The *rpoH* gene encoding heat shock sigma factor σ^{32} of psychrophilic bacterium *Colwellia maris*

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Abstract

The *rpoH* gene encoding a heat shock sigma factor, σ^{32}, was cloned from the psychrophilic bacterium *Colwellia maris*. The deduced amino acid sequence of σ^{32} from *C. maris* is more than 60% homologous to that of σ^{32} from mesophilic bacteria. The RpoH box, a 9-amino-acid sequence region (QRKLFFNLR) specific to σ^{32}, and two downstream box sequences complementary to a part of 16S rRNA were identified. Primer extension analysis showed that the *C. maris rpoH* is expressed from only one σ^{70}-type promoter. Northern blot analysis showed that the level of *rpoH* mRNA was clearly increased at 20^{o}C, a temperature that induces heat shock in this organism. In the presence of an inhibitor of transcriptional initiation, the degradation of *rpoH* mRNA was much slower at 20^{o}C than at 10^{o}C. Thus, increased stability of the *rpoH* mRNA might be responsible for the *rpoH* mRNA accumulation. The predicted secondary structure of the 5'-region of *C. maris rpoH* mRNA was different from the conserved patterns reported for most mesophilic bacteria, and the base pairing of the downstream boxes appeared to be less stable than that of *E. colii rpoH* mRNA. Thus, essential features that ensure the HSP expression at relatively low temperature are embedded in the *rpoH* gene of psychrophiles.

Introduction

All organisms synthesize a set of highly conserved proteins called heat-shock proteins (HSPs) in response to changes in the external environment, such as high temperature or other stresses (Parsell and Lindquist 1993; Yura and Nakahigashi 1999). These proteins include the molecular chaperones, such as GroES, GroEL, DnaK, and DnaJ, which help maintaining proper folding of cellular proteins, and proteases, such as Lon, Clp, and FtsH, which act to degrade denatured proteins (Walter and Buchner 2002; Hengge and Bukau 2003). Although various types of environmental stress promote the expression of HSP genes, high temperature is a major factor in the induction of these genes. Because each organism has its own optimal growth temperature, the temperature range for the induction of HSP genes varies depending on the organism. For example, the optimal temperature of the mesophilic bacterium *Escherichia coli* is 37^{o}C and HSPs are induced at 42^{o}C (Bukau 1993), while the optimal temperature of the thermophilic bacterium *Thermus thermophilus* is 75^{o}C and HSPs are induced at 85^{o}C (Osipiuk and Joachimiak 1997). Thus, the mechanism of the regulation of HSP expression should be modulated according to the optimal temperatures of individual organisms.

Colwellia maris strain ABE-1 (JCM 10085) is a psychrophilic bacterium that grows at 10-15^{o}C, and its lethal temperature is about 25^{o}C (Yumoto et al. 1998). In this bacterium, relatively low temperature, such as 20^{o}C, causes high-temperature stress, whereas these temperatures induce cold-shock responses in most mesophilic organisms (Yamanaka 1999; Sakamoto et al. 1997). Recently, we cloned the *groESL*, *dnaK* and *dnaJ* genes from *C. maris* (Yamauchi et al. 2003 and 2004). The GroES, GroEL, DnaK, and DnaJ proteins of *C. maris* were found to be evolutionarily conserved with those of mesophilic and thermophilic bacteria, and the levels of the mRNAs encoding these proteins increased at much lower temperatures than those of the corresponding mRNAs .of mesophilic bacteria. These results indicate that psychrophiles, like other bacteria, respond to high-temperature stress by producing HSPs.

In *E. coli*, HSP expression is positively controlled at the transcriptional step by the product of the

rpoH gene, the heat shock promoter-specific σ^{32} subunit of RNA polymerase (Arsene et al. 2000). The HSP expression in *E. coli* is induced as a consequence of a rapid increase of the stability and of the synthesis of σ^{32}. The DnaK chaperone system and several proteases, mainly FtsH, an ATP-dependent metallo-protease associated with the inner membrane, are among the major regulatory factors of σ^{32} instability under optimal growth conditions (Tatsuta et al. 1998). The DnaK chaperone system inactivates σ^{32} by direct association and mediates its degradation by proteases (Tatsuta et al. 2000). σ^{32} synthesis is also regulated by the secondary structure of the 5' region of *rpoH* mRNA (Morita et al. 1999a and 1999b). In this case, the base pairing must be strong enough to suppress the translation step at low temperature, whereas it must be weak enough to allow the induction of translation at the elevated temperature.

Although many reports have described the mechanisms of the regulation of HSP expression of mesophilic and thermophilic bacteria (Segal and Ron 1996), little is known about the mechanism of the response of psychrophilic bacteria to high-temperature stress. In *C. maris*, a putative σ^{32} promoter is located in the upstream region of the *groES* and *dnaK* genes, as is often the case in mesophilic bacteria, such as *E. coli* (Yamauchi et al. 2003, 2004). These results indicate that the synthesis of HSPs is regulated by the σ^{32} system in *C. maris*, as in several mesophilic bacteria. Accordingly, studying the regulatory mechanism of σ^{32} synthesis is important for understanding the molecular mechanism that regulates HSP expression in psychrophiles.

In this study, we cloned the gene for the σ^{32} homologue from *C. maris*, and compared its deduced amino acid sequence and the nucleotide sequence in its promoter region with those of mesophilic bacteria. Furthermore, we analyzed the accumulation of *rpoH* mRNA and the predicted secondary structure of the *C. maris rpoH* mRNA to identify the factor which controls σ^{32} expression.

