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Difference in NaCl tolerance of membrane-bound 5'-nucleotidases purified from deep-sea and brackish water *Shewanella* species

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Keyword:	Deep-sea, Growth environment, 5'-Nucleotidase, NaCl tolerance, <i>Shewanella</i>

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6 **Difference in NaCl tolerance of membrane-bound 5'-nucleotidases purified from**
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8 **deep-sea and brackish water *Shewanella* species**
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43 **Abbreviations**

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45 NTase 5'-nucleotidase

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47 SANTase *Shewanella amazonensis* 5'-nucleotidase

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49 SVNTase *Shewanella violacea* 5'-nucleotidase
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6 **Abstract** *Shewanella* species are widely distributed in sea, brackish, and fresh
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8 water areas, growing psychrophilically or mesophilically, and piezophilically or
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10 piezo-sensitively. Here, membrane-bound 5'-nucleotidases (NTases) from deep-sea
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12 *Shewanella violacea* and brackish water *Shewanella amazonensis* were examined from
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14 the aspect of NaCl tolerance in order to gain an insight into protein stability against salt.
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16 Both NTases were single polypeptides with molecular masses of ~59 kDa, as determined
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18 on mass spectroscopy. They similarly required 10 mM MgCl₂ for their activities, and
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20 they exhibited the same pH dependency and substrate specificity for 5'-nucleotides.
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22 However, *S. violacea* 5'-nucleotidase (SVNTase) was active enough in the presence of
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24 2.5 M NaCl, whereas *S. amazonensis* 5'-nucleotidase (SANTase) exhibited significantly
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26 reduced activity with the same concentration of the salt. Although SVNTase and
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28 SANTase exhibited high sequence identity (69.7%), differences in the ratio of acidic to
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30 basic amino acid residues and the number of potential salt bridges maybe being
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32 responsible for the difference in the protein stability against salt. 5'-Nucleotidases from
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34 these *Shewanella* species will provide useful information regarding NaCl tolerance,
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36 which may be fundamental for understanding bacterial adaptation to growth
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38 environments.
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45 **Keywords** Deep-sea · Growth environment · 5'-Nucleotidase · NaCl tolerance ·

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Introduction

Shewanella species belonging to class *Gammaproteobacteria* are widely distributed in sea, brackish, and fresh water areas, growing psychrophilically or mesophilically, and piezophilically or piezo-sensitively. *Shewanella* species are also known to utilize a wide range of extracellular organic and inorganic compounds in both soluble and insoluble forms, which makes these widely distributed species important players in biogeochemical cycles (Hau and Gralnick 2007). Such diversity has evoked our interest in using *Shewanella* for the bioremediation of contaminated water and the development of microbial fuel cells.

The wide distribution of *Shewanella* species also makes them suitable bacteria for investigating not only the enzymatic reactions that drive biogeochemical cycles (Falkowski et al. 2008), but also the protein stability mechanism as to such various environments. In this context, we have carried out protein thermal stability studies using cytochromes *c* isolated from a variety of *Shewanella* species (Ogawa et al. 2007; Takenaka et al. 2010; Masanari et al. 2011; Kato et al. 2015). Recently, we found a positive correlation between the optimal growth pressures of four *Shewanella* species and the stabilities of their highly homologous cytochromes *c*₅ (Masanari et al. 2014; Masanari et al. 2016). As *Shewanella* cytochromes *c*₅ are monomeric proteins, we have gained fundamental insights into the structure and stability relationships in a single polypeptide.

5'-Nucleotidase (NTase) is a widespread enzyme and appears to function as a nutrient-acquiring enzyme at the periphery of bacteria. It is also an enzyme involved in extracellular nucleotide signaling and degradation in mammals (Yegutkin et al. 2006; Pettengill et al. 2013) and in human pathogenic bacteria (Zagursky et al. 2000; Firon et al.

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6 2014). *Escherichia coli* NTase (ECNTase) has been extensively characterized as a
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8 model protein for bacterial and mammal homologues. Its crystal structure shows that it
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10 is a monomeric two-domain protein (Knöfel and Sträter 1999), which undergoes
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12 hinge-bending domain rotation during the catalytic cycle (Knöfel and Sträter 2001).
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14 Therefore, similar to and in addition to *Shewanella* cytochromes *c*₅, NTases from
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16 *Shewanella* species are useful target proteins for investigating relationships among
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18 structure, stability, and enzymatic activity.
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21 NTases from *Vibrio* species have unveiled basic enzymology such as substrate
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23 and cation specificities (Bengis-Garber and Kushner 1981; Itami et al. 1989). As most
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25 *Vibrio* species had been isolated from sea water environments, the effects of salts such as
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27 NaCl on their NTase activities were investigated, but a comparative study on the salt
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29 tolerance of the enzymes has not been performed. Taking account of the wide
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31 distribution of *Shewanella* species, a molecular insight into the salt tolerance of
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33 *Shewanella* NTases will be of interest. So far, fresh water *Shewanella oneidensis* NTase
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35 has only been characterized at the cellular and crude extract levels, its importance in
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37 ecology and physiology being revealed (Pinchuk et al. 2008; Covington et al. 2010).
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39 However, little is known about the salt tolerance of purified *Shewanella* NTases obtained
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41 from various environments.
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45 In this study, we purified two homologous membrane-bound NTases. One
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47 (SVNTase) was from deep-sea psychrophilic *Shewanella violacea* strain DSS12 isolated
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49 from sea water sediments of the Ryukyu Trench, which grows optimally with ~0.51 M
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51 NaCl, but shows no growth in the absence of NaCl (Nogi et al. 1998). The other one
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53 (SANTase) is from brackish water mesophilic *Shewanella amazonensis* strain SB2B
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55 isolated from deposits of the Amazon River Delta, which grows optimally with ~0.2 M
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6 NaCl and even grows in the absence of NaCl (Venkateswaran et al. 1999), but not with
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8 ~0.51 M (Venkateswaran et al. 1998). These growth properties related to the NaCl
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10 concentration fit well with the previous definitions of 'halophilic' and 'halotolerant', which
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12 are defined as ones that optimally grow with more than 0.2 M NaCl and 0–0.2 M,
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14 respectively (Venkateswaran et al. 1999). The NTases of *S. violacea* and *S. amazonensis*
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16 derived from such distinctive salt environmental niches were both characterized in this
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18 study. Although they exhibited high amino acid sequence identity (69.7%), they showed
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20 a remarkable difference in NaCl tolerance, which will be discussed as to protein stability
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22 against salt. The findings can be expanded to bacterial environmental adaptation.
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30 **Materials and methods**

31 32 33 **Preparation of *S. violacea* and *S. amazonensis* membranes**

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38 *S. violacea* DSS12 was grown in liquid marine broth 2216 (Difco) routinely containing
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40 0.33 M NaCl at 8 °C as described previously (Nogi et al. 1998). *S. amazonensis* SB2B
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42 was grown in LB broth routinely containing 0.17 M NaCl at 37 °C as described
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44 previously (Venkateswaran et al. 1998). Frozen cells (10 g) of these species were
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46 suspended in 30 ml of TKG buffer [20 mM Tris-HCl (pH 8.0, adjusted at 4 °C), 140 mM
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48 KCl, 10% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 5 µg ml⁻¹ leupeptin, 5
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50 µg ml⁻¹ pepstatin A, and 1 µg ml⁻¹ DNase I]. The following steps were performed at 4
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52 °C. Cells were disrupted by four passages through a precooled French pressure cell at
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54 147 MPa. Then streptomycin sulfate was added to each cell extract, which was gently
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6 stirred for 15 min on ice, followed by centrifugation ($12,600 \times g$ for 10 min) to remove
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8 unbroken cell debris and the nucleic acid moiety; this step was repeated twice.
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10 The membrane protein fractions derived from the cell extracts were precipitated
11
12 by centrifugation at $200,000 \times g$ for 70 min, and then washed with membrane wash buffer
13
14 [50 mM Tris-HCl (pH 8.0, adjusted at 4°C), 2 mM MgCl_2 , 1 mM dithiothreitol, and 10%
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16 (v/v) glycerol], followed by centrifugation at $220,000 \times g$ for 80 min. The washed
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18 membranes were resuspended in the membrane wash buffer to a protein concentration of
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20 $\sim 30 \text{ mg ml}^{-1}$, and then stored at -80°C or used for ATP hydrolysis assaying and
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22 purification of NTase.
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25 26 27 **Purification of NTases from *S. violacea* and *S. amazonensis* membranes**

