PsCPR.

Effects of mutations in the SD-like sequence in the two-cistronic expression plasmid on protein accumulation

As described above, it was suggested that the SD-like sequence in pCTCR-42 disturbs the accumulation of PsCPR. Therefore, plasmids pCTCR-42-GAG, -GGA, -GAA and -AAA, which have silent mutations in the region encoding Leu^{40}–Glu^{42} in pCTCR-42, and pCTCR-41-GA, -AG and -AA, which have silent mutations in the region encoding $Leu^{40}Glu^{41}$ in pCTCR-41, were constructed to analyse whether silent mutations in the SD-like sequence affect the accumulation level of the second cistronic product (Fig. 2). Although the thickness of the band at the position of PsCPR from *E. coli* BL21/pCTCR-42-GGA was similar to that from BL21/pCTCR-42, the other mutant plasmids gave obviously thicker bands than that from BL21/pCTCR-42 (Fig. 2A). The thickness of the bands from BL21/pCTCR-41, BL21/pCTCR-41-AG, -GA and -AA slightly increased in this order (Fig. 2B). Strains carrying plasmids containing the SD-like sequence, which has low homology with the SD2 sequence of AGGAGG, tended to accumulate a larger amount of PsCPR protein. These results clearly indicated that the SD-like sequence at the near-upstream region of the SD2-sequence of the two-cistronic plasmids disturbs the accumulation of the second cistronic product.

The accumulation level of the PsCPR protein in *E. coli* BL21 cells containing pCbSTCR (same as pCTCR-93 in this paper) was higher than that containing pCbSTCR-3 (same as pCTCR-45) but lower than that containing pCbSTCR-4 (same as pCTCR-21) (*15*). The SD-like sequence AGGAG in pCTCR-93 is positioned at approximately 150 bases upstream of SD2. Although the near-upstream region of the SD2 sequence of the two-cistronic plasmids disturbs the accumulation of the second cistronic product as described above (Fig. 2), the disturbing effect of the SD-like sequence in pCTCR-93, which is positioned at far-upstream of SD2, was unclear. To investigate whether the SD-like sequence at the far-upstream region of the SD2 sequence disturbs the accumulation of the second cistronic product, mutant plasmids of

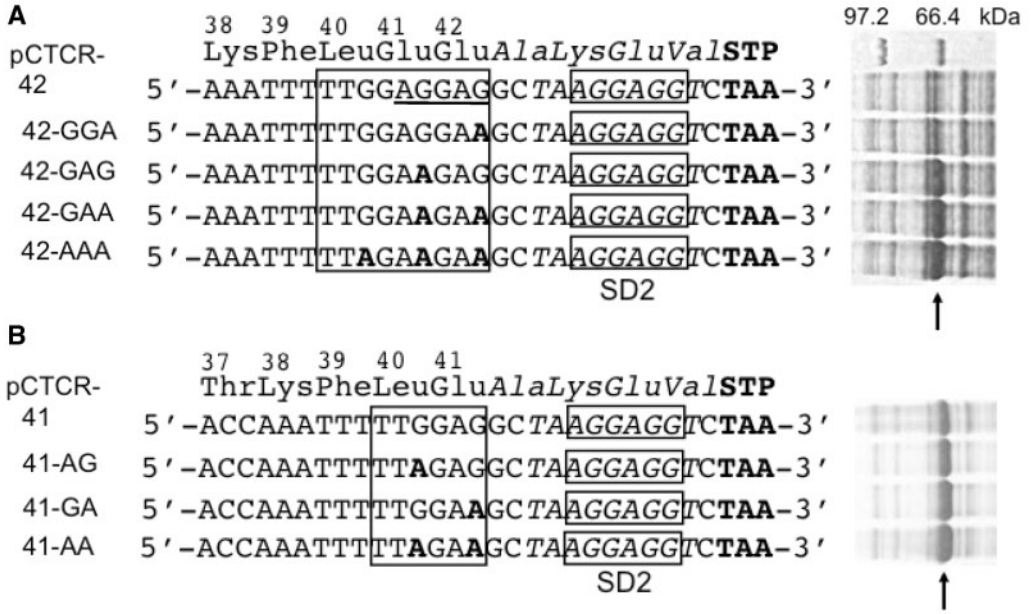

Fig. 2 Partial nucleotide sequences of pCTCR-42, pCTCR-41 and their mutant plasmids, which have silent mutations, and SDS–PAGE of whole cell extracts of *E. coli* cells containing these plasmids. (A) Mutant plasmid derived from pCTCR-42. The nucleotide region encoding Leu^{40}–Glu^{42} is boxed. (B) Mutant plasmid derived from pCTCR-41. The nucleotide region encoding $Leu^{40}Glu^{41}$ is boxed. In both (A) and (B), silent mutations are indicated in bold. The representation of the partial amino acid and nucleotide sequences are the same as those in the legend to Fig. 1B. Proteins on the 7.5% (w/v) polyacrylamide gel were stained with CBB R-250. The arrows indicate the positions of the bands of PsCPR.

