

Cloning, sequence analysis and expression of the sigma factor σ^{54} (*rpoN*) of deep-sea barophilic *Shewanella violacea* strain DSS12

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The *rpoN* gene (encoding the sigma factor σ^{54}) of barophilic *Shewanella violacea* strain DSS12 was cloned and its nucleotide sequence was determined. The *rpoN* gene was found to encode a putative protein consisting of 492 amino acid residues with a predicted molecular mass of 55,359 Da. Significant homology was evident comparing the *rpoN* sequence of *S. violacea* with that of *E. coli* (62.8% identity), *V. anguillarum* (61.7% identity) and *P. putida* (57.0% identity). The DNA-binding domain at the C-terminus of σ^{54} is well conserved in the case of the *S. violacea rpoN* gene product and the helix-turn-helix motif and the RpoN box are also present. In addition, the conserved glutamine-rich domain is present at the N-terminus. In *S. violacea*, σ^{54} was expressed at a relatively constant level under various growth conditions as determined by western blotting analysis using antiserum containing polyclonal antibodies against *P. putida* σ^{54} .

Key Words : barophilic bacteria, σ^{54} , transcription, *Shewanella violacea*

深海由来好圧性細菌 *shewanella violacea* DSS12株の シグマ因子 σ^{54} のクローニング、 塩基配列決定とその発現

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深海由来好圧性細菌 *Shewanella violacea* DSS12株の σ^{54} 因子遺伝子をクローニングし、その塩基配列を決定した。本菌株の σ^{54} 因子は492アミノ酸、推定分子量55,359 Daのタンパクであることを確認した。また、本菌株の σ^{54} 因子のアミノ酸配列は他の細菌のそれらの配列と高い相同性が見られ、既知の σ^{54} 因子と同様にその因子としての機能の特徴づける領域がよく保存されていた。さらに、*P. putida*の σ^{54} 因子の抗体を用いたウエスタンブロット解析から、本菌株が σ^{54} 因子を生育条件によらず常に一定に発現していることも確認した。

キーワード : 好圧性細菌, σ^{54} 転写, *Shewanella violacea*

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1 Introduction

The deep-sea is an extreme environment subject to high pressure and low temperature. Microorganisms living in the deep-sea are adapted to this environment. To investigate how these bacteria can adapt to extremely high pressure conditions, our laboratory has isolated several barophilic bacteria from deep-sea mud samples^{1), 2)}.

We are interested in examining the molecular mechanisms of gene regulation in deep-sea microorganisms, because some gene expression systems in these bacteria may be regulated by high pressure. A pressure-regulated operon has been found in barophilic *Shewanella violacea* strain DSS12 and the expression of this operon was shown to be controlled by elevated pressure at the level of transcription³⁾. The sigma factor σ^{54} of *Escherichia coli* was found to bind to a consensus sequence in the promoter region of this operon, suggesting that such a factor may exist in strain DSS12⁴⁾.

The *rpoN* gene encodes the RNA polymerase sigma factor σ^{54} which in enteric bacteria has been shown to be responsible for transcription of nitrogen-regulated genes such as glutamine synthetase and nitrogen fixation genes^{5)~7)}. However, it should be understood that *rpoN* is not a nitrogen-regulated gene per se. Neither the expression nor the activity of σ^{54} is normally subject to nitrogen control⁸⁾. Furthermore, in a number of cases, σ^{54} is required for expression of genes that are not subject to nitrogen control, e. g., hydrogenase genes in *E. coli* and xylene degradation genes in *Pseudomonas putida*^{9), 10)}. The *rpoN* gene has been cloned and sequenced from a number of bacteria including *Klebsiella pneumoniae*¹¹⁾, *Azotobacter vinelandii*¹²⁾, *Rhizobium meliloti*¹³⁾, *Pseudomonas putida*¹⁴⁾, *Thiobacillus ferrooxidans*¹⁵⁾, *Escherichia coli*¹⁶⁾, *Salmonella typhimurium*¹⁷⁾ and *Vibrio anguillarum*¹⁸⁾. All show a high degree of homology.

In this report, we describe the cloning and sequencing of the *rpoN* gene encoding σ^{54} from barophilic *S. violacea* strain DSS12. We also investigated the expression of the *rpoN* gene product under various growth conditions by Western blot analysis.

