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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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Abbreviations

| | |
|---------|---|
| NTase | 5'-nucleotidase |
| SANTase | <i>Shewanella amazonensis</i> 5'-nucleotidase |
| SVNTase | <i>Shewanella violacea</i> 5'-nucleotidase |

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

Keywords Deep-sea · Growth environment · 5'-Nucleotidase · NaCl tolerance ·

Shewanella

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.

2014). *Escherichia coli* NTase (ECNTase) has been extensively characterized as a model protein for bacterial and mammal homologues. Its crystal structure shows that it is a monomeric two-domain protein (Knöfel and Sträter 1999), which undergoes hinge-bending domain rotation during the catalytic cycle (Knöfel and Sträter 2001). Therefore, similar to and in addition to *Shewanella* cytochromes *c*₅, NTases from *Shewanella* species are useful target proteins for investigating relationships among structure, stability, and enzymatic activity.

NTases from *Vibrio* species have unveiled basic enzymology such as substrate and cation specificities (Bengis-Garber and Kushner 1981; Itami et al. 1989). As most *Vibrio* species had been isolated from sea water environments, the effects of salts such as NaCl on their NTase activities were investigated, but a comparative study on the salt tolerance of the enzymes has not been performed. Taking account of the wide distribution of *Shewanella* species, a molecular insight into the salt tolerance of *Shewanella* NTases will be of interest. So far, fresh water *Shewanella oneidensis* NTase has only been characterized at the cellular and crude extract levels, its importance in ecology and physiology being revealed (Pinchuk et al. 2008; Covington et al. 2010). However, little is known about the salt tolerance of purified *Shewanella* NTases obtained from various environments.

In this study, we purified two homologous membrane-bound NTases. One (SVNTase) was from deep-sea psychrophilic *Shewanella violacea* strain DSS12 isolated from sea water sediments of the Ryukyu Trench, which grows optimally with ~0.51 M NaCl, but shows no growth in the absence of NaCl (Nogi et al. 1998). The other one (SANTase) is from brackish water mesophilic *Shewanella amazonensis* strain SB2B isolated from deposits of the Amazon River Delta, which grows optimally with ~0.2 M

NaCl and even grows in the absence of NaCl (Venkateswaran et al. 1999), but not with ~0.51 M (Venkateswaran et al. 1998). These growth properties related to the NaCl concentration fit well with the previous definitions of 'halophilic' and 'halotolerant', which are defined as ones that optimally grow with more than 0.2 M NaCl and 0–0.2 M, respectively (Venkateswaran et al. 1999). The NTases of *S. violacea* and *S. amazonensis* derived from such distinctive salt environmental niches were both characterized in this study. Although they exhibited high amino acid sequence identity (69.7%), they showed a remarkable difference in NaCl tolerance, which will be discussed as to protein stability against salt. The findings can be expanded to bacterial environmental adaptation.

Materials and methods

Preparation of *S. violacea* and *S. amazonensis* membranes

S. violacea DSS12 was grown in liquid marine broth 2216 (Difco) routinely containing 0.33 M NaCl at 8 °C as described previously (Nogi et al. 1998). *S. amazonensis* SB2B was grown in LB broth routinely containing 0.17 M NaCl at 37 °C as described previously (Venkateswaran et al. 1998). Frozen cells (10 g) of these species were suspended in 30 ml of TKG buffer [20 mM Tris-HCl (pH 8.0, adjusted at 4 °C), 140 mM KCl, 10% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 5 µg ml⁻¹ leupeptin, 5 µg ml⁻¹ pepstatin A, and 1 µg ml⁻¹ DNase I]. The following steps were performed at 4 °C. Cells were disrupted by four passages through a precooled French pressure cell at 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
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12 by centrifugation at $200,000 \times g$ for 70 min, and then washed with membrane wash buffer
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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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42 The solubilized protein fractions were loaded onto a HiTrap Q column (1.6×2.5
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44 cm, **GE Healthcare**) equilibrated with buffer A [20 mM Tris-HCl (pH 8.0, adjusted at 30
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46 °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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48 temperature. The column was first washed with 20 ml of the same buffer, and then the
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50 protein fractions exhibiting NTase activity (examined by ATP hydrolysis activity
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52 assaying as described below) were eluted with a linear gradient of NaCl (0.3 to 1.0 M for
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54 *S. violacea* membrane and 0.2 to 1.0 M for *S. amazonensis* membrane) in the same buffer.
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This HiTrap Q column chromatography step was repeated. The resulting protein fractions were then loaded onto a Superdex 200 column (1.6 × 60 cm, GE Healthcare) equilibrated with buffer A without dithiothreitol containing 0.15 M NaCl. The fractions exhibiting ATP hydrolysis activity were pooled and concentrated with an Amicon Ultra-15 10K (Millipore).

Protein analysis

Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. The purity of an NTase was routinely checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 15% polyacrylamide using the buffer system described by Laemmli (1970). The purified NTase protein bands separated on SDS-PAGE were electrophoretically transblotted onto a polyvinylidene fluoride membrane (Millipore) and visualized with Coomassie Brilliant Blue G-250. The protein band corresponding to the NTase was excised and directly subjected to N-terminal sequencing with an automatic peptide sequencer, Procise 492HT (Applied Biosystems).

Mass spectroscopy was also performed for the purified NTases to determine their molecular masses. The proteins were spotted on the target plates after mixing with the matrix solution, i.e., saturated sinapic acid in 0.1% (v/v) TFA and 40% (v/v) acetonitrile, and then positive ion mass spectra were obtained with a MALDI-TOF mass spectrometer, Autoflex II (Bruker).

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

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

Results and discussion

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

Preliminary experiments revealed that the ATP hydrolysis activities of *S. violacea* and *S. amazonensis* membranes were maximal with and without 2.0 M KCl, respectively, in the presence of 10 mM MgCl₂ and 4 mM ATP in the buffer containing 20 mM Tris-HCl (pH 8.0) at 30 °C. Therefore, these conditions were used for monitoring ATP hydrolysis activity during enzyme purification. Starting from ~400 mg of *S. violacea* and *S. amazonensis* total membrane proteins, 0.3 mg of each NTase was obtained at the end of the purification procedure (Table 1). The final SVNTase and SANTase preparations exhibited specific activities of 65 and 175 units (mg protein)⁻¹, respectively. The difference in specific activity may reflect the intrinsic properties of these enzymes, although the SVNTase preparation contained minor impurities whereas SANTase one did

not (Lanes 5 in Fig. 1ab), thus the former exhibited lower specific activity than the latter.

