

Research Article

Cloning, Identification, and Characterization of the *rpoS*-Like Sigma Factor *rpoX* from *Vibrio alginolyticus*

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Vibrio alginolyticus ZJ-51 displays phase variation between opaque/rugose colonies (Op) and translucent/smooth colonies (Tr). These colony variants show great differences in biofilm formation and motility. In this study, a gene encoding for an *rpoS*-like sigma factor, *rpoX*, has been cloned and characterized. The absence of *rpoX* did not affect colony switching rate but did decrease biofilm formation in both the Op and the Tr variants. When challenged with hydrogen peroxide, the $\Delta rpoX$ in the Op background showed a slightly higher survival rate compared with the wild type, whereas survival was decreased in the Tr background. Deletion of *rpoX* in the Tr background resulted in a higher ability to resist ethanol challenges and to survive hyperosmolarity challenges, and in the Op background the opposite phenotype was observed. This indicates that the *rpoX* gene is involved in biofilm formation and stress response but the effects are controlled by colony phase variation in *V. alginolyticus*.

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1. Introduction

Sigma factors are a class of proteins constituting essential dissociable subunits that confer sequence-specific DNA binding properties to RNA polymerase (RNAP). Within the RNAP holoenzyme, sigma factors provide promoter recognition specificity to the polymerase and contribute to DNA strand separation, then they dissociate from RNAP core enzyme and transcription initiation follows [1]. Besides the vegetative sigma factor, which recognizes the “standard” promoter, many bacteria possess alternative sigma factors that confer altered promoter specificity upon RNAP [2]. Alternative sigma factors are synthesized or activated in response to certain environmental conditions. They partially and/or temporarily replace the vegetative sigma factor and are thus crucial for many stress responses [2].

Sigma factors can be divided into two structurally unrelated families: the σ^{70} and the σ^{54} families. The σ^{70} family consists of primary sigma factors, as well as related alternative sigma factors [3, 4]. These alternative sigma factors are further classified by the physiological processes that they control, for example, stress response and flagella

biosynthesis [3, 4]. The extracytoplasmic function factors (ECF) belong to the σ^{70} family of sigma factors, but are phylogenetically distinct. Many ECF members are responsible for sensing and reacting to signals from the extracytoplasmic environment [3–5]. The σ^{70} family proteins generally contain four regions, region 1 to region 4, but only region 2 and 4 are well conserved in all members [3, 4]. *rpoS* or σ^s , a member of the σ^{70} alternative sigma factors subfamily, is important for survival under various stress conditions including starvation, oxidative stress, osmotic shock, extreme temperature, low pH (acidic stress), and exposure to UV (DNA damage) [6–15].

V. alginolyticus is an opportunistic pathogen of marine fish, shellfish, as well as human [16]. On the coast of Southern China, *V. alginolyticus* is the dominant *Vibrio* species found in the seawater and in farmed marine animals [17]. *V. alginolyticus* ZJ-51, isolated from diseased grouper, has shown colony phase variation between opaque/rugose (Op, opaque variant) and translucent/smooth (Tr, translucent variant). The two variants exhibit different capability to form biofilms and to express genes involved in polysaccharide biosynthesis, flagellar synthesis, and the AI-2 quorum-sensing system [18]. Recently, genome sequences revealed

TABLE 1: Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics ^a	Reference or source
E. coli		
SY327	$\Delta(lac\ pro)\ argE(Am)\ rif\ malA\ recA56\ \lambda pir$ lysogen	[19]
S17-1	<i>thi pro hsdR hsdM⁺ recA</i> RP4-2-Tc::Mu-Km::Tn7 λpir lysogen	[20]
V. alginolyticus		
ZJ-O	Opaque variant of wild strain, isolated from diseased cultured fish in Guangdong province on the Southern China coast. Ap ^r	[17]
ZJ-T	Translucent variant of wild strain; isolated from diseased cultured fish in Guangdong province on the Southern China coast. Ap ^r	[17]
$\Delta rpoX$ -O	ZJ-O carrying an in-frame deletion of <i>rpoX</i> . Ap ^r	This study
$\Delta rpoX$ -T	ZJ-T carrying an in-frame deletion of <i>rpoX</i> . Ap ^r	This study
CrpoX-O	$\Delta rpoX$ -O complemented in trans with wild-type <i>rpoX</i> gene. Ap ^r and Cm ^r	This study
CrpoX-T	$\Delta rpoX$ -T complemented in trans with wild-type <i>rpoX</i> gene. Ap ^r and Cm ^r	This study
Plasmids		
pDM4	Cm ^r , suicide vector with an R6K origin(<i>pir</i> requiring) and <i>sacBR</i> of <i>Bacillus subtilis</i>	[21]
pDMrpoX	Cm ^r , pDM4 containing a mutant allele of <i>rpoX</i>	This study
pDMCrpoX	Cm ^r , pDM4 containing the wild-type allele of <i>rpoX</i>	This study

^aCm^r, chloramphenicol resistant

Ap^r, ampicillin resistant.

that in addition to *rpoS*, an *rpoS*-like sigma factor was found in *V. alginolyticus* 12G01. The protein is composed of 279 amino acids. *Amino acid 10 to 277* contains an *rpoS*-proteo region with three conserved sigma 70 regions. However, the function of this protein has not been determined yet. Based on this, we wanted to identify this protein in *V. alginolyticus* ZJ-51 and study whether it has a role in biofilm formation and stress response in the two colony phase variants.