Materials and methods

Bacteria and culture conditions

The psychrophilic bacterium *C. maris* strain ABE-1 (JCM 10085) was cultured at 10^{o}C for 3 days in nutrient medium containing 1% Bacto peptone, 1% meat extract and 0.5 M NaCl (Ochiai et al. 1979). *Escherichia coli* JM109 was grown at 37^{o}C in LB medium supplemented with 50 μg ml^{-1} ampicillin when required.

DNA preparation

The genomic DNA of *C. maris* was isolated and purified according to the method described by Williams (1988). Cells from 80-ml cultures were harvested by centrifugation at 1,800 g at 4^{o}C for 15 min. They were suspended in 5 ml of buffer containing 50 mM Tris-HCl pH 8.5, 50 mM NaCl, and 5 mM NaEDTA and were then treated with 7 mg ml^{-1} lysozyme at 37^{o}C for 45 min. The cells were disrupted by incubation with 0.8% N-lauroylsarcosine at 37^{o}C for 20 min. The lysate was extracted three times with phenol: chloroform: isoamylalcohol (25:24:1) and then DNA was precipitated with 99.5% ethanol. The DNA pellet was dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

Amplification of the *rpoH* fragment by PCR

Degenerate primers were designed to encode amino acid sequences conserved among 10 gram-negative-bacterial σ^{32} homologues. The nucleotide sequence of the sense primer, 5'-ATHGTNAARGTNGC-3' (FW), corresponding to amino acid residues 124 (I) to 128 (A) of *E. coli* σ^{32}, and the anti-sense primer, 5'- CATNGCRTTYTTYTC-3' (RV), corresponding to amino acid residues 271 (E) to 275 (M) of *E. coli* σ^{32}, were used for PCR. Amplification was carried out in a 50-μl reaction mixture containing 1×PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, each FW and RV primer at 250 μM, and 1.25 U of Taq DNA polymerase (Gibco BRL, Gaithersburg, Md., USA) in a DNA thermal cycler 480 (Applied Biosystems, Foster city, Calif., USA) for 30 cycles.

Cycling conditions were as follows: an initial 3 cycles of denaturation at 95°C for 1 min, annealing at 33°C for 1 min, and extension at 72°C for 1 min, followed by 27 cycles of incubation at 95°C for 1 min, 38°C for 1 min, and 72°C for 1 min. The amplified DNA fragment was cloned in a T-vector (Novagen, Darmstadt, Germany) and sequenced using an automatic DNA sequence analyzer (ABI 310, Applied Biosystems).

Cloning of the *rpoH* gene

Genomic DNA of *C. maris* was digested with restriction enzymes *Hinc*II, *Hind*III and *Pst*I (New England Biolabs, Beverly, Mass., USA). The digested DNA was separated by electrophoresis in a 1% agarose gel and transferred to a nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK). The DIG-labeled probes were hybridized with the digested fragments at 48°C overnight. The DNA fragments used as probes were as follows: a 462-bp PCR amplified fragment of *rpoH* (Fig. 1, probe 1), and 140-bp *Hind*III-*Pst*I fragment corresponding to the first half of *rpoH* (Fig. 1, probe 2). Labeling of each probe was carried out using a PCR DIG probe synthesis Kit (Boeringer Mannheim, Mannheim, Germany). The membrane was washed twice in SSC solutions containing 0.1% SDS as follows: 2×SSC at room temperature for 15 min and 0.1×SSC at 48°C for 20 min, and detection of hybridized bands was carried out according to the method recommended for the DIG Detection Kit (Boeringer Mannheim).

To construct a *C. maris* partial genomic library, *Hinc*II-, *Hind*III- and *Pst*I- digested DNA fragments having the sizes corresponding to those of the hybridized fragments were ligated with the plasmid vector pBluescript (pBS) II KS (+) (Stratagene, La Jolla, Calif., USA) and resultant constructs were introduced into *E. coli* JM109. The plasmids carrying the *rpoH* were identified by hybridization with the DNA probes and sequenced as described above.

RNA preparation and Northern blot analysis

Total RNA was purified from cells that had been incubated at 20°C for the indicated time. After incubation, 10 ml of culture was mixed with an equal volume of ethanol containing 10%

(W/V) phenol. Cells were collected by centrifugation and resuspended in 600 μl of TE-SDS buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, and 0.5% SDS). After addition of 500 μl of acid phenol, the suspension of cells was incubated at 65^{o}C for 10 min and extracted with phenol: chloroform: isoamylalcohol (25:24:1) three times. Contaminating DNA was removed by digestion with DNaseI (Nippon Gene, Tokyo, Japan), and total RNA was precipitated with 99.5% ethanol. To remove impurities, one-fourth of the sample volume of 10 M LiCl was added to each 1 μg μl^{-1} RNA sample and the mixture was incubated at 4^{o}C overnight. RNA pellets were dissolved in about 20 μl of H_2O and stored at –80^{o}C.

Total RNA (20 μg) was separated by electrophoresis in a 1% agarose gel containing 0.6 M formaldehyde and then transferred onto a nylon membrane. A DIG-labeled DNA probe including a part of the *rpoH* gene (Fig. 1A, probe 1) was used for hybridization. The membrane was washed and detection of hybridized bands was carried out according to the method recommended for the DIG Detection Kit, and signals from hybridized mRNA were detected with a chemiluminescence analyzer (Las-1000, Fuji Film, Tokyo, Japan).

Analysis of the *rpoH* mRNA stability

C. maris cells that had been grown at 10^{o}C were incubated either at 10^{o}C or 20^{o}C after addition of rifampicin at 500 μg ml^{-1} at time zero. Aliquots of cultures were withdrawn at the various time periods and the levels of *rpoH* mRNA were determined by Northern blot analysis as described above.