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32 Suspensions of membrane protein fractions from *S. violacea* and *S. amazonensis* cells
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34 ($\sim 20 \text{ mg protein ml}^{-1}$) were supplemented with 2% (w/v) detergent, **Octaethylene glycol**
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36 **monododecyl ether (C_{12}E_8 , Tokyo Chemical Industry)** After 15 min stirring at 4°C ,
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38 each suspension was centrifuged at $220,000 \times g$ for 90 min to separate the solubilized
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40 proteins.
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43 The solubilized protein fractions were loaded onto a HiTrap Q column (1.6×2.5
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45 cm, **GE Healthcare**) equilibrated with buffer A [20 mM Tris-HCl (pH 8.0, adjusted at 30
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47 $^\circ\text{C}$), 2 mM MgCl_2 , 1 mM dithiothreitol, 10% (w/v) glycerol, and 0.1% C_{12}E_8] at room
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49 temperature. The column was first washed with 20 ml of the same buffer, and then the
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51 protein fractions exhibiting NTase activity (examined by ATP hydrolysis activity
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53 assaying as described below) were eluted with a linear gradient of NaCl (0.3 to 1.0 M for
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55 *S. violacea* membrane and 0.2 to 1.0 M for *S. amazonensis* membrane) in the same buffer.
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6 This HiTrap Q column chromatography step was repeated. The resulting protein
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8 fractions were then loaded onto a Superdex 200 column (1.6 × 60 cm, GE Healthcare)
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10 equilibrated with buffer A without dithiothreitol containing 0.15 M NaCl. The fractions
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12 exhibiting ATP hydrolysis activity were pooled and concentrated with an Amicon
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14 Ultra-15 10K (Millipore).
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17 18 19 **Protein analysis**

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23 Protein concentrations were determined by the method of Lowry et al. (1951) with bovine
24
25 serum albumin as the standard. The purity of an NTase was routinely checked by
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27 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 15%
28
29 polyacrylamide using the buffer system described by Laemmli (1970). The purified
30
31 NTase protein bands separated on SDS-PAGE were electrophoretically transblotted onto
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33 a polyvinylidene fluoride membrane (Millipore) and visualized with Coomassie Brilliant
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35 Blue G-250. The protein band corresponding to the NTase was excised and directly
36
37 subjected to N-terminal sequencing with an automatic peptide sequencer, Procise 492HT
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39 (Applied Biosystems).
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43 Mass spectroscopy was also performed for the purified NTases to determine their
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45 molecular masses. The proteins were spotted on the target plates after mixing with the
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47 matrix solution, i.e., saturated sinapic acid in 0.1% (v/v) TFA and 40% (v/v) acetonitrile,
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49 and then positive ion mass spectra were obtained with a MALDI-TOF mass spectrometer,
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51 Autoflex II (Bruker).
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Activity measurements

ATP hydrolysis activity assaying during the protein purification process was routinely performed at 30 °C in a mixture comprising 20 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, and 1 µg ml⁻¹ bovine serum albumin, 0.6 ml, according to the modified method of Fiske and Subbarow (1925). In preliminary experiments for the membrane fractions, the proteins from *S. violacea* and *S. amazonensis* exhibited maximal ATP hydrolysis activity with 2.0 M and 0 M KCl, respectively, these concentration being therefore used for the respective reaction solutions during purification procedures. The reaction was initiated by adding 4 mM disodium ATP and terminated by the addition of 0.33 N trichloroacetic acid. ATP hydrolysis activity was calculated by means of a colorimetric assay, at an absorbance of 740 nm, that estimates the amount of Pi liberated from ATP. One unit of ATP hydrolysis activity was defined as the amount of enzyme that liberated 1 µmol of Pi per min.

For the purified SVNTase and SANTase (1.0 µg protein), ATP hydrolysis activity was examined at various pHs using 20 mM morpholinepropanesulfonic acid-NaOH buffer (pH 5.0–7.0, adjusted at 25 °C), 20 mM Tris-HCl buffer (pH 7.0–9.0, adjusted at 25 °C), or 20 mM glycine-NaOH buffer (pH 9.0–11.0, adjusted at 25 °C) at 30 °C in the presence of 10 mM MgCl₂. At the resulting optimal pH, the ATP hydrolysis activity of each purified enzyme was further examined for divalent cation dependency using 2 or 10 mM MgCl₂, MnCl₂, CaCl₂, and ZnCl₂ at 30 °C in the buffer containing 20 mM Tris-HCl (pH 8.0).

Substrate specificity was also examined at 30 °C with 5'-ATP, 5'-AMP, 5'-GTP, and 3'-AMP in the presence of 10 mM MgCl₂ in the buffer containing 20 mM Tris-HCl

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6 (pH 8.0). The procedure for the activity measurements involving these substrates was
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8 the same as that used for the ATP hydrolysis activity described above. Under the
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10 conditions of 10 mM MgCl₂ in the buffer containing 20 mM Tris-HCl (pH 8.0), the ATP
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12 hydrolysis activities of the two purified enzymes were further measured in the
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14 temperature range of 10 to 60 °C in order to determine the optimal reaction temperature.
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16 Finally, the salt dependency of the ATP hydrolysis activities of the purified SVNTase and
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18 SANTase were measured with 0–2.5 M NaCl and 10 mM MgCl₂ in the buffer containing
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20 20 mM Tris-HCl (pH 8.0) at 30 °C.
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27 **Results and discussion**

28 **Purification of NTases from the *S. violacea* and *S. amazonensis* membranes**

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32 Preliminary experiments revealed that the ATP hydrolysis activities of *S. violacea* and *S.*
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34 *amazonensis* membranes were maximal with and without 2.0 M KCl, respectively, in the
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36 presence of 10 mM MgCl₂ and 4 mM ATP in the buffer containing 20 mM Tris-HCl (pH
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38 8.0) at 30 °C. Therefore, these conditions were used for monitoring ATP hydrolysis
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40 activity during enzyme purification. Starting from ~400 mg of *S. violacea* and *S.*
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42 *amazonensis* total membrane proteins, 0.3 mg of each NTase was obtained at the end of
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44 the purification procedure (Table 1). The final SVNTase and SANTase preparations
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46 exhibited specific activities of 65 and 175 units (mg protein)⁻¹, respectively. The
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48 difference in specific activity may reflect the intrinsic properties of these enzymes,
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50 although the SVNTase preparation contained minor impurities whereas SANTase one did
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6 **not** (Lanes 5 in Fig. 1ab), thus the former exhibited lower specific activity than the latter.
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8 The protein bands separated by SDS-PAGE, which appeared to correspond to
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10 SVNTase and SANTase, exhibited molecular masses of ~60 kDa (Fig. 1ab). Mass
11 spectroscopy analysis consistently confirmed their molecular masses: ~59,200 Da for
12 SVNTase and ~59,600 Da for SANTase (Fig. 1cd). These molecular masses were
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14 similar to those reported previously for membrane-bound or periplasmic NTases isolated
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16 from other bacteria (Tamao et al. 1991).
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23 **Calculated molecular masses of SVNTase and SANTase**

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28 The N-terminal amino acid residues of the SVNTase and SANTase proteins separated by
29 SDS-PAGE (Fig. 1ab) could not be determined, indicating that their N-terminal residues
30 were **modified**. Further analysis revealed sequences XGSDNDD and XMDAD for
31 SVNTase and SANTase, respectively. These sequences except for the N-termini exactly
32 corresponded with those of mature NTases deduced from the respective gene sequences
33 deposited in genome databases (Fig. 2), **which exhibited that the N-termini were Cys**
34 **residues**. The mature SVNTase and SANTase **including N-terminal Cys** consisted of
35 549 and 551 amino acids, respectively. From the entire primary structures of the mature
36 proteins, the molecular masses of SVNTase and SANTase were calculated to be 58,828
37 and 59,194 Da, respectively. The differences between the mass spectroscopic molecular
38 masses (Fig. 1cd) and the ones calculated from the amino acid sequences, about 400 Da,
39 should be due to **modification of the side chain of N-terminal Cys residue with lipid**.
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The modification may not occur at the amino group, consistent with the previous study (Weyer et al. 1987), thus Edman degradation proceeded during N-terminal peptide

sequencing.