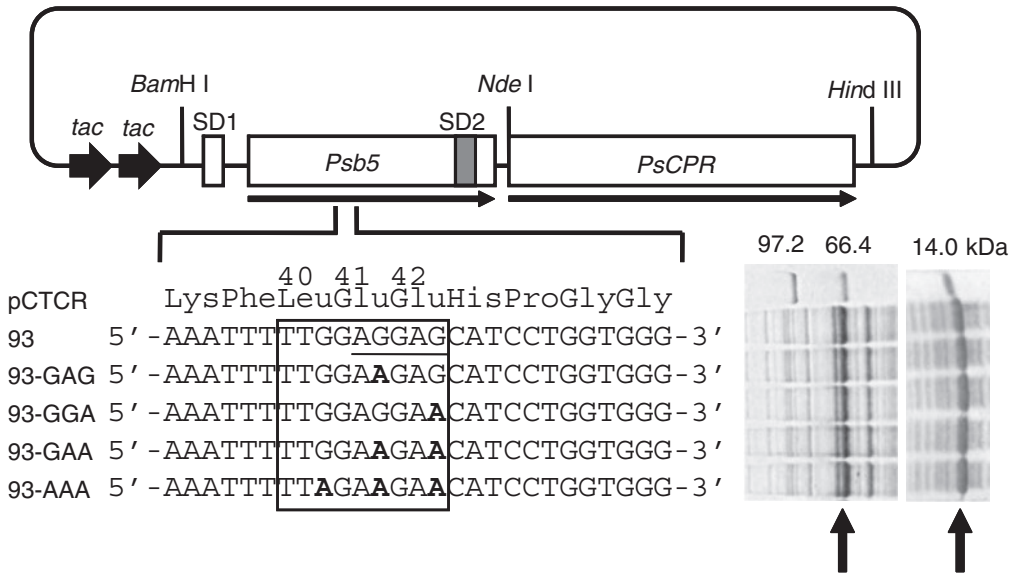

Fig. 3 Partial nucleotide sequences of pCTCR-93 and its mutant plasmids, which have silent mutations, and SDS–PAGE of whole cell extracts of *E. coli* cells containing these plasmids. The nucleotide region encoding Leu^{40}–Glu^{42} is boxed. Silent mutations are shown in bold. The underlined AGGAG is complementary to the 3′-terminus of *E. coli* 16S rRNA. Proteins on the gel were stained with CBB R-250. The polyacrylamide concentrations of gels for the analyses of PsCPR (left) and Psb5 (right) were 7.5% (w/v) and 15% (w/v), respectively. The left and right arrows indicate the positions of the bands of PsCPR and Psb5, respectively.

pCTCR-93, which have silent mutations in the region encoding $Leu^{40}Glu^{41}Glu^{42}$, were constructed (Fig. 3). The thicknesses of the bands at the position of PsCPR from *E. coli* BL21/pCTCR-93-GAG, -GGA, -GAA and -AAA were similar to that from BL21/pCTCR-93. The bands produced by these *E. coli* cells at the position of the product of the first cistron were also very similar. The distantly positioned SD-like sequence from SD2 in pCTCR-93 minimally disturbed the accumulation of both the first and second cistronic products. This result indicates that the lower accumulation level of PsCPR protein in *E. coli* BL21/pCTCR-93 cells cannot be attributed to the SD-like sequence. Another nucleotide region that affects the accumulation level of PsCPR may exist in the deleted regions of pCTCR-93.