Primer extension analysis

For primer extension analysis, 5'-DIG-labeled oligonucleotide 5' TAAGTTGCATTGCTTGACTCAT 3', which is complementary to nucleotides +1 to +22 relative to the *rpoH* initiation codon, was used. Total RNA was isolated from *C. maris* cells incubated at 10 or 20^{o}C for 30 min as described above. Twenty micrograms of total RNA was mixed with 4 μl of 5× first-strand buffer, 1 μl of 0.1 M DTT, and 2 pmol of oligonucleotide, 10 mM dNTP, and 200 U

of Superscript III RNaseH$^-$ reverse transcriptase (Invitrogen, Carlsbad, Calif., USA) in a final volume of 20 μl. The extension reaction was carried out at 55^{o}C for 1 hour, following incubation at 70^{o}C for 15 min to inactivate the reverse transcriptase. The reaction mixture was heat-denatured at 95^{o}C for 3 min, electrophoresed on a 6% sequencing gel and then transferred onto a nylon membrane. The extension product was detected with a DIG-detection kit (Boeringer Mannheim). DNA sequencing reactions carried out with the same primer were used to identify the transcriptional start site.

Prediction of mRNA secondary structure

Potential secondary structures for the 5'-region of the *rpoH* mRNA coding region were predicted using the Vienna RNA secondary structure prediction program on the Internet (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi/) (Hofacker et al. 1994; Zuker and Stiegler 1981; McCaskill 1990).

Nucleotide sequence accession number

The nucleotide sequence reported here has been deposited in the DDBJ, GenBank and EMBL databases (AB109565).

Results

Nucleotide and deduced amino acid sequence of *Colwellia maris rpoH*

Figure 1 shows the restriction map of the genomic region including the *rpoH* gene of *C. maris*. Using two DNA probes, we identified three DNA fragments containing the *rpoH* gene (Fig. 1).

The *rpoH* gene was 855-bp long and encoded a polypeptide consisting of 285 amino acid residues with a deduced molecular weight of 32637 and pI of 6.82. A putative ribosome-binding site (GAGGAG) was found 6-bp upstream from the translational initiation codon (Fig. 2A). Fifty-one- and 86-bp downstream from the termination codon, two potential inverted repeat sequences were found. These inverted repeat sequences might function as transcription terminator signals.

In most γ-proteobacteria, such as *E. coli* and *Vibrio cholerae*, the *rpoH* genes have a sequence region called the 'downstream box' immediately downstream of the initiation codon. This region is complementary to the 3'-region of 16S rRNA, and probably acts as a positive regulator of σ^{32} translation (Nagai et al. 1991). In *C. maris rpoH* mRNA, two putative downstream box regions were found (Fig. 2A). The first downstream box (ds-box 1) was located one nucleotide downstream from the initiation codon and extended for 18 nucleotides. Approximately 80% of the nucleotides were complementary to the 3' sequence of *C. maris* 16S rRNA from nt 1443 to 1461 (accession no. AB002630) (Fig. 2B). The second downstream box (ds-box 2) was found starting six nucleotides downstream from the first downstream box and extended for 16 nucleotides. This region was also complementary to the *C. maris* 16S rRNA sequence from nt 1437 to 1452 (88% matching) (Fig. 2B). These two downstream box regions might play a role in the positive regulation of σ^{32} translation in *C. maris*, like the downstream box regions in mesophilic bacteria.

The deduced amino acid sequence of *C. maris* σ^{32} was highly homologous to those of other bacteria. The overall identities with σ^{32} of *E. coli* (accession no. M20668), *Pseudomonas aeruginosa* (Naczynski et al., 1995; Benvenisti et al., 1995), *Serratia marcescens* (Nakahigashi et al., 1995) and *V. cholerae* (Sahu et al., 1997) were 64%, 60%, 66% and 64%, respectively (Fig. 3).

The best-conserved region was region 2, particularly sub-region 2.3-2.4, which exhibited the highest similarity among these five σ^{32} proteins. The stretch of nine amino acids QRKLFFNLR (residues 131-139), called the RpoH box, was perfectly conserved in *C. maris* σ^{32} (Fig. 3).

Identification of the *rpoH* promoter region

The start site of *rpoH* transcription was identified by the primer extension reaction with 5' DIG-labeled primer complementary to the 5' end of the *rpoH* gene and total RNA from *C. maris* cells under heat shock (20^{o}C) and non-heat shock (10^{o}C) conditions. As shown in Figure 4A, we found only one extension product. Although we searched over 307 nucleotides of the region upstream of the translational start codon of *rpoH*, no other extension product was found. This means that there is only one transcriptional start site in the upstream region of *C. maris rpoH*. The level of extension product was clearly increased after temperature upshift from 10^{o}C to 20^{o}C. The 5' terminal nucleotide of *rpoH* mRNA was G, which was located 162 nucleotides upstream of the translational initiation site. The putative promoter region for *C. maris rpoH* appeared to have the nucleotide sequences of TTGACA and TATTAC at –35 and –10, similar to the σ^{70}-type promoter of *E. coli* (Fig. 4B).

Level of *C. maris rpoH* mRNA

To investigate the level of *rpoH* mRNA at high temperature, we carried out Northern blot analysis. Total RNA was extracted from *C. maris* cells incubated at 20^{o}C and hybridized with DIG-labeled probe (Fig. 1, probe 2). The DIG-labeled probe hybridized to a 0.9-kb transcript (Fig. 5A). Judging from the size, this transcript could be assigned as the full-length *rpoH* transcript. Although the *rpoH* mRNA was found constitutively in *C. maris* cells grown at 10^{o}C, it was clearly accumulated after heat shock treatment at 20^{o}C, and this result was consistent with that of the primer extension reaction (Fig. 4A). The increase of *rpoH* mRNA reached a maximum of about 3-fold around 15 min after heat shock treatment (Fig. 5B). These results indicate that the level of *rpoH* mRNA of *C. maris* is increased by heat shock.

