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Increased number of Arginine-based salt bridges contributes to the thermotolerance of thermotolerant acetic acid bacteria, *Acetobacter tropicalis* SKU1100

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ABSTRACT

Thermotolerant acetic acid bacteria (AAB), *Acetobacter tropicalis* SKU1100, can grow above 40 °C. To investigate the basis of its thermotolerance, we compared the genome of *A. tropicalis* SKU1100 with that of mesophilic AAB strain *Acetobacter pasteurianus* IFO3283-01. The comparative genomic study showed that amino acid substitutions from large to small residue and Lys to Arg occur in many orthologous genes. Furthermore, comparative modeling study was carried out with the orthologous proteins between SKU1100 and IFO3283-01 strains, indicating that the number of Arg-based salt bridges increased in protein models. Since it has been reported that Arg-based salt bridges are important factor for thermo-stability of protein structure, our results strongly suggest that the increased number of Arg-based salt bridges may contributes to the thermotolerance of *A. tropicalis* SKU1100 (the thermo-stability of proteins in *A. tropicalis* SKU1100).

1. Introduction

Acetic acid bacteria (AAB) are Gram-negative strictly aerobic bacteria, which are classified into 10 genera, of which the major genera are *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter* [1–5]. AAB oxidize various sugars and alcohols into the corresponding acids. In particular, *Acetobacter* sp. has been used from ancient times for industrial vinegar fermentation because of their high ethanol-oxidizing ability. Industrial acetic acid fermentation is carried out at 25–30 °C, and thus requires a cooling system to maintain the temperature of the culture from the fermentative heat generation. Recent global warming leads to further expenses for the cooling. Therefore, favorable AAB that can work properly near or above 40 °C are needed.

Thermotolerant bacteria are so-called mesophilic bacteria but grow at temperatures 5–10 °C higher than typical mesophilic strains of the same genus or the same species [6,7]. These strains are different from so-called thermophilic bacteria, which are defined as strains with an innate ability to grow above 60 °C [8,9]. Therefore, it is conceivable that these thermotolerant strains have acquired their growth phenotype by adapting to a habitat with a higher temperature, such as tropical regions [7,8].

Previously, we isolated a thermotolerant strain *Acetobacter* sp. SKU1100 from fruits in Thailand, which is now called *Acetobacter tropicalis* SKU1100 [10–12]. This strain can grow in shaking culture above 40 °C, which is higher than that of *Acetobacter pasteurianus* NBRC3283, which can grow up to 38 °C (non-acetic acid fermentation condition). In addition to the complete genome of *A. pasteurianus* IFO3283-01 [13], we have recently obtained the draft genome of *A. tropicalis* SKU1100 (GenBank Accession Nos. BABS01000001–BABS01000773), which may enable us to elucidate the thermotolerance mechanism of AAB. It has been reported that Lys to Arg substitutions are the important factor to generate thermo-stability in protein structure of thermophilic bacteria [14]. These substitutions are also thought to be the important factor to generate thermo-stability in protein structure of thermotolerant bacteria [15]. In addition, it has been suggested that salt bridges formed with Arg residues, rather than Lys residues, are more stable and thus contribute to the thermo-stability of proteins [16–18].

In the present study, to investigate the relationship between thermotolerance and amino acid mutation of orthologous proteins for AAB, *A. tropicalis* SKU1100 genome was compared with that of mesophilic AAB strain *A. pasteurianus* IFO3283-01 in terms of amino acid substitutions. In addition, 3D models of the orthologous

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proteins were constructed to elucidate salt bridge-forming residues, because of Lys to Arg substitutions being increased in the thermotolerant strain.

The results obtained in our study clearly suggested that Arg-based salt bridge have contributed to evolutional change in this strain in terms of its thermotolerance.