Introduction of SD-like sequences in mono-cistronic expression plasmids

In order to analyse whether a SD-like sequence positioned at the near-upstream region of the SD sequence also affects the accumulation level of the heterologous

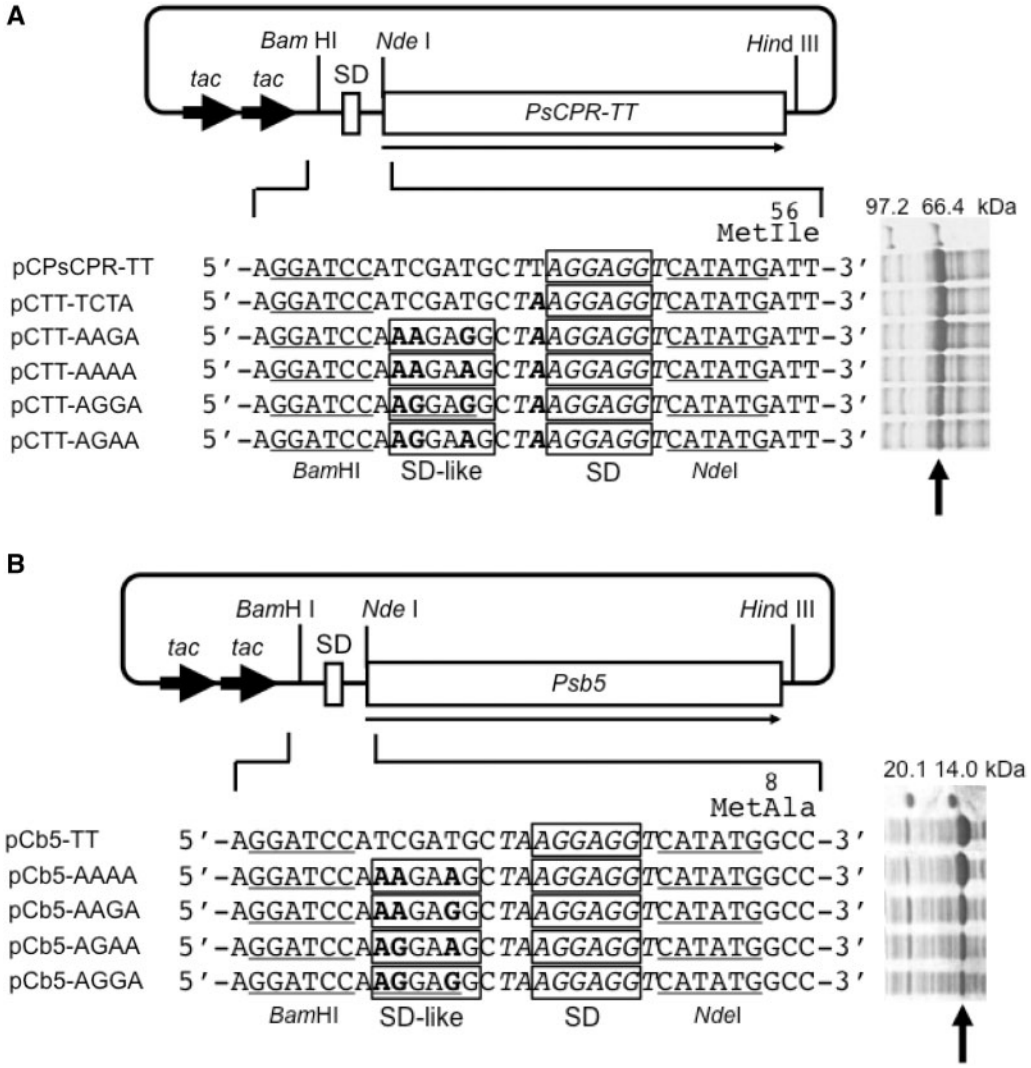

Fig. 4 Mono-cistronic expression plasmids for PsCPR and Psb5, and effects of the introduction of SD-like sequences on protein accumulation in *E. coli* cells. (A) Plasmid pCPsCPR-TT and its mutant plasmids (left), and 7.5% (w/v) SDS–PAGE gel of whole cell extracts of *E. coli* cells containing these plasmids (right). The arrow under the gel indicates the position of the band of PsCPR. (B) Mono-cistronic plasmid pCPsCPR-TT and its mutants (left), and SDS–PAGE gel of whole cell extracts of *E. coli* cells containing these plasmids using a 15% (w/v) polyacrylamide gel. The mutated nucleotides are shown in bold. The arrow under the gel indicates the position of the band of Psb5. In both (A) and (B), proteins on the gel were stained with CBB R-250. The nucleotides that are complementary to the 3′-terminal region of *E. coli* 16S rRNA are shown in italics. The SD-like sequence and native SD-sequence are boxed. The underlined AGGAGs in the SD-like sequences are complementary to the 3′-terminus of *E. coli* 16S rRNA. *Bam*HI and *Nde*I sites are also underlined.