Stability of the *rpoH* mRNA of *C. maris*

Because the promoter region of *C. maris rpoH* was similar to the promoter of the housekeeping-type σ^{70} of *E. coli*, the accumulation of *C. maris rpoH* mRNA at high temperature might not be due to a difference of transcriptional rate, but rather to a difference of the stability of the *rpoH* mRNA. To examine whether stabilization causes the mRNA accumulation, we analyzed the rate of degradation of the *rpoH* mRNA after the addition of an inhibitor of transcription, rifampicin (Fig. 6). The degradation of *rpoH* mRNA was much slower at 20°C than at 10°C (Fig. 6A, B). The half-decay time of *rpoH* mRNA was increased from about 15 min at 10°C to 80 min at 20°C (Fig. 6C). From these results, we conclude that the *rpoH* gene of *C. maris* is transcribed from only one σ^{70}-type promoter and the level of *rpoH* mRNA is mainly regulated by a difference of the mRNA degradation rate.

Analysis of the secondary structure of *C. maris rpoH* mRNA

The secondary structures of the 5'-region of *rpoH* mRNA, which contains the ribosome-binding site, the translational initiation codon and the downstream box, from different gram-negative bacteria were predicted to form conserved patterns (Nakahigashi et al. 1995). The formation of these secondary structures is hypothesized to repress the translation of *rpoH* mRNA at the optimal temperature by preventing translation initiation, because ribosomes are inaccessible to the ribosome-binding site, translational initiation codon, and downstream box of the *rpoH* mRNA.

To compare the secondary structure of *rpoH* mRNA of *C. maris* with those of mesophilic bacteria, we used a computer program to examine the secondary structure produced from the promoter (Fig. 7). In the predicted structure of the *rpoH* mRNA of *C. maris*, some important features involved in thermal regulation of *E. coli rpoH* were conserved: (i) both downstream boxes 1 and 2 formed base pairs and, (ii) G-C pairings found in *rpoH* of various mesophilic bacteria were perfectly conserved (15G-50C and 16C-49G) (Nakahigashi et al. 1995). These results suggest that the model proposed for the mRNA secondary structure-based translational regulation in

mesophilic bacteria might also function in *C. maris*.

A specific feature of also secondary structure of *rpoH* mRNA of *C. maris* was found in the downstream box 1 region, namely, the stem formed in this region was interrupted by one or more short unpaired regions (+8A and +112A-+115C). In contrast, this region was sequestered by base pairing in *E. coli rpoH* (Morita et al. 1999a). In *E. coli*, blocking the downstream box by the base pairing is the most important factor regulating the translation efficiency of *rpoH* (Morita et al.1999a). In *C. maris* it seems that the base pairing of downstream box 1 should be less stable than that in *E. coli*.

Discussion

In this study, we cloned the *rpoH* gene of a psychrophilic bacterium, *C. maris*, and examined its transcriptional regulation The deduced amino acid sequence of *C. maris* σ^{32} is highly homologous to those of mesophilic bacteria, suggesting that the function and post-translational regulation of *C. maris* σ^{32} are similar to those of σ^{32} from mesophilic bacteria. Various functionally important regions are conserved in *C. maris* σ^{32} (Fig. 3). The best-conserved region in σ^{32} is region 2, and sub-region 2.3-2.4 shows the strongest conservation (Fig. 3). It is proposed that the best-conserved sub-regions, 2.3 and 2.4, participate in strand opening and –10 recognition, respectively (Lonetto et al. 1992). Furthermore, several genes encoding HSPs of *C. maris*, such as *groESL* and *dnaK* genes, have the consensus sequence of the σ^{32}-dependent promoters in *E. coli* (Yamauchi et al. 2003, 2004). These results indicate that *C. maris* σ^{32} might have functions similar to the σ^{32} of mesophilic bacteria, namely, recognizing heat shock promoters and transcribing the downstream genes.

In *C. maris* σ^{32}, the RpoH box, a nine-amino-acid-sequence region (QRKLFFNLR; residues 131-139), is conserved between regions 2.4 and 3.1 (Fig. 3). The RpoH box is highly conserved in all σ^{32} homologues, but is not present in other σ factors (Nakahigashi et al., 1995). It has been postulated that this region may be involved in the DnaK-DnaJ-mediated negative control of σ^{32} stability (Nagai et al., 1994). Therefore, it is reasonable to speculate that chaperone-mediated negative control of the activity and/or stability of σ^{32} might be conserved among mesophilic and psychrophilic bacteria.

Primer extension experiments showed that *C. maris rpoH* had only one σ^{70}-type promoter. This promoter structure largely differs from those of other bacterial species. In most γ-proteobacteria, transcription of *rpoH* is strictly regulated by the use of several promoters (Reisenauer 1996; Manzanera 2001; Ramirez-Santos et al. 2001). For example, transcription of *E. coli rpoH* is likely regulated by four promoters: three are σ^{70}-type and one is a σ^{E}-type promoter. Because the transcription regulated by σ^{E}-type promoters is known to be strongly stimulated at high

temperatures, synthesis of *rpoH* mRNA should increase at high temperature (Wang and Kaguni 1989). In contrast, only a temperature-independent promoter was found in *C. maris*, indicating that the transcription of *rpoH* mRNA is constitutive. However, as shown by Northern blot analysis, the level of *rpoH* mRNA increased after temperature shift from 10^{o}C to 20^{o}C (Fig. 5). Further experiments using an inhibitor of transcription, rifampicin, explained this discrepancy, namely, the rate of degradation *C. maris rpoH* mRNA was much slower at 20^{o}C than at 10^{o}C. From these results, we conclude that *rpoH* of *C. maris* is transcribed from only one σ^{70}-type promoter and the level of *rpoH* mRNA is mainly regulated by the difference in degradation rate.