2. Materials and Methods

2.1. Strains, Plasmids, and Growth Conditions. Bacterial strains and plasmids are described in Table 1. ZJ-O is the opaque/rugose variant of the wild type ZJ-51 and ZJ-T is the translucent/smooth variant derived from ZJ-O. Bacteria were routinely grown in Trypticase Soy Broth (TSB) (Becton,

Dickinson and company), or 1.5% TSB agar plate (TSA) at 30°C, with the following antibiotics: 100 µg/mL ampicillin (Ap) and 10 µg/mL chloramphenicol (Cm). When necessary, Luria-Bertani (LB) and 1.5% LB agar plate (LA) were used. Biofilm growth medium (BF medium) contains minimal 1 × M63 salts supplemented with 1% NaCl, 1.5% Casamino acids, 1% glucose, 1 mM MgSO₄, and 10 µg Thiamine per liter. For selection of conjugants, Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) (HUANKAI MICROBIAL SCI.&TECH., GD, China) was used with Cm at 10 µg/mL. For sucrose selection, TSA and TSB containing 10% sucrose were used.

2.2. Cloning the *rpoX* Gene from *V. alginolyticus*. To clone *rpoX* from *V. alginolyticus* ZJ-51, a pair of primers derived from the *rpoS*-like sigma factor gene (NZ_AAPS01000024) in *V. alginolyticus* 12G01 were designed as follows: *rpoX*-I (5'-CCCCACCTGCTCACG-3') and *rpoX*-II (5'-GAGACA-CAAGCGAA-3'). PCR was performed at 95°C for 5 min, 35 cycles at 94°C for 30 sec, 30 sec at 55°C, 1 min at 72°C, followed by incubation at 72°C for 10 min. A 1.199-bp DNA fragment from the *V. alginolyticus* ZJ-51 genomic DNA was amplified and sequenced. Nucleotide and deduced protein sequences were analyzed with BLAST and CLUSTAL (version 1.81).

2.3. Mutant and Complementation Strains. To create null mutants of *rpoX* in both Op and Tr backgrounds, an in-frame deletion was made by allelic exchanges as described previously [21]. Briefly, the allele was created by two rounds of PCR. The first round of PCR was carried out to generate two fragments flanking the coding region of *rpoX*. The first fragment was amplified using primers *rpoX*-A (ATAGAT-CTCCCCACCTGCTCACG), which contains a *Sall* site at the 5'-end and *rpoX*-B (GGGCTAAAAATCTTATGACAG-TTCATCGT); *rpoX*-D (ATGTCGACTCGAGACACAAGC-GCAA), which contains a *Bgl*III site at the 5'-end and *rpoX*-C (ACGGATGAACTGTCATAAGATTTTTAGCCC) were used to amplify the second fragment. Both fragments contain a 10-bp overlapping sequence and were used as templates for the second PCR using the primers *rpoX*-A and *rpoX*-D. The resulting fragment was ligated into the suicide vector pDM4 that had been cut with *Sall* and *Bgl*III to generate the plasmid pDMrpoX. The recombinant suicide plasmid was then conjugated into *V. alginolyticus* ZJ-O and ZJ-T. Conjugants were selected on the TCBS plates containing 10 µg/mL Cm, followed by sucrose selection. The in-frame deletions, where the wild-type coding sequence of *rpoX* was completely removed by allelic exchange, were confirmed by sequencing.

To construct complemented strains, the open reading frame (ORF) of *rpoX* and flanking DNA were amplified using the primers *rpoX*-A and *rpoX*-D. The fragment was ligated into pDM4, resulting in pDMCrpoX. The plasmid was introduced into the *rpoX* null mutants by conjugation. Cm- and Ap-resistant transconjugants were selected, and the presence of the plasmid was confirmed by PCR analysis and sequencing.

2.4. Growth Rate Assay. Overnight cultures of the bacteria were inoculated in TSB with the initial OD₆₀₀ of 0.01. The optical density was measured every 2 hours.

2.5. Colony Phase Variation Assay. Strains of the Op background were incubated statically in LB at 30°C for 24 hours as described before [18]. Then the cultures were serially diluted and plated on LA. Colony phase was observed and the variation rate was calculated as percentage of Tr cells in the total number of cells. The experiment was repeated three times.

2.6. Biofilm Assay. Biofilm formation was quantitatively analyzed with crystal violet staining as described previously [18]. Biofilm structures were observed with scanning electron microscope (SEM) as described previously [22]. Briefly, biofilms formed on sterilized glass coverslips that were placed in 50 mL centrifuge tubes containing 10 mL BF medium. After 6-hour static incubation, the coverslips were removed and biofilm structures on the coverslip were observed with SEM S-3400N.

2.7. Stress Response Assays. Bacterial viability was quantitatively determined using the LIVE/DEAD BacLight Bacterial Viability Kit for microscopy and quantitative assay (Molecular Probes, OR) according to the manufacture instruction. Fluorescence output was measured with a Victor 3 multiple label microplate reader (PE, USA) with the excitation and emission wavelengths of 485 nm and 535 nm, respectively.