protein in a mono-cistronic expression system, SD-like sequences were introduced into the untranslated region at the near-upstream region of the SD sequence in the plasmid pCPsCPR-TT (Fig. 4A). pCPsCPR-TT is a mono-cistronic high-level expression plasmid for PsCPR, which was constructed in the process of introducing silent mutations into the N-terminal region of the wild-type *PsCPR* gene to prevent translational initiation inhibition caused by the local secondary structure at the RBS in the mRNA (*9*). At first, only one nucleotide substitution of T to A was introduced into the region just upstream of the SD sequence to make the nucleotide sequence in this region completely complementary to the 3′-end region of the 16S rRNA in the 30S subunit of the *E. coli* ribosome. The thickness of the band at the position of PsCPR from cells containing pCTT-TCTA, which has a T to A mutation, was almost identical to that from *E. coli* BL21/pCPsCPR-TT, indicating that this single nucleotide substitution did not affect the accumulation level. The band from BL21/pCTT-AAGA was similar to that from BL21/pCTT-TCTA. The bands shown by BL21/pCTT-AAAA, -AGGA and -AGAA were obviously thinner than that produced by BL21/pCTT-TCTA. In particular, BL21/pCTT-AGGA and -AGAA, which have plasmids containing the additional SD-like sequences AGGAGG and AGGAAG upstream of the original SD sequence, respectively, had significantly decreased band thickness. These results indicate that the SD-like sequences positioned in the near-upstream region of the SD sequence also disturbed the accumulation of the PsCPR protein in the mono-cistronic expression plasmid.

To confirm whether the accumulation of other heterologous proteins besides the PsCPR protein is disturbed by the SD-like sequences in a mono-cistronic

expression plasmid, plasmids pCb5-TT, pCb5-AAAA, -AAGA, -AGAA and -AGGA, which are derivatives of the mono-cistronic expression plasmid pCPb5 containing the *Psb5* gene (*16*), were constructed (Fig. 4B). *E. coli* BL21 cells containing plasmid pCb5-AAAA, which has the less similar SD-like sequence, produced thick bands like that from the strain containing plasmid pCb5, indicating that the SD-like sequences disturb not only the accumulation of the PsCPR protein but also that of the Psb5 protein. However, the effects of the AAGAAG sequences in pCTT-AAAA and pCb5-AAAA were different. The AAGAAG in pCTT-AAAA decreased the thickness of the band at the position of PsCPR (Fig. 4A), while that in pCb5-AAAA did not decrease that at the position of Psb5 (Fig. 4B). Since the AAGAAG at near-upstream of the SD sequence in the mRNA derived from pCTT-AAAA is complementary to the CUUCUU in the ACUUCUUCC, which encodes $Thr^{60}Ser^{61}Ser^{62}$ in PsCPR, the formation of the intramolecular double-stranded structure of mRNA may disturb the effective complementary recognition of the SD sequence by 30S ribosomal subunit and cause the decrease in the accumulation level of PsCPR protein through the prevention of translational initiation. Another difference was the effect of the AAGAGG. Introduced AAGAGG in pCb5-AAGA decreased the thickness of the band at the position of Psb5 (Fig. 4B), while that in pCTT-AAGA did not decrease the band at the position of PsCPR. The reason of this difference remains to be clarified.

Effect of a SD-like sequence on the accumulation levels of PsCPR protein in the soluble and insoluble fractions of E. coli *cells*

Formation of inclusion bodies is often observed when heterologous genes are over-expressed in *E. coli* cells. Therefore, the distributions of the synthesized PsCPR protein in the soluble and insoluble fractions of *E. coli* BL21 cells containing pCTT-TCTA, -AAGA, -AAAA, -AGGA and -AGAA were analysed (Fig. 5). In these strains, the thickness of the band at the position of PsCPR in the whole cell extract increased until 10 h after the addition of IPTG and decreased between 24 h and 36 h (data not shown), although the bands that were produced by *E. coli* BL21/pCTT-AAAA, -AGGA and -AGAA cells were less thick than those from *E. coli* BL21/pCTT-TCTA and -AAGA (Fig. 5A). The distributions of the synthesized PsCPR protein at 24 h after the addition of IPTG are shown in Figs. 5B and C. In *E. coli* BL21/pCTT-TCTA and -AAGA cells, the bands in the insoluble fraction were much thicker than those in the soluble fraction, indicating that large amounts of PsCPR accumulated in an insoluble form (Fig. 5B and C, lanes 3 and 4). However, for the *E. coli* cells containing pCTT-AAAA, -AGGA and -AGAA, the bands in the insoluble fractions (Fig. 5C, lanes 5–7) were similar or less thick than those in the soluble fractions (Fig. 5B, lanes 5–7). Protein amounts in the soluble fractions (Fig. 5B, lanes 5–7) were similar to those produced by the other two *E. coli* strains (Fig. 5B, lanes 3 and 4). The amounts of PsCPR protein in the