As the secondary structure of the 5'-region of *rpoH* mRNA is an important factor for regulating σ^{32} translation (Morita et. al 1999a and 1999b), we examined the secondary structure of *rpoH* mRNA of *C. maris* by computer analysis. Because *C. maris rpoH* had only one transcriptional start site, we analyzed the secondary structure of mRNA from the –162 to +144 region. In the predicted mRNA secondary structure, the base pairing of the downstream box 1 region was much different from those of mesophilic bacteria (Fig. 7). It is easy to imagine that imperfect base pairing is necessary to guarantee melting within the appropriate temperature range. In *E. coli*, not only forming base pairs but also achieving the appropriate instability of the mRNA secondary structure of the downstream box is a critical factor to regulate the translation efficiency of *rpoH* (Morita et al. 1999a). In *Bradylhizobium japoniccum*, several heat shock proteins are under the control of a highly conserved cis-element called ROSE (repression of heat shock gene expression), which adopts a similar pattern of mRNA secondary structure. In this case, one highly conserved bulged nucleotide opposite the ribosome-binding site might facilitate temperature-mediated melting of ROSE mRNA (Nocker et al. 2001). Additional unpaired regions in the downstream box 1 of *C. maris rpoH* might serve as 'wedges' conferring more instability on the stem structure compared to those of mesophilic bacteria. These features of the secondary structure of *C. maris rpoH* mRNA should contribute to HSP expression at relatively low temperatures, such as 20^{o}C.

This report is the first to describe the DNA sequence and transcriptional analysis of *rpoH* of psychrophilic bacteria. The analysis of the *rpoH* gene encoding heat shock sigma factor σ^{32} of *C.*

maris presented here provides important information for understanding the regulatory mechanisms of HSP expression in psychrophilic bacteria.

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Figure legends

Figure 1 Restriction map of the genomic region containing the *rpoH* in *C. maris*. The DNA fragments used for Southern and Northern blot analysis are shown as black boxes: probe 1 (pb1), for Southern and Northern blot analyses; probe 2 (pb2), for Southern blot analysis. The three DNA fragments used for DNA sequencing are indicated by bars a, b, and c. The location of *rpoH* is shown as a hatched box.

Figure 2 Partial nucleotide sequence of *rpoH*. (A) Upstream sequence of *C. maris rpoH*. The ribosome-binding site (RBS) is double-underlined. The translation initiation site of σ^{32} is shown by bold face type. Putative downstream boxes (ds-box 1 and ds-box 2) are indicated by boxes. (B) The two putative downstream boxes of *rpoH*. Two putative downstream boxes (upper sequence) were shown to be complementary to part of the 3'-end of 16S rRNA (lower sequence). Lines indicate canonical base pairing, and two dots denote allowed U-G pairing. Numbers below the 16S rRNA sequence indicate the positions of nucleotides.

Figure 3 Comparison of the amino acid sequence of σ^{32} of *C. maris* with those of other bacterial species. The deduced amino acid sequence of *C. maris* (Cma) σ^{32} is aligned with those from *E. coli* (Eco), *P. aeruginosa* (Pae), *S. marcescens* (Sma) and *V. cholerae* (Vch). The four typical regions conserved among the known sigma factors of the σ^{70} family are indicated above the amino acid sequences. The RpoH box is shown by a box. Amino acid residues identical to those of *C. maris* proteins are indicated by dots. Asterisks indicate amino acid residues identical in all five bacteria.

Figure 4 Identification of the transcriptional start site of *rpoH*. (A) The product of the primer extension reaction is indicated by an arrow (+1). Lane 1, cells grown at 10^{o}C; Lane 2, cells grown at 10^{o}C and exposed to 20^{o}C for 30 min. (B) Sequence of the promoter region of *C.*

maris rpoH. The vertical arrow indicates the transcriptional start site. Potential –10 and –35 sequences of the promoters are underlined.

Figure 5 Transcription of *rpoH* under heat shock conditions. (A) Northern blot analysis of *rpoH*. Total RNA was extracted from *C. maris* cells exposed to heat stress at 20^{o}C for various periods (lane, 0 min; lane 2, 5 min; lane 3, 15 min; lane 4, 30 min; lane 5, 45 min; lane 6, 60 min; lane 7, 90 min; lane 8, 120 min). The RNA was transferred to a membrane and hybridized with a DIG-labeled probe (Fig. 1, pb 1). (B) Relative increase of *rpoH* mRNA level. The level of the mRNA is expressed as a percentage relative to the amount prior to the shift to 20^{o}C.

Figure 6 Stability of *rpoH* mRNA at 10 or 20^{o}C. (A) (B) Total RNA was extracted from *C. maris* cells at various times after the addition of 500 μg ml^{-1} rifampicin at 10^{o}C (A) or 20^{o}C (B) (lane1, before addition of rifampicin at 10^{o}C; lane 2, 15 min; lane 3, 30 min; lane 4, 45 min; lane 5, 60 min; lane 6, 90 min). The RNA was transferred to a membrane and hybridized with DIG-labeled probe (Fig. 1, pb 1). (C) The difference of *rpoH* mRNA stability at 10^{o}C (closed circles) and 20^{o}C (open circles). The level of *rpoH* mRNA was expressed as a percentage relative to the amount before addition of rifampicin at 10^{o}C.

Figure 7 Possible secondary structure of the 5'-region of *C. maris rpoH* mRNA. The 5'-region of *rpoH* mRNA (nt -20 to +144) was analyzed using the Vienna RNA secondary structure prediction program. The initiation codon and ribosome-binding site (RBS) are boxed. The two putative downstream boxes (ds-box 1 and ds-box 2) are indicated by lines. The conserved base pairing is indicated by a dotted box.

