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MINI-REVIEW

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Transcriptional regulation under pressure conditions by RNA polymerase σ^{54} factor with a two-component regulatory system in *Shewanella violacea*

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Abstract Deep-sea bacteria have unique systems for gene and protein expression controlled by hydrostatic pressure. One of the σ factors, σ^{54}, was found to play an important role in pressure-regulated transcription in a deep-sea piezophilic bacterium, *Shewanella violacea*. A glutamine synthetase gene (*glnA*) has been targeted as a model for the pressure-regulated promoter to investigate transcriptional regulation by the σ^{54} factor. Recognition sites for σ^{54} and σ^{70} factors were observed at an upstream region of the *glnA*, and NtrC-binding sites were also identified at the same region. Primer extension analyses revealed that the transcription initiation sites of both promoters were determined and that transcription from the σ^{54} site was regulated by elevated pressure. The σ^{54} promoter is known to be activated by a two-component signal transduction system, the NtrB–NtrC phosphorylation relay. Our results suggested that this system might be regulated by deep-sea conditions and that the gene expression controlled by the σ^{54} promoter was actually regulated by pressure. We propose a possible model of the molecular mechanisms for pressure-regulated transcription.

Key words Gene expression · Piezophilic bacterium · Piezoresponse · *Shewanella violacea* · σ^{54} promoter · Transcription · Two-component signal transduction system

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Introduction

The psychrophilic, moderately piezophilic bacterium *Shewanella violacea* DSS12 is a deep-sea isolate from a mud sample collected at the Ryukyu Trench (depth, 5,110 m) that grows optimally at 30 MPa and 8°C but also grows at atmospheric pressure (0.1 MPa) and 8°C (Kato et al. 1995; Nogi et al. 1998). We have targeted this strain to elucidate the molecular basis of gene regulation in piezophilic bacteria at different pressure conditions because it is useful as a model bacterium for comparing the various features of bacterial physiology under pressure conditions (Nakasone et al. 1998). Isolation and characterization of several pressure-regulated *cis*-acting elements have been conducted (Kato et al. 1997; Nakasone et al. 1999; Ikegami et al. 2000b). Through the sequence analyses of these *cis* elements in pressure-regulated genes, the consensus sequence for RNA polymerase sigma factor σ^{54} was found upstream of the genes and the *S. violacea* σ^{54} was shown to bind to this region (Nakasone et al. 1999; Ikegami et al. 2000a, b).

Thus, we have focused on the molecular mechanisms of σ^{54}-dependent transcription under different pressure conditions. The σ^{54}-containing RNA polymerase has been shown to be responsible for transcription of several genes, e.g., nitrogen metabolic genes such as the *glnA* operon (Merrick and Edwards 1995). Our approach to understand the basis of gene expression under defined conditions is by detailed characterization of the components of the transcriptional machinery and the accessory factors involved. In previous studies, this regulation has been shown to be mediated by one of the σ factors, σ^{54}, and a two-component regulatory system composed of the bacterial signal-transducing protein NtrB and the bacterial enhancer-binding protein NtrC (Ikegami et al. 2000a, b, c).

This review summarizes our recent work in transcriptional regulation under pressure conditions by the σ^{54} factor in *S. violacea*. We describe the upstream *cis* elements isolated from this strain, which are involved in transcriptional regulation under high-pressure conditions. Characterization of several *trans*-acting factors, such as σ factors and two-component transcriptional regulators contributing to

pressure regulation in this piezophilic bacterium, is also discussed.

Gene expression of glutamine synthetase at elevated hydrostatic pressure in the piezophilic bacterium *Shewanella violacea*

We have reported previously on the isolation and characterization of a glutamine synthetase gene (*glnA*) from *S. violacea* (Ikegami et al. 2000c). Figure 1A shows the gene organization around the *glnA* operon composed of five open reading frames (ORFs) and the nucleotide sequence of the *glnA* promoter region. To determine the transcription initiation sites and the effect of cultivation under elevated hydrostatic pressure on the *glnA* transcription, primer extension analysis was conducted. Through analysis of the 5′-ends of the mRNA, two distinct transcription products were identified (Fig. 1B). The σ^{70} consensus sequence (Cowing et al. 1985), –10 [TTGACA] and –35 [TATAAT], was identified upstream of the region corresponding to transcript #2, and the σ^{54} consensus sequence (Dixon 1984), [TGGYAYR-N4-TTGCA], was found upstream of the region corresponding to transcript #1 (see Fig. 1A). Therefore, it is evident that there are two tandem promoters of *glnA* in *S. violacea*, as seen in the case of *glnA* in *Escherichia coli* (Reitzer and Magasanik 1986; Hunt and Magasanik 1985).