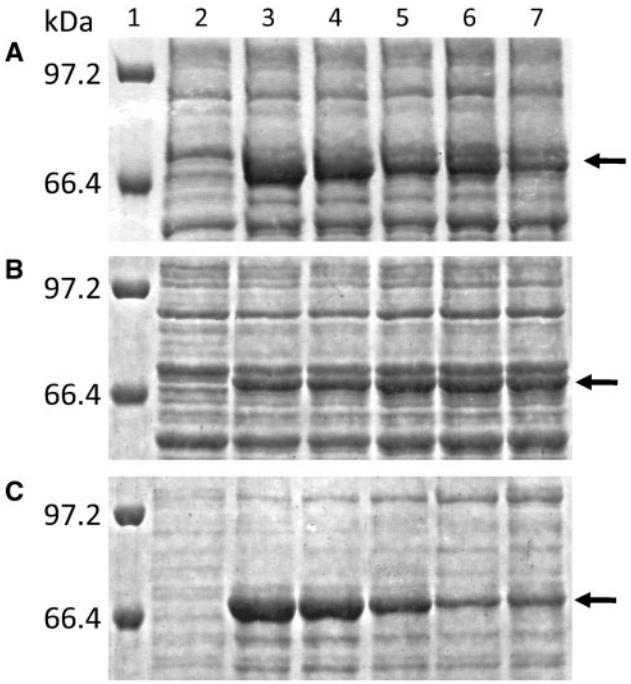

Fig. 5 Distribution analysis of synthesized PsCPR in *E. coli* cell extracts by SDS–PAGE. The SDS–PAGE gels of the whole cell extracts (A), soluble fraction (B) and insoluble fraction (C) of *E. coli* BL21 cells containing pCW^{+}_{ori} (lane 2), pCTT-TCTA (lane 3), pCTT-AAGA (lane 4), pCTT-AAAA (lane 5), pCTT-AGGA (lane 6) and pCTT-AGAA (lane 7), and marker protein (lane 1) are shown. The arrows indicate the positions of the PsCPR bands. The *E. coli* cells were cultivated for 24 h at 37°C after the addition of 0.2 mM IPTG. Proteins from the same amounts of cells were analysed. The amount of cells was adjusted using the absorbance of the culture fluid at 600 nm.

soluble fractions from *E. coli* BL21/pCTT-TCTA and -AGAA cells, which were estimated by the thickness of the bands on the gels, were ~20 and 60% of the total amounts of accumulated PsCPR protein, respectively. These results indicate that the SD-like sequence, which decreased the overall accumulation level of PsCPR, is effective in decreasing the insoluble form of PsCPR protein.

Discussion

In this study, we investigated a nucleotide region in the first cistron of a two-cistronic expression plasmid that caused a decrease in the accumulation level of PsCPR (the product of the second cistron), by systematic deletions of nucleotides (Fig. 1). From these analyses, we found that the presence of a SD-like sequence in the region significantly affected the accumulation level (Fig. 2). In the two-cistronic expression plasmid, the SD-like sequences positioned at the near-upstream region of the SD sequence disturbed the accumulation level of PsCPR, but the SD-like sequences at the far-upstream region of the SD sequence minimally disturbed the accumulation level of both the first and second cistronic products (Fig. 3). The SD-like sequences positioned at the near-upstream region of the SD sequence in mono-cistronic expression plasmids for PsCPR and Psb5 also affected protein accumulation (Fig. 4). The more similar the nucleotide sequence of the SD-like region was to the typical SD sequence, which is complementarily recognized by the 3′-end region of the 16S rRNA in the 30S subunit of the *E. coli* ribosome, the roughly larger the disturbing effects were (Figs. 1, 2 and 4).

