Characterization of *Lactobacillus salivarius* alanine racemase: short-chain carboxylate-activation and the role of A131

Jyumpei Kobayashi1*, Jotaro Yukimoto2, Yasuhiro Shimizu1, Taketo Ohmori3, Hirokazu Suzuki4, Katsumi Doi5 and Toshihisa Ohshima1,5

**Abstract**

Many strains of lactic acid bacteria produce high concentrations of d-amino acids. Among them, *Lactobacillus salivarius* UCC 118 produces d-alanine at a relative concentration much greater than 50% of the total d, l-alanine (100%/9, l-alanine). We characterized the *L. salivarius* alanine racemase (ALR) likely responsible for this d-alanine production and found that the enzyme was activated by carboxylates, which is an unique characteristic among ALRs. In addition, alignment of the amino acid sequences of several ALRs revealed that A131 of *L. salivarius* ALR is likely involved in the activation. To confirm that finding, an *L. salivarius* ALR variant with an A131K (ALR^A131K^) substitution was prepared, and its properties were compared with those of ALR. The activity of ALR^A131K^ was about three times greater than that of ALR. In addition, whereas *L. salivarius* ALR was strongly activated by low concentrations (e.g., 1 mM) of short chain carboxylates, and was inhibited at higher concentrations (e.g., 10 mM), ALR^A131K^ was clearly inhibited at all carboxylate concentrations tested (1–40 mM). Acetate also increased the stability of ALR such that maximum activity was observed at 35 °C and pH 8.0 without acetate, but at 50 °C in the presence of 1 mM acetate. On the other hand, maximum ALR^A131K^ activity was observed at 45 °C and around pH 9.0 with or without acetate. It thus appears that A131 mediates the activation and stabilization of *L. salivarius* ALR by short chain carboxylates.

**Keywords:** d-amino acid, Alanine racemase, *Lactobacillus salivarius*, Lactic acid bacteria, Short-chain carboxylate

**Background**

With the exception for glycine, which contains no asymmetric carbon, all proteogenic amino acids exist as l-α-form molecules. Indeed, up to around 1980, d-amino acids as enantiomers of corresponding l-amino acids were generally considered to have no important role in living organisms, and so attracted little attention, though it was acknowledged that some d-amino acids were among the main constituents of bacterial cell walls (Heijenoort 2001). However, advances in the analytical techniques used to study amino acid enantiomers have shown that, in fact, d-amino acids are widely distributed among a variety of organisms, including plants, fish and mammals (Kullman et al. 1999; Erbe and Brückner. 2000; Pätzold et al. 2005). In addition, it was recently reported that d-amino acids exert inhibitory effects on biofilm formation (Kolodkin-Gal et al. 2010; Hochbaum et al. 2011). Such evidence of the physiological importance of d-amino acids has prompted investigation into their metabolism and the enzymes involved.

D-Alanine is well known to be a component of bacterial cell walls and to be synthesized from l-alanine by alanine racemase (ALR). Our group recently found that many lactic acid bacteria secrete d-alanine into their medium (Mutaguchi et al. 2013) and the relative percentage (100b/(d + l)) of d-alanine per total d, l-alanine in many lactic acid bacterial cells is more than 50%; the value for *Lactobacillus salivarius* is 89.6%, for example. ALR is thought to be pivotal for d-alanine production in lactic acid bacteria, but this racemase generally catalyzes the conversion of l-alanine to a racemic mixture, and...
the d-alanine percentage does not normally exceed 50%. Thus it may be postulated that an unknown mechanism accounts for the excess d-alanine production seen in L. salivarius.