2 Materials and Methods

2.1 Bacterial strains, plasmids, media and culture conditions

Barophilic *Shewanella violacea* strain DSS12 was the source of chromosomal DNA used to clone the *rpoN* gene. *E. coli* JM109 and BL21 (DE3) were used as the host strains for cloning and overexpression of *P. putida* σ^{54} , respectively. Plasmid vector pCRII-TOPO (Invitrogen) was used for the cloning of PCR products. Plasmid vector pUC18 was used for constructing sequencing clones. For overexpression of *P. putida* σ^{54} , the expression vector pGEMEX-1 (Promega) was used. *S. violacea* strain DSS12 was cultured in Marine broth 2216 (Difco). *E. coli* was cultivated in LB medium at 37°C. When

required, the following compounds were added to the media : ampicillin (50 mg/ml), 5-bromo-4-chloro-3-indolyl- β -D-galactoside (50 mg/ml), and isopropyl-1-thio- β -D-galactopyranoside (IPTG, 1 mM).

2.2 Cloning and sequence analysis of the *rpoN* gene from *S. violacea*

The deduced amino acid sequences of the *rpoN* gene from the other eubacteria were aligned, and synthetic degenerate oligonucleotide primers were synthesized based on highly conserved regions. These oligonucleotide primers corresponded to the amino acid residues TPQLQQAI (N1, 5'- ACN CCN CAR YTN CAR CAR GCN AT -3') and HESTVSRV (N2, 5'- ACY CTN SWN ACN GTN SWY TCR TG -3'). The degenerate oligonucleotide primers were then used for PCR amplification of the corresponding region of the chromosomal DNA of *S. violacea* strain DSS12. The PCR product (approx. 1,155 bp) was cloned into the vector pCRII-TOPO. The sequence of a part of the *rpoN* gene was determined by the dye terminator method using an ABI-Prism 377 automatic DNA sequencer (Applied Biosystems).

In order to clone the full-length *rpoN* gene, Southern hybridization and colony hybridization were carried out. The synthetic oligonucleotide primers (Fig. 2) N3 (5'- ATC CAA CAA GCG TTA GAC -3') and N4 (5'- GAG ATG CTC ATA CAA ACC -3') were synthesized for use as hybridization probes and each was labeled with digoxigenin (Boehringer Mannheim). At first, the chromosomal DNA from *S. violacea* was digested with *Hind*III. The digestion mixture was then separated on a 1% agarose gel and the DNA was transferred to Hybond-N+ (Amersham). The *Hind*III fragments were cloned into pUC18 and screened by colony hybridization with the digoxigenin-labeled probes. The insert DNA was subcloned into pUC18. The nucleotide sequence of the full-length *rpoN* gene was determined as described above.

2.3 Overexpression and purification of *P. putida* σ^{54} for use in antibody production

The *rpoN* gene of *P. putida* was excised from the plasmid pTS441¹⁴⁾ with *Sa*I and *Hind*III, and cloned between the *Sa*I and *Hind*III sites of the expression vector pGEMEX-1. This plasmid (pGENTR22), encoding the full-length *rpoN* gene, was digested with *sac*I and self-linked. The resulting plasmid (pGENTR22 Δ *Sac*I), encoding a modified σ^{54} , was used for expression of *P. putida* σ^{54} . An *E. coli* BL21 (DE3) transformant carrying pGENTR22 Δ *Sac*I was grown in LB medium containing ampicillin (50mg/ml) at 37°C and expression of the chimeric gene was induced in the presence of 1 mM IPTG. The modified σ^{54} was purified in two steps by DEAE-cellulose and Bio Gel P-100 column chromatography. The protein was analyzed by SDS-PAGE on a 10% acrylamide gel and visualized by staining with Coomassie blue. Antiserum containing

polyclonal antibodies against *P. putida* σ^{54} was obtained by injecting the antigen into an albino rabbit. The blood collected was left to clot at room temperature. After storage overnight at 4°C, aggregated material was removed by centrifugation. The supernatant was used as crude antiserum.