The level of transcript #1 was elevated at 50 MPa; however, the level of transcript #2 was not enhanced at this pressure (see Fig. 1B). Transcripts #1 and #2 were expressed at a relatively low consistent level under atmospheric pressure conditions. In contrast, the expression of transcript #1 from the σ^{54}-dependent promoter was highly induced under high-pressure conditions (50 MPa) as compared to the expression of transcript #2 from the σ^{70}-dependent promoter. The σ^{54}-dependent promoter appears to play an important role in the transcription of *glnA* under high-pressure conditions in *S. violacea*, just as for the pressure-regulated operon (Nakasone et al. 1999). However, the level of expression of

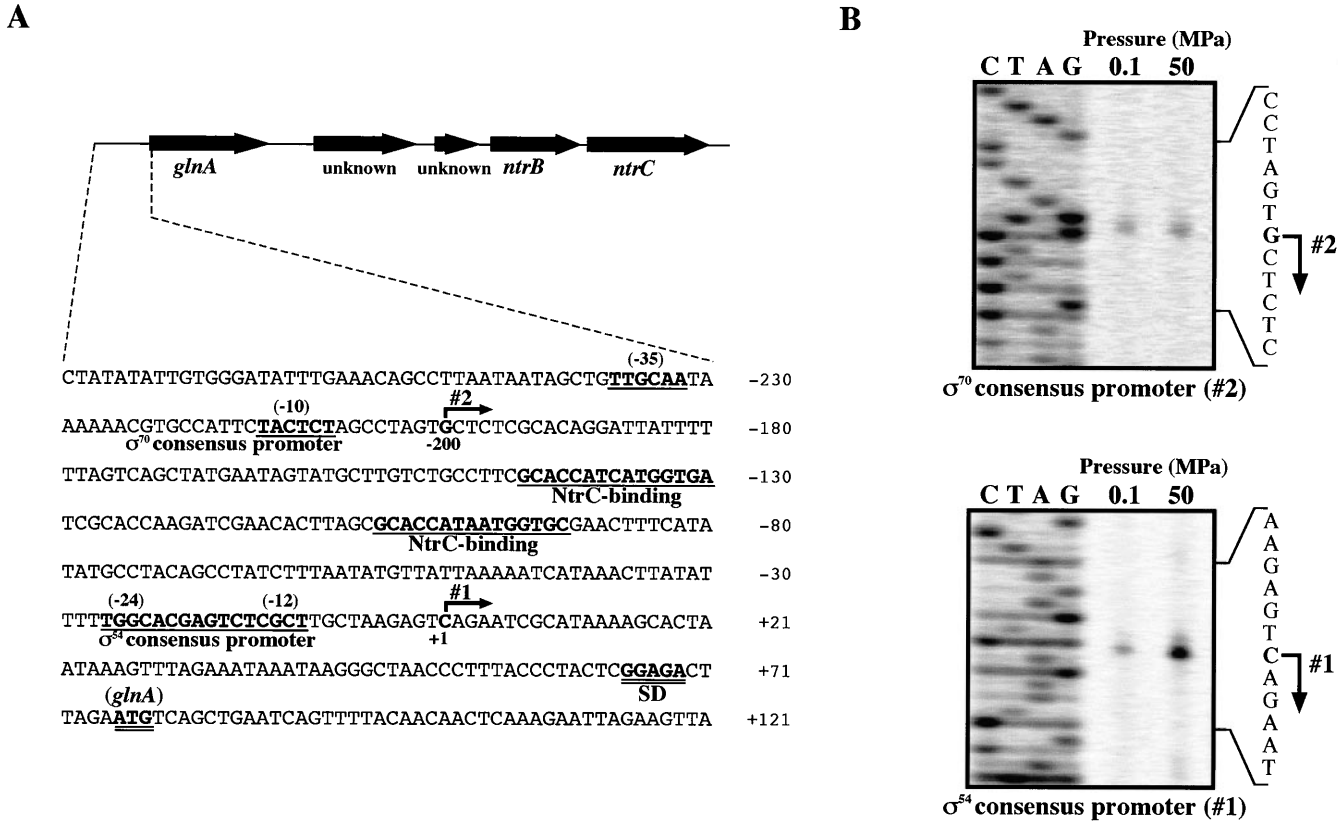

Fig. 1A,B. Gene organization around the *glnA* gene, nucleotide sequence of the *glnA* promoter region, and primer extension analysis of the *glnA* gene under different pressure conditions. **A** The coding regions and their direction of transcription are indicated by *arrows*. The gene names are shown under the corresponding coding regions. Transcription initiation sites (*#1* and *#2*) are marked by *arrows* above the nucleotide sequence, and the coordinate +1 is defined as the first nucleotide of transcript #1. The *numbers* shown at the *right* side correspond to the nucleotide numbers from position +1. The consensus promoter sequences (σ^{70} type, –35 and –10; σ^{54} type, –24 and –12) and the consensus sequences of the NtrC-binding site are *underlined*. The ATG start codon and potential ribosome-binding site (*SD*) are indicated by *double-underlining*. **B** Primer extension analysis of the *glnA* gene in *Shewanella violacea* under different pressure conditions. Total cellular RNA was prepared from cells grown at 0.1 and 50 MPa. These pressure conditions are shown at the top of the corresponding lanes. The transcripts (#1 and #2) are shown by *arrows*

transcript #2 (σ^{70}-dependent promoter) was also observed to be enhanced by elevated pressure up to 30 MPa, which is the optimal pressure for growth of *S. violacea* (Ikegami et al. 2000c), showing that the σ^{54}-dependent promoter is also involved in the transcription under high-pressure conditions, just as for the pressure-regulated α (*rpoA*) operon (Nakasone et al. 2000). In other bacteria, transcription from the σ^{54}-dependent promoter, such as in the case of *glnAp2*, is regulated by an enhancer-binding protein, NtrC (Ninfa et al. 1987). The NtrC-binding sites [consensus: TGCACCA-N3-TGGTGCA] are essential for regulation of transcription by the σ^{54}-containing RNA polymerase (Reitzer and Magasanik 1986). Two NtrC-binding sites were also found from the promoter region of *glnA* in *S. violacea* (see Fig. 1A).

Isolation and characterization of the RNA polymerase σ factor, σ^{54}, of the piezophilic bacterium *Shewanella violacea*