Before exposure to various stresses, overnight cultures were centrifuged and washed twice with 0.9% NaCl. Then the equal cell numbers, corresponding to an OD₆₀₀ of 0.5/mL, were diluted in 0.9% NaCl containing either 18% ethanol (Ethanol challenge) or 30 mM H₂O₂ (oxidative challenge) and 2.5 M NaCl (osmotic challenge). Incubations were carried out at 30°C. The heat challenge was performed in pre-warmed (45°C) 0.9% NaCl. At selected time points, samples were withdrawn, centrifuged, and resuspended in the same volume of 0.9% NaCl, then fluorescence was measured. All experiments were done three times (**Gene Accession Number** FJ358498).

3. Results

3.1. Identification of *rpoX* of *V. alginolyticus* ZJ-51. A fragment of 1.199 kb in length was amplified from chromosomal DNA of *V. alginolyticus* ZJ-51 using a pair of primers derived from the sequence of the *V. alginolyticus* 12G01 *rpoS*-like gene. The sequence contained the ORF of the *rpoX* gene (840 bp) and the flanking regions. BlastN analysis showed a 73% identity of the DNA fragment to the *rpoS*-like gene of *Vibrio splendidus* LGP329 (FM954973), in addition to a 98% identity to that of *V. alginolyticus* 12G01 (NZ_AAAPS01000024). The sequence of the deduced protein was the same as the *rpoS*-like sigma factor of *V. alginolyticus* 12G01 and showed 80% identity to the *rpoS*-like sigma factor of *Vibrio sp.* MED222 and 79% identity to the *rpoS*-like sigma factor of *V. splendidus* LGP32. Interestingly, RpoQ of

V. fischeri ES114 and *rpoS* of *V. alginolyticus* VIB283 were also picked up in the search with 40% and 36% identity, respectively (Figure 1).

The predicted protein sequence of *rpoX* contains three conserved domains: (i) sigma 70 region 1.2 (amino acid 10 to 45), (ii) sigma 70 region 2 (amino acid 48 to 108), and (iii) sigma region 4 (amino acid 219 to 271) (Figure 2). Protein sequence alignment revealed that the reason for a 40% identity to the *rpoS* of *V. alginolyticus* was due to the second conserved region, which belongs to the sigma 70 region 2 superfamily. In contrast to *rpoS* and RpoQ, *rpoX* does not contain the sigma 70 region 3, but it possesses sigma 70 region 1.2, which is absent in the *rpoS*-like sigma factors of *Vibrio sp.* MED222 and *V. splendidus* LGP32 (Figure 2).

3.2. Growth Ability. Bacterial growth was investigated for wild types, the *rpoX* mutants, and the complemented strains. All the mutants showed the same growth rate as the wild types in both Op and Tr backgrounds (Figure 3). Deletion of *rpoX* does not interfere with the cell growth.

3.3. Colony Phase Variation Ability. In the previous study [18], transition from Tr to Op has never been observed, thus variation from Op to Tr colony was examined only in the Op background (ZJ-O, $\Delta rpoX$ -O, and *CrpoX*-O). After incubation in LB without shaking for 24 hours, the switching rates from Op to Tr were 26.00% (ZJ-O), 27.12% ($\Delta rpoX$ -O), and 26.78% (*CrpoX*-O), respectively (Figure 4). It suggested that *rpoX* has no effect on colony phase variation rate.

3.4. Biofilm Assay. Biofilm formation was investigated for wild types, the *rpoX* mutants, and the complemented strains. As shown in Figure 5(a), all strains exhibited similar biofilm formation kinetics. The amount of attached cells peaked when the bacteria were in late exponential growth phase (6 hours). The peak was followed by a dramatic decrease to a basal level of attached cells. However, compared to their parental strains, *rpoX* mutants in both backgrounds were reduced in their maximal attached biomass. The structure of the biofilms was observed using SEM. As shown in Figure 5(b), ZJ-O and $\Delta rpoX$ -O attached to the surface with single cells, but cells of ZJ-T and $\Delta rpoX$ -T were stacked together and formed structures resembling microcolonies. Surprisingly, the $\Delta rpoX$ -T cell had a pilus-like structure on the cell surface, which is absent in the other three strains.

3.5. Stress Response. The *rpoS* mutants in *Vibrio* species have been shown to be more susceptible to environmental stresses than the wild type [9, 12–15]. Therefore we examined the effect of the *rpoX* deletion in *V. alginolyticus* ZJ-O and ZJ-T on the survival of these cells under various stress conditions.

As shown in Figure 6(a), when challenged with heat (45°C) for 90 minutes, more than 60% of the cells had died in all strains. The survival rate for all strains was similar. This suggests that *rpoX* is not involved in the heat shock response in *V. alginolyticus*.

When exposed to hydrogen peroxide (30 mM H₂O₂), the *rpoX* mutant in the Op background was slightly better than

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V. alg ZJ-51      -----MKE S L SVGKDPYAHY L K D I
V. alg 12G01     -----MKE S L SVGKDPYAHY L K D I
V. sp MED222     -----M N N D N F A A F Q G L T N E T P I K E S F S T A T E P Y A H Y L K D I
V. fis ES114     -----M A Y A K L D M R V C E E Q G L N D S Y S L Y L K E I
V. alg RpoS      M S I S N T V T K V E E F A F D N A S V K T I D N E L E R S S S T E S K A T A R E E F D A S S K S L D A T Q L Y L G E I