2.4 Western blot analysis

S. violacea strain DSS12 was cultured in Marine Broth 2216 under various growth conditions, in terms of both pressure (0.1 and 50 MPa) and temperature (4, 8 and 15°C). The cells obtained were suspended in buffer A [10 mM MgCl₂, 0.1 mM EDTA, 0.1 mM DTT, 10 mM p-APMSF, 10 mM Tris-HCl, pH 7.8]. These suspensions were treated with a sonic oscillator for 10 min at 4°C and centrifuged at 18000 x g for 15 min. An equivalent amount (20 mg) of each of the cell lysates was fractionated by 10% SDS-PAGE, the proteins were then blotted onto a PVDF membrane and the membrane was treated with antiserum containing polyclonal antibodies against *P. putida* σ^{54} , using a 1:400 dilution of the crude antiserum.

3 Results and Discussion

3.1 Cloning and sequence analysis of the *rpoN* gene from *S. violacea*

To clone a portion of the *rpoN* gene, a highly conserved region of amino acids in σ^{54} , found in several Gram-negative bacteria, was used as the basis for preparing a pair of degenerate oligonucleotide primers, N1 and N2, for PCR. A 1,155 bp fragment of the expected size, based on the positions of the primers in the sequence of *rpoN* gene, was obtained by PCR. The product was cloned into the vector pCRII-TOPO and its sequence was determined. A database homology search using FASTA, based on the nucleotide sequence of the amplified product, resulted in high probability matches with other eubacterial *rpoN* genes. Significant homology was also found between the deduced amino acid sequence of the product and that of the *rpoN* gene of *E. coli* (60.0% identity), *V. anguillarum* (55.6% identity) and *P. putida* (50.6% identity). These findings show that the cloned product is a *rpoN* homolog.

A 312 bp fragment amplified from the chromosomal DNA of strain DSS12 by PCR using the primers N3 and N4 was DIG-labeled and used as a hybridization probe to isolate a clone containing the full-length *S. violacea rpoN* gene. Southern hybridization was performed to investigate the distribution of the *rpoN* gene in the *S. violacea* chromosome (Fig. 1). As shown in the restriction map in Fig. 2A, the *rpoN* gene was present in a 3.1 kb *Hind*III fragment, as detected by colony hybridization. The insert DNA was subcloned into pUC18 and the sequence of the entire *rpoN* gene, consisting of 1,476 bp, was determined. This gene was found to encode a putative protein consisting of 492 amino acid residues with a predicted molecular mass of 55,359 Da. The nucleotide sequence is shown in Fig. 2B; a potential ribosome-binding site is also shown. Comparing the sequences of bacterial *rpoN* genes, a high degree of conserva-

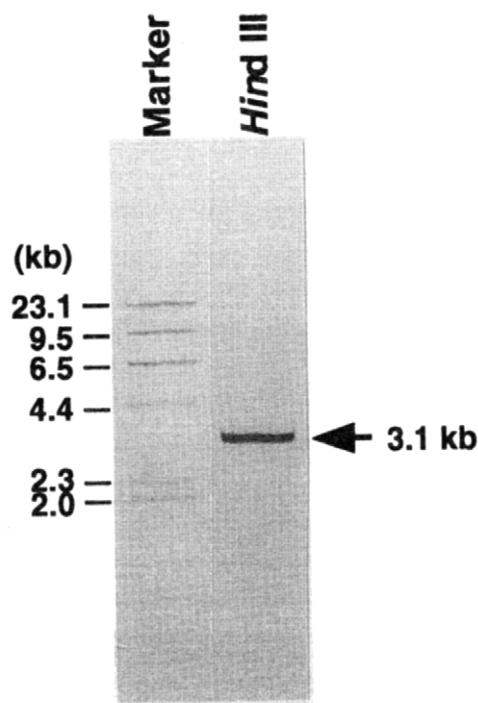


Fig. 1 Southern hybridization analysis of genomic DNA from *S. violacea* strain DSS12. Chromosomal DNA was digested with restriction endonuclease *Hind*III and fractionated on a 1% agarose gel. The probe used corresponded to a portion of the *rpoN* gene amplified by PCR from chromosomal DNA of *S. violacea* strain DSS12. The sizes of the *Hind*III markers are indicated in kb.