In addition to the results for the characterization of the *glnA* operon already discussed, we have characterized other genes, including the pressure-regulated operon cloned from *S. violacea*, which is controlled by elevated pressures at the level of transcription (Nakasone et al. 1999). In this report, we have analyzed a σ^{54}-like factor that recognizes a DNA element, designated as region A, upstream of the pressure-regulated operon. The RNA polymerase σ factor, σ^{54}, has been shown to be responsible for transcription of nitrogen-regulated genes such as glutamine synthetase and nitrogen fixation genes (Merrick and Edwards 1995). 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* (Lutz et al. 1990; Kohler et al. 1989). In *S. violacea*, transcription of these pressure-responsible genes may be dependent on the σ^{54}-containing RNA polymerase and its related transcription factors. From these backgrounds, we have isolated the *rpoN* gene encoding σ^{54} from the piezophilic bacterium *S. violacea* and also investigated the expression of the *rpoN* gene product under different pressure conditions. The structure of this fragment, containing eight ORFs, is shown in Fig. 2A. This *rpoN* gene, consisting of 1,476 bp, was found to encode a putative protein consisting of 492 amino acid residues with a predicted molecular mass of 55,359 Da (Ikegami et al. 2000b). The nucleotide sequence of the upstream region is also shown in Fig. 2A.

To determine the transcriptional initiation site and the effect of cultivation under elevated hydrostatic pressure on *rpoN* transcription, primer extension was conducted. The single major product appeared as an "A" residue at the transcription initiation site under both atmospheric and high-pressure conditions (Fig. 2B). Putative core promoter sequences [–35; TAGCCT] and [–10; GAGAAG], which might be recognized by the typical σ^{70}-containing RNA polymerase, were observed (see Fig. 2A). The analysis also showed that each transcript was detected with the same prominence under both atmospheric and high-pressure conditions, indicating the *rpoN* gene is expressed at a constant level under both pressure conditions (Ikegami et al. 2000b).

Western blot analysis was also performed to examine the expression of σ^{54} in *S. violacea*. The cells were cultured at a pressure of 0.1 or 50 MPa, and cell lysates were prepared and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was then incubated with antiserum against *Pseudomonas putida* σ^{54} (Ikegami et al. 2000b). As shown in Fig. 2C, bands of equivalent intensity were detected in the case of cells grown at either 0.1 or 50 MPa. These bands each corresponded to a mass of 55 kDa consistent with the predicted molecular mass of *S. violacea* σ^{54}. The results of primer extension and Western blot analyses together indicated that σ^{54} is expressed at a relatively constant level in *S. violacea* under both pressure conditions, 0.1 and 50 MPa, at both the transcriptional and translational levels. These observations are consistent with the finding that the intracellular concentration of σ^{54} in *E. coli* remained consistent under various growth conditions (Jishage et al. 1996). Our findings suggest the possibility that the level of functional σ^{54} molecules may be controlled by the availability of certain regulatory factors such as NtrC. The role of this activator is to catalyze the isomerization of closed complexes between the σ^{54}-containing holoenzyme and the promoter and to open the complexes (Sasse-Dwight and Gralla 1990). In *S. violacea*, transcription by the σ^{54}-containing RNA polymerase may be highly dependent on such transcription factors.