We previously identified a d-amino acid aminotransferase (d-AAT) in L. salivarius that exhibited characteristics different from those of known d-AATs from strains of Bacillus and Geobacillus species (Kobayashi et al. 2013). In that context, we were interested in the inherent characteristics of ALR of L. salivarius and identified an ALR homolog gene in an L. salivarius data base. In the present study, we expressed this L. salivarius ALR gene strongly activated in the presence of carboxylates such as acetate and propionate. By contrast, ALRs from Geobacillus and Bacillus species are reportedly inhibited by acetate and propionate (Morollo et al. 1999; Kanodia et al. 2009). In addition, ALRs from L. fermentum and Pseudomonas putida are known to be stabilized by acetate, although the mechanism for this effect is not yet known (Johnston and Diven. 1969; Rosso et al. 1969). We therefore compared the primary sequences of ALRs from L. salivarius, L. fermentum and P. putida with those of various Bacillus and Geobacillus species. Nearly all important residues for the execution of catalytic activity are well conserved, except for a single residue: A131 in L. salivarius, L. fermentum and P. putida ALRs, and K129 in Bacillus and Geobacillus ALRs. K129 is thought to stabilize R136 binding to the carboxylate group through carbamate formation (Morollo et al. 1999). Here we examined the role of A131 in the regulation of L. salivarius ALR by the carboxylates by preparing an L. salivarius ALR variant (A131K) and comparing its properties with those of the parental wild-type ALR.

Methods
Sequence analysis of ALR
The primary amino acid sequence of L. salivarius ALR was analyzed and compared with those of previously reported ALRs (Johnston and Diven 1969; Inagaki et al. 1986; Kanda-Nambu et al. 2000; Ju et al. 2009; Kanodia et al. 2009; Liu et al. 2012). The amino acid sequences of L. salivarius UCC118, B. pseudofirmus OF4 and G. stearothermophilus IFO 12550 ALRs were retrieved from the UniProt data base (http://www.uniprot.org/), and that of P. putida YZ-26 was obtained from the report by Liu et al. (2012). The sequences of B. anthracis Sterne 34F2 (Kanodia et al. 2009), B. subtilis PCI219 (Kanda-Nambu et al. 2000) and L. fermentum ATCC9330 (Johnston and Diven. 1969) ALRs were not found in a data base, but those of B. anthracis ATCC14578, B. subtilis 168 and L. fermentum ATCC14931 were obtained from the UniProt data base and used as alternatives. Multiple alignments were performed using TCoffee (http://www.tcoffee.org/Projects/tcoffee/).

Plasmid construction
The ORF containing the ALR gene in L. salivarius (UniProt ID: Q1WV14) was amplified using PCR with chromosomal DNA from L. salivarius UCC118 and primers ALR-F 5'-ATATCATATGTTAATTGGAAGACATCG-3' (Ndel site is underlined) and ALR-R 5'-ATTCTCGAGC-TACCTGTAATTTCTGGAAC-3' (Xhol site is underlined). The PCR product was cloned between the Ndel and Xhol sites of pET-28a (Novagen, Darmstadt, Germany) to obtain pET-ALR.

To generate the ALR\textsuperscript{A131K} mutant gene, the upstream and downstream regions of ALR were amplified from pET-ALR using primers T7 promoter-F 5'-TAATAC GACCTACTATAGG-3' and ALR-A131K-R 5'-CATACCTGTGTCTATAATTTAGGATCTTATTGTTG-3' (mutation bases are underlined) for the upstream, and primers ALR-A131K-F 5'-CAAAGATTTAAGATCACC TAAATTTAGACACACGTATG-3' (mutation bases are underlined) and T7 terminator-R 5'-GCTAGTATTGCTCAAGCGG-3 for the downstream. The two fragments were subsequently fused using overlap extension PCR to give the mutant gene ALR\textsuperscript{A131K}. The fused fragment was cloned between the Ndel and Xhol sites of pET-28a to give pET-ALR\textsuperscript{A131K}.