tion of the primary sequence is evident and three distinct regions (Regions I, II and III) can be identified. Region III contains two notable motifs, a potential helix-turn-helix motif and a RpoN box^{8, 12}. DNA-binding and core-binding domains together comprise Region III of σ^{54} ¹⁹. A comparative alignment of the deduced amino acid sequence of the *S. violacea rpoN* gene product with that of *E. coli*⁶, *V. anguillarum*¹⁸) and *P. putida*¹⁴) is presented in Fig. 3A. The *S. violacea rpoN* (σ^{54}) sequence shows significant homology with that of *E. coli* (62.8% identity), *V. anguillarum* (61.7% identity) and *P. putida* (57.0% identity). The three distinct regions mentioned above were identified in the σ^{54} of *S. violacea*. Region I is a conserved glutamine-rich stretch at the N-terminus. Region II is a non-conserved acidic domain that extends from amino acid residue 48 to 98. Region III (containing the C-terminal DNA-binding and core-binding domains) is conserved and contains two motifs, the helix-turn-helix DNA-binding motif extending from amino acid residue 383 to 402 and the RpoN box extending from amino acid residue 470 to 479 (Fig. 3A, B). These features are the same as observed in the case of σ^{54} from other species.

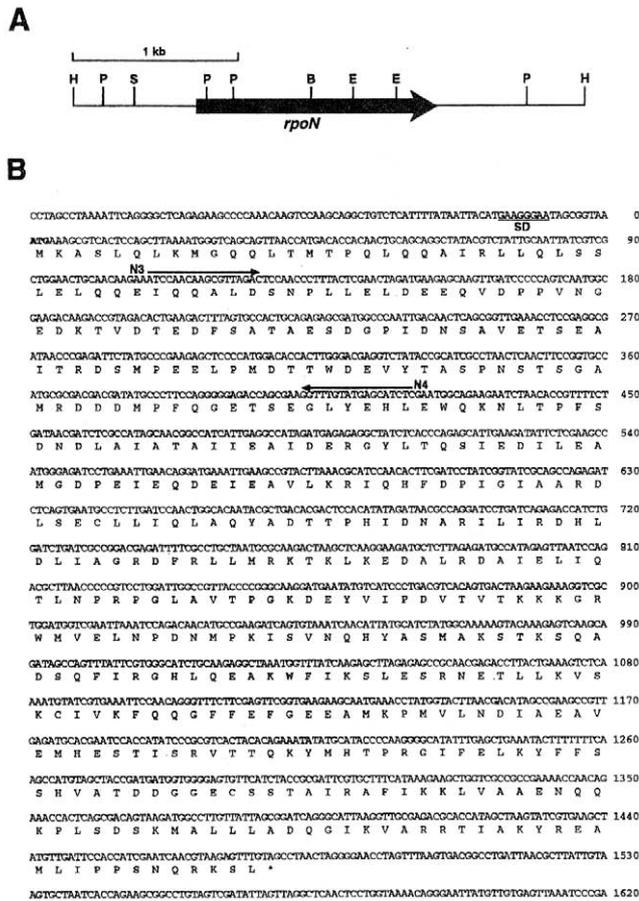


Fig. 2 Restriction map, the nucleotide sequence and deduced amino acid sequence of the *rpoN* gene. (A) Restriction map showing the location of the *rpoN* gene on the chromosome of *S. violacea* strain DSS12. The coding region and its direction of transcription are indicated by arrows. Restriction sites are shown as follows : H, *Hind*III ; P, *Pst*I ; S, *Sa*I ; B, *Bam*HI ; E, *Eco*T221. (B) Nucleotide sequence and deduced amino acid sequence of the *rpoN* gene of strain DSS12. The deduced amino acid sequence is shown in single-letter code below the nucleotide sequence. The putative start codon is shown in bold, and the stop codon is indicated by an asterisk. A putative ribosome-binding site (SD) is underlined. The nucleotide sequences corresponding to the two primers, N3 and N4, are indicated by arrows.

The conserved DNA-binding domain of σ^{54} is involved in promoter recognition in bacteria such as *E. coli*⁽⁶⁾. Physical contact between σ^{54} and the promoter sequence occurs via the C-terminal one-third of the protein⁽²⁰⁾. This region was highly conserved in *S. violacea* (Fig. 3A). This observation suggests that the *S. violacea* σ^{54} has the ability to recognize promoter sequences, like the σ^{54} of *E. coli*. In a previous study, the possible interaction between a σ^{54} factor and promoter regions in *S. violacea* was discussed in relation to transcription of the pressure-regulated operon. EMSA showed that recombinant *E. coli* σ^{54} is capable of binding to the promoter region of the pressure-regulated operon in strain DSS12⁽⁴⁾. It seems likely that the σ^{54} of *S. violacea* interacts with the pressure-regulated operon. Therefore, to analyze the transcription of this operon initiated by the σ^{54} containing RNA polymerase, we focused on the expression of σ^{54} in *S. violacea*.

