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Newly idetified sigma factors of RNA Polymerase from deep-sea bacterium *Shewanella violacea*

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Two genes for alternative σ factors, σ^{E2} and σ^{E3}, classified in the extracytoplasmic function σ family for RNA polymerases, were identified in the deep-sea piezophilic bacterium *Shewanella violacea* DSS12. Amino acid alignments revealed that the domains for transcriptional functions are were comparatively conserved compared with *E. coli* σ^{E} in both proteins. Core-binding analysis suggests suggested that both proteins function as σ factors in this piezophilic bacterium.

Key words: *Shewanella violacea* DSS12; alternative σ factor; extracytoplasmic function; ECF family; core binding analysis

Introduction

Deep-sea bacteria developed microbial activities in order to adapt to high hydrostatic pressure and low temperature conditions.[1-3] Among such organisms, *Shewanella violacea* strain DSS12, a moderately piezophilic and psychrophilic deep-sea bacterium, grows optimally at 30 MPa and at 8°C, but also at 0.1 MPa (atmospheric pressure) and 8°C.[4] So farTo date, various genes have been shown found to be regulated by hydrostatic pressure in this strain.[5] One approach toward understanding the basis for of gene expression under defined conditions is *via* detailed characterization of the transcriptional machinery involved. RNA polymerase is an important enzyme in transcription composed of , , and ' (core enzyme, $_2$') and one of several σ proteins (holoenzyme, $_2$'σ).[6] Switching σ proteins and altering gene-expression changes allows bacterial cells to adapt to environmental changes.[7] In *Escherichia coli,* one of the σ proteins, σ^{E}, belongs belonging to the extracytoplasmic function (ECF) σ family has been studied for its role in combating extracytoplasmic stress.[8] In *S. violacea* cells, the σ^{E} coding-gene, *rpoE*, has been identified. and Its coding protein showed high similarity with *E. coli* σ^{E}.[9] Recently, genome analysis of several *Shewanella* strains has been performed, and some ECF σ type proteins have been identified in the database.

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Among these sequencesproteins, we focused on two types of ECF σ genes proteins having low similarity with *E. coli* σ^E, and we designated them these encoding genes as *rpoE2* and *rpoE3*, respectively. In this report, the isolation of the *rpoE2* and *rpoE3* genes from *S. violacea* and their characterization of these encoding proteins are is described.

Materials and methods

First, to clone a partial regions of the *rpoE2* and *rpoE3* genes from *S. violacea*, two sets of degenerate oligonucleotides, (primer 1 [(5'-CATAAGGGBGGYYTRTATCG-3')] and primer 2 [(5'-TCATCTTTCATTKASRACCTCC-3')] for *rpoE2* and primer 3 [(5'-CGATACGATAGCCTTGTCAGGGC-3')] and primer 4 [(5'-TCATCCATTGGATTGCCMTC-3')] for *rpoE3*) , were designed and synthesized based on highly conserved sequences from several *Shewanella* strains.