The protein bands separated by SDS-PAGE, which appeared to correspond to SVNTase and SANTase, exhibited molecular masses of ~60 kDa (Fig. 1ab). Mass spectroscopy analysis consistently confirmed their molecular masses: ~59,200 Da for SVNTase and ~59,600 Da for SANTase (Fig. 1cd). These molecular masses were similar to those reported previously for membrane-bound or periplasmic NTases isolated from other bacteria (Tamao et al. 1991).

Calculated molecular masses of SVNTase and SANTase

The N-terminal amino acid residues of the SVNTase and SANTase proteins separated by SDS-PAGE (Fig. 1ab) could not be determined, indicating that their N-terminal residues were modified. Further analysis revealed sequences XGSDNDD and XMDAD for SVNTase and SANTase, respectively. These sequences except for the N-termini exactly corresponded with those of mature NTases deduced from the respective gene sequences deposited in genome databases (Fig. 2), which exhibited that the N-termini were Cys residues. The mature SVNTase and SANTase including N-terminal Cys consisted of 549 and 551 amino acids, respectively. From the entire primary structures of the mature proteins, the molecular masses of SVNTase and SANTase were calculated to be 58,828 and 59,194 Da, respectively. The differences between the mass spectroscopic molecular masses (Fig. 1cd) and the ones calculated from the amino acid sequences, about 400 Da, should be due to modification of the side chain of N-terminal Cys residue with lipid. 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.
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19 **Similarity between the purified SVNTase and SANTase activities**

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23 Enzymatic properties were analyzed and compared using the purified SVNTase and
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25 SANTase. The optimal pH for the ATP hydrolysis activity of the purified SVNTase and
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27 SANTase was between 8.0 and 9.0 in the presence of 10 mM MgCl₂ at 30 °C (Fig. 3a).
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29 This range of optimal pH obtained in the present study is also consistent with those of
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31 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
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40 Kushner 1981). With 10 mM MnCl₂, significant activities, although less than those with
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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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and divalent cations, and substrate specificity, consistent with typical features of bacterial NTases, as reported previously (Itami et al. 1989; Sakai et al. 1989).

Difference between the purified SVNTase and SANTase activities

As the two source bacteria, *S. violacea* and *S. amazonensis*, have optimal growth temperatures, 8 °C for the former and 37 °C for the latter, the temperature dependencies of the ATP hydrolysis activities of the two NTases purified from them were examined. In the buffer of 20 mM Tris-HCl (pH 8.0 at 25 °C) with 10 mM MgCl₂, maximal ATP hydrolysis activities were obtained at 30 °C for SVNTase and 45 °C for SANTase (Fig. 4a), reflecting the difference in the optimal growth temperatures of the source bacteria.

NaCl concentration dependencies were also examined under the same buffer conditions as those used for the temperature dependency experiments at 30 °C. SVNTase exhibited almost the same level of ATP hydrolysis activity with 2.0–2.5 M NaCl of that with 0 M NaCl (Fig. 4b), but showing a slightly inhibitory effect of NaCl. The inhibitory effect was prominent for SANTase, which exhibited ~30% activity with 2.0–2.5 M NaCl of that with 0 M (Fig. 4b). Therefore, the SVNTase activity is clearly more tolerant of NaCl than that of SANTase. This difference also reflects the growth environments of the source bacteria, i.e., *S. violacea* optimally grows with a high NaCl concentration compared with *S. amazonensis*, both being defined as 'halophilic' and 'halotolerant' in the previous literature, respectively (Venkateswaran et al. 1999).

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

investigated their primary structures. The resulting whole mature sequences (Fig. 2) demonstrated that mature SVNTase contained 83 acidic residues (Asp and Glu), and mature SANTase 79 acidic residues. Although the former enzyme contained more acidic residues than the latter, the content of acidic residues of SVNTase (15.1%) was far less than those of extremely halophilic enzymes (usually over 20%, DasSarma and DasSarma 2015; Fukuchi et al. 2003), which might contribute to the stabilization of extremely halophilic enzymes under molar order salt concentrations. Therefore, the absolute content of acidic residues in SVNTase may not contribute to its salt stability, as generally observed in extremely halophilic enzymes.

However, SVNTase contained 45 basic residues (Arg, His, and Lys), resulting in a ratio of acidic to basic residues of 1.84, which was higher than that of SANTase (ratio of 1.49) that had 53 basic residues. A similar tendency for an excess of acidic residues being compensated for by fewer basic residues was also indicated for other enzymes from halophiles as a molecular signature of high-salt environmental adaptation through genome-wide and environmental surveys (Fukuchi et al. 2003; Paul et al. 2008; Rhodes et al. 2010). Such a proteomic feature may be related with the stability and functionality in high-salt environments, for which biochemical evidence could be obtained in the present study using SVNTase and SANTase.

Three-dimensional structural insight into the difference in protein stability as to NaCl between SVNTase and SANTase

Since the amino acid sequences of SVNTase and SANTase were more than 50% identical 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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14 In the model structure of SVNTase (Fig. 5a), nine salt bridges were formed
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16 between acidic and basic amino acid residues; Glu-195 and Arg-150, Glu-195 and
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18 Lys-198, Asp-547 and Arg-387, Asp-479 and Lys-467, Asp-223 and Arg-238, Glu-290
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20 and Lys-293, Asp-278 and His-220, Asp-64 and Lys-60, and Asp-153 and Lys-156. In
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22 contrast, in the model structure of SANTase (Fig. 5b), 14 salt bridges were formed;
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24 Glu-186 and Lys-145, Asp-223 and Arg-238, Asp-278 and His-220, Glu-290 and Lys-293,
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26 Glu-48 and Lys-346, Asp-64 and Lys-60, Asp-361 and Arg-421, Asp-528 and Lys-533,
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28 Asp-51 and Lys-332, Asp-547 and Arg-387, Glu-195 and Lys-198, Asp-153 and Lys-156,
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30 Glu-69 and Arg-297, and Asp-299 and Arg-297. These results of comparison of the
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32 number of potential salt bridges implied that the lower the number is, as observed in
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34 SVNTase, the less the contribution of salt bridges to the protein stability is. Therefore,
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36 SANTase, which contained a higher number of potential salt bridges than that in
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38 SVNTase, exhibited vulnerability in the presence of high concentrations of NaCl, whose
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40 ions may be able to reduce the strength of the salt bridges, thus resulting in reduced
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42 activity, as observed in Fig. 4b. *Vice versa*, SVNTase, which contained a lower number
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44 of potential salt bridges, exhibited high NaCl tolerance compared with SANTase, because
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46 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

these NTases could again be classified into two types (Table 2). The NTases derived from 'halophilic' *Shewanella* species including SVNTase showed an average number of 9.2 ± 0.45 and those from 'halotolerant' ones including SANTase one of 15.4 ± 0.89 , $p < 0.01$, indicating significant difference.