2. Materials and methods

2.1. Bacterial strains

A. tropicalis SKU1100 (NBRC 101654) and *A. pasteurianus* NBRC3283 were used in this study. These strains were cultured in potato medium (0.5% glucose [D], 2% glycerol [G], 1% yeast extract [Y], 1% peptone [P], and 150 mL/L of potato extract) or YPGD medium (0.5% each of Y, P, G, and D) [11].

2.2. Comparative analysis of orthologous genes

The draft genome assembly (GenBank Accession Nos. BABS01000001–BABS01000773) of *A. tropicalis* SKU1100 (NBRC 101654) and the previously published complete genome sequence of *A. pasteurianus* IFO3283-01 were used for the comparative genome analysis [13]. Although *A. pasteurianus* IFO3283-01 have six plasmid sequences, only the chromosome sequence was used for this analysis. Homologous genes were identified by homology searches in an amino acid sequence using the BLASTP filtering expectation value of identity $\geqslant$70%, *e*-value $\leqslant$1.0 E−30, and sequence overlap $\geqslant$70% [19]. When the best hit was identical to the query, the ORFs were regarded as being orthologous genes. Putative localization of orthologous proteins was evaluated using the PSORTb program [20,21]. Multiple sequence alignment between orthologous proteins was performed using ClustalW [22]. Nucleotide sequence alignment of orthologous genes was achieved from amino acid sequence alignment using house-written ruby script. Amino acid sequences of predicted orthologous genes are provided in Supplemental Table 1.

2.3. Comparative modeling of orthologous proteins

Initially, BLASTP search of *A. pasteurianus* orthologous proteins were performed against pdbaa database (identity $\geqslant$20%, *e*-value $\leqslant$1.0 E−10, and sequence overlap $\geqslant$70%) to find homologous sequences of known protein structures. The pdbaa database was downloaded from the NCBI FTP site at ftp://ftp.ncbi.nih.gov/blast/db/. Satisfying the BLASTP criteria, sequence alignments between query and top hit protein were constructed using ClustalW [22]. From sequence alignment, 3D models of *A. pasteurianus* orthologous proteins were built using Modeller 9v7 [23–26]. In addition, 3D models of *A. tropicalis* were constructed with *A. pasteurianus* 3D model structure as template. The resulting 3D models were used for the detection of salt bridge interaction. The criterion for determining salt bridges was as follows: distance between carboxyl oxygen atoms on the side chain of Glu or Asp and ε-nitrogen atoms on the side chain of Arg within 5.00 Å[18].

2.4. Molecular dynamics simulations of modeled structures

Structural models of NAD-dependent alcohol dehydrogenases (*A. tropicalis* SKU1100: ATPR_0541 and *A. pasteurianus* IFO3283-01: APA01_00250) were used for molecular dynamics (MD) simulation as the initial structures. Two starting structures were solvated in a cubic box using SPC water molecules. Box dimensions for ATPR_0541 and APA01_00250 were chosen by the criterion that the distance of protein atoms from the wall was greater than 0.9 nm. The system was neutralized by adding Na^+ ions. Initially, to equilibrate the position of water molecules, 10 ps of MD simulation was performed with protein position fixed. Thousand steps of steepest descent energy minimization was carried out with protein heavy chain fixed. Next, 1000 steps of steepest descent energy minimization was carried out without any restraint. Initial velocities were assigned according to the Maxwell–Boltzmann distributions at 300 K, and no restraints 2 ns MD simulations were performed. All the simulations were carried out at standard pressure of 1 bar with a coupling constant of 0.5 ps for pressure and 0.1 ps for temperature making use of Berendsen coupling algorithm [27]. Periodic boundary conditions were applied. Non-bonded list was updated every 10 steps. The time step for the integration algorithm was kept at one femto-second. Simulation trajectories were saved every 1000 steps. System temperature was kept at 300 K. The GROMACS 4.5.4 package with force field GROMOS96 G53a6 was used for the simulations [28–30]. Particle mesh Ewald electrostatics was applied using Lennard–Jones cutoff of 1.4 nm and coulomb cutoff of 1.0 nm. Maximum spacing for the fast Fourier transform grid was kept at 0.12 nm and cubic interpolation order was applied [31,32]. The resulting trajectories of two proteins were analyzed using the standard software provided by the GROMACS 4.5.4 package. Last 800 trajectories were used for the analysis of protein flexibility.