Results and discussions

Upon Based on the DNA sequences of the PCR products and following screening with a *S. violacea* -phage library, two types of positive clones of approximately 10 kb size were obtained. As shown in Fig. 1AB, the gene organization of the open reading frames (ORFs) in the fragment containing *S. violacea rpoE2* (AB449872) consisted of ORFs of an unknown protein gene, a phosphoribosylaminoimidazole carboxylase subunit gene, a von willebrand factor type A domain protein gene, *rpoE2*, another unknown protein gene, and a putative protease-coding gene, whereas the *rpoE3* (AB449873) gene fragment contained ORFs of a von willebrand factor type A domain protein gene, a TRP domain protein gene, an unknown protein gene, *rpoE3,* another unknown protein gene, and a sodium/proline symporter gene in an order which is similar with that of *S. oneidensis*. Two ORFsCloned genes, 687 bp (*rpoE2*), and 510 bp (*rpoE3*), respectively in length, were found to encode proteins consisting of 229, and 170 amino acid residues, with molecular masses of 25,608, and 20,103 Da, respectively (Fig. 1C). Amino acid alignment of RpoE2 (σ^{E2}) and RpoE3 (σ^{E3}) proteins with those of *S. oneidensis* and σ^E proteins from *S. violacea*, and *E. coli* was also performeddone. As shown in Fig. 2, evolutionarily conserved regions (1.2 to 4.2) defined for based on a comparison of divergent the principal σ proteins[10] were found (Fig. 2). Region 1.2 is known to prevent the σ protein from binding to promoter DNA without binding to the RNA polymerase core enzyme.[10] *S. violacea* σ^{E2} and σ^{E3} contain this region (σ^{E3} has a partial region). suggesting that *S. violacea* σ^{E2} and σ^{E3} could bind with the core enzyme prior to transcriptional initiation. CThe conserved region 2.1, also designated the RpoD box,[11] is involved in binding to the RNA polymerase core enzyme.[10] These regions were partially conserved in the *S. violacea* σ^{E2} and σ^{E3} proteins. In the case of the σ^A protein of *Bacillus subtilis*, region 2.3, which is rich in aromatic residues, was shown found to mediate DNA promoter melting.[1211] Several conserved aromatic residues were observed in these regions of the σ^{E2} and σ^{E3} proteins of *S. violacea*. Regions 2.4 and 4.2, which are involved in physical contact between the –10 and –35 promoter boxes and σ, were also highly conserved in *S. violacea* σ^{E2} and σ^{E3}. To classify the σ^{E2} and σ^{E3} proteins as true σ factors, phylogenetic analysis with other σ^{E2} and σ^{E3} homologus proteins from *Shewanellla* strains and some other gram-negative bacteria was performeddone by the neighbor-joining method (Fig.3A). The analysisresults revealed suggest that both proteins can are likely to be classified classifiable in the ECF σ family, like other closely related proteins from *Shewanella* strains., as indicated by taxonomic studies of 16S rRNA sequences. In order to characterize the σ^{E2} and σ^{E3} proteins, we constructed recombinant-expression plasmids using expression plasmid pQE30Xa (QIAGENQiagen, Valencia, CA) for hexahistidine-tagged derivatives of the σ^{E2} and σ^{E3}