If we assume that NTases from 'halophilic' *Shewanella* are more stable as to NaCl than ones from 'halotolerant' ones, as has been biochemically proved for the representative SVNTase and SANTase in this study, the NaCl tolerance difference between the two types of *Shewanella* NTases can be attributed to the differences in the ratio of acidic to basic amino acid residues and the number of potential salt bridges.

Environmental adaptation of *Shewanella* species

The present *Shewanella* NTases are peripheral proteins, which renders them constantly exposed to the external NaCl environment. Therefore, their activities must be finely tuned through changes in the ratio of acidic to basic residues and the number of salt bridges, which are fundamental for cell growth. In sea water environments, where 'halophilic' *Shewanella* species live, normal salt concentrations are submolar and thus the NTases would never encounter the molar salt concentrations examined in the present *in vitro* assays. Nonetheless, from the present study, it can be at least concluded that SVNTase is more stable as to NaCl than SANTase, which might reflect a sea water adaptation mechanism. Not only the molecular mechanism for bacterial adaptation to salt environments, but also the present findings regarding the molecular basis of salt stability will facilitate further progress as to genetic improvement of salinity tolerance in future agriculture.

The present structure analysis of *Shewanella* NTases indicated that the *Shewanella* species have evolved into two distinct groups, in accordance with the growth conditions, i.e., NaCl concentration, for the source *Shewanella* species (Table 2). This *Shewanella* grouping with regard to halophilicity is also applicable to the piezophilicity of *Shewanella* growth: 'halophilic' and 'halotolerant' species are piezophilic and piezo-sensitive during growth, respectively (Nogi et al. 1998; Kato and Nogi 2001). Therefore, the NTases of these *Shewanella* species might also have had chance to evolve in accordance with hydrostatic pressure. The effect of pressure on the activity of *Shewanella* enzymes will be of interest for a future study. Not only individual effects on the stability of *Shewanella* enzymes, but also their collective impacts attributed to both salt and pressure will provide substantial clues as to protein stability, which may be fundamental for understanding bacterial adaptation to harsh conditions.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

- Bengis-Garber C, Kushner DJ (1981) Purification and properties of 5'-nucleotidase from the membrane of *Vibrio costicola*, a moderately halophilic bacterium. J Bacteriol 146:24–32
- Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordli L, Schwede T (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res 42:W252–W258
- Brettar I, Christen R, Höfle MG (2002) *Shewanella denitrificans* sp. nov., a vigorously denitrifying bacterium isolated from the oxic-anoxic interface of the Gotland Deep in the central Baltic Sea. Int J Syst Evol Microbiol 52:2211–2217
- Covington ED, Gelbmann CB, Kotloski NJ, Gralnick JA (2010) An essential role for UshA in processing of extracellular flavin electron shuttles by *Shewanella oneidensis*. Mol Microbiol 78:519–532
- DasSarma S, DasSarma P (2015) Halophiles and their enzyme: negativity put to good use. Curr Opin Microbiol 25:120–126
- Falkowski PG, Fenchel T, Delong EF (2008) The microbial engines that drive Earth's biogeochemical cycles. Science 320:1034–1039
- Firon A, Dinis M, Raynal B, Poyart C, Trieu-Cuot P, Kaminski PA (2014) Extracellular nucleotide catabolism by the Group B *Streptococcus* ectonucleotidase NudP increases bacterial survival in blood. J Biol Chem 289:5479–5489
- Fiske CH, Subbarow Y (1925) The colorimetric determination of phosphorus. J Biol Chem 66:375–400

- Fukuchi S, Yoshimune K, Wakayama M, Moriguchi M, Nishikawa K (2003) Unique amino acid composition of proteins in halophilic bacteria. *J Mol Biol* 327:347–357
- Hau HH, Gralnick JA (2007) Ecology and biotechnology of the genus *Shewanella*. *Annu Rev Microbiol* 61:237–258
- Hirokawa T, Boon-Chieng S, Mitaku S (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* 14:378–379
- Huang J, Sun B, Zhang X (2010) *Shewanella xiamenensis* sp. nov., isolated from coastal sea sediment. *Int J Syst Evol Microbiol* 60:1585–1589
- Itami H, Sakai Y, Shimamoto T, Hama H, Tsuda M, Tsuchiya T (1989) Purification and characterization of membrane-bound 5'-nucleotidase of *Vibrio parahaemolyticus*. *J Biochem* 105:785–789
- Kabsch W, Sander C (1983) Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* 22:2577–2637
- Kato Y, Fujii S, Kuribayashi T, Masanari M, Sambongi Y (2015) Thermal stability of cytochrome *c'* from mesophilic *Shewanella amazonensis*. *Biosci Biotech Biochem* 79:1125–1129
- Kato C, Nogi Y (2001) Correlation between phylogenetic structure and function: examples from deep-sea *Shewanella*. *FEMS Microbiol Ecol* 35:223–230
- Knöfel T, Sträter N (1999) X-ray structure of the *Escherichia coli* periplasmic 5'-nucleotidase containing a dimetal catalytic site. *Nat Struct Biol* 6:448–453
- Knöfel T, Sträter N (2001) *E. coli* 5'-nucleotidase undergoes a hinge-bending domain rotation resembling a ball-and-socket motion. *J Mol Biol* 309:255–266

- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Leonardo MR, Moser DP, Barbieri E, Brantner CA, MacGregor BJ, Paster BJ, Stackebrandt E, Nealson KH (1999) *Shewanella pealeana* sp. nov., a member of the microbial community associated with the accessory nidamental gland of the squid *Loligo pealei*. *Int J Syst Bacteriol* 49:1341–1351
- Liu Y, Gao W, Wang Y, Wu L, Liu X, Yan T, Alm E, Arkin A, Thompson DK, Fields MW, Zhou J (2005) Transcriptome analysis of *Shewanella oneidensis* MR-1 in response to elevated salt conditions. *J Bacteriol* 187:2501–2507
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Masanari M, Fujii S, Kawahara K, Oki H, Tsujino H, Maruno T, Kobayashi Y, Ohkubo T, Wakai S, Sambongi Y (2016) Comparative study on stabilization mechanism of monomeric cytochrome *c*₅ from deep-sea piezophilic *Shewanella violacea*. *Biosci Biotechnol Biochem* 80:2365–2370
- Masanari M, Wakai S, Ishida M, Kato C, Sambongi Y (2014) Correlation between the optimal growth pressures of four *Shewanella* species and the stabilities of their cytochromes *c*₅. *Extremophiles* 18:617–627
- Masanari M, Wakai S, Tamegai H, Kurihara T, Kato C, Sambongi Y (2011) Thermal stability of cytochrome *c*₅ of pressure-sensitive *Shewanella livingstonensis*. *Biosci Biotechnol Biochem* 75:1859–1861
- Nogi Y, Kato C, Horikoshi K (1998) Taxonomic studies of deep-sea barophilic *Shewanella* strains and description of *Shewanella violacea* sp. nov. *Arch Microbiol* 170:331–338

- Ogawa K, Sonoyama T, Takeda T, Ichiki S, Nakamura S, Kobayashi Y, Uchiyama S, Nakasone K, Takayama SJ, Mita H, Yamamoto Y, Sambongi Y (2007) Roles of a short connecting disulfide bond in the stability and function of psychrophilic *Shewanella violacea* cytochrome *c*₅. *Extremophiles* 11:797–807
- Paul S, Bag SK, Das S, Harvill ET, Dutta C (2008) Molecular signature of hypersaline adaptation: Insights from genome and proteome composition of halophilic prokaryotes. *Genome Biol* 9:R70
- Pettengill M, Robson S, Tresenriter M, Millán JL, Usheva A, Bingham T, Belderbos M, Bergelson I, Burl S, Kampmann B, Gelinas L, Kollmann T, Bont L, Levy O (2013) Soluble ecto-5'-nucleotidase (5'-NT), alkaline phosphatase, and adenosine deaminase (ADA1) activities in neonatal blood favor elevated extracellular adenosine. *J Biol Chem* 288:27315–27326
- Pinchuk GE, Ammons C, Culley DE, Li SM, McLean JS, Romine MF, Nealson KH, Fredrickson JK, Beliaev AS (2008) Utilization of DNA as a sole source of phosphorus, carbon, and energy by *Shewanella* spp.: Ecological and physiological implications for dissimilatory metal reduction. *Appl Environ Microbiol* 74:1198–1208
- Rhodes ME, Fitz-Gibbon ST, Oren A, House CH (2010) Amino acid signatures of salinity on an environmental scale with a focus on the Dead Sea. *Environ Microbiol* 12:2613–2623
- Sakai Y, Tamao Y, Shimamoto T, Hama H, Tsuda M, Tsuchiya T (1989) Cloning and expression of the 5'-nucleotidase gene of *Vibrio parahaemolyticus* in *Escherichia coli* and overproduction of the enzyme. *J Biochem* 105:841–846
- Takenaka S, Wakai S, Tamegai H, Uchiyama S, Sambongi Y (2010) Comparative analysis

- of highly homologous *Shewanella* cytochromes c_5 for stability and function. *Biosci Biotechnol Biochem* 74:1079–1083
- Tamao Y, Noguchi K, Sakai-Tomita Y, Hama H, Shimamoto T, Kanazawa H, Tsuda M, Tsuchiya T (1991) Sequence analysis of *nutA* gene encoding membrane-bound Cl^- -dependent 5'-nucleotidase of *Vibrio parahaemolyticus*. *J Biochem* 109:24–29
- Venkateswaran K, Dollhopf ME, Aller R, Stackebrandt E, Nealson KH (1998) *Shewanella amazonensis* sp. nov., a novel metal-reducing facultative anaerobe from Amazonian shelf muds. *Int J Syst Bacteriol* 48:965–972
- Venkateswaran K, Moser DP, Dollhopf ME, Lies DP, Saffarini DA, MacGregor BJ, Ringelberg DB, White DC, Nishijima M, Sano H, Burghardt J, Stackebrandt E, Nealson KH (1999) Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. *Int J Syst Bacteriol* 49:705–724
- Weiner RM, Coyne VE, Brayton P, West P, Raiken SF (1988) *Alteromonas colwelliana* sp. nov., an isolate from oyster habitats. *Int J Syst Bacteriol* 38:240–244
- Weyer KA, Schäfer W, Lottspeich F, Michel H (1987) The cytochrome subunit of the photosynthetic reaction center from *Rhodopseudomonas viridis* is a lipoprotein. *Biochemistry* 26:2909–2914
- Xiao X, Wang P, Zeng X, Bartlett DH, Wang F (2007) *Shewanella psychrophila* sp. nov. and *Shewanella piezotolerans* sp. nov., isolated from west Pacific deep-sea sediment. *Int J Syst Evol Microbiol* 57:60–65
- Yegutkin GG, Samburski SS, Jalkanen S, Novak I (2006) ATP-consuming and ATP-generating enzymes secreted by pancreas. *J Biol Chem* 281:29441–29447
- Zagursky RJ, Ooi P, Jones KF, Fiske MJ, Smith RP, Green BA (2000) Identification of a *Haemophilus influenzae* 5'-nucleotidase protein: cloning of the *nucA* gene and

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immunogenicity and characterization of the NucA protein. Infect Immun 68:2525–
2534

Zhao JS, Manno D, Beaulieu C, Paquet L, Hawari J (2005) *Shewanella sediminis* sp. nov.,
a novel Na⁺-requiring and hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading
bacterium from marine sediment. Int J Syst Evol Microbiol 55:1511–1520

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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*.