Localization of SVNTase and SANTase

The N-termini of both mature SVNTase and SANTase comprised a Cys residue in the deduced amino acid sequences, which were preceded by a Sec-dependent periplasmic targeting peptide of 20 amino-acid residues (Fig. 2). These results indicate that both NTases are synthesized as precursors and that their signal peptides are cleaved off during translocation to the periplasmic side of membranes. Although the NTases were successfully purified from the membrane protein fractions of *S. violacea* and *S. amazonensis* in this study, the whole amino acid sequences of the mature proteins appeared to be soluble without any membrane-embedded helices with the SOSUI program (Hirokawa et al. 1998). Thus, both NTases may be lipoproteins that are anchored to membranes through modified N-terminal Cys residues. Mature N-terminal Cys residues are also found in various lipoproteins anchored to membranes through the lipid portion attached to their Cys residues, as observed for *Vibrio parahaemolyticus* NTase (Tamao et al. 1991), which was also purified from the membrane protein fraction, similar to in the present study.

Previously, the mature *S. oneidensis* NTase, which also contains an N-terminal Cys residue similar to SVNTase and SANTase, and exhibits 64.3 and 76.8% sequence identities with the respective NTases, has been detected in periplasmic (Covington et al. 2010) and extracellular (Pinchuk et al. 2008) protein fractions on FAD hydrolysis activity assaying and proteome analysis, respectively. These enzyme location results are not consistent with our present results; it is unknown whether or not this is due to the

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5 differences in the species, culture conditions, and/or protein molecules examined.

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8 However, at least SVNTase and SANTase from the two *Shewanella* species were
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10 examined at a substantial level using purified enzymes, giving consistent membrane
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12 localization.

13 14 15 16 17 18 19 **Similarity between the purified SVNTase and SANTase activities**

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22 Enzymatic properties were analyzed and compared using the purified SVNTase and
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24 SANTase. The optimal pH for the ATP hydrolysis activity of the purified SVNTase and
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26 SANTase was **between 8.0 and 9.0** in the presence of 10 mM MgCl₂ at 30 °C (Fig. 3a).
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28 This **range of** optimal pH obtained in the present study is also consistent with those of
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30 NTases purified from other bacteria (Bengis-Garber and Kushner 1981; Itami et al. 1989).
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34 Both purified NTases similarly showed the highest activities with 10 mM MgCl₂,
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36 not with 2 mM, at pH 8.0 (Fig. 3b). Requirement of 10 mM MgCl₂ has also been
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38 observed for *Vibrio costicola* (renamed *Salinivibrio costicola*) NTase (Bengis-Garber and
39
40 Kushner 1981). With 10 mM MnCl₂, significant activities, although less than those with
41
42 10 mM MgCl₂, were detected for SVNTase and SANTase, but not with CaCl₂ and ZnCl₂.
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44 These divalent cation specificities are also consistent with the previous results obtained
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46 for other bacterial NTases (Bengis-Garber and Kushner 1981; Itami et al. 1989; Sakai et
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48 al. 1989; Zagursky et al. 2000). Similar to ATP hydrolysis, 5'-AMP and 5'-GTP could
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50 be hydrolyzed by the purified SVNTase and SANTase, but 3'-AMP could not (Fig. 3c).
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52 In summary, the two NTases purified from *S. violacea* and *S. amazonensis* membranes
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54 exhibited the same enzymatic properties from the aspects of the dependencies as to pH
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6 and divalent cations, and substrate specificity, consistent with typical features of bacterial
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8 NTases, as reported previously (Itami et al. 1989; Sakai et al. 1989).
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10 11 12 **Difference between the purified SVNTase and SANTase activities** 13

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16 As the two source bacteria, *S. violacea* and *S. amazonensis*, have optimal growth
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18 temperatures, 8 °C for the former and 37 °C for the latter, the temperature dependencies
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20 of the ATP hydrolysis activities of the two NTases purified from them were examined.
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22 In the buffer of 20 mM Tris-HCl (pH 8.0 at 25 °C) with 10 mM MgCl₂, maximal ATP
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24 hydrolysis activities were obtained at 30 °C for SVNTase and 45 °C for SANTase (Fig.
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26 4a), reflecting the difference in the optimal growth temperatures of the source bacteria.
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30 NaCl concentration dependencies were also examined under the same buffer
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32 conditions as those used for the temperature dependency experiments at 30 °C.
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34 SVNTase exhibited almost the same level of ATP hydrolysis activity with 2.0–2.5 M
35
36 NaCl of that with 0 M NaCl (Fig. 4b), but showing a slightly inhibitory effect of NaCl.
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38 The inhibitory effect was prominent for SANTase, which exhibited ~30% activity with
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40 2.0–2.5 M NaCl of that with 0 M (Fig. 4b). Therefore, the SVNTase activity is clearly
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42 more tolerant of NaCl than that of SANTase. This difference also reflects the growth
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44 environments of the source bacteria, i.e., *S. violacea* optimally grows with a high NaCl
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46 concentration compared with *S. amazonensis*, both being defined as 'halophilic' and
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48 'halotolerant' in the previous literature, respectively (Venkateswaran et al. 1999).
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Primary structural insight into the difference in NaCl tolerance between SVNTase and SANTase

The whole amino acid sequences of mature SVNTase and SANTase were then compared with that of well-characterized ECNTase. High overall sequence identity (69.7%) was observed between SVNTase and SANTase, confirming their phylogenetic proximity. Both enzymes exhibited high sequence identities (nearly 50%) with that of ECNTase. Cys-258 and Cys-275 that form a disulfide bond in ECNTase (Knöfel and Sträter 1999) were found in SVNTase and SANTase (Fig. 2; for convenience, the amino acid numbering system for ECNTase including its signal peptide is adopted throughout the text). His-117 and Asp-120 forming a catalytic Asp-His dyad in the active site found in ECNTase were also conserved in the present *Shewanella* enzymes. Gly-407, Phe-429, and Phe-498, which interact with the substrate adenine ring, were all conserved in the present *Shewanella* enzymes. In addition, two metal ions were found in the ECNTase crystal. The crystal structure further exhibited metal ion binding residues (Asp-41, His-43, Asp-84, Asn-116, His-217, His-252, and Gln-254), which were also conserved in the present *Shewanella* enzymes. All these findings together indicate that the SVNTase and SANTase have structures and catalytic mechanisms similar to those of ECNTase.