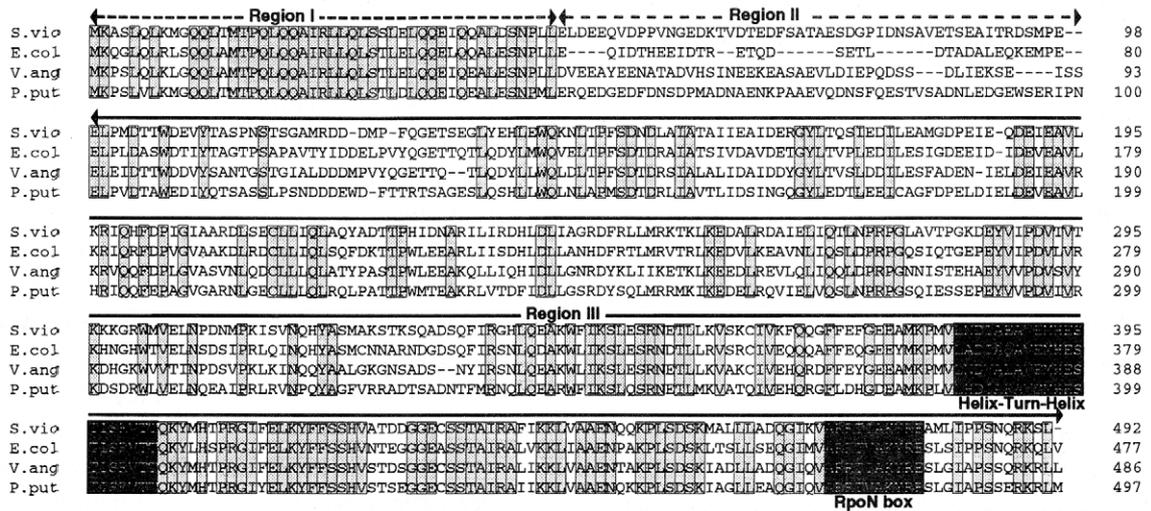
3.2 Expression of σ^{54} in *S. violacea*

To examine the expression of σ^{54} in *S. violacea*, Western blot analysis was carried out using antiserum against *P. putida* σ^{54} . After the cells had been cultured under various growth conditions, in terms of both pressure (0.1 and 50 MPa) and temperature (4, 8 and 15°C), cell lysates were prepared and fractionated by SDS-PAGE and the gel was incubated with the antiserum.

As shown in Fig. 4A, bands of equivalent intensity were detected in the case of cells grown at 0.1 or 50 MPa. These bands corresponded to sizes of 55 and 95 kDa, respectively. The 55 kDa band represents a protein consistent in size with the predicted molecular mass of *S. violacea* σ^{54} . The 95 kDa band represents an unknown protein, which appears to have a domain with the same immunoreactivity as σ^{54} . Our analysis showed that the levels of expression of σ^{54} were similar at 4, 8 or 15°C (Fig. 4B). In addition, a similar level of expression was detected at various times during the growth period (data not shown). These results together indicate that σ^{54} is expressed at a relatively constant level in *S. violacea* under various growth conditions. These observations are consistent with the finding that the intracellular concentration of σ^{54} in *E. coli* remains constant under several growth conditions⁽²¹⁾. It has also been reported that the number of σ^{54} molecules is high compared with the number of genes subject to transcription by σ^{54} -containing RNA polymerase. This raises the possibility that the level of functional σ^{54} molecules may be controlled by the availability of certain regulatory factors such as NtrC.

The alternative sigma factor σ^{54} requires an enhancer-binding activator protein, NtrC, which activates the expression of various genes including nitrogen assimilation and nitrogen fixation genes in a number of bacteria⁽²²⁾. The response regulator NtrC is σ^{54} -dependent. The role of the activator is to catalyze the isomerization of closed complexes between the σ^{54} -containing holoenzyme and the promoter, to open the complexes⁽²³⁾. In *S. violacea*, transcription by the σ^{54} -containing RNA polymerase may be highly dependent on such transcription factors. It is possible also that expression of the pressure-regulated operon may be controlled by several transcription factors. To understand the mechanisms of transcription of the pressure-regulated operon in *S. violacea* under different pressure conditions, isolation and characterization of the transcription factors and several sigma factors from this strain are essential.