Conserved residues mentioned in the text and others in the mature regions are highlighted by black and gray shadowing, respectively.

Fig. 3. Similarity of purified enzyme activities. (a) Effects of pH on the ATP hydrolysis activities of SVNTase (filled symbols) and SANTase (open symbols). The buffers used were 20 mM morpholinepropanesulfonic acid-NaOH (pH 5.0–7.0, triangles), 20 mM Tris-HCl (pH 7.0–9.0, circles), and 20 mM glycine-NaOH (pH 9.0–11.0, squares). The activity relative to the maximal levels of the respective enzymes is shown. (b) Effects of divalent cations on the ATP hydrolysis activities of SVNTase (filled bars) and SANTase (open bars). The activity relative to that of the respective enzymes with 10 mM $MgCl_2$ is shown. (c) Substrate specificity of the ATP hydrolysis activities of SVNTase (filled bars) and SANTase (open bars). The activity relative to that of the respective enzymes for ATP is shown. The symbols and bar heights represent the mean values obtained for four measurements in each panel. Error bars represent the standard deviations.

Fig. 4. Difference in the purified enzyme activities. (a) Effects of temperature on the ATP hydrolysis activities of purified SVNTase (filled circles) and SANTase (open circles). The activity values are plotted as a function of the activity assay temperature, where the pH perturbation due to the temperature difference appeared to be at most 0.1 pH units under the buffer conditions used. The activity relative to the maximal levels of the respective enzymes is shown. The symbols represent the mean values obtained for four

measurements. Error bars represent the standard deviations. **(b)** Effects of NaCl concentration on the ATP hydrolysis activities of SVNTase (filled circles) and SANTase (open circles). The activity relative to that of the respective enzymes with 0 M NaCl is shown. The symbols represent the mean values obtained for four measurements using two different enzyme preparations. Error bars represent the standard deviations.

Fig. 5. Distribution of potential salt bridges in model structures of SVNTase (a, magenta) and SANTase (b, cyan). The side chains of acidic (red) and basic (blue) amino acid residues that possibly form salt bridges (green dots) are indicated.

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 et al. 1998 | Halophilic | 549 | 100 | 1.84 | 9 |
| <i>S. pealeana</i> (WP_012155150.1) | — | 0.5 | Leonardo et al. 1999 | Halophilic | 552 | 85.3 | 1.73 | 9 |
| <i>S. piezotolerans</i> (ACJ29571.1) | — | 0.51— 0.68 | Xiao et al. 2007 | Halophilic | 550 | 87.1 | 1.74 | 9 |
| <i>S. sediminis</i> (WP_012143007.1) | — | 0.34 | Zhao et al. 2005 | Halophilic | 549 | 89.3 | 1.93 | 9 |
| <i>S. colwelliana</i> (WP_028764957.1) | — | 0.34— 0.68 | Weiner et al. 1988 | Halophilic | 546 | 84.9 | 1.68 | 10 |
| SANTase type | | | | | | | | |
| <i>S. amazonensis</i> (WP_011760134.1) | + | 0.17 | Venkatesw aran et al. 1998 | Halotolerant | 551 | 69.7 | 1.49 | 14 |
| <i>S. oneidensis</i> (NP_717608.1) | + | 0.1— 0.3 | Liu et al. 2005 | Partially halotolerant | 549 | 64.3 | 1.16 | 16 |
| <i>S. denitrificans</i> (WP_011496574.1) | + | 0.17— 0.51 | Brettar et al. 2002 | Partially halotolerant | 551 | 69.6 | 1.21 | 16 |
| <i>S. xiamenensis</i> (WP_037417707.1) | + | 0.17— 0.34 | Huang et al. 2010 | Partially halotolerant | 549 | 64.9 | 1.18 | 16 |
| <i>S. putrefaciens</i> (WP_014610752.1) | + | ~0.2 | Venkatesw aran et al. 1999 | Halotolerant | 552 | 66.0 | 1.22 | 15 |