Hereafter, we will discuss the difference in protein stability as to NaCl between SVNTase and SANTase from the aspect of primary structure. In general, extremely halophilic enzymes that act at molar order salt concentrations exhibit unique molecular features (DasSarma and DasSarma 2015), e.g., a more than 20% content of acidic amino acid residues (Fukuchi et al. 2003). In order to determine whether or not there is a similar tendency for the NaCl-tolerant SVNTase as compared with SANTase, we then

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5 investigated their primary structures. The resulting whole mature sequences (Fig. 2)
6 demonstrated that mature SVNTase contained 83 acidic residues (Asp and Glu), and
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8 mature SANTase 79 acidic residues. Although the former enzyme contained more
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10 acidic residues than the latter, the content of acidic residues of SVNTase (15.1%) was far
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12 less than those of extremely halophilic enzymes (usually over 20%, [DasSarma and](#)
13
14 [DasSarma 2015; Fukuchi et al. 2003](#)), which might contribute to the stabilization of
15
16 extremely halophilic enzymes under molar order salt concentrations. Therefore, the
17
18 absolute content of acidic residues in SVNTase may not contribute to its salt stability, as
19
20 generally observed in extremely halophilic enzymes.
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25 However, SVNTase contained 45 basic residues (Arg, His, and Lys), resulting in
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27 a ratio of acidic to basic residues of 1.84, which was higher than that of SANTase (ratio
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29 of 1.49) that had 53 basic residues. A similar tendency for an excess of acidic residues
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31 being compensated for by fewer basic residues was also indicated for other enzymes from
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33 halophiles as a molecular signature of high-salt environmental adaptation through
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35 genome-wide and environmental surveys ([Fukuchi et al. 2003; Paul et al. 2008; Rhodes et](#)
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37 [al. 2010](#)). Such a proteomic feature may be related with the stability and functionality in
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39 high-salt environments, for which biochemical evidence could be obtained in the present
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41 study using SVNTase and SANTase.
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47 **Three-dimensional structural insight into the difference in protein stability as to** 48 49 **NaCl between SVNTase and SANTase** 50

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53 Since the amino acid sequences of SVNTase and SANTase were more than 50% identical
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55 to that of ECNTase, their three-dimensional model structures could be successfully
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6 simulated by using the crystal structure of substrate-bound ECNTase (PDB ID: 1HPU) as
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8 a template by the SWISS-MODEL workplace (Biasini et al. 2014). The simulated main
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10 chain structures of SVNTase and SANTase showed a major difference in the number of
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12 residues potentially forming a salt bridge.
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15 In the model structure of SVNTase (Fig. 5a), nine salt bridges were formed
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17 between acidic and basic amino acid residues; Glu-195 and Arg-150, Glu-195 and
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19 Lys-198, Asp-547 and Arg-387, Asp-479 and Lys-467, Asp-223 and Arg-238, Glu-290
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21 and Lys-293, Asp-278 and His-220, Asp-64 and Lys-60, and Asp-153 and Lys-156. In
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23 contrast, in the model structure of SANTase (Fig. 5b), 14 salt bridges were formed;
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25 Glu-186 and Lys-145, Asp-223 and Arg-238, Asp-278 and His-220, Glu-290 and Lys-293,
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27 Glu-48 and Lys-346, Asp-64 and Lys-60, Asp-361 and Arg-421, Asp-528 and Lys-533,
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29 Asp-51 and Lys-332, Asp-547 and Arg-387, Glu-195 and Lys-198, Asp-153 and Lys-156,
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31 Glu-69 and Arg-297, and Asp-299 and Arg-297. These results of comparison of the
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33 number of potential salt bridges implied that the lower the number is, as observed in
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35 SVNTase, the less the contribution of salt bridges to the protein stability is. Therefore,
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37 SANTase, which contained a higher number of potential salt bridges than that in
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39 SVNTase, exhibited vulnerability in the presence of high concentrations of NaCl, whose
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41 ions may be able to reduce the strength of the salt bridges, thus resulting in reduced
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43 activity, as observed in Fig. 4b. *Vice versa*, SVNTase, which contained a lower number
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45 of potential salt bridges, exhibited high NaCl tolerance compared with SANTase, because
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47 the salt bridges might contribute less to the stability of its tertiary structure.
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Structural insights into other *Shewanella* NTases

The issues of the differences in the ratio of acidic to basic amino acid residues and the number of potential salt bridges between SVNTase and SANTase were expanded to other *Shewanella* NTases. Based on information obtained from the literature, the *Shewanella* species listed in Table 2 were classified into two groups; one including *S. violacea* could not grow with 0 M NaCl and while the other one including *S. amazonensis* could. In addition, the former *Shewanella* species required a higher NaCl concentration than the latter for optimal growth. These growth properties regarding halophilicity more or less fitted well with the definitions of 'halophilic' and 'halotolerant', as described previously (Venkateswaran et al. 1999).

These *Shewanella* species each had homologous NTases that consisted of ~550 residues in mature forms exhibiting more than 64% and up to 89% sequence identity with that of SVNTase (Table 2). Using these amino acid sequences of the mature forms, the numbers of acidic and basic residues were determined, and the ratios of acidic to basic residues were calculated as described for the SVNTase and SANTase sequences. It was revealed that these NTases could also be classified into two types (Table 2). The NTases derived from 'halophilic' *Shewanella* species including SVNTase showed an average ratio of 1.78 ± 0.10 and those from 'halotolerant' ones including SANTase one of 1.25 ± 0.14 , where $p < 0.01$, indicating significant difference.

The three-dimensional structures of these NTases could be modeled, as for SVNTase and SANTase in this study, using the ECNTase crystal structure as a template (data not shown). Using the resulting model structures for the various *Shewanella* NTases, the numbers of potential salt bridges could be determined, it being revealed that

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6 these NTases could again be classified into two types (Table 2). The NTases derived
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8 from 'halophilic' *Shewanella* species including SVNTase showed an average number of
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10 9.2 ± 0.45 and those from 'halotolerant' ones including SANTase one of 15.4 ± 0.89 , $p <$
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12 0.01 , indicating significant difference.
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15 If we assume that NTases from 'halophilic' *Shewanella* are more stable as to
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17 NaCl than ones from 'halotolerant' ones, as has been biochemically proved for the
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19 representative SVNTase and SANTase in this study, the NaCl tolerance difference
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21 between the two types of *Shewanella* NTases can be attributed to the differences in the
22
23 ratio of acidic to basic amino acid residues and the number of potential salt bridges.
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26 27 **Environmental adaptation of *Shewanella* species**

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32 The present *Shewanella* NTases are peripheral proteins, which renders them constantly
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34 exposed to the external NaCl environment. Therefore, their activities must be finely
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36 tuned through changes in the ratio of acidic to basic residues and the number of salt
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38 bridges, which are fundamental for cell growth. In sea water environments, where
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40 'halophilic' *Shewanella* species live, normal salt concentrations are submolar and thus the
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42 NTases would never encounter the molar salt concentrations examined in the present *in*
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44 *vitro* assays. Nonetheless, from the present study, it can be at least concluded that
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46 SVNTase is more stable as to NaCl than SANTase, which might reflect a sea water
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48 adaptation mechanism. Not only the molecular mechanism for bacterial adaptation to
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50 salt environments, but also the present findings regarding the molecular basis of salt
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52 stability will facilitate further progress as to genetic improvement of salinity tolerance in
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54 future agriculture.
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6 The present structure analysis of *Shewanella* NTases indicated that the
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8 *Shewanella* species have evolved into two distinct groups, in accordance with the growth
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10 conditions, i.e., NaCl concentration, for the source *Shewanella* species (Table 2). This
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12 *Shewanella* grouping with regard to halophilicity is also applicable to the piezophilicity
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14 of *Shewanella* growth: 'halophilic' and 'halotolerant' species are piezophilic and
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16 piezo-sensitive during growth, respectively (Nogi et al. 1998; Kato and Nogi 2001).
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18 Therefore, the NTases of these *Shewanella* species might also have had chance to evolve
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20 in accordance with hydrostatic pressure. The effect of pressure on the activity of
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22 *Shewanella* enzymes will be of interest for a future study. Not only individual effects on
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24 the stability of *Shewanella* enzymes, but also their collective impacts attributed to both
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26 salt and pressure will provide substantial clues as to protein stability, which may be
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28 fundamental for understanding bacterial adaptation to harsh conditions.
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43
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51 **Conflict of interest**

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53 The authors declare that they have no conflict of interest.
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For Peer Review

Figure legends

Fig. 1. Purification and molecular masses. SDS-PAGE analysis for purification steps for SVNTase (**a**) and SANTase (**b**). Lanes 1, total membrane protein fractions; lanes 2, fractions solubilized with C₁₂E₈; lanes 3, first HiTrap Q elution; lanes 4, second HiTrap Q elution; and lanes 5, Superdex 200 elution. Three to ten micrograms of protein was loaded per lane, and the gels were stained with Coomassie Brilliant Blue G-250. *Arrows* indicate the positions of NTases. Molecular mass markers are also indicated in kDa. Mass spectroscopy of the purified enzyme preparations of SVNTase (**c**) and SANTase (**d**). The materials used for SDS-PAGE analysis (lanes 5 in Fig. 1ab) were subjected to mass spectroscopy. *Arrows* indicate the peaks of NTases. Impurities corresponding to the two bands observed on SDS-PAGE of SVNTase (lane 5 in Fig. 1a) were also detected in this analysis (molecular masses lower than that of SVNTase, Fig. 1c).