Although the disturbing effects of SD-like sequences on gene expression at the translation level were reported previously (*17*, *18*), the mechanism causing these effects has not been clearly elucidated. However, it is undoubtedly due to the complementary misrecognition of the SD-like sequence in the mRNA by the 3′-end region of the 16S rRNA in the 30S subunit of the *E. coli* ribosome, which causes translational initiation inhibition. A possible mechanism for translational expression inhibition by the SD-like sequence is considered below.

In mono-cistronic mRNA that has a SD-like sequence at the near-upstream region of the native SD sequence, such as those from pCTT-AAAA, -AGGA, -AGAA, pCb5-AGAA, -AAGA and -AGGA, the 30S ribosomal subunit can bind to either the SD-like sequence or the native SD sequence together with IF-3, IF-2GTPfMet–tRNA^{fMet} and IF-1 (Fig. 6A-a). The 30S subunit, which binds to the native SD sequence positioned at an optimal distance from the initiation codon (AUG), is joined by the 50S subunit to initiate effective translation (*19*) (Fig. 6A-b). When the 30S subunit binds to the SD-like sequence, inefficient formation of the 70S initiation complex with the 50S subunit occurs because of the inappropriate distance between the SD-like sequence and the initiation codon (Fig. 6A-c). It is well known that the effectiveness of a SD sequence is generally determined both by its base-pairing potential with the anti-SD sequence in 16S rRNA and its spacing from the start codon (*20*, *21*). Aligned spacing of the SD sequences generally varies from 5 to 13 bases, with optimal spacing of ~8–10 bases for *E. coli* genes (*22*, *23*). However, when the 30S ribosomal subunit binds to a SD-like sequence that is positioned more than 13 bases upstream from an initiation codon as in the mono-cistronic mRNAs described above, fMet-tRNA^{fMet} cannot be positioned correctly in the P-site of the initiation complex with the start codon. Moreover, once a 30S subunit binds to a SD-like sequence that is located within approximately 35 bases of the native SD-sequence, binding of another 30S subunit molecule on the SD-sequence is blocked by steric hindrance between the two 30S subunit molecules, since a single ribosome can span 35 bases on the mRNA (*24*) (Fig. 6A-c). As a result, the efficiency of translational initiation is decreased. This competitive mechanism is consistent with the previous reports (*17*, *18*). Alexandrova *et al*. observed that the translational expression of a chloramphenicol acetyltransferase reporter gene was inhibited by in-frame and out-of-frame SD-like sequences in a mono-cistronic expression system (*17*). Jin *et al*. reported that a significant negative effect on the expression of a peptide fragment of the *Staphylococcus aureus* protein A gene product was observed when the SD-like sequence was placed either 15 bases upstream or 15 bases downstream of the initiation codon, and that the negative effect caused by the SD-like sequence at the upstream site was weakened when the distance between the SD-like sequence and the initiation codon was increased from 15 to 39 bases (*18*). Since reduced ribosome binding to mRNA by inefficient translational initiation increases mRNA exposure to nuclease digestion (*25*, *26*), the instability of the mRNA also decreases the expression level.

In the case of a two-cistronic expression system such as pCTCR-45, -44, -43, -42, pCTCR-42-GGA and -AAA (Figs. 1, 2 and 6B-a), two main pathways of translational initiation inhibition of the second cistron are considered (Fig. 6B). One is *de novo* initiation inhibition (Fig. 6B-b and c). In this pathway, a recycled 30S subunit, which is supplied by the dissociation of the 70S ribosome with a termination factor (RF-1, 2 or 3) and a ribosome-recycling factor (RRF) at the termination codon of the first cistron, newly misrecognizes the SD-like sequence at the near-upstream region of SD2, resulting in translational initiation inhibition as in the above-mentioned case of translational initiation inhibition of mono-cistronic mRNA. The other is re-initiation inhibition by a 30S subunit and/or 70S ribosome that remained on the mRNA after translational termination of the first cistron (Fig. 6B-b, d and e). The attached ribosome can scan the mRNA in a bi-directional manner. It is known that the scanning ribosome can re-initiate translation at a nearby initiation codon of a downstream cistron that is positioned a few nucleotides away or overlapping with the termination codon of the preceding cistron (*27*). Martin and Webster suggested that both the 30S subunit and the 70S ribosome are involved in translational re-initiation (*28*). Yoo and