V. alg ZJ-51      V G - - I D L L S R E E E L H Y A K L N R A G D E E A R E M L I E S N L R L V V K I A K G Y L K R P G N N F T L L D L I
V. alg 12G01     V G - - I D L L S R E E E L H Y A K L N R A G D E E A R E M L I E S N L R L V V K I A K G Y L K R P G N N F T L L D L I
V. sp MED222     V G - - I D L L S R E D E L Y Y A K L N R A G D K H A R D I M I E S N L R L V V K I A K S Y L K R K G N N F T L L D L I
V. fis ES114     N R - - Y Q L L T A E E E L A Y S R L Y K K G D V S A R N T L I E S N L R L V V K V A N K Y R K R N Q T D L A I L D I I
V. alg RpoS      G F S P L L I L T A E E E V L Y A R R A L R G D E A A R K R M I E S N L R L V V K I S R R Y S N R - - - G L A L L D L I

V. alg ZJ-51      E E G N I G L I K A V D K F D P E P G Y R F S T Y A V W W I R E N I E A A L M N N G R T V R L P A H V C K E I N S L A L
V. alg 12G01     E E G N I G L I K A V D K F D P E P G Y R F S T Y A V W W I R E N I E A A L M N N G R T V R L P A H V C K E I N S L A L
V. sp MED222     E E G N I G L I K A I D K Y D P E P G Y R F S T Y A V W W I R E N I E A A L M N K G R T V R L P A H V C K E I N S L A S
V. fis ES114     E E G N L G L I K A I D K F N P E L G Y R F S T Y A V W W I R E S I E S A L F N H S R T V R L P V H I T K E L N T Y L R
V. alg RpoS      E E G N L G L I R A V E K F D P E R G F R F S T Y A T W W I R Q T I E R A L M N Q T R T I R L P I H V V K E L N I Y L R

V. alg ZJ-51      K K L D A S K S E S K E L S I S E L S R C S G V K A E R I D E L V A L S G - F I D V S T T A D L H R V P F V L E L C E S
V. alg 12G01     K K L D A S K S E S K E L S I S E L S R C S G V K A E R I D E L V A L S G - F I D V S T T A D L H R V P F V L E L C E S
V. sp MED222     K K L D V S K K M R K E I S I S E L S N I S G V K A E R V D Q L V A L S G - F I D V T T T V G I S H A P L V L E Q C E S
V. fis ES114     A A R E L S K S L K R E P S I R D I S T Y C E E E Q I K V S K I I S L I P N Q L T C H S A C S D E L S P K F E L S S N
V. alg RpoS      T A R E L S Q K L D H E P T A E E I A A Q L D I P V E D V S K M L R L N E R I S S V D T P I G D G E K A L L D I P D

V. alg ZJ-51      E Y T P D P Q Q D C E N Q C F T Q A I E N I I K T L P T K L Q K V L I H R F G L F D Q R V K T L S E I G - - L L L N V S
V. alg 12G01     E Y T P D P Q Q D C E N Q C F T Q A I E N I I K T L P T K L Q K V L I H R F G L F D Q R V K T L S E I G - - L L L N V S
V. sp MED222     E Y I P D P Q Q D C E D Q S F T H A L E Q I I N T L P P K L Q K V L I H R F G F F D N K V K T L S E V G Q M L D V N V S
V. fis ES114     N K A L E P D S S L S Q N N L E M N L S S W I D S L N Y R E K E I I I N R Y G L F G C K V K T L E D L G - - K D L D L S
V. alg RpoS      A N N S D P E V S T Q D D D I K S S L I H W L E E L N P K Q K E V L A R R F G L L G Y E P S T L E E V G - - R E I G L T

V. alg ZJ-51      N E R V R Q M Q I E A V E R V Q K R L R F D G W V - - - - -
V. alg 12G01     N E R V R Q M Q I E A V E R V Q K R L R F D G W V - - - - -
V. sp MED222     N E R V R Q M Q K E A V E R I Q K R L K F D G W V - - - - -
V. fis ES114     K E R V R Q I Q S E T L I K L N T I F K N K L D L E I A L G - - - -
V. alg RpoS      R E R V R Q I Q V E G L R R L R E V L I K Q G L N M E N L F N V E D D

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FIGURE 1: Alignment of the amino acid sequences of *rpoX* from *V. alginolyticus* ZJ-51 (this study, the *rpoX* gene gb: FJ358498), *rpoS*-like sigma factor from *V. alginolyticus* 12G01 (ZP_01261551), *Vibrio sp.* MED222 (ZP_01063551) and *V. splendidus* LGP32 (YP_002395435), RpoQ from *V. fischeri* ES114 (YP_206973), and *rpoS* from *V. alginolyticus* (ABW86844) with the accession number of the databases indicated in parentheses. Identical amino acid residues present in three or more proteins are boxed in black. Similar residues are highlighted in gray. The *V. alginolyticus rpoX* translated sequence overall identity ranges from 36% to 100% for the other sequences shown.

the wild type to survive the first 60 minutes, but after 90 minutes, no differences could be observed. $\Delta rpoX$ in the Tr background resulted in cells more sensitive to hydrogen peroxide, compared to wild type. This phenotype could be complemented (Figure 6(b)).