Fig. 2. Sequence comparison of NTases from *S. violacea*, *S. amazonensis*, and *E. coli*. The amino acid sequences were obtained from a database: SVNTase, WP_041419915.1; SANTase, WP_037417707.1; and ECNTase, NP_415013. The deduced sequences of signal peptides and mature proteins are depicted in lower and upper case letters, respectively. Gaps in the alignment are indicated by dashes. The residue numbers of ECNTase including its signal peptide are indicated above the sequence. The secondary structure elements assigned using the substrate-bound ECNTase crystal structure (PDB ID: 1HPU) with the program *DSSP* (Kabsch and Sander 1983) are schematically shown under the sequences, α -helices being represented by *ribbons* and β strands by *arrows*.

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6 Conserved residues mentioned in the text and others in the mature regions are highlighted
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8 by black and gray shadowing, respectively.
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14 **Fig. 3.** Similarity of purified enzyme activities. (a) Effects of pH on the ATP hydrolysis
15 activities of SVNTase (filled symbols) and SANTase (open symbols). The buffers used
16 were 20 mM morpholinepropanesulfonic acid-NaOH (pH 5.0–7.0, triangles), 20 mM
17 Tris-HCl (pH 7.0–9.0, circles), and 20 mM glycine-NaOH (pH 9.0–11.0, squares). The
18 activity relative to the maximal levels of the respective enzymes is shown. (b) Effects
19 of divalent cations on the ATP hydrolysis activities of SVNTase (filled bars) and
20 SANTase (open bars). The activity relative to that of the respective enzymes with 10
21 mM MgCl₂ is shown. (c) Substrate specificity of the ATP hydrolysis activities of
22 SVNTase (filled bars) and SANTase (open bars). The activity relative to that of the
23 respective enzymes for ATP is shown. The symbols and bar heights represent the mean
24 values obtained for four measurements in each panel. Error bars represent the standard
25 deviations.
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45 **Fig. 4.** Difference in the purified enzyme activities. (a) Effects of temperature on the
46 ATP hydrolysis activities of purified SVNTase (filled circles) and SANTase (open circles).
47 The activity values are plotted as a function of the activity assay temperature, where the
48 pH perturbation due to the temperature difference appeared to be at most 0.1 pH units
49 under the buffer conditions used. The activity relative to the maximal levels of the
50 respective enzymes is shown. The symbols represent the mean values obtained for four
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6 measurements. Error bars represent the standard deviations. **(b)** Effects of NaCl
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8 concentration on the ATP hydrolysis activities of SVNTase (filled circles) and SANTase
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10 (open circles). The activity relative to that of the respective enzymes with 0 M NaCl is
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12 shown. The symbols represent the mean values obtained for four measurements **using**
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14 **two different enzyme preparations.** Error bars represent the standard deviations.
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21 **Fig. 5.** Distribution of potential salt bridges in model structures of SVNTase (a, magenta)
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23 and SANTase (**b**, cyan). The side chains of acidic (red) and basic (blue) amino acid
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25 residues that possibly form salt bridges (green dots) are indicated.
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Table 1. Purification steps for SVNTase and SANTase.

	Total protein (mg)		Total activity (U)		Recovery (%)		Specific activity (U mg ⁻¹)		Purification (fold)	
	SV	SA	SV	SA	SV	SA	SV	SA	SV	SA
Membrane	400	403	552	1530	100	100	1.4	3.8	1.0	1.0
C ₁₂ E ₈ extract	109	109	309	837	56.0	54.7	2.8	7.7	2.0	2.0
First Hi Trap Q	14.0	22.1	355	500	64.3	32.7	25.4	22.6	18.1	5.9
Second Hi Trap Q	8.2	5.5	213	173	38.6	11.3	26.0	31.5	18.6	8.3
Gel filtration	0.3	0.3	19.5	52.5	3.5	3.4	65.0	175	46.4	46.1

Materials from *S. violacea* and *S. amazonensis* cells are designated as SV and SA, respectively.

Table 2. *Shewanella* NTases.

<i>Shewanella</i> species (Accession number of NTase)	Halophilicity		Reference	Definition according to Venkateswaran <i>et al.</i> , 1999	Total amino acid residues of mature NTase	Sequence identity with SVNTase (%)	Ratio of amino acid residues (acidic / basic)	Number of potential salt bridges
	Growth on 0 M NaCl	NaCl required for optimal growth (M)						
SVNTase type								
<i>S. violacea</i> (WP_041419915.1)	–	0.51	Nogi <i>et al.</i> 1998	Halophilic	549	100	1.84	9
<i>S. pealeana</i> (WP_012155150.1)	–	0.5	Leonardo <i>et al.</i> 1999	Halophilic	552	85.3	1.73	9
<i>S. piezotolerans</i> (ACJ29571.1)	–	0.51– 0.68	Xiao <i>et al.</i> 2007	Halophilic	550	87.1	1.74	9
<i>S. sediminis</i> (WP_012143007.1)	–	0.34	Zhao <i>et al.</i> 2005	Halophilic	549	89.3	1.93	9
<i>S. colwelliana</i> (WP_028764957.1)	–	0.34– 0.68	Weiner <i>et</i> <i>al.</i> 1988	Halophilic	546	84.9	1.68	10
SANTase type								
<i>S. amazonensis</i> (WP_011760134.1)	+	0.17	Venkatesw aran <i>et al.</i> 1998	Halotolerant	551	69.7	1.49	14
<i>S. oneidensis</i> (NP_717608.1)	+	0.1– 0.3	Liu <i>et al.</i> 2005	Partially halotolerant	549	64.3	1.16	16
<i>S. denitrificans</i> (WP_011496574.1)	+	0.17– 0.51	Brettar <i>et</i> <i>al.</i> 2002	Partially halotolerant	551	69.6	1.21	16
<i>S. xiamenensis</i> (WP_037417707.1)	+	0.17– 0.34	Huang <i>et</i> <i>al.</i> 2010	Partially halotolerant	549	64.9	1.18	16
<i>S. putrefaciens</i> (WP_014610752.1)	+	~0.2	Venkatesw aran <i>et al.</i> 1999	Halotolerant	552	66.0	1.22	15