3. Results and discussion

3.1. Characterization of A. tropicalis SKU1100 and A. pasteurianus NBRC3283

The growth abilities of the two closely related species, *A. tropicalis* SKU1100 and *A. pasteurianus* NBRC3283, were compared at high temperatures. As shown in Fig. 1, it was found that both species could grow well on YPGD agar plate up to 39 °C, although SKU1100 grew better than NBRC3283. However, over 40 °C, the growth of NBRC3283 was largely repressed, while SKU1100 could

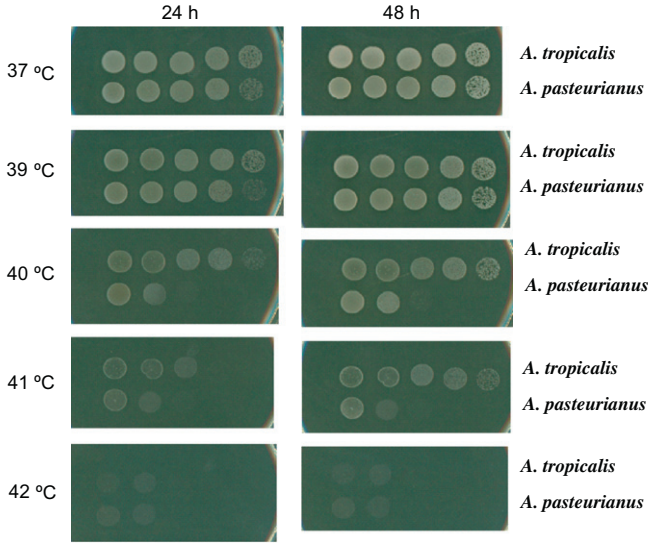


Fig. 1. Growth comparison of *A. tropicalis* SKU1100 and *A. pasteurianus* NBRC3283 at various temperatures under non-fermentation condition. These strains were precultured in potato medium until its turbidity reached 150 Klett units at 30 °C. Then, the cultures were diluted 10^{-1}, 10^{-2}, 10^{-3}, or 10^{-4} times. Seven microliters of the diluted solutions was spotted onto YPGD plates, which were incubated at 37, 39, 40, 41, or 42 °C for 24 and 48 h. In each panel, the upper lane shows the spots of *A. tropicalis* and the lower lane shows those of *A. pasteurianus*.

grow even at 41 °C. Thus, *A. tropicalis* SKU1100 was considered as a more thermotolerant AAB strain than *A. pasteurianus* NBRC3283.

3.2. Comparison of amino acid composition of orthologous groups

The 1242 highly conserved orthologous groups between *A. tropicalis* and *A. pasteurianus* were determined (see Section 2) and used for the comparison of amino acid composition (Supplemental Table 1). The number of orthologous genes as a function of GC content is shown (Supplemental Fig. 1). The peak of ORF number in *A. tropicalis* shifts to a higher GC content than that of *A. pasteurianus*. The differences in average GC content between the two microorganisms are directly reflected in the GC content of the ORFs.

The amino acid composition of the protein coding regions was analyzed, as shown in Table 1. Ala, Arg, Asp, Gly, Leu, Thr, and Val are more frequently used in *A. tropicalis* than *A. pasteurianus*, which results in the higher GC content of *A. tropicalis*. The number of codons per 1000 bases (fraction values) of GCC (Ala), GCG (Ala), GAC (Asp), GGA (Gly), GGC (Gly), GGG (Gly), CTC (Leu), CTG (Leu), CGG (Arg), Thr (ACC), and Thr (ACG) are increased (data not shown). Nishio and coworkers have reported that thermotolerant corynebacterium, *Corynebacterium efficiens*, has more rare codons, GGG (Gly) and CGG (Arg), than mesophilic *Corynebacterium glutamicum* [15]. Our results showed the same tendency with respect to CGG (Arg) codon. Increased number of CGG codons may be correlated with thermotolerance of bacteria, as described below.