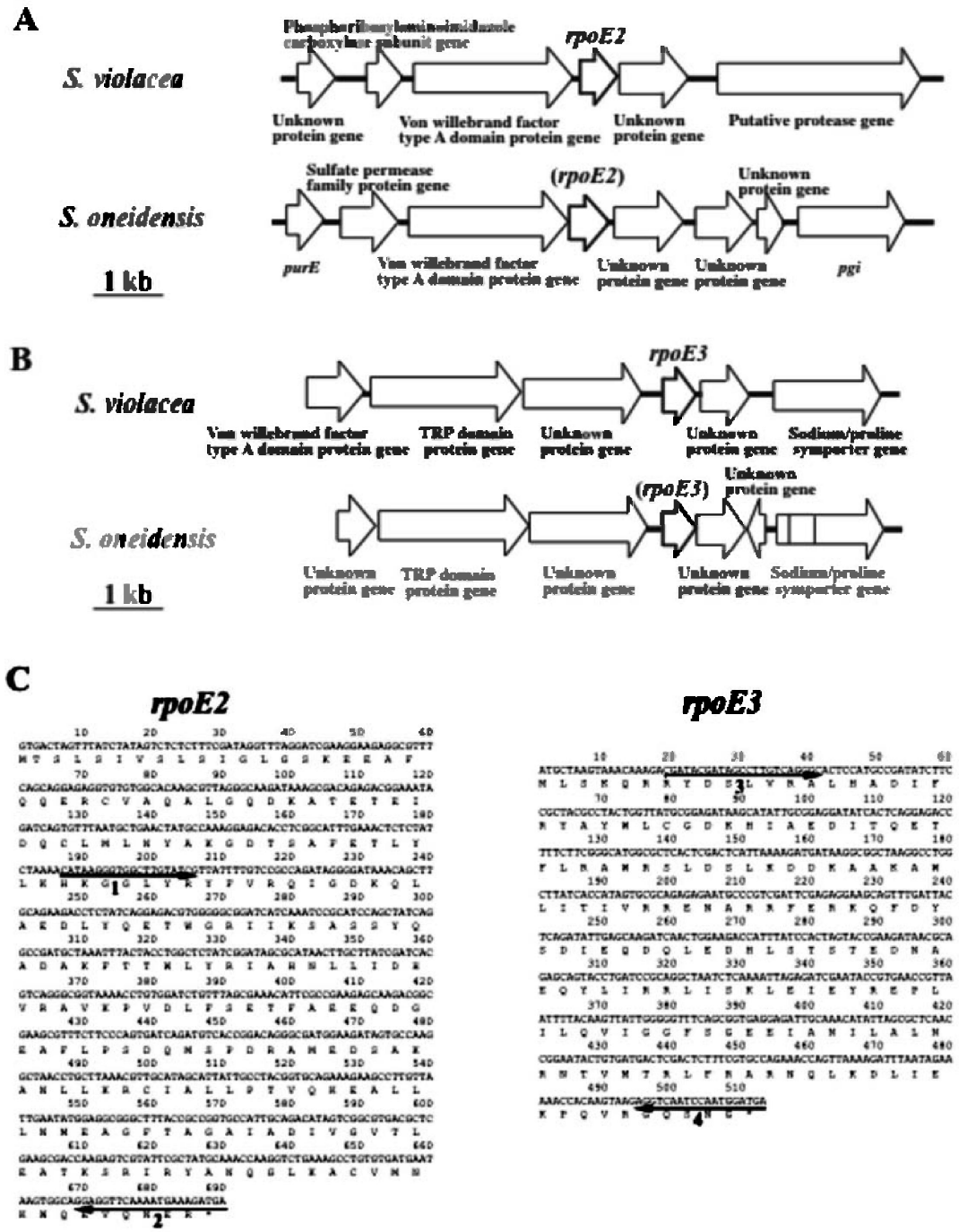

Fig. 1. Comparison of the gene ORF organizations Organizations of the *the RNA polymerase* σ^{E2} *and* σ^{E3} *genesrpoE2* and *rpoE3* Regions of *S. violacea* strain Strain DSS12.

The gene sStructures of the *S. violacea rpoE2* (A) and *rpoE3* (B) 10-kb fragments. The orientation of the genes ORFs is shown by the arrows. (C) , Nucleotide and deduced amino acid sequences of the σ^{E2} and σ^{E3} proteins. The nucleotide sequences corresponding to degenerate PCR primers are shown by arrows.