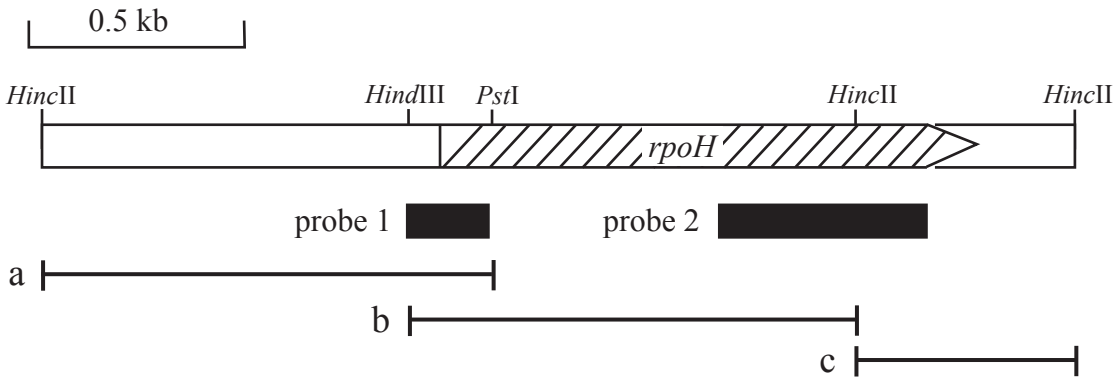


Fig. 1 Yamauchi et al.
Colwellia maris rpoH gene

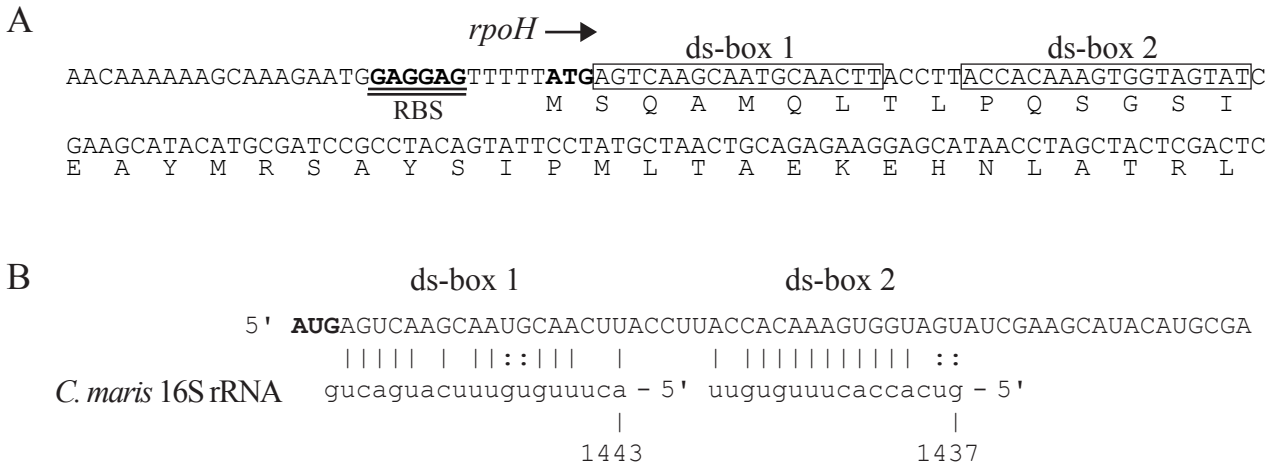


Fig. 2 Yamauchi et al.
Colwellia maris rpoH gene

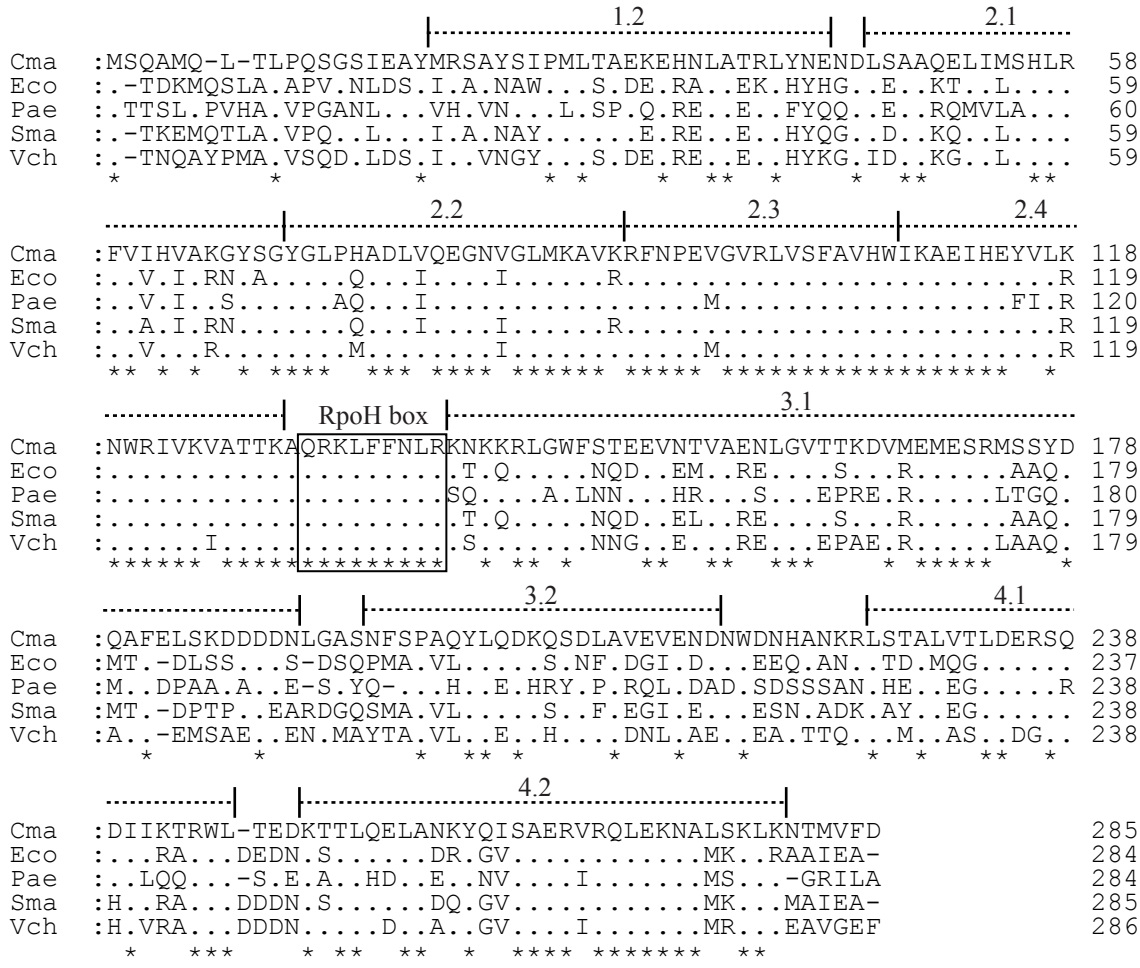
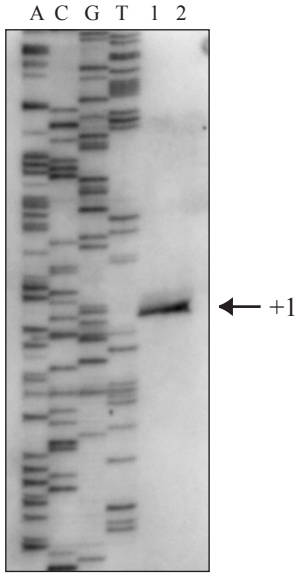