3.3. Comparison of amino acid substitution of orthologous groups

To investigate the direction of amino acid mutation, the biased amino acid substitution of 1242 orthologous genes between *A. pasteurianus* and *A. tropicalis* was calculated. The amino acid sequences of 1242 orthologous proteins were aligned using ClustalW [22]. Amino acid substitutions between *A. pasteurianus* and *A. tropicalis* were extracted and compared (Table 2).

The results clearly showed high substitution frequency of large to small residues (Met-Leu, Glu-Asp, Lys-Arg, Glu-Ala, and Ile-Val) from *A. pasteurianus* to *A. tropicalis*. It has been reported that large to small hydrophobic residue substitutions of subunit–subunit interface stabilize the thermal stability of protein [33]. However, some previous studies have suggested that large to small hydrophobic residue substitutions of buried hydrophobic core destabilize the thermal stability of protein [14,34]. Thus, although such many amino acid substitutions occur between each two orthologous proteins, it is difficult to evaluate the effect of each amino acid substitution. Even though, these mutations can be speculated to make more compact packing structure, and to decrease the conformational flexibility. The decreasing of conformational flexibility is important factor for the thermal stability of protein [18]. Therefore, we hypnotized that the decreasing of conformational flexibility due to such the amino acid exchange stabilizes the protein structure in thermotolerant strain. Although it is difficult to evaluate this idea, MD simulations of orthologous proteins may be useful for the analysis of conformational flexibility. Therefore, as described in the next section, the MD simulations were actually tried in case of one orthologous enzymes.

Of the amino acid substitutions occurred, Lys to Arg substitutions from *A. pasteurianus* to *A. tropicalis* were paid attention. Some previous studies have suggested that Lys to Arg substitutions affect the thermal stability of proteins in both thermophilic and thermotolerant bacteria [14,15]. Several properties of Arg residues are thought to be better adapted to high temperatures than Lys residues [14], including the fact that the Arg-δ-guanido moiety has a reduced chemical reactivity due to its high pK_a and its resonance stabilization. Indeed, it has been reported that thermotolerant *C. efficiens* have more Arg residues than mesophilic *C. glutamicum* [15]. In addition, Arg or Lys residue has been known to form a salt bridge with either Glu or Asp residue, and it has been suggested that the salt bridge formed with Arg and Glu (or Asp) confers a strong stabilizing mechanism for hyperthermophilic proteins [14]. In addition, many other substitutions to Asp and Arg were also observed (Asn-Asp, Gln-Arg, and His-Arg). Increasing of Asp and Arg residue may contribute to the increasing of the Arg-based salt bridges, which contribute to the thermo-stability of *A. tropicalis* proteins.

3.4. Evaluation of the number of salt bridge-forming Arg residues

To evaluate the number of salt bridge-forming Arg residues, comparative models of orthologous proteins were constructed. Comparative modeling predicts the three-dimensional structure of a given target protein sequence primarily on the basis of its alignment to proteins of known structure as templates. The prediction process consists of fold assignment, target-template alignment, and model building. Initially, we performed a BLAST search against the pdbaa database (identity $\geqslant$20%, *e*-value $\leqslant$1.0 E−10, and sequence overlap $\geqslant$70%) to find sequences homologous to proteins of known three-dimensional structures. Satisfying the BLAST criteria, 737 orthologous groups were found to have close homologs in Protein Data Bank (Supplemental Fig. 2). Using ClustalW, 737 orthologous amino acid sequences of *A. pasteurianus* were

Table 1
Amino acid composition of protein coding regions.