Extremophiles

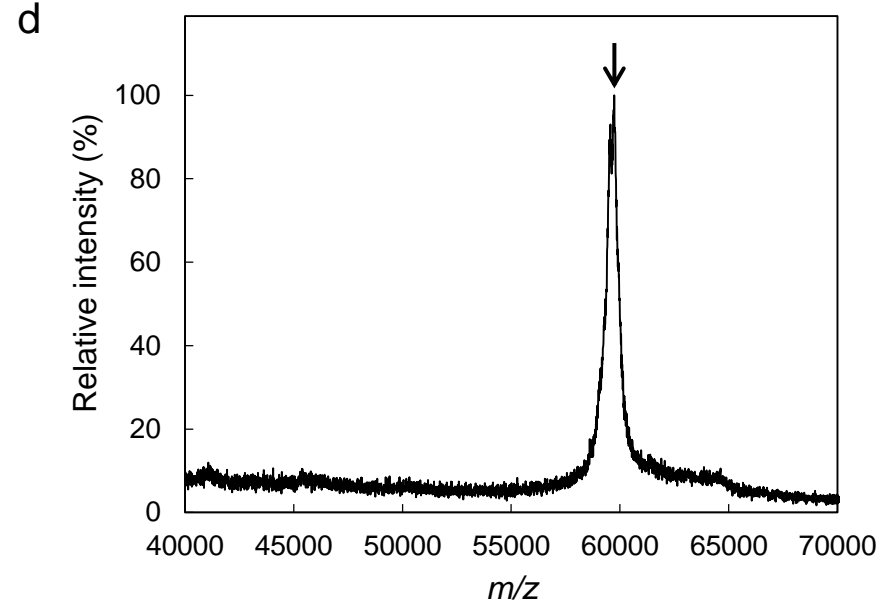
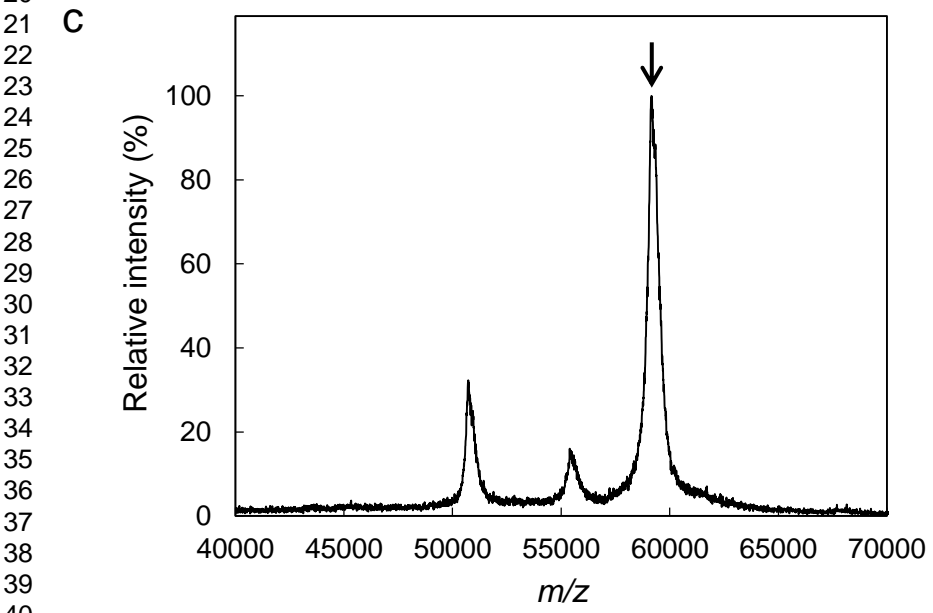
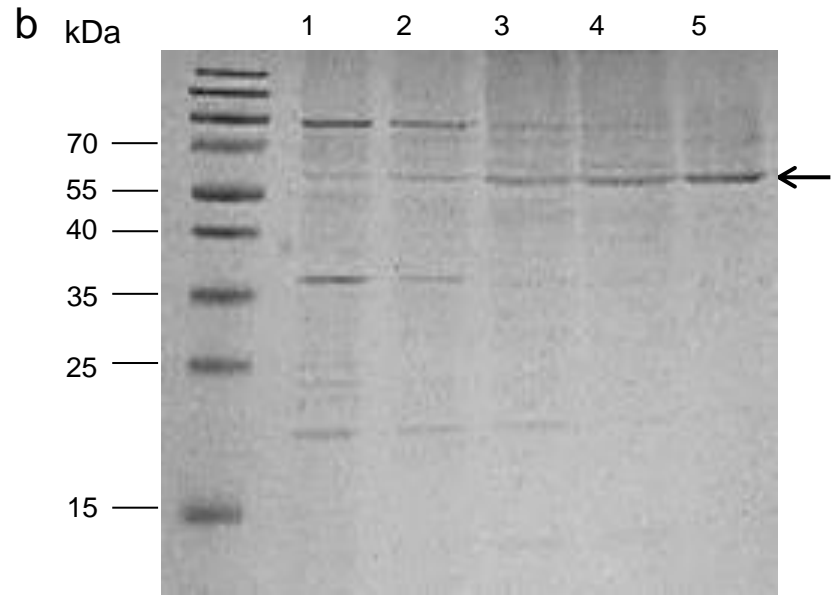
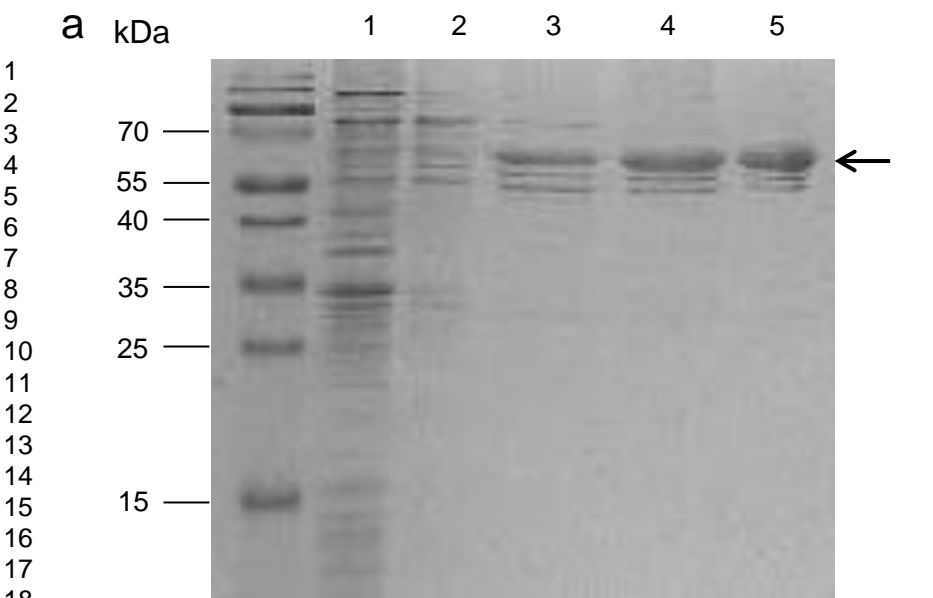