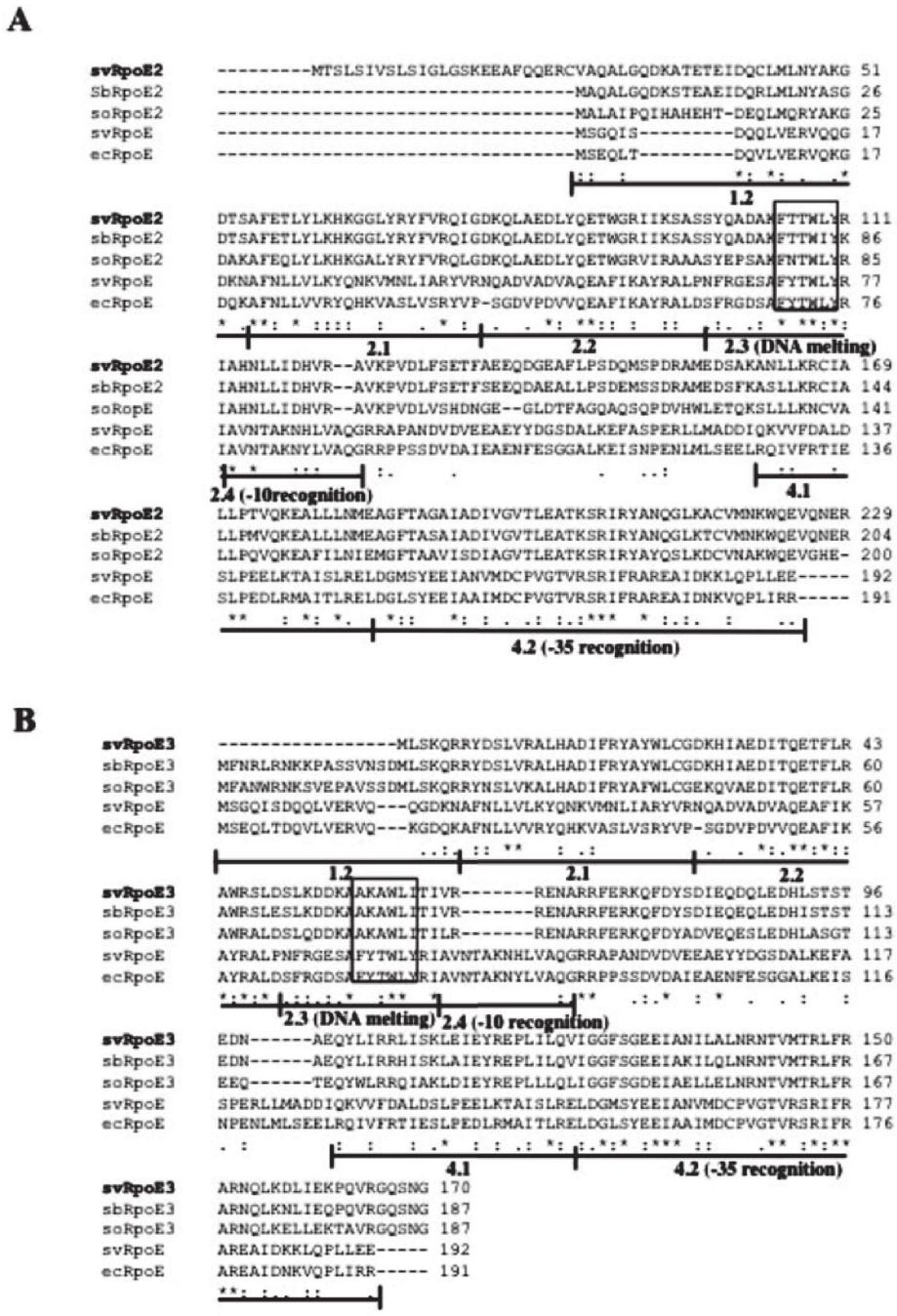

Fig. 2. Characterization of the *S. violacea* σ^{E2} and σ^{E3} proteinsProteins.

Comparison of the amino acid sequences of *S. violacea* σ^{E2} (A) and σ^{E3} (B) with those of *S. benthica, S. oneidensis,* and σ^{E} from *S. violacea* and *E. coli.* Completely conserved residues are indicated by asterisks (*), while dots (.) represent conserved substitutions between sequences. Dashes (-) represent gaps introduced for optimization of the alignment. Evolutionarily conserved regions (1.2 to 4.2) defined in the σ protein responsible for core binding, DNA melting, and –10 and –35 recognition are indicated under the amino acid sequence. The region rich in aromatic residues suggested to be responsible for DNA melting is boxed. Legends of to strains: sv, *S. violasea*; sb, *S. benthica*; so, *S. oneidensis*; ec, *E. coli.*