Fig. 3 Yamauchi et al.
Colwellia maris rpoH gene

A



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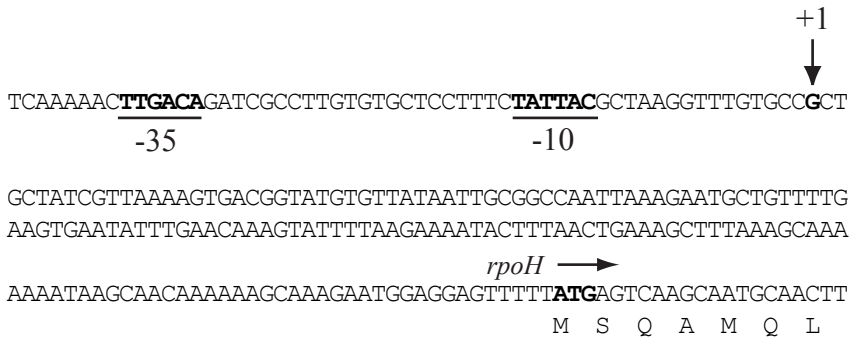

Fig. 4 Yamauchi et al.
Colwellia maris rpoH gene

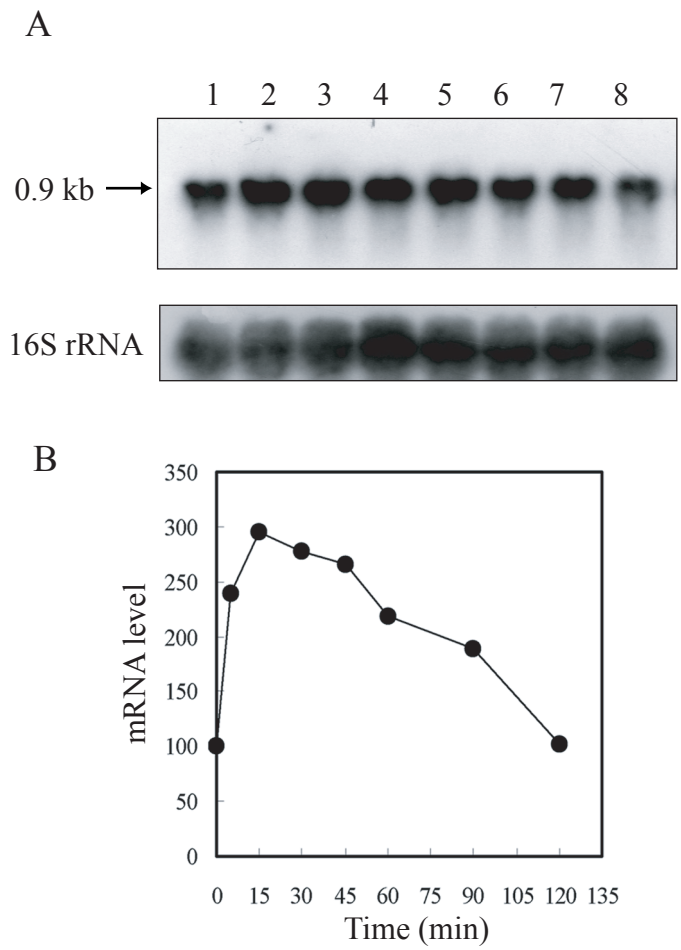