Preparation of recombinant proteins
Escherichia coli BL21-CodonPlusTM(DE3)-RIPL cell (Strategene, CA, USA) harboring pET-ALR was cultured in 5 mL of LB medium containing 50 mg/L kanamycin and 50 mg/L chloramphenicol overnight at 37 °C. The cells were then subcultured and induced using 100 mL of Overnight Expression medium containing 45 g/L Overnight Express™ Instant LB Medium (Novagen), 50 mg/L kanamycin and 50 mg/L chloramphenicol for 18 h at 25 °C with shaking (180 rpm). The cells were then pelleted by centrifugation (8500×g for 10 min at 4 °C) and resuspended in 20 mM MOPS buffer (pH 7.0) containing 500 mM NaCl, after which the suspension was sonicated and again centrifuged as described above. The supernatant was then filtered (0.22 µm pore size), and the enzyme was purified on a Ni–NTA Agarose column (QIAGEN, Venlo, The Netherlands). The purified enzyme was dialyzed against 100 volumes of 20 mM MOPS buffer (pH 7.0) containing 500 mM NaCl and 2.0 mM EDTA for 12 h at 4 °C with two changes of the buffer solution. The resultant dialysate was then concentrated by ultrafiltration using an Amicon Ultra (Merck Millipore, MS, USA). The resultant enzyme solution was stored in the presence of 50% glycerol at −20 °C. ALR\textsuperscript{A131K} protein was prepared using the same procedure with pET-ALR\textsuperscript{A131K}.
ALR assays
ALR and ALRA131K activities were determined by measuring the initial velocity of d-alanine formation from L-alanine using ultra-performance liquid chromatography (UPLC) (Waters, Tokyo, Japan). The standard reaction mixture (0.1 mL) containing 100 mM MES buffer (pH 7.0), 20 mM L-alanine, 0.05 mM pyridoxal-5′-phosphate (PLP) and 10 % (v/v) purified enzyme solution. The enzyme reaction was run for 5 min at 30 °C and was then stopped by adding 0.1 mL of 20 % trichloroacetic acid. The UPLC analysis was performed with an ACQUITY UPLC TUV system consisting of a Waters Binary Solvent Manager, Sample Manager, FLR Detector and AccQ-Tag Ultra 2.1 × 100-mm column (Waters). The eluent flow rate was 0.20 mL/min, the column temperature was 45 °C, and the fluorescent wavelengths of the FLR detector were 350 and 450 nm. The eluent was linearly graduated using 80 % sodium acetate buffer (50 mM, pH 5.9) and 20 % methanol. All enzyme assays were performed more than three times under the same conditions. Mean values and standard deviations were calculated from each assay.

Effect of temperature on activity
The effect of temperature on ALR and ALR A131K activities was examined using the standard reaction mixture and varying the temperature between 20 and 60 °C in presence and absence of 1 mM acetate.

Effect of pH on activity
The effect of pH on ALR and ALR A131K activities was examined by measuring their activities under standard reaction conditions using different pH buffers in presence and absence of 1 mM acetate. The buffers used for the assays were 100 mM citrate (pH 4.0–5.5), 100 mM MES (pH 5.5–7.0), 100 mM Tris–HCl (pH 7.0–9.0) and 100 mM sodium carbonate (pH 9.0–11.0).

Effect of various carboxylates on enzyme activity
The effects of various carboxylates and related carboxylates containing acetate, propionate, butyrate, d,L-lactate, citrate, L-glutamate, d,L-aspartate, pyruvate, 2-oxoglutarate, oxaloacetate succinate and fumarate on ALR and ALR A131K activities were examined. All carboxylates used in this study were sodium salts or were neutralized with sodium hydroxide before use. The carboxylates (1 mM) were added to the standard reaction mixture, and the rate of L-alanine racemization was determined using the UPLC method as described above.

Kinetic analysis
Steady state kinetic analyses of ALR and ALR A131K were performed using various concentrations of L-alanine in the presence of several concentrations of acetate. Initial velocity was measured using UPLC to determine the rate of conversion of L-alanine to d-alanine. The L-alanine concentrations used were 6.67, 10.0, 13.3, 20.0 and 40.0 mM, and the acetate concentrations were 1.00, 10.0, 20.0 and 40.0 mM.