Recognition of the σ^{54} factor and NtrC protein by the *glnA* promoter region

As previously mentioned (see Fig. 1A), we have identified potential regulatory elements, consensus sequences for σ^{70}, σ^{54}, and NtrC binding, upstream of the *glnA* operon (Ikegami et al. 2000c). Furthermore, an electrophoretic mobility shift assay (EMSA) was performed to confirm whether purified recombinant *S. violacea* σ^{54} recognized the DNA fragment containing the σ^{54} consensus promoter sequence of the *glnA* operon. A [γ-^{32}P]DNA fragment containing the σ^{54} consensus sequence was incubated with various concentrations of purified *S. violacea* σ^{54} protein in the presence or absence of antiserum against σ^{54} (Ikegami et al. 2000c). An increase in the retarded band in proportion to the amount of protein was observed (lanes 2, 3, and 4 in Fig. 3A) and a decrease in the retarded band as a result of treatment with the antiserum against σ^{54} was also detected (lane 5 in Fig. 3A). These results indicated that a specific binding of the *S. violacea* σ^{54} to the promoter region of *glnA* occurred, as observed previously in the case of the pressure-regulated operon (Nakasone et al. 1999; Ikegami et al. 2000c). In this bacterium, the intracellular concentrations of σ^{54} were similar under several pressure conditions as already described. Transcription from σ^{54}-dependent promoters is known to be regulated by the enhancer-binding protein NtrC, and the initiation of transcription is responsi-