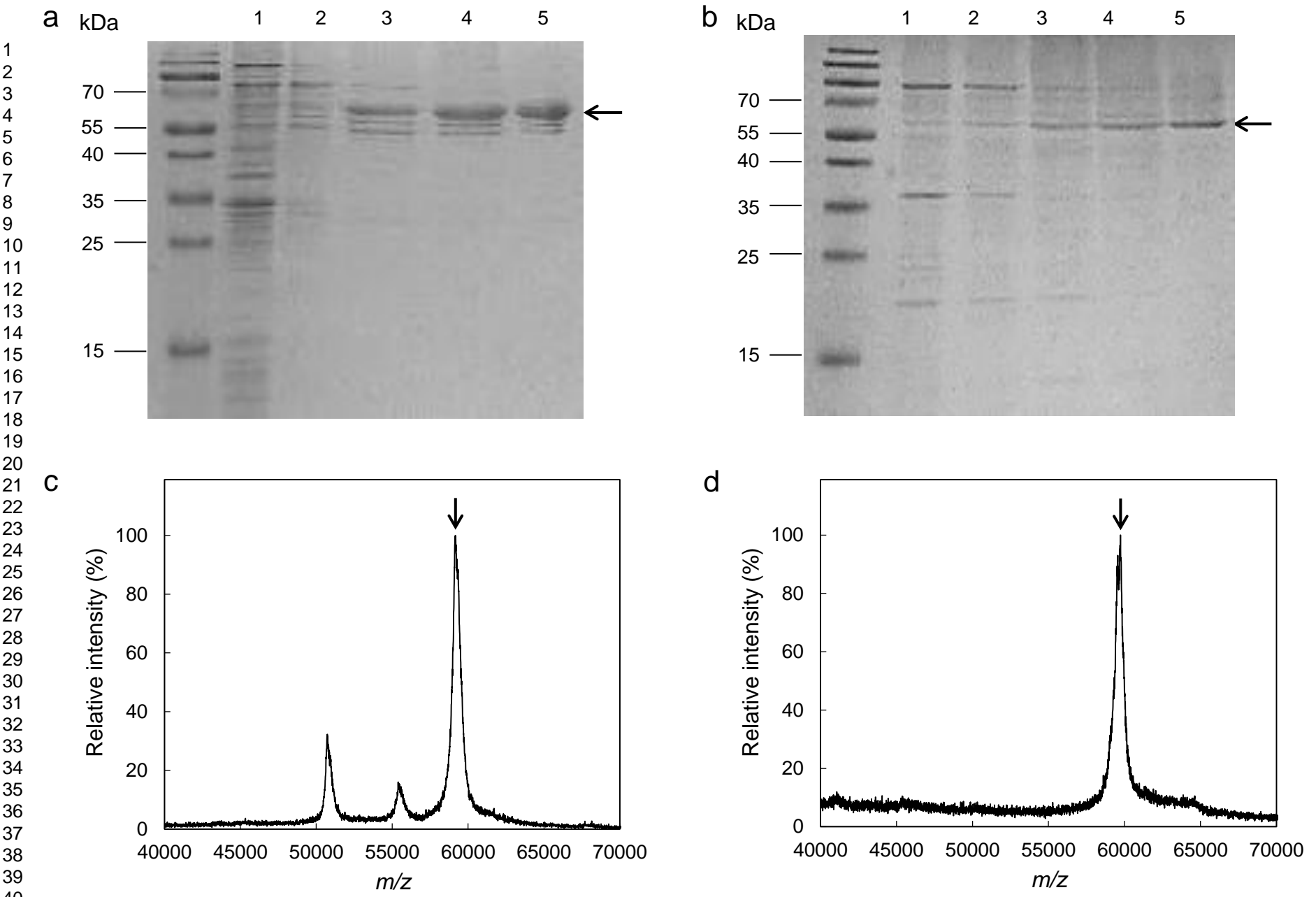
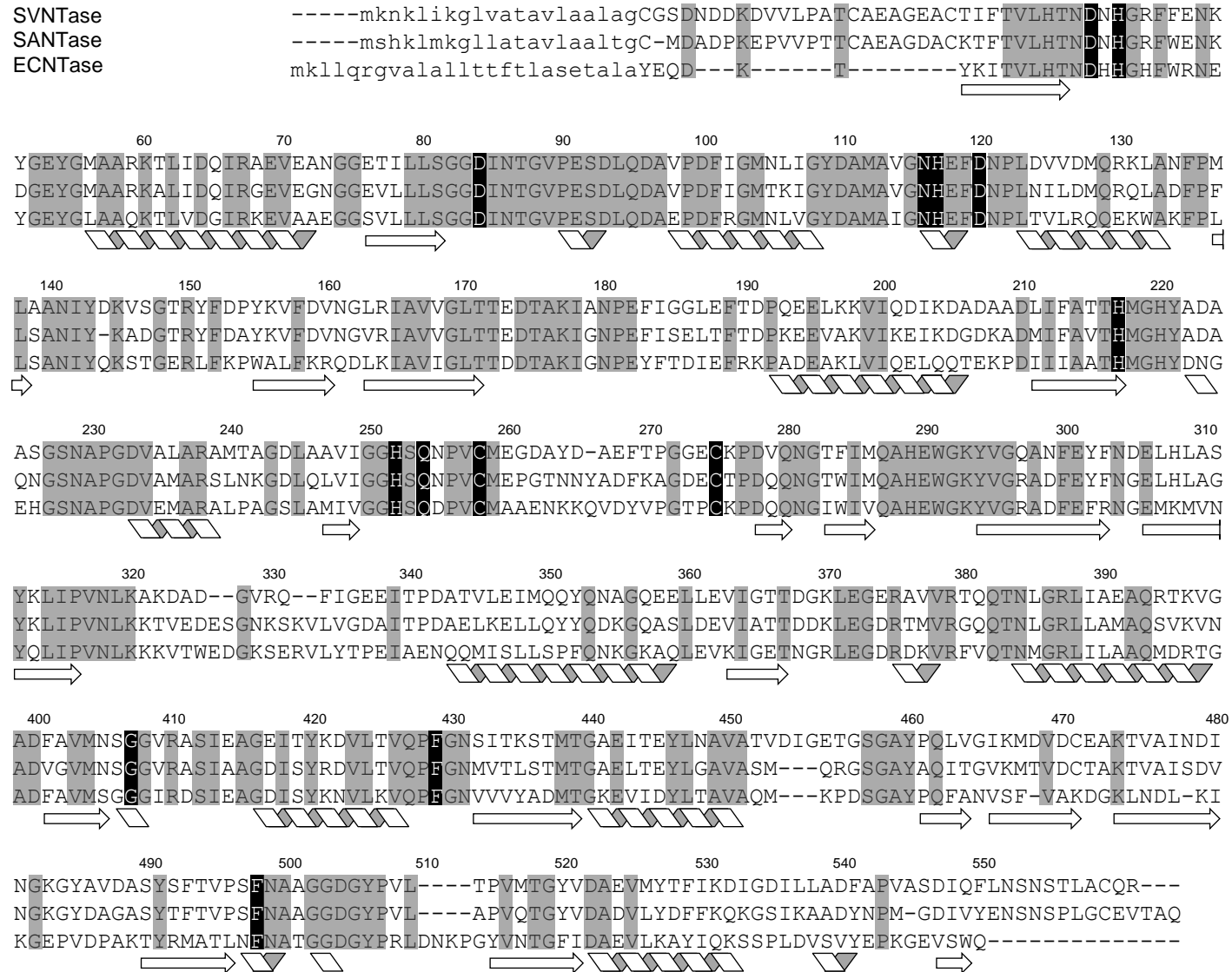