Results
Primary sequence and amino acid composition of ALRs from L. salivarius and other sources
We found a gene homologous (Q1WV14 in Uniprot) with ALR in an L. salivarius DNA database, and compared amino acid compositions of L. salivarius with other sources of ALRs (Table 1). The amino acid composition of the L. salivarius ALR was different from those of other ALRs in contents of arginine and lysine. However, affections to characteristics of L. salivarius ALR by these differences are unclear. Thus we aligned and compared the deduced amino acid sequence with those of ALRs from six other sources (Fig. 1). Although L. salivarius ALR showed relatively low overall sequence homology with ALRs from B. anthracis Sterne 43F2 (35.0 %), B. pseudofirmus OF4 (26.1 %), B. subtilis 168 (34.6 %), G. stearothermophilus IFO 12550 (35.6 %) and P. putida YZ-26 (20.4 %), the two catalytic bases in the active site, K40 and Y267, were strictly conserved (Tanizawa et al. 1988; Shaw et al. 1997; Watanabe et al. 2002). In addition, among the four residues (K129, R138, M314 and D315) reportedly responsible for the binding of carboxylates such as acetate and propionate, which are specific inhibitors in the case of ALRs from Bacillus and Geobacillus species (Morollo et al. 1999; Kanodia et al. 2009), R138, M314 and D315 are conserved in the sequence of L. salivarius ALR. On the other hand, K129 is replaced by A131 in L. salivarius ALR, which suggests carboxylates may exert a different effect on L. salivarius ALR.

Purification of L. salivarius ALR and ALR A131K from recombinant E. coli
We cloned the L. salivarius ALR gene (Q1WV14 in Uniprot) and its substitution mutant, ALR A131K, to obtain recombinant E. coli BL21-CodonPlus(TM)(DE3)-RIPL cells containing a hybrid plasmid harboring Q1WV14 or its ALR A131K mutant gene. Both expression products were purified to homogeneity using one-step Ni–NTA agarose column chromatography with high yields of 60.7 and 51.6 %, respectively. Both his-tagged recombinant proteins were exhibited single band located with estimated molecular mass of 43 kDa (molecular mass of L. salivarius ALR from primary amino acid sequence is about 41 kDa). The activity of ALR A131K was 438 ± 6.25 μmol/min mg, much higher than that of the ALR (161 ± 5.83 μmol/min mg) under the standard assay conditions.
Table 1 Amino acid composition of *L. salivarius* ALR with other ALRs

| Amino acid | Number of residues per subunit |
|------------|-------------------------------|
|            | *L. sal* | *L. fer* | *P. put* | *B. ant* | *B. pse* | *B. sub* | *G. ste* |
| Alanine    | 33       | 41       | 51       | 32       | 36       | 37       | 39       |
| Arginine   | 14       | 23       | 27       | 20       | 17       | 21       | 30       |
| Asparagine | 15       | 8        | 19       | 15       | 11       | 14       | 9        |
| Aspartate  | 19       | 16       | 21       | 19       | 21       | 18       | 22       |
| Cysteine   | 6        | 4        | 2        | 1        | 5        | 5        | 4        |
| Glutamate  | 14       | 15       | 14       | 8        | 9        | 8        | 10       |
| Glutamine  | 27       | 27       | 22       | 30       | 27       | 28       | 24       |
| Glycine    | 31       | 32       | 34       | 28       | 25       | 30       | 26       |
| Histidine  | 10       | 7        | 11       | 8        | 13       | 11       | 16       |
| Isoleucine | 29       | 15       | 14       | 33       | 24       | 23       | 24       |
| Leucine    | 30       | 36       | 42       | 31       | 35       | 38       | 39       |
| Lysine     | 28       | 15       | 15       | 20       | 17       | 29       | 13       |
| Methionine | 11       | 12       | 11       | 6        | 11       | 14       | 10       |
| Phenylalanine | 12        | 13        | 13       | 21       | 13       | 14       | 16       |
| Proline    | 11       | 16       | 12       | 17       | 14       | 15       | 23       |
| Serine     | 20       | 15       | 25       | 16       | 29       | 19       | 17       |
| Threonine  | 18       | 23       | 25       | 25       | 22       | 20       | 19       |
| Tryptophan | 4        | 3        | 4        | 6        | 0        | 4        | 5        |
| Tyrosine   | 12       | 15       | 9        | 16       | 12       | 13       | 14       |
| Valine     | 27       | 37       | 38       | 37       | 28       | 28       | 28       |