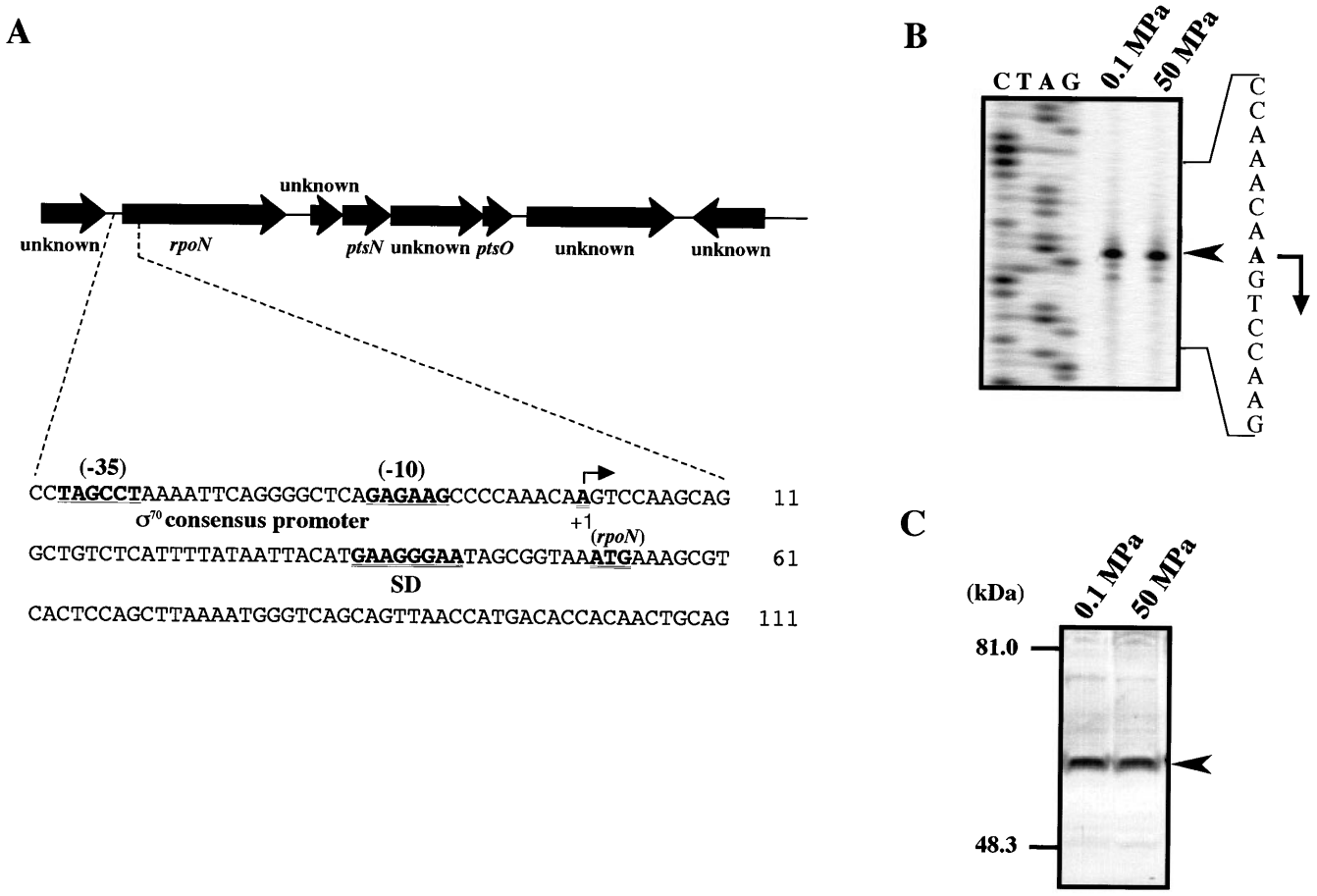

Fig. 2A–C. Gene organization around the *rpoN* gene, nucleotide sequence of the *rpoN* promoter region, primer extension analysis of transcription of the *rpoN* gene, and Western blot analysis of σ^{54} under different pressure conditions. **A** The coding regions and their direction of transcription are indicated by *arrow*s. The transcription initiation site is marked by the *arro*w above the nucleotide sequence, and the consensus promoter sequence is *underlined*. The start codon and potential ribosome-binding site (*SD*) are indicated by *double-underlining*. The *numbers* shown at the *right* side correspond to the nucleotide numbers from position +1. **B** Primer extension analysis of the transcription initiation site of the *rpoN* gene in *S. violacea*. Total cellular RNA was prepared from cells grown at 0.1 and 50 MPa. These pressure conditions are shown at the top of the corresponding lanes. *Arrow*, transcription start site. **C** Western blot analysis of the σ^{54} protein under different growth conditions. *Arrowhead*, position of the σ^{54} protein with a mass of 55 kDa. Sizes of molecular mass standards are indicated in kDa

ble for the conversion of NtrC to active NtrC phosphate by the protein kinase NtrB (Ninfa et al. 1987).

Pressure-regulated transcription from the σ^{54}-dependent promoter in *S. violacea* may be controlled by several transcription factors, as in the case of other nitrogen regulation systems (Alvarez-Morales et al. 1984). In addition, we performed an EMSA to confirm whether the purified *S. violacea* NtrC (SvNtrC) recognizes the DNA element containing the NtrC consensus sequence. In the EMSA (see lane 2, Fig. 3B), a DNA–protein complex was formed when the ^{32}P-labeled DNA probe containing the NtrC consensus sequence of the upstream region of *glnA* (from position –221 to +60; see Fig. 1A) was incubated with the purified SvNtrC protein. The complex was eliminated in the presence of a nonradioactive excess amount (×30) of target DNA (self-competitor) (see lane 3, Fig. 3B). Thus, the analysis demonstrated that the purified SvNtrC protein specifically recognizes the element containing the NtrC consensus sequence on the *S. violacea glnA* operon.

In vitro reconstitution and characterization of NtrB and NtrC in *Shewanella violacea*

To reconstitute the two-component regulatory system and characterize autophosphorylation of the SvNtrB and *trans*-phosphorylation of the SvNtrB–P to the SvNtrC in vitro, we constructed the recombinant expression plasmids harboring *S. violacea ntrB* and *ntrC* genes. Overexpression experiments of the hexahistidine-tagged derivatives of SvNtrB and SvNtrC were conducted, and these fusion proteins were purified using a Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) column. To confirm if the SvNtrB protein is a protein kinase capable of autophosphorylation, SvNtrB was incubated with [γ-^{32}P]ATP from 0° to 37°C for 15 min. As shown in lanes 1 to 3 of Fig. 4A, the SvNtrB became marked, indicating that it was autophosphorylated. The autophosphorylation of SvNtrB was observed only at low temperatures (maximum, 10°C), whereas activity was not detected at