Interestingly, when exposed to 18% ethanol and/or to 2.5 M NaCl (hyperosmolarity), the $\Delta rpoX$ in the Op background showed decreased survival rates than the wild type and complemented strain. However, deletion of *rpoX* in the Tr background resulted in higher survival ability to ethanol and NaCl than the wild and complemented cells (Figures 6(c) and 6(d)). This suggests that the function of *rpoX* is connected to the colony morphotype: in the Op background, *rpoX* is required for resistance towards ethanol

and hyperosmolarity, whereas in the Tr background, *rpoX* may be detrimental, as its loss increases resistance.

4. Discussion

In this study, the gene *rpoX*, an *rpoS*-like sigma factor from *V. alginolyticus* ZJ-51, was identified and sequenced. The nucleotide sequence showed no similarity with other genes, except for *rpoS* of *V. splendidus*. In contrast the deduced protein exhibited a certain level of identity to *rpoS*-like sigma factors in a few *Vibrio* species. *rpoX* contains three conserved domains: (i) sigma 70 region 1.2, (ii) region 2, and (iii) region 4. Sequence alignments revealed that the similarity to *rpoS* originated from region 2. All members of the σ^{70}

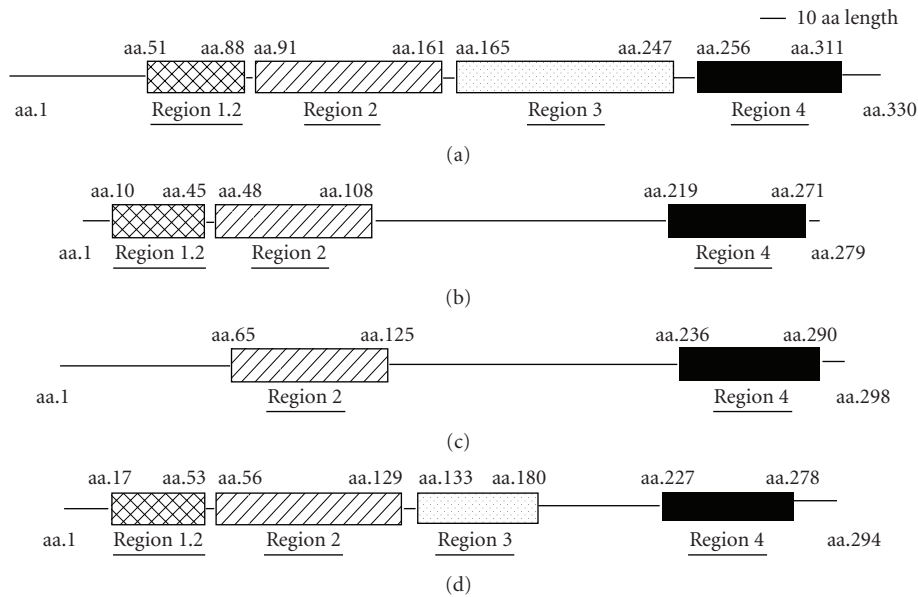


FIGURE 2: Comparison of the conservative domains among *rpoS*, *rpoX*, and the other *rpoS*-like sigma factors. (a) Conserved *rpoS* domains in *V. alginolyticus*, (b) *rpoX* conserved domains in *V. alginolyticus* 12G01 and ZJ-51, (c) conserved domains in *rpoS*-like sigma factors in *Vibrio sp.* MED222 and *V. splendidus* LGP32, and (d) RpoQ conserved domains in *V. fischeri* ES114. Compared with *rpoS* and RpoQ, *rpoX* does not have the sigma 70 region 3, but it contains sigma 70 region 1.2, a region that is absent in the *rpoS*-like sigma factors of *Vibrio sp.* MED222 and *V. splendidus* LGP32.

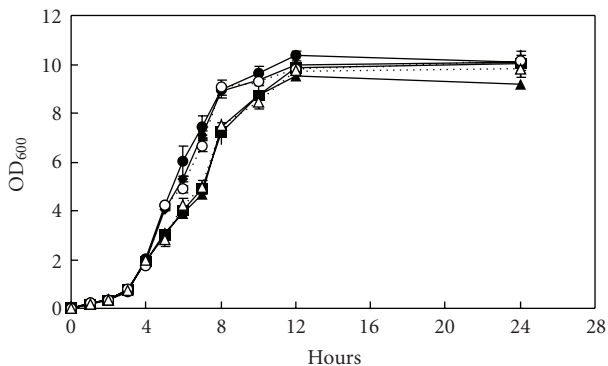


FIGURE 3: Kinetics of cell growth of both mutant and wild type of *V. alginolyticus* ZJ-51, wild type (Op: closed circle; Tr: closed triangle), *rpoX* null mutant (Op: closed diamond; Tr: closed square), and complemented strains (Op: open circle; Tr: open triangle). Bacteria were grown in TSB medium in Erlenmeyer flasks, which were incubated at 30°C with 240 rpm. Samples were taken at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 hours. For every sample, the OD at 600 nm was measured.

family contain regions 2 and 4 [3, 4]. Region 2 is the most conserved region in the family since it contains both the -10 promoter recognition residues and the primary core RNAP binding domain, which is essential for the specificity and initiation of transcription [4, 23]. Compared to *rpoS* and RpoQ, *rpoX* does not have the sigma 70 region 3. Instead a sigma 70 region 1.2 is present, which is absent in the *rpoS*-like sigma factors of *Vibrio sp.* MED222 and *V. splendidus* LGP32. The σ^{70} region 1.2 is a subregion of region