A



B

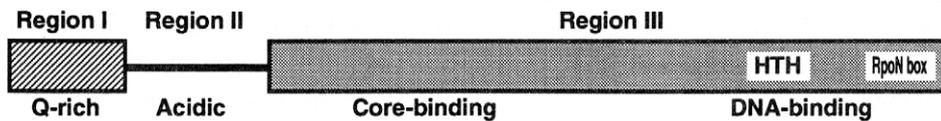


Fig. 3 Alignment and diagrammatic representation of the *S. violacea* σ^{54} protein. (A) Alignment of the deduced amino acid sequence of *S. violacea* σ^{54} protein with three other σ^{54} proteins. Amino acid sequences of σ^{54} proteins from *E. coli* (accession no. U12684), *V. anguillarum* (accession no. U86585), and *P. putida* (accession no. M24916) are compared with that of *S. violacea*. Identical amino acids are boxed. Region I (glutamine-rich), Region II (acidic) and Region III (DNA-binding, core-binding) are indicated in the upper sequence. The helix-turn-helix DNA binding motif and the RpoN box in σ^{54} are indicated below the sequence. The abbreviations used for the species are as follows: S.vio, *S. violacea*, E. col, *E. coli*, V. ang, *V. anguillarum*, P. put, *P. putida*. (B) Diagrammatic representation of the domain structure of *S. violacea* σ^{54} . The glutamine-rich domain (Region I) is separated from the DNA-binding and core-binding domains (Region III) by an acidic domain (Region II). Region III possesses a helix-turn-helix DNA-binding motif (HTH) and a conserved sequence termed the RpoN box.

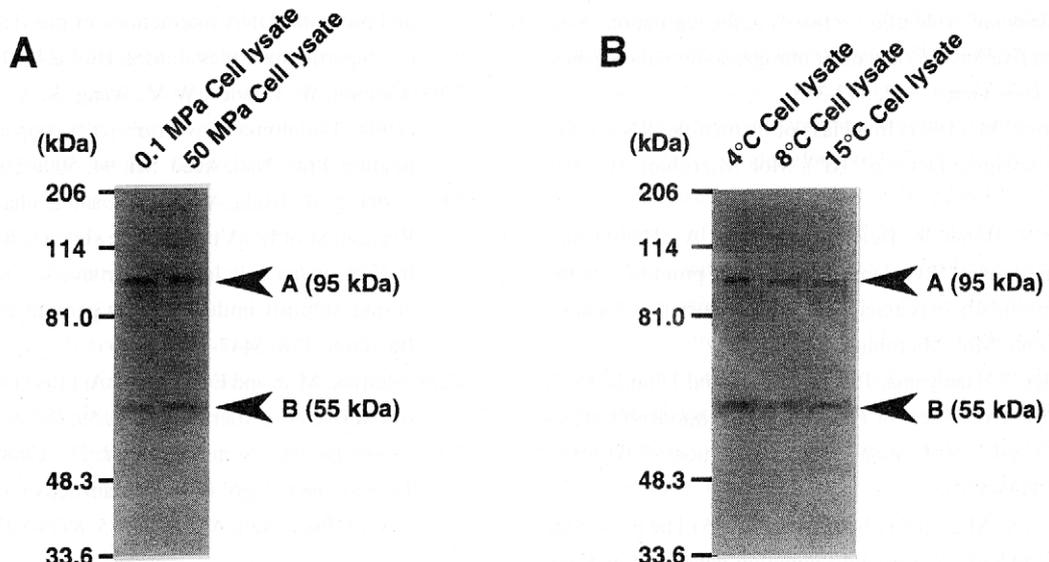


Fig. 4 Western blot analysis of expression of the σ^{54} protein in *S. violacea* under various growth conditions. Cell lysates, prepared from cells cultured under various growth conditions, in terms of both pressure (A: 0.1 and 50 MPa) and temperature (B: 4, 8 and 15°C), were fractionated by 10% SDS-PAGE, and then blotted onto a PVDF membrane, and the membrane was treated with antiserum containing polyclonal antibodies against *P. putida* σ^{54} . The sizes of the molecular mass standards are indicated in kDa.

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