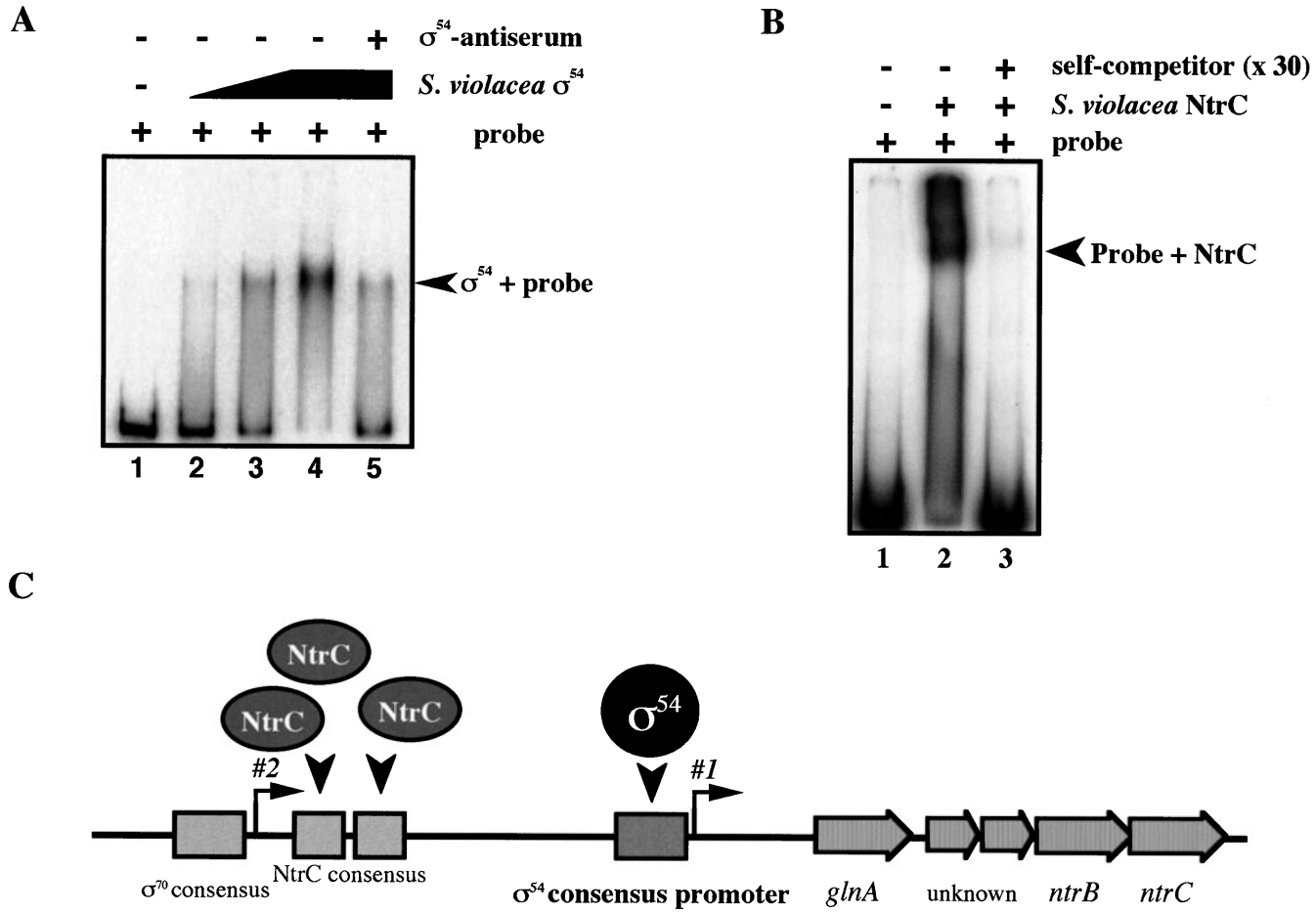

Fig. 3A–C. Interaction of the σ^{54} and the NtrC factors with the promoter region of the *glnA–ntrBC* operon. **A** An electrophoretic mobility shift assay (EMSA) of the interaction of the σ^{54} protein from *S. violacea* with the promoter region of the *glnA* gene and the effect of antiserum containing anti-σ^{54} polyclonal antibodies. The labeled fragment was incubated with increasing concentrations of *S. violacea* σ^{54} protein: 0, 30, 60, 150, and 150 ng (*lanes 1, 2, 3, 4,* and *5*, respectively). Antiserum containing σ^{54} polyclonal antibodies was simultaneously added to the reaction mixture (*lane 5*). **B** EMSA of the interaction of the NtrC protein from *S. violacea* with the promoter region of the *glnA* gene. *Lane 1*, free probe (0.1 pmol); *lanes 2* and *3*, the probe incubated with the σ^{54} protein (120 ng); *lane 3*, the same mixture as in lane 2 with self-competitor (×30). **C** Cartoon model for interaction of the σ^{54} and the NtrC factors with the promoter region of the *glnA–ntrBC* operon

37°C. Autophosphorylation activity of NtrB at low temperatures (10°C) was not detected in *E. coli*. Furthermore, we have detected transcriptional activity at low temperatures in *S. violacea* (data not shown). Therefore, this bacterium adapts to the psychrosphere (low-temperature environment) and may have evolved a low-temperature-adapted system in the deep-sea environment. This observation is also the first to detect autophosphorylation at low temperatures (Ikegami et al. 2002).