Fig. 1. Kuribayashi *et al.*

Extremophiles

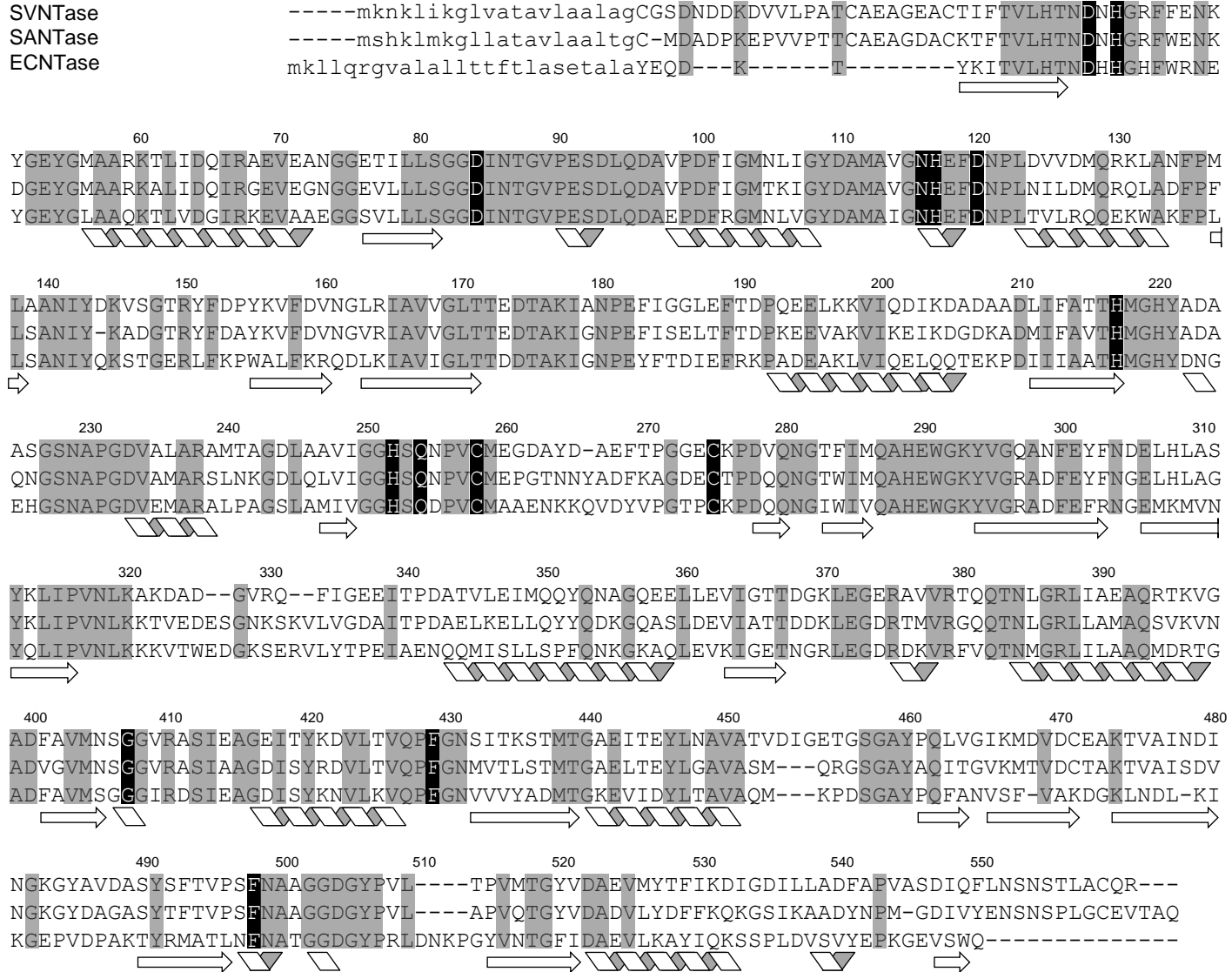


Fig. 2. Kuribayashi et al.

Extremophiles

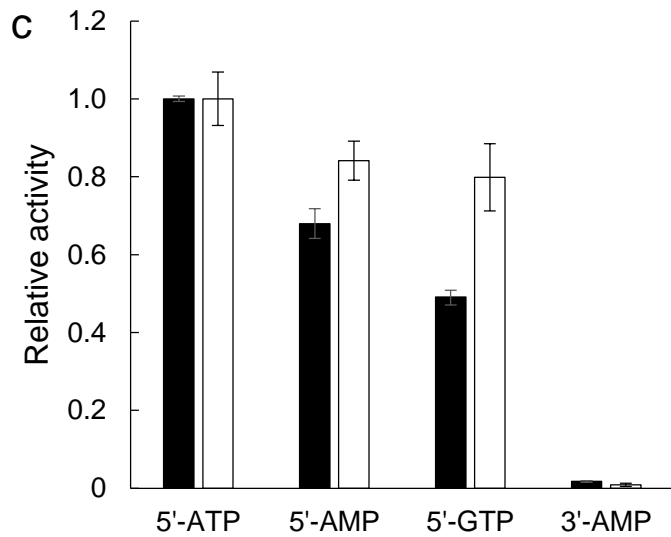
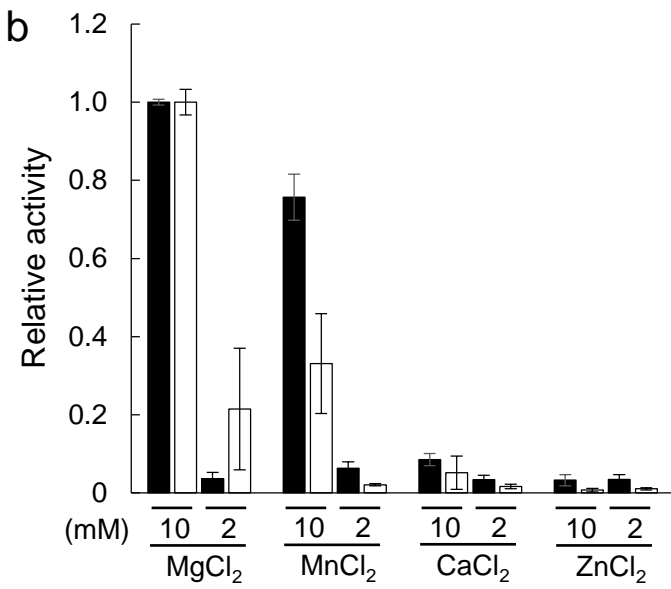
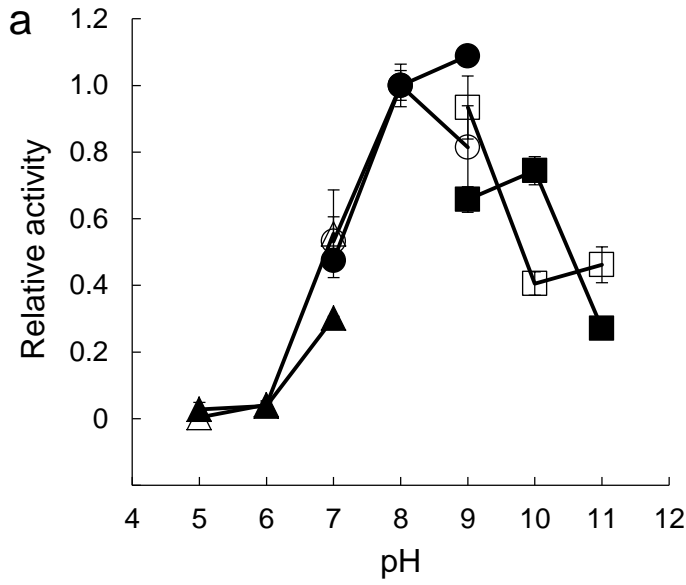
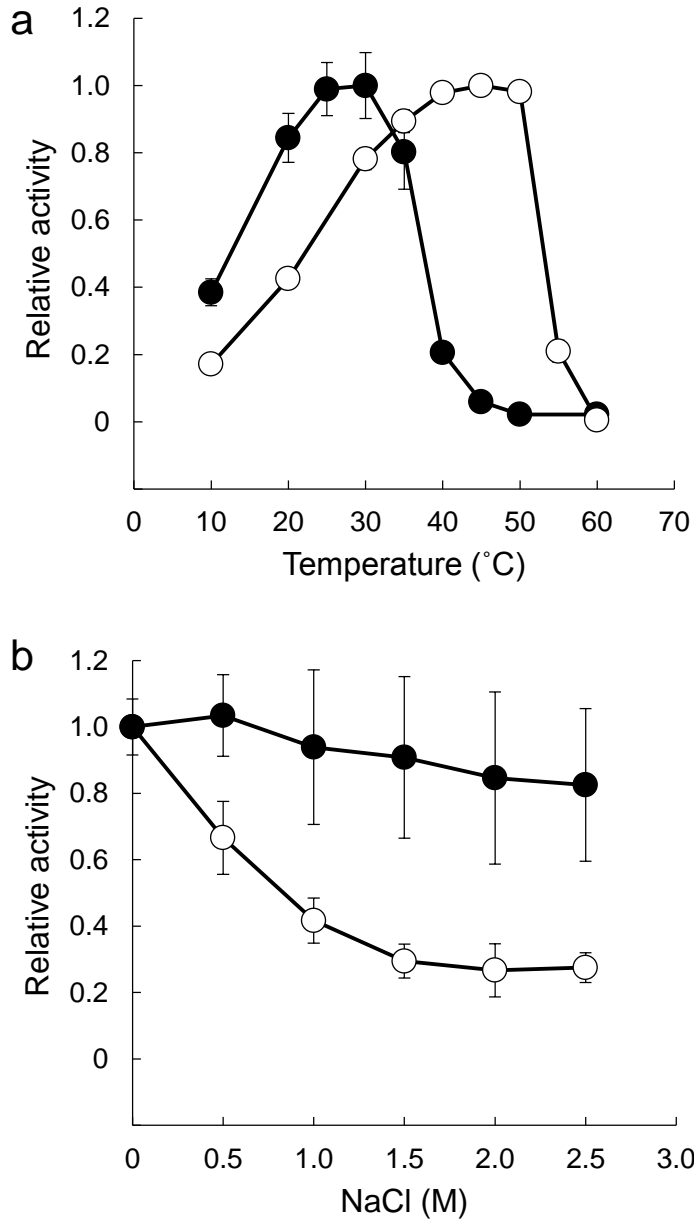