Fig. 1. Kuribayashi *et al.*

Fig. 2. Kuribayashi *et al.*

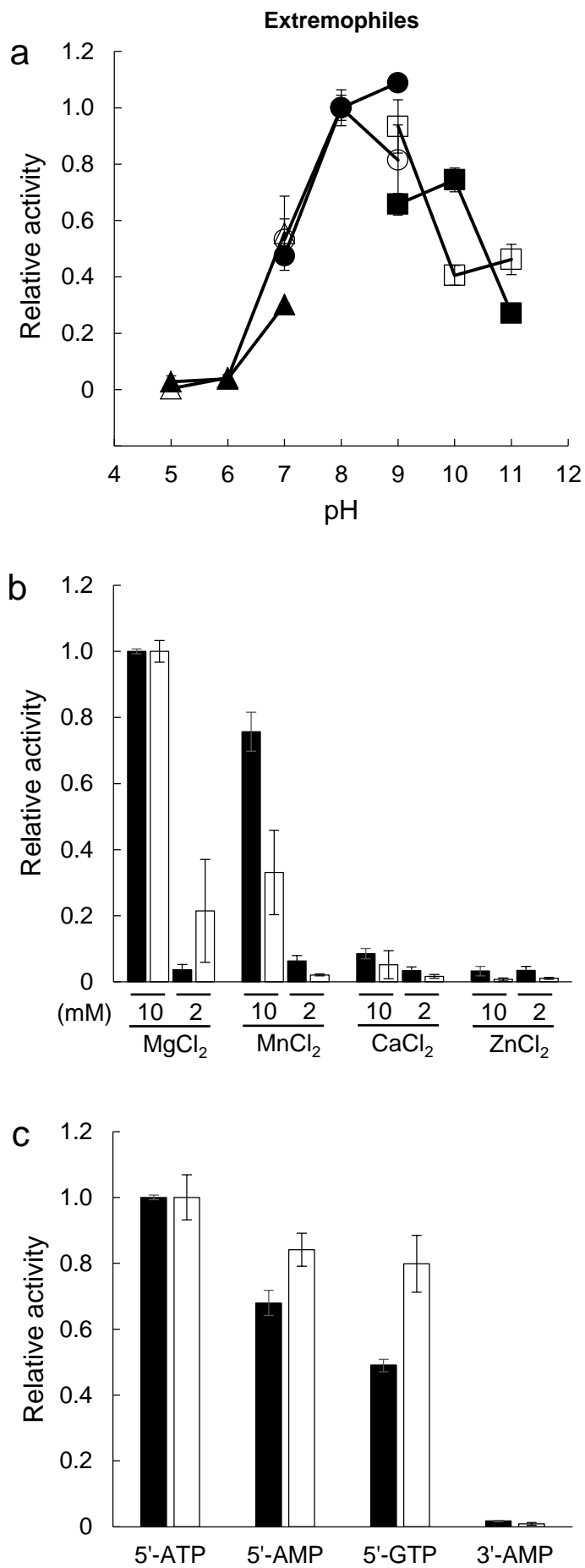


Fig. 3. Kuribayashi *et al.*

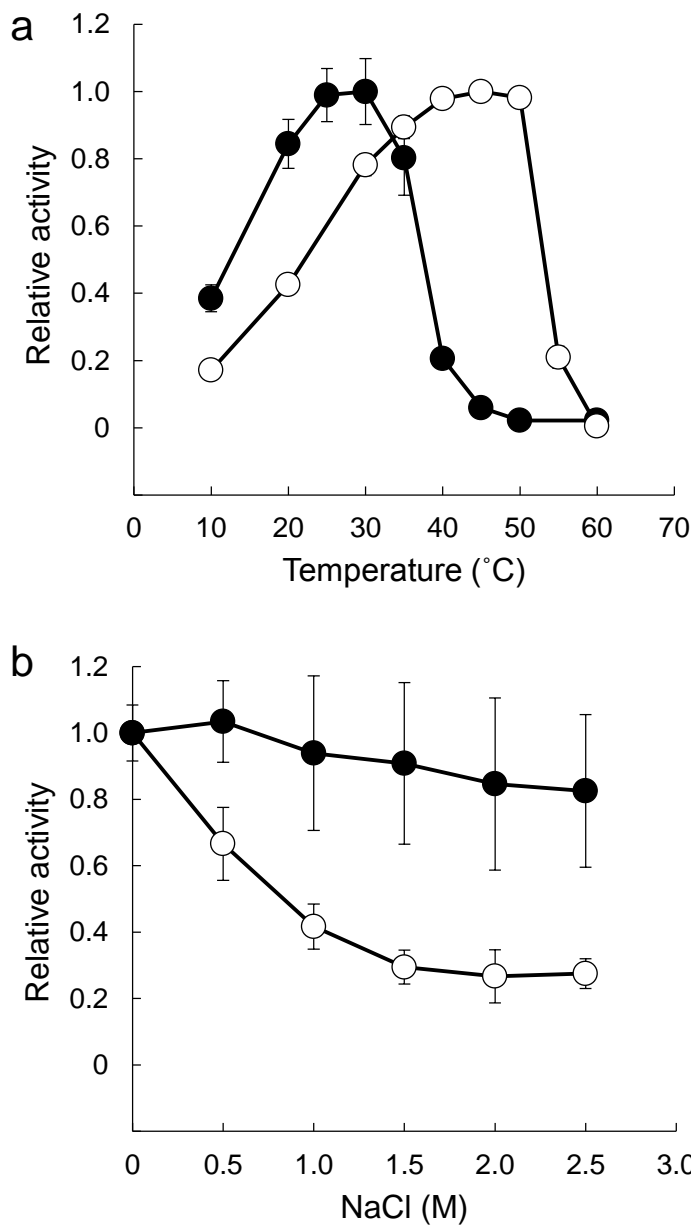