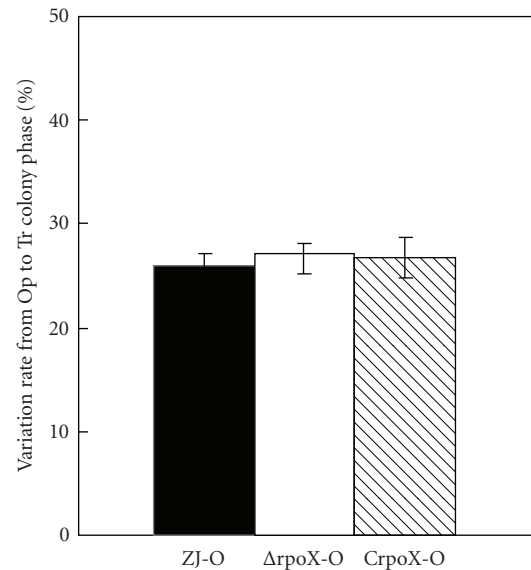


FIGURE 4: Variation rate from the opaque to translucent colony phase was examined in ZJ-O, $\Delta rpoX$ -O, and CrpoX-O of *V. alginolyticus* ZJ-51. After static incubation in LB for 24 hours, percentages of switched colonies were 26.00%, 27.12%, and 26.78% in the wild, mutant, and complemented strains, respectively.

1 and is found in the σ^{70} family except for the Sigma B of *Myxococcus xanthus* and the FliA protein of *Salmonella typhimurium*. Region 3 forms a discrete compact three-helix domain within the sigma factor. This region is usually not involved in the recognition of promoter DNA. But region 3

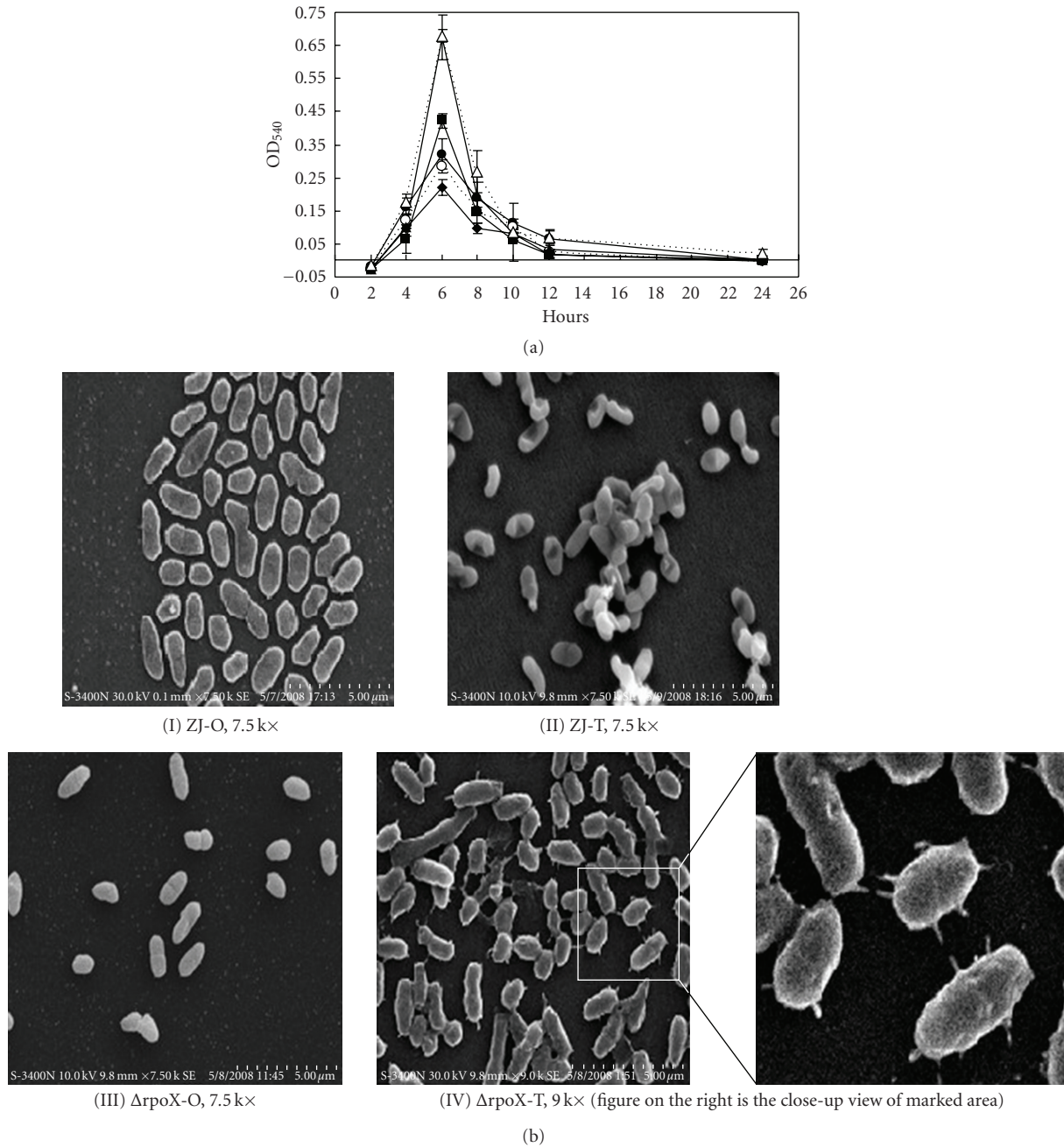
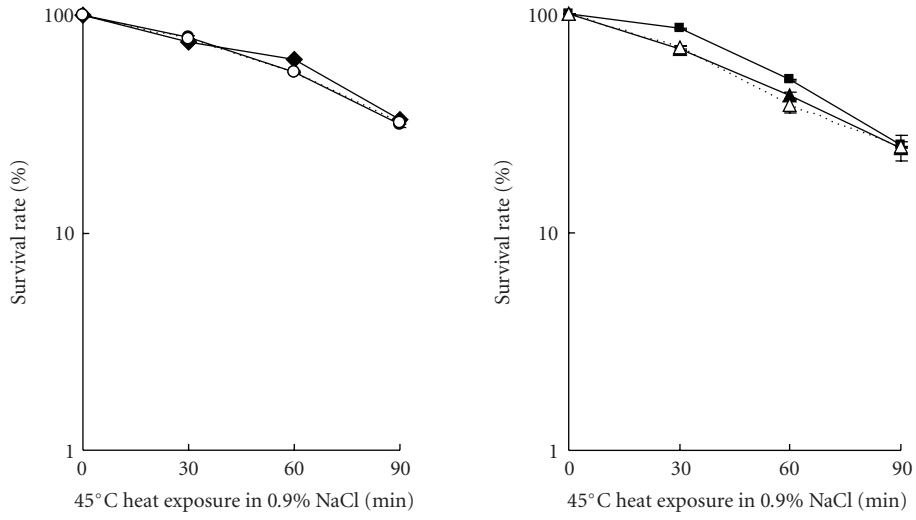