Fig. 3. Kuribayashi *et al.*

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Fig. 4. Kuribayashi *et al.*

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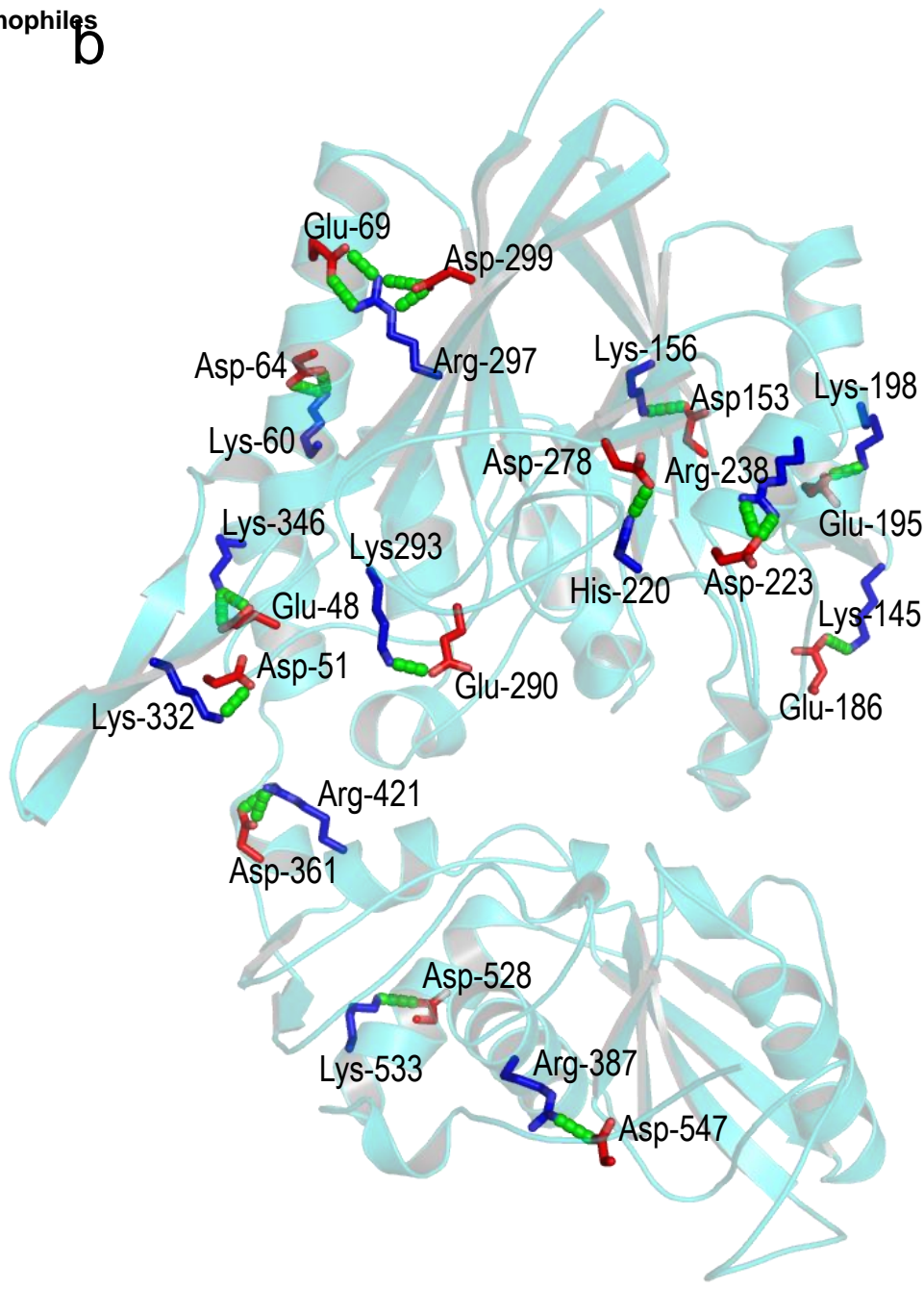
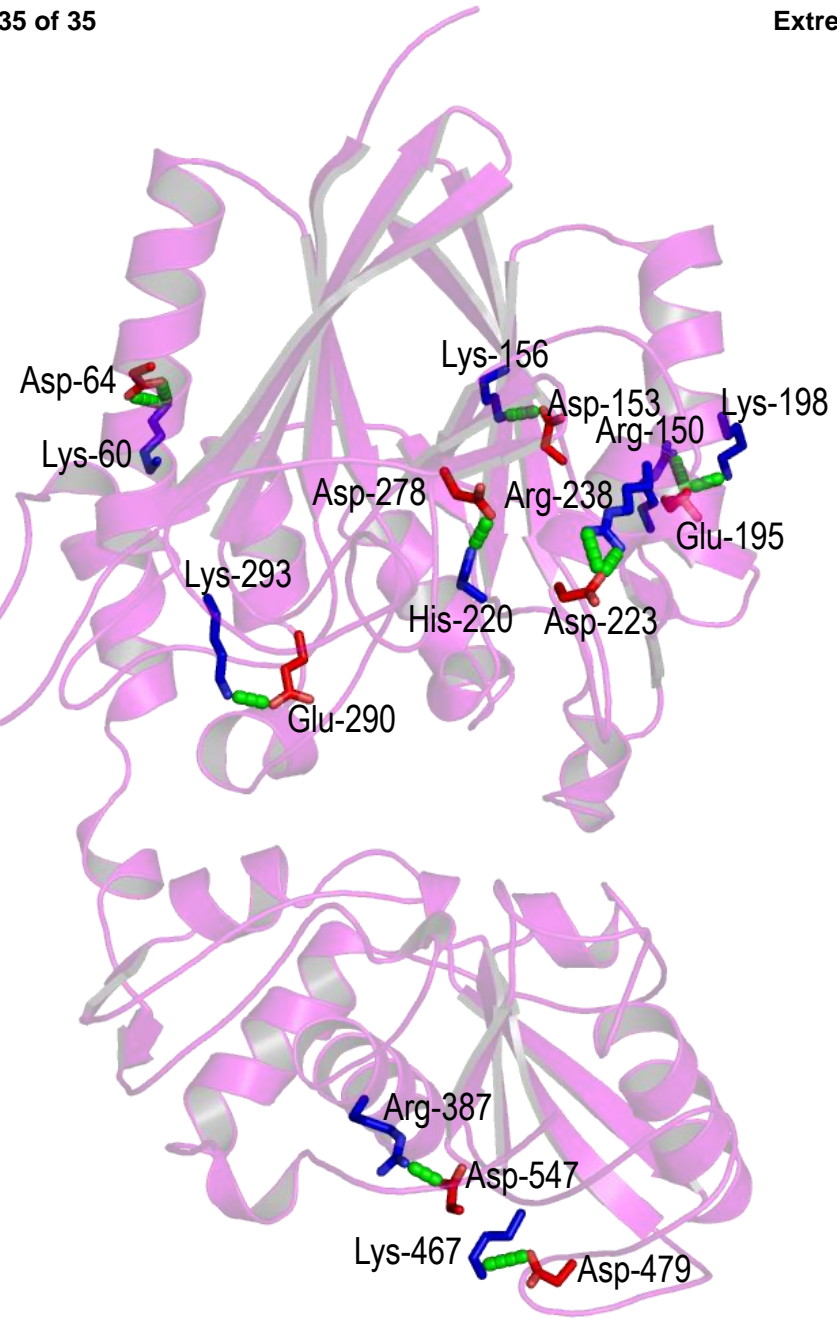


Fig. 5. Kuribayashi et al.