To determine if SvNtrB–P could phosphorylate SvNtrC (*trans*-phosphorylation), SvNtrB–P was incubated with SvNtrC at 10°C. SvNtrC was labeled in the presence of SvNtrB–P (lane 6, Fig. 4B), indicating that it is a substrate for SvNtrB. To develop an in vitro transcription system for the σ^{54}-dependent promoter, we must consider the temperature dependency of the reaction, especially at low-temperature conditions. The phosphorylated relay of the two-component system should also be performed under high-pressure conditions. Thus, we are now endeavoring to assay the systems under high-pressure.

Possible model of molecular mechanisms of pressure-regulated transcription by the σ^{54} factor

In transcription of the σ^{54}-dependent promoter such as the *glnA* operon, σ^{54}-containing RNA polymerase holoenzyme activates transcription at the promoter, assisted by the activity of NtrC that is controlled by NtrB (Keener and Kustu 1988; Buck et al. 2000). In previous studies, we suggested that these *trans*-acting factors (σ^{54}, NtrC, or NtrB) may play an important role in pressure-regulated transcription at the σ^{54}-dependent promoter in this piezophilic bacterium (Ikegami et al. 2000a, b, c). We

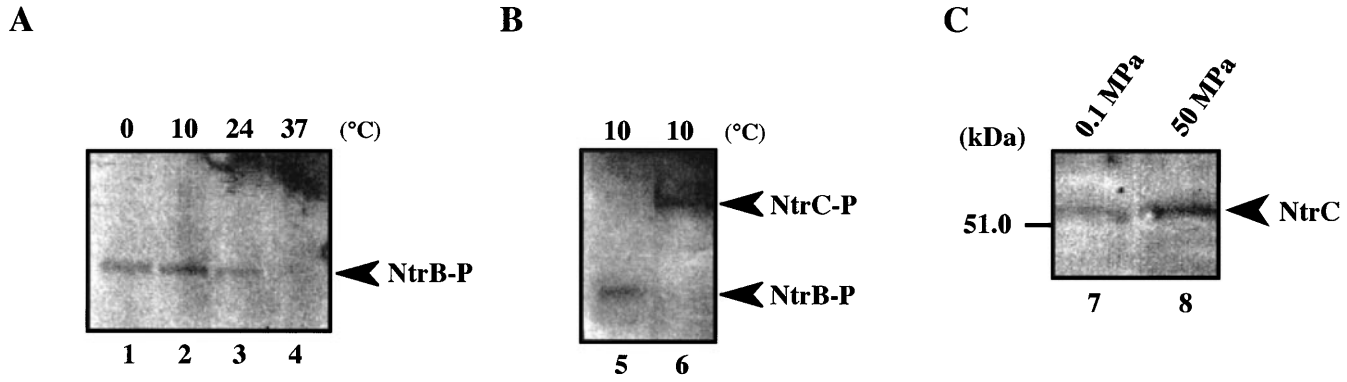

Fig. 4A–C. Autophosphorylation of the SvNtrB protein, *trans*-phosphorylation of the SvNtrB–P to the SvNtrC protein in vitro, and Western blot analysis of expression of the SvNtrC under different pressure conditions. **A** Autophosphorylation of SvNtrB incubated in the presence of [γ-^{32}P]ATP at several temperature conditions. *Lane 1*, 0°C; *lane 2*, 10°C; *lane 3*, 24°C; *lane 4*, 37°C. **B** *trans*-Phosphorylation to SvNtrC incubated with the phosphorylated SvNtrB–P at 10°C for 1 min. *Lane 5*, phosphorylated SvNtrB; *lane 6*, phosphorylated SvNtrC. **C** Cell lysates prepared from cells cultured at 0.1 or 50 MPa were fractionated by 10% SDS-PAGE and then blotted onto a polyvinylidene fluoride (PVDF) membrane. The membrane was treated with antiserum against SvNtrC