Fig. 4. Kuribayashi *et al.*

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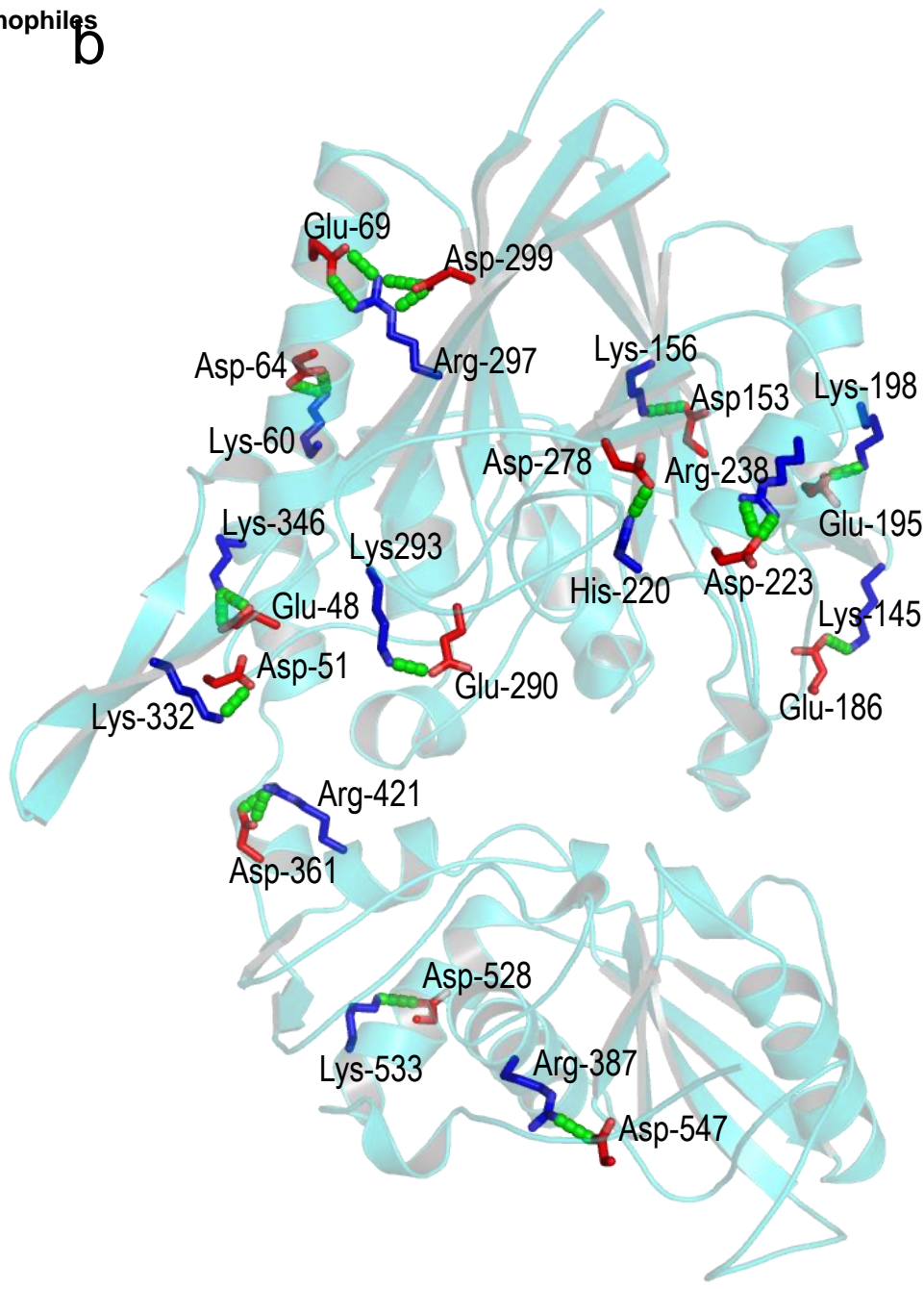
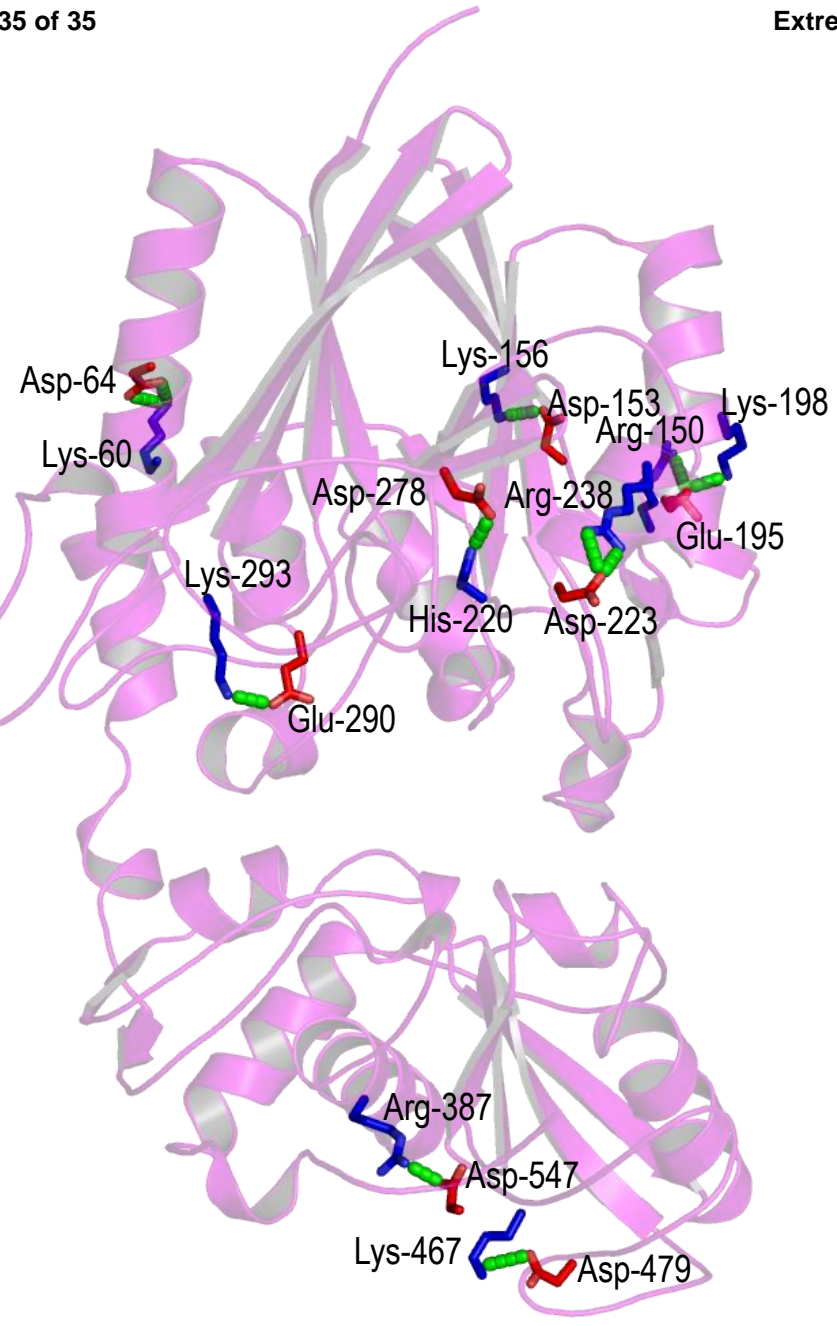


Fig. 5. Kuribayashi *et al.*