Amino acid	Number (%)		Percentage	
	A. pasteurianus	*A. tropicalis*	*A. pasteurianus*	*A. tropicalis*
Ala	52,531	53,062	11.55	11.66
Arg	30,006	30,584	6.60	6.72
Asn	13,086	12,292	2.88	2.70
Asp	24,580	25,095	5.40	5.52
Cys	4565	4393	1.00	0.97
Gln	16,433	15,724	3.61	3.46
Glu	26,011	25,716	5.72	5.65
Gly	38,044	38,690	8.36	8.50
His	10,739	10,504	2.36	2.31
Ile	23,081	22,544	5.07	4.96
Leu	46,125	46,647	10.14	10.25
Lys	16,680	16,109	3.67	3.54
Met	11,880	11,506	2.61	2.53
Phe	16,331	16,258	3.59	3.57
Pro	24,569	24,745	5.40	5.44
Ser	24,504	24,815	5.39	5.45
Thr	25,525	25,927	5.61	5.70
Trp	5913	5867	1.30	1.29
Tyr	10,362	10,278	2.28	2.26
Val	33,984	34,193	7.47	7.52
Total	454,949	454,949	–	–

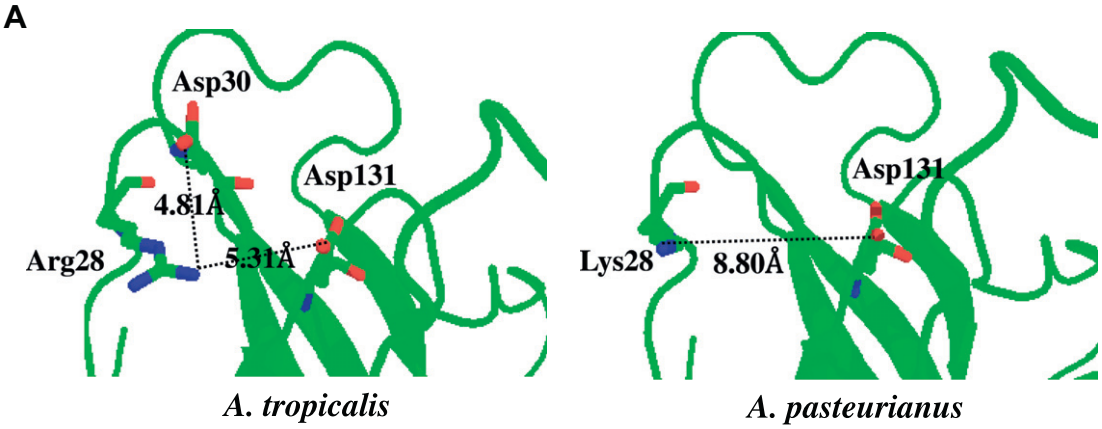
Table 2
Comparison of amino acid substitution patterns in orthologous genes between *A. pasteurianus* IFO3283-01 and *A. tropicalis* SKU1100.

A. pasteurianu	*A. tropicalis*	Forward	Reverse
Glu	Asp	1889	1621
Lys	Arg	1477	1227
Met	Leu	1077	842
Asn	Asp	781	548
Ile	Leu	1310	1101
Ile	Val	2535	2356
Gln	Arg	738	574
Glu	Ala	1027	872
Asn	Thr	375	235
His	Arg	513	382