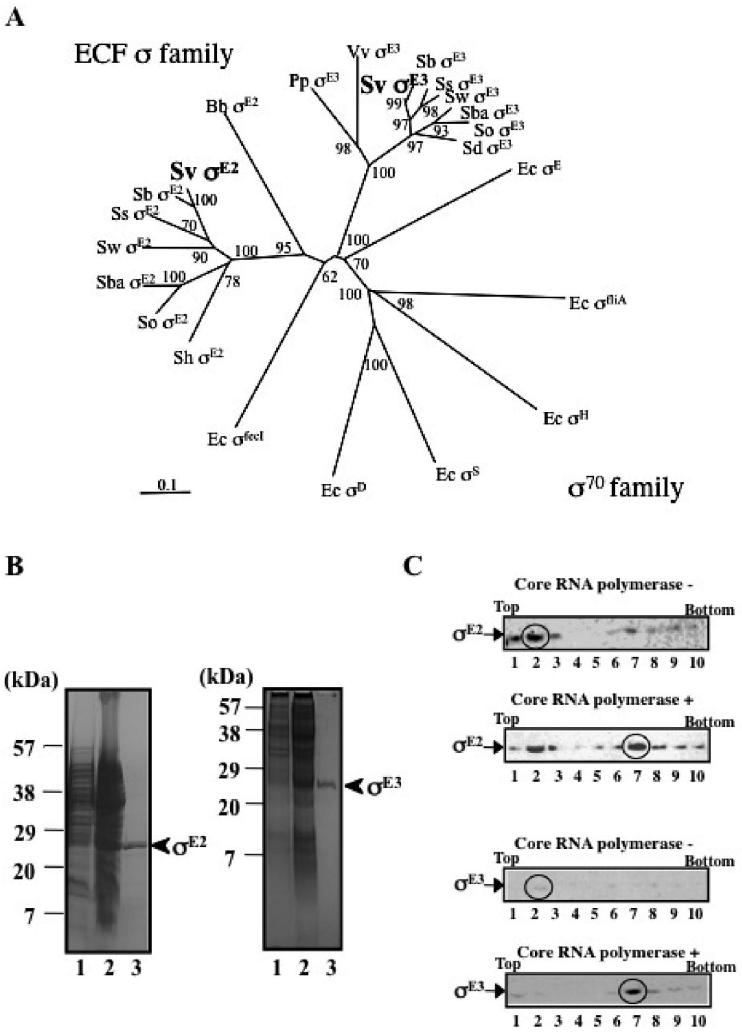

Fig. 3. Phylogenetic analysisAnalysis, purificationPurification, and core Core binding Binding assay Assay of the *S. violacea* σ^{E2} and σ^{E3} proteinsProteins.

(A) , Phylogenetic analysis of *S. violacea* σ^{E2} and σ^{E3} proteins with several homologus proteins and *E. coli* defined σ proteins. The neighbor-joining tree was drawn based on amino acid sequences from several gram-negative bacteria. The numbers indicate estimated confidence levels, expressed as percentages, for the positions of the branches as determined by bootstrap analysis with 1,000 replications. The bar indicates 0.1 nucleotide substitutions per site. Legends of to strains: Sv, *S. violasea*; Sb, *S. benthica*; Ss, *S. sediminis*; So, *S. oneidensis*; Sw, *S. woodyi*; Sba, *S. baltica*; Sh, *S. halifaxensis*; Sd, *S. denitrificans*; Bp, *Bordetella pertussis*; Vv, *Vibrio vulnificus*; Pp, *Photobacterium profundom*; Ec, *E. coli.*

(B) , Overexpression and purification of *S. violacea* σ^{E2} and σ^{E3} proteins. SDS-PAGE analysis of the *S. violacea* σ^{E2} and σ^{E3} hexahistidine-tagged fusion proteins. Lane 1, before induction of expression; lane 2, after overexpression of the *rpoE2* and *rpoE3* genes induced by treatment with IPTG at 1 mM; lane 3, purified hexahistidine-tagged-σ^{E2} and σ^{E3} proteins using a Ni^{2+}-chelating column. The molecular sizes are indicated in kDa on the left.

(C) , Results of core-binding analysis of the *S. violacea* σ^{E2} and σ^{E3} proteins.

Lanes 1–-10, sedimented fractions from top to bottom of the centrifugation tube; Arrows arrows indicate the positions of the recombinant σ^{E2} and σ^{E3} proteins.

proteins. PCR fragments of *rpoE2* and *rpoE3* were cloned into pQE30Xa and transformed to *E. coli* JM109. Cells harboring these expression plasmids were cultured for the log-phase, and production of the N-terminal hexahistidine-tagged proteins was done by the addition of IPTG. As shown in Fig. 3B, recombinant σ^{E2} and σ^{E3} proteins were purified to homogeneity by a simple chromatographic step, . and tThe molecular weights of the *S. violacea* σ^{E2} and σ^{E3} proteins as estimated by SDS-PAGE were 27 kDa and 26 kDa, respectively. We finally carried out core binding analysis using the glycerol gradient sedimentation technique according to the procedure described by Joo *et al.*[1312] *S. violacea* core RNA polymerase purified from cells[2] and each the purified σ^{E2} and σ^{E3} proteins were mixed, and was loaded on the top of a linear 15–35% (vol/vol) glycerol gradient, and centrifuged at 49,000 rpm. Ten fractions were subsequently analyzed by Western blotting analysis using tetra-His antibody (QIAGENQiagen). As shown in Fig. 3C, the σ^{E2} and σ^{E3} proteins were sedimented together with the core enzyme, indicating that both cloned σ^{E2} and σ^{E3} proteins have core-binding ability as σ factors, as predicted by the conserved region 2.1 (Fig. 2). Therefore, theseThe results confirm that σ^{E2} and σ^{E3} function as σ factors in *S. violacea.* Characterization Identification of the σ^{E2}- and σ^{E3}-regulatory binding sequencesgenes in this organism is now in progress.

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