Biochemical and Biophysical Research Communications 409 (2011) 120–124

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 23 April 2011
Available online 30 April 2011

Keywords:
Thermotolerant acetic acid bacteria
Acetobacter pasteurianus
Acetobacter tropicalis
Comparative modeling

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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doi:10.1016/j.bbrc.2011.04.126

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

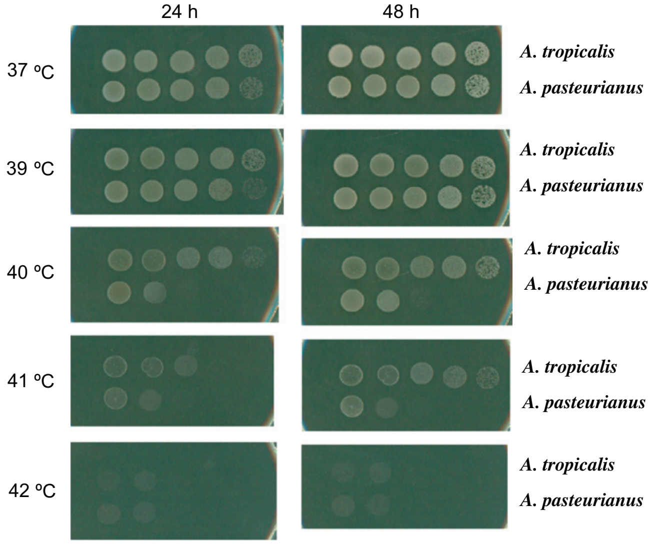

Fig. 1. Growth comparison of *A. tropicalis* SKU1100 and *A. pasteurianus* NBRC3283 at various temperatures under non-fermentation condition. These strains were precultured in potato medium until its turbidity reached 150 Klett units at 30 °C. Then, the cultures were diluted 10^{-1}, 10^{-2}, 10^{-3}, 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*.