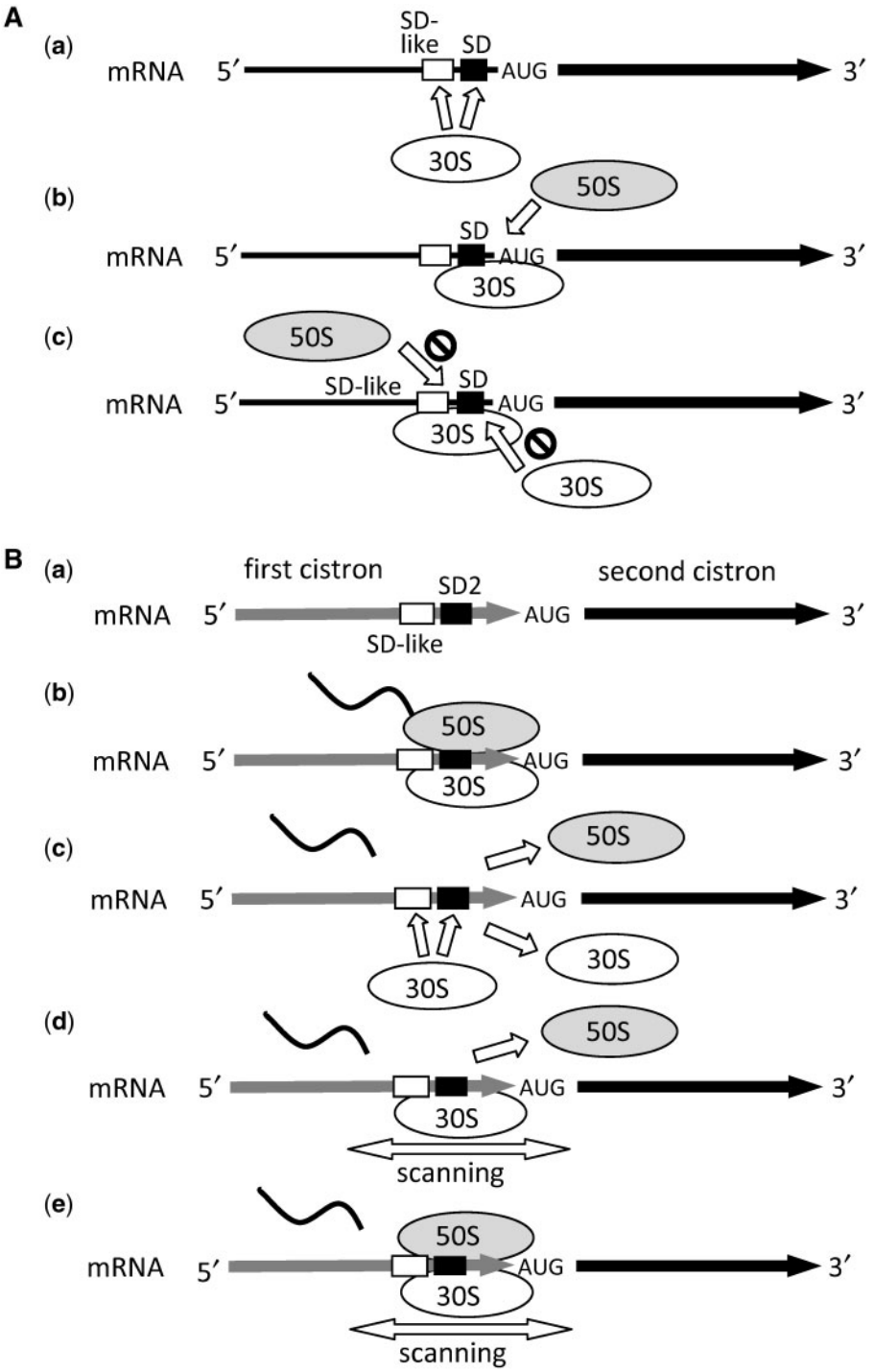

Fig. 6 Hypothetical models of translational initiation by RBS-interference. (A) RBS-interference on mono-cistronic mRNA. (a) A 30S ribosome binds to either the SD-like sequence (open box) or native SD sequence (closed box) for the expression of the structural gene (closed arrow), which starts from the initiation codon (AUG). (b) When a 30S ribosomal subunit (open ellipse) binds to a native SD sequence that is located a suitable distance from the initiation codon, a 70S initiation complex is formed by binding of the 50S ribosomal subunit (shadowed ellipse), and translational initiation occurs efficiently. (c) When a 30S subunit binds to a SD-like sequence located at an inappropriate distance from the initiation codon, the formation of a 70S initiation complex does not occur. Other 30S subunits cannot bind to the native SD sequence because of the steric hindrance of the bound 30S subunit molecule. As a result, efficient translational initiation is disturbed. (B) RBS-interference on two-cistronic mRNA. (a) Two-cistronic mRNA, which consists of a structural gene (first cistron; shadowed arrow) containing a SD-like sequence (open box), a SD sequence (SD2; closed box) for the expression of a structural gene (second cistron) and the termination codons of the first cistron (shadowed arrow) and the second cistron (closed arrow), which start from the initiation codon (AUG). When both the SD-like and SD sequence (SD2) are accessible to the 30S ribosomal subunit, the same type of RBS-interference as that on mono-cistronic mRNA shown in (A) occurs. (b) Recognition of the termination codon by the 70S ribosome complex, which has translated the first cistron. The curved line on the 50S subunit represents the synthesized polypeptide of the first cistron. (c) After the complete dissociation of the polypeptide and the 70S ribosomal complex from mRNA, *de novo* binding of a 30S ribosomal subunit to mRNA occurs. In this case, *de novo* initiation inhibition occurs, since the same RBS-interference as that on the mono-cistronic mRNA shown in (A) occurs. (d) Re-initiation inhibition by the 30S subunit after dissociation of the 50S subunit. (e) Re-initiation inhibition by the 70S complex after translational termination of the first cistron. In (d) and (e), misrecognition of the SD-like sequence caused by scanning results in translational initiation inhibition of the second cistron.

RajBhandary described that accurate translational re-initiation would begin with a 30S ribosome-mRNA complex (*29*). Although it is unclear whether scanning is carried by the 30S subunit or the 70S ribosome, the misrecognition of a SD-like sequence during scanning causes re-initiation inhibition (Fig. 6B-d and e). However, further study of the inhibition mechanism at the junction of two cistrons is required.