FIGURE 5: (a) Kinetics of biofilm formation of the *V. alginolyticus* ZJ-51, wild type (Op: closed circle; Tr: closed triangle), *rpoX* null mutant (Op: closed diamond; Tr: closed square), and complemented strains (Op: open circle; Tr: open triangle). Bacteria were grown in BF medium in a 24-well polystyrene plate that was incubated statically at 30°C for 2, 4, 6, 8, 10, 12, and 24 hours. Cells were processed as mentioned in Materials and Methods section. Biofilms were quantified by measuring the OD at 540 nm. (b) Observation of biofilm structures by SEM S-3400N: (I) ZJ-O, 7.5 k \times , (II) ZJ-T, 7.5 k \times , (III) $\Delta rpoX$ -O, 7.5 k \times , (IV) $\Delta rpoX$ -T, 9.0 k \times . (figure on the right is the close-up view of marked area)

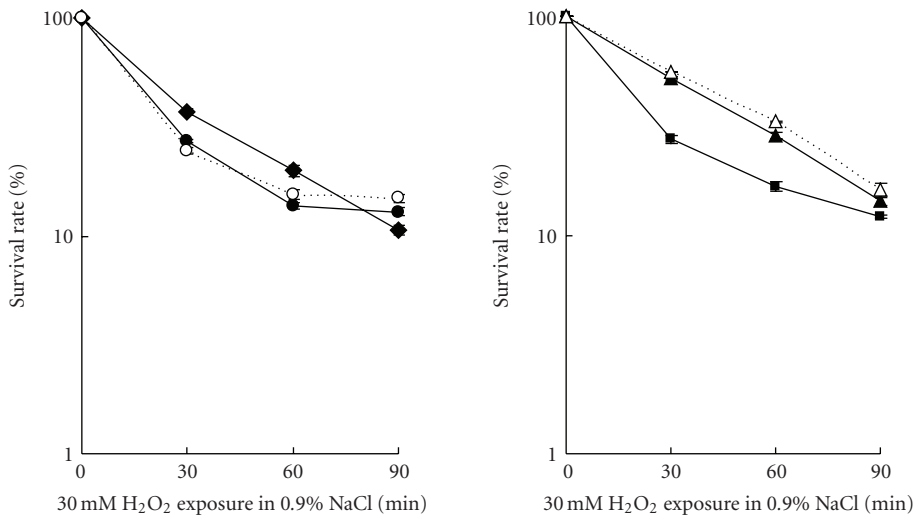
plays an important role in binding the core RNA polymerase in the holoenzyme for some specific bacterial promoters that contain an extended -10 promoter element [4, 23]. This region is weakly conserved or virtually absent from most of the sigma factors including the ECF subfamily [4]. The difference in the conserved domains suggests that *rpoX* may have functions other than those exerted by *rpoS*.

In a previous study we have shown that the Tr variant produced copious biofilms with mature three-dimensional