aligned with template sequences. Model building was performed using Modeller 9v7 [23–26]. To reduce alignment error between two orthologous proteins, 3D models of *A. tropicalis* were constructed with *A. pasteurianus* 3D model structure as a template. We found 253 substitutions from Lys to Arg in *A. tropicalis* in the 737 orthologous proteins. Fig. 2 shows one example of a substitution in the structural model between orthologous NAD-dependent alcohol dehydrogenases of *A. pasteurianus* and *A. tropicalis*. In case of the latter enzyme, Arg28, which substitutes for Lys of the protein in *A. pasteurianus*, forms a salt bridge with two Asp residues, Asp30 and Asp131. In contrast, original Lys28 in *A. pasteurianus* have forms a Lys-based salt bridge with only Asp 131 residue. In addition, to evaluate the flexibility of two modeled structures, we performed MD simulations of the NAD-dependent alcohol dehydrogenases (*A. tropicalis* SKU1100: ATPR_0541 and *A. pasteurianus* IFO3283-01: APA01_00250). It was shown that the flexibility of salt-bridge forming regions in *A. tropicalis* is more rigid than that of *A. pasteurianus* (Supplemental Fig. 3). In particular, both Arg 28 and Asp 131 regions in ATPR_0541 showed rather low root-mean-square (RMS) fluctuations with respect to Lys 28 and Asp 131 in APA01_00250. It is obvious that Arg-based salt-bridge contribute to the low flexibility of these regions. Since the decreasing of conformational flexibility is important factor for the thermal stability of protein, these results suggest that the increased number of Arg-based salt bridges contributes to the thermo-stability of proteins in *A. tropicalis*.

Thus, the total numbers of Arg-based salt bridges were compared in the 737 proteins between *A. tropicalis* and *A. pasteurianus*. As shown in Table 3, of the 737 proteins, 363 proteins have a higher number of Arg-based salt bridges in *A. tropicalis* when compared with those of *A. pasteurianus*, while 241 proteins have a lower number of salt bridges in *A. tropicalis*. Another 134 proteins have the same number of residues between *A. tropicalis* and *A. pasteurianus*. When examining the subcellular localization of these proteins, the proteins with a higher number of Arg-based salt bridges in *A. tropicalis* are observed among the soluble proteins, cytoplasmic and periplasmic proteins, while the proteins with less salt bridges are membrane proteins.

Due to the alignment error and incorrect side chain rotamers, modeling structure is not very accurate in some cases. However, more broad range cut-off value of salt bridges (6–7 Å) also showed same tendency (data not shown). Therefore, it had been thought that the increased number of Arg-based salt bridges contributes to the thermo-stability of proteins. Side chain structure of protein



B

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A. pasteurianus      1 -MSGKMKAAVVHEFGKPLTIEELDIPPIKPTQILVKMIACGVCHTDLHAASGDWPKKPHL 59
A. tropicalis        1 -MSGKMKAAVVHEFGKPLTIEELDIPDIRPDQILVKMTACGVCHTDLHAASGDWPKKPTV 59
Template (1LLU_A)    2 TLPQTMKAAVVHAYGAPLRIEEVKVPLPGPGQVLVKIEASGVCHTDLHAAEGDWPVKPPL 61

A. pasteurianus     60 PFIPGHEGVGTVVQVGSEVDWVKEGDVVGVPWLYSACGHCEHCLAGWETLCAKQEETGYS 119
A. tropicalis       60 PFIPGHEGVGVVTKVGSNVTWVKEGDEVGVPWLYSACGHCEHCLGGWETLCAKQQETGYS 119
Template (1LLU_A)   62 PFIPGHEGVGYVAAVGSGVTRVKEGDRVGIPWLYTACGCCEHCLTGWETLCESQQNTGYS 121

A. pasteurianus    120 VNGCFAEYVVADPNYIAHLPKGVDPVKVAPVLCAGLTVYKGLKMTDTRAGNWVAISGVGG 179
A. tropicalis      120 VNGCFAEYVVADPNYVARLPKGIDPVKVAPVLCAGLTVYKGLKMTDTKAGNWVAISGVGG 179
Template (1LLU_A)  122 VNGGYAEYVLADPNYVGILPKNVEFAEIAPILCAGVTVYKGLKQTNARPGQWVAISGIGG 181