The silent mutations introduced into the SD-like sequence in the first cistron, which is positioned far (~150 bases) upstream of SD2 did not decrease the accumulation level of either Psb5 or PsCPR (Fig. 3). Since the accumulation level of the first cistronic product, Psb5, was high, the SD-like sequence region is probably covered by the actively translating 70S ribosome. Once the codons at the SD-like sequence are translated, a new 30S subunit no longer has access to the SD-like sequence, because the continuously moving ribosomes shield the SD-like sequence. Even if a 30S ribosomal subunit could bind to the SD-like sequence, it would not be easy for the resulting mRNA–30S complex to find the distant SD2 and its associated initiation codon. The weakly bound 30S subunit on the mRNA may be removed by the actively translating 70S ribosome, which comes from the upstream mRNA. Recently, Wen *et al*. (*25*) reported that internal SD-like sequences tend to slightly pause translational elongation, presumably by binding to 16S rRNA in the 30S subunit of the active 70S ribosome. It is likely that this pausing effect of the 70S ribosome on the mRNA is not critical *in vivo* during protein synthesis.

The significant inhibitory effect of SD-like sequences located at the near-upstream region of the native SD-sequence is of interest for the design of novel techniques to control the production of recombinant proteins. The mutation or elimination of unfavourable SD-like sequences and the introduction of SD-like sequences can cause a change in the accumulation level of heterologous proteins. The protein accumulation level can be changed by modulating the nucleotide sequence of the SD-like sequence and by changing its position relative to the native SD sequence. The more complementary a SD-like sequence is to the anti-SD sequence, the larger the inhibitory effect is expected to be. This 'ribosome-binding site interference' (RBS interference) is considered to be useful for precise modulation of protein accumulation of heterologous genes.

The modulation of protein accumulation using SD-like sequences is important not only for controlling the protein accumulation level but also for the production of correctly folded recombinant proteins. Rapidly synthesized heterologous recombinant proteins often accumulate as insoluble aggregates (inclusion bodies) in *E. coli* cells. In order to prevent the formation of inclusion bodies, cultivation at lower temperatures has been employed as a way to slow the rate of protein synthesis (*30*). In this study, the amount of insoluble PsCPR protein was successfully decreased at 37°C by using an additional SD-like sequence (Fig. 5). RBS interference is expected to be a new technique for preventing the formation of inclusion bodies without the need to lower the cultivation temperature.

In conclusion, we demonstrated here that the protein accumulation levels of heterologous genes can be modulated by the use of SD-like sequences upstream of the SD sequence. RBS interference is expected to be useful for the construction of improved expression systems for heterologous genes in *E. coli* cells.

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Conflict of interest

None declared.

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