Fig. 5 Yamauchi et al.
Colwellia maris rpoH gene

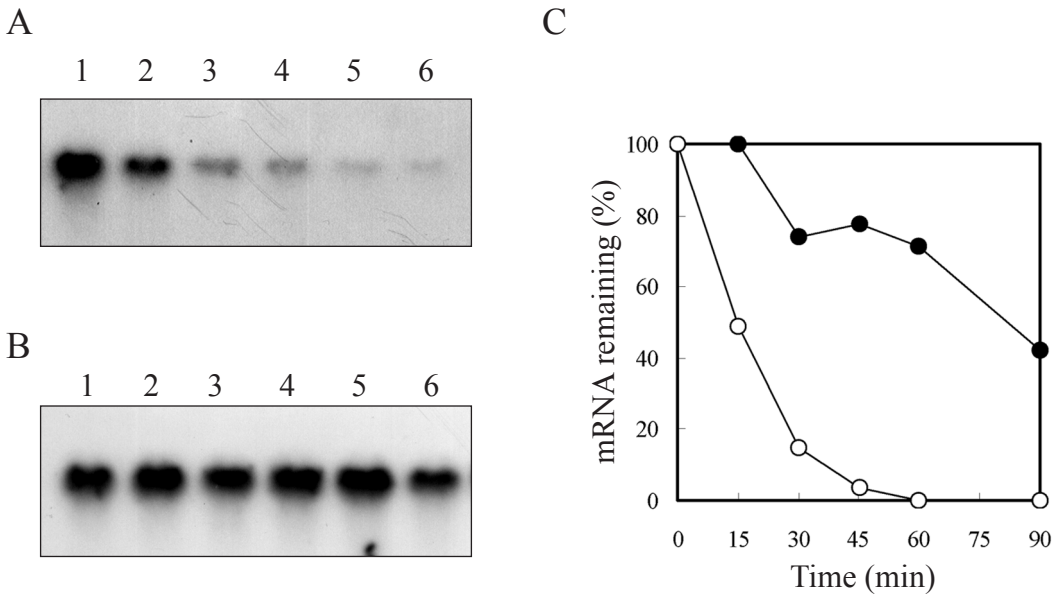

Fig. 6 Yamauchi et al.
Colwellia maris rpoH gene

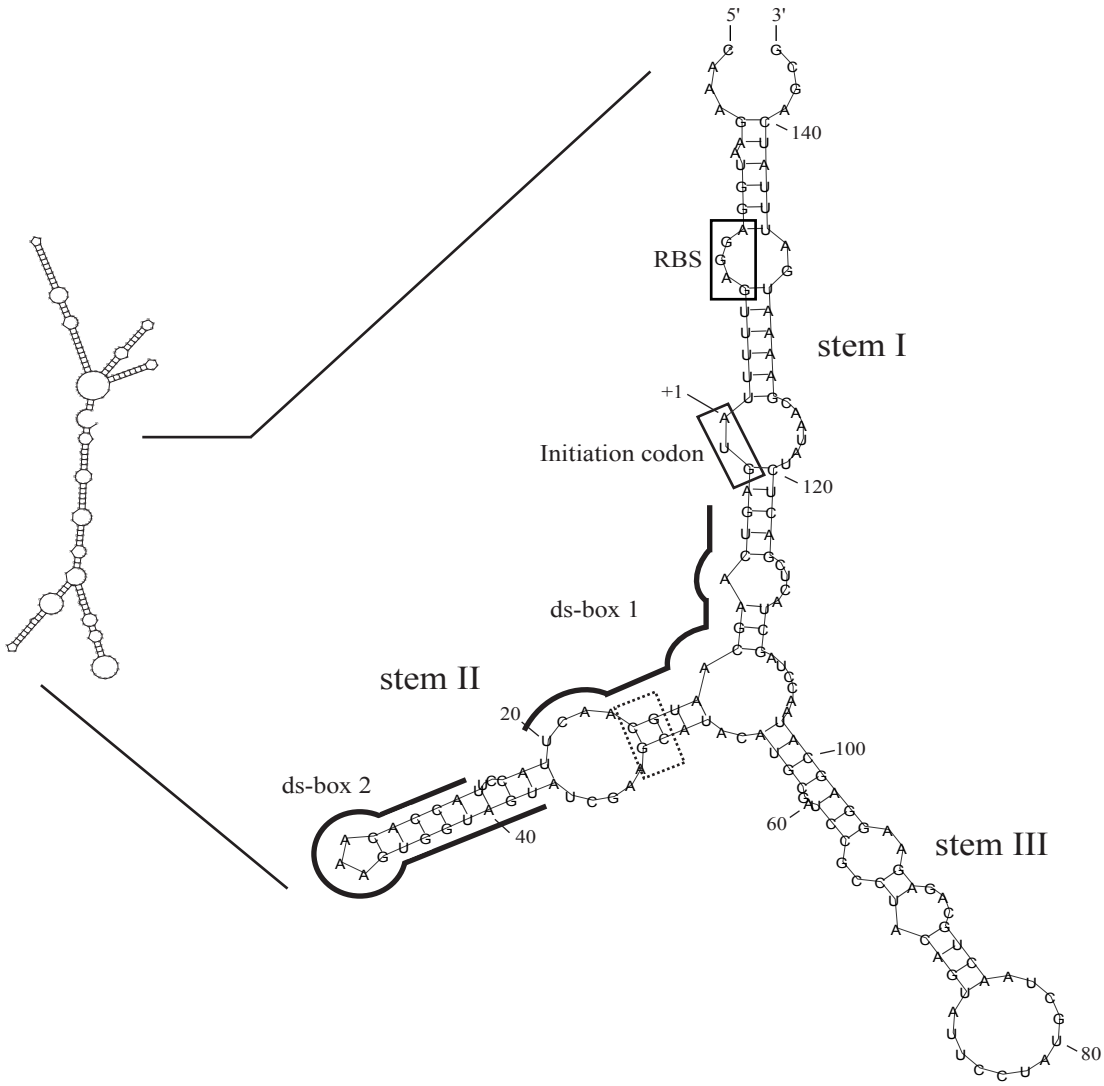

Fig. 7 Yamauchi et al.
Colwellia maris rpoH gene