A. pasteurianus    180 LGQMAVQYAVAMGLNVVAVDIDDEKLATAKKLGATYTVNARNTDPAAFMQEKVGGVHGGL 239
A. tropicalis      180 LGQMAVQYGVAMGLNVIAVDIDDQKLATARKLGAALTVNARTTDPAAFIQKEVGGVHGGL 239
Template (1LLU_A)  182 LGHVAVQYARAMGLHVAAIDIDDAKLELARKLGASLTVNARQEDPVEAIQRDIGGAHGVL 241

A. pasteurianus    240 ITAVSTKAFSQAMGYVRAGGTLVLNGLPPGDFPISIFDMVMNAITIRGSIVGTRLDMIEA 299
A. tropicalis      240 ITAVSTKAFSQAMGYVRAGGTLVLNGLPPGDFPISIFDMVMKGTTIRGSIVGTRLDMIEA 299
Template (1LLU_A)  242 VTAVSNSAFGQAIGMARRGGTIALVGLPPGDFPTPIFDVVLKGLHIAGSIVGTRADLQEA 301

A. pasteurianus    300 LSFFAEGKVTSVTTTDRIDNINAIFDALKNGRVEGRVVLDFRN 342
A. tropicalis      300 LSFFAEGKVTSVTQTDRLENINNIFTRLEEGKVEGRIVIDFRA 342
Template (1LLU_A)  302 LDFAGEGLVKATIHPGKLDDINQILDQMRAGQIEGRIVLEM-- 342
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Fig. 2. 3D modeled structure and multiple alignments with template sequence. (A) Structural model of NAD-dependent alcohol dehydrogenase (*A. tropicalis* SKU1100: ATPR_0541 and *A. pasteurianus* IFO3283-01: APA01_00250). Salt bridge-forming residues are represented by stick model. In *A. tropicalis* modeled structure, Arg28 forms salt bridges with two Asp residues, Asp30 and Asp131. In contrast, Lys28 in *A. pasteurianus* modeled structure have forms a Lys-based salt bridge with Asp 131 residue. The figure was prepared using PyMOL 0.99 [36]. (B) Salt bridge-forming residue pairs are shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Table 3
Comparison of the number of salt bridge-forming Arg residues in the orthologous genes. Three-dimensional structure model was constructed for 737 orthologous pairs between *A. tropicalis* and *A. pasteurianus*. In each structural pair, number of Arg-based salt bridges were compared. If the *A. tropicalis* ortholog has higher salt bridge number than *A. pasteurianus* ortholog, it was counted as "Higher in *A. tropicalis*", and vice versa as "Higher in *A. pasteurianus*". Furthermore, these comparisons were separated in their localization.

Subcellular localization	Total number of orthologous groups	Number of salt bridge-forming Arg residues		
		Higher in *A. tropicalis*	Higher in *A. pasteurianus*	No difference
Cytoplasmic	394	205	123	66
Cytoplasmic membrane	57	21	28	8
Outer membrane	13	4	5	4
Periplasmic	17	11	3	3
Extracellular	1	0	1	0
Unknown	255	121	81	53
Total	737	362	241	134

sometime has high flexibility in solution. Therefore, to evaluate the more precious Arg-based salt bridge interactions, MD simulations of orthologous proteins should be performed.

In the present study, we investigated the amino acid substitutions between *A. tropicalis* and *A. pasteurianus*. Analysis of amino acid substitutions from *A. pasteurianus* to *A. tropicalis* showed that a large number of large to small residue substitutions and Lys to Arg substitutions has occurred, both of which may contribute to increase the thermal stability of proteins. Comparative modeling study of the orthologous proteins showed that the increased number of Arg in *A. tropicalis* orthologous proteins is largely involved in a salt bridge formation. Recently, Kanchanarach et al. have reported that quinoprotein alcohol dehydrogenases of thermotolerant *Acetobacter* strains have slightly higher optimal temperature and heat stability than those of mesophilic strains [35]. Therefore, we suggest that at least the increased number of Arg-based salt bridges may contribute to the thermo-stability of proteins and thus to the thermotolerance of *A. tropicalis* SKU1100.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.04.126.

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