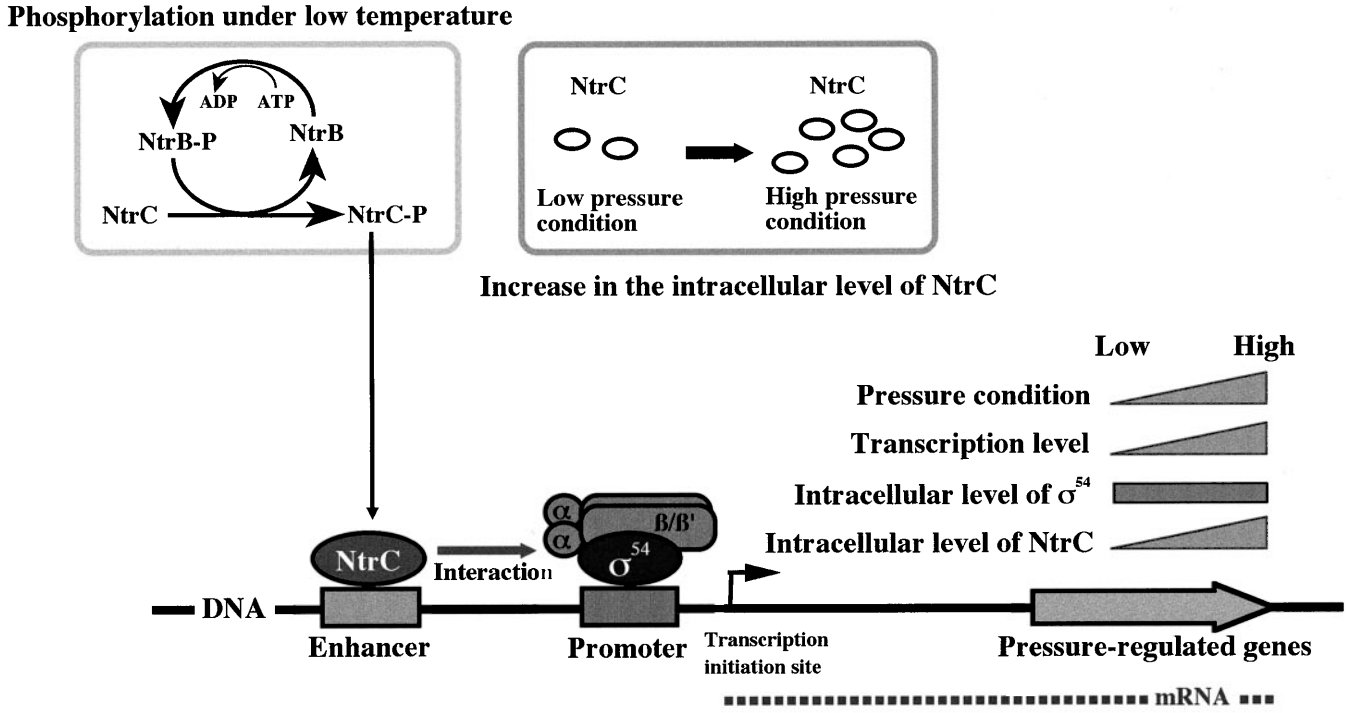

Fig. 5. Diagrammatic representation of the pressure-regulated transcription mechanisms in piezophilic *S. violacea*

discussed here that σ^{54} in *S. violacea* is expressed at a relatively constant level under both atmospheric and high-pressure conditions as determined by both primer extension and Western blotting analyses, suggesting that the level of functional σ^{54} molecules is possibly regulated by the availability of NtrC. In this regard, we tried to examine the expression of the NtrC protein in *S. violacea* by Western blot analysis (Ikegami et al. 2002). The cells were cultured at a pressure of 0.1 or 50 MPa, and cell lysates were prepared and fractionated by SDS-PAGE. The gel was then incubated with antiserum against the SvNtrC. The amount of this factor expressed under high-pressure conditions (lane 2, Fig. 4C; 50 MPa) is greater than that at atmospheric pressure conditions (lane 1, Fig. 4C; 0.1 MPa).

Consideration of the results presented here together with previous work leads us to suggest a possible model for the mechanism of regulated expression of the *glnA* operon in the deep-sea piezophilic bacterium *S. violacea* (Fig. 5). *S. violacea* σ^{54}, expressed at a consistent level at both atmospheric and high pressure, suggests that the intracellular level of σ^{54}-containing RNA polymerase holoenzyme under both conditions is constant. This observation also strongly suggests that the transcriptional activity at this σ^{54}-dependent promoter is proportional to the amount of

SvNtrC factors and that it is regulating the gene expression of the *glnA* operon under high-pressure conditions. This model for explaining gene expression under high pressure should be tested by molecular genetic approaches analyzing several pressure- and low temperature-sensitive mutants in these transcription systems.

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