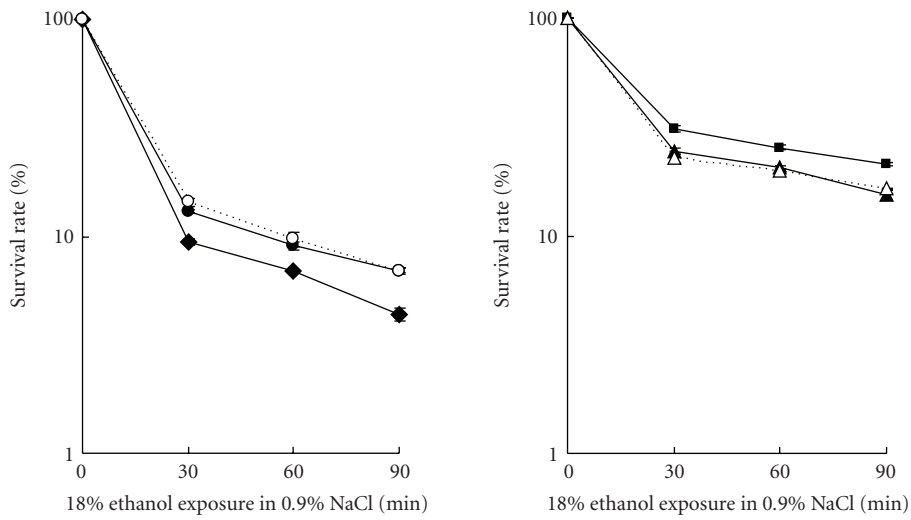
structures in contrast to the basic level of biofilm with mostly individual cells attached on the glass that is formed by the Op variant [18]. A deletion of *rpoX* did not affect the kinetics of biofilm formation. However, this gene could be involved in the attachment and not in the maturation of the biofilm, most likely through a change in cell surface properties. This theory is supported by accessory filamentous-like structures observed on the attached cells of $\Delta rpoX$ -T, though we do not yet know the role of these structures in the biofilm formation



(a)



(b)



(c)

FIGURE 6: Continued.

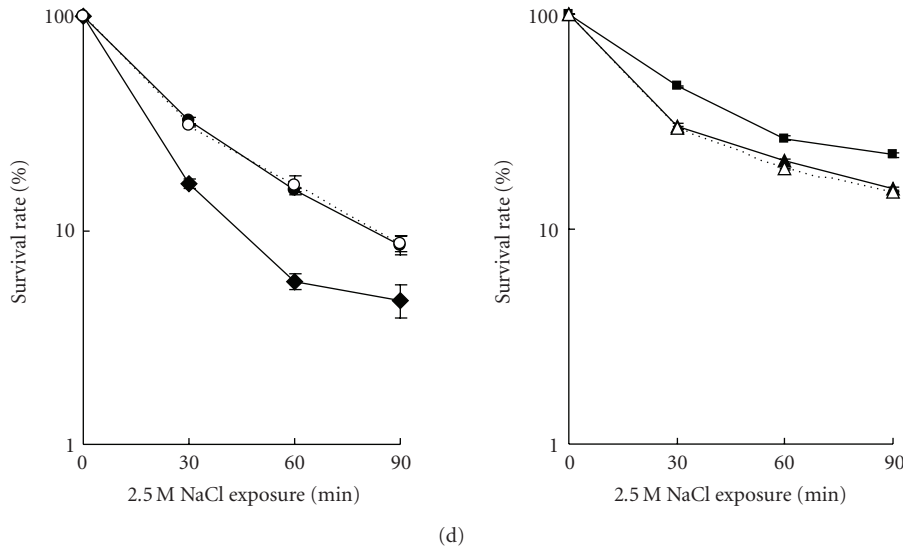


FIGURE 6: Stress survival of stationary-phase *V. alginolyticus* cells. Wild type (Op: closed circle; Tr: closed triangle), *rpoX* null mutant (Op: closed diamond; Tr: closed square), and complemented strains (Op: open circle; Tr: open triangle) were exposed to various stress conditions and the survival rate was determined as percentage of viable cells at the indicated time points. One hundred percent survival corresponds to number of viable cells prior to exposure to the indicated stress: (a) 45°C heat shock, (b) 30 mM H₂O₂, (c) 18% ethanol (d) and 2.5 M NaCl.

of *V. alginolyticus*. In comparison, the *rpoS* gene was reported to be important for the maturation of a biofilm via regulation of the energy metabolism and motility [24]. Deletion of the *rpoS* gene decreased the attachment of *E. coli* to the surface and the attached cells maintained as microcolonies and did not develop a mature biofilm [25].

Although the *rpoX* gene is important for survival of *V. alginolyticus* in high concentrations of hydrogen peroxide, the effect is not as strong as in an *rpoS* deletion [6, 9, 15, 26]. This suggests that *rpoX* might assist *rpoS* to ensure survival in the presence of hydrogen peroxide. However, the *rpoX* was not required for heat shock, although *rpoS* is important for that [15]. In the present study, *rpoX* had no effect on the rate of colony phase variation, but interestingly, when the cells were treated with ethanol and or sodium chloride, the effect of the *rpoX* gene on survival ability depended upon the strain background (translucent versus opaque). In our study on ValR, the master regulator of the quorum sensing signaling pathway, we observed that ValR regulated flagella and exopolysaccharide biosynthesis genes in a colony phase dependent way (submitted manuscript). Sequence alignment revealed that *rpoX* shared 43% identity with the *rpoS*-like sigma factor (RpoQ) of *Vibrio fischeri* ES114. This sigma factor was shown to be regulated by quorum sensing system [27]. Therefore, we suggest that the *rpoX* gene is regulated by the quorum sensing system.

In conclusion, the *rpoX* sigma factor in *V. alginolyticus* has functions that are different from those of the *rpoS* sigma factor. Although both sigma factors are involved in biofilm formation and stress response, the regulatory mechanism of *rpoX* and the relationship between *rpoX* and *rpoS* require further investigation. Moreover, it is important to elucidate the effect of colony phase variation on the regulation of *rpoX*, which may help this pathogen to survive changes in the environment.

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