L. sal *L. salivarius* UCC 118, L. fer *L. fermentum* ATCC14931, P. put *P. putida* YZ-26, B. ant *B. anthracis* Sterne 43F2, B. pse *B. pseudofirmus* OF4, B. sub *B. subtilis* PCI 219, G. ste *G. stearothermophilus* IF0 12550

Effect of temperature on activity
The effects of temperatures between 20 and 60 °C on the activities of purified ALR and ALR<sup>A131K</sup> were examined using 20 mM l-alanine as the substrate in the presence and absence of 1 mM acetate (Fig. 2a, b). The maximum activity of ALR without acetate was observed at 35 °C, but shifted to 50 °C in the presence of 1 mM acetate (Fig. 2a). Moreover, the activity of ALR in the presence of 1 mM acetate at 50 °C was much higher than in its absence at 35 °C (317 ± 5.72 μmol/min mg vs. 178 ± 2.04 μmol/min mg). In the absence of acetate, ALR activity was completely lost at 55 °C, but it exhibited about 71 % relative activity in the presence of 1 mM acetate. By contrast, 1 mM acetate had little or no effect on the temperature dependence of ALR<sup>A131K</sup> activity, and maximum ALR<sup>A131K</sup> activity was observed at 45 °C in presence or absence of acetate (Fig. 2b).

Effect of pH on activity
The effect of pH on ALR and ALR<sup>A131K</sup> activities was examined at pH 4.0–11.0 in the presence and absence of 1 mM acetate (Fig. 3a, b). ALR showed maximum activity at around pH 8.0 in presence and absence of 1 mM acetate. In addition, the relative activity of ALR was slightly increased by the addition of acetate. On the other hand, ALR<sup>A131K</sup> exhibited maximum activity at around pH 9.0, and ALR<sup>A131K</sup> activity was unaffected by 1 mM acetate at any pH.

Kinetic analyses of *L. salivarius* ALR
The initial velocity of d-alanine formation from l-alanine catalyzed by ALR was measured at several l-alanine concentrations in the presence of five concentrations of acetate. The resultant Lineweaver–Burk plots of the relation between the l-alanine concentration and the initial velocity showed five straight lines (Fig. 4a, b). Although ALR was strongly activated by the addition of 1 mM acetate, the activation level was reduced by the addition of acetate concentrations above 10, and 40 mM acetate greatly inhibited ALR activity at all l-alanine concentrations (Fig. 4a). By contrast, ALR<sup>A131K</sup> activity was inhibited or unaffected at all the acetate concentrations tested (1–40 mM). The *K<sub>m</sub>* values of ALR and ALR<sup>A131K</sup> for l-alanine in absence of acetate were 11.5 and 9.20 mM, respectively. The *V<sub>max</sub>* of ALR and ALR<sup>A131K</sup> in absence of acetate were 272 and 751 μmol/min mg, respectively.

Effect of different carboxylates on the activity
We also tested the effect of carboxylates other than acetate on the activities of ALR and ALR<sup>A131K</sup> (Fig. 5).
ALR$^{\text{A131K}}$ was inhibited to a greater degree than ALR by several of the carboxylates tested. Although propionate and butyrate are reportedly strong inhibitors of B. pseudofirmus OF4; B. subtilis Sterne 43F2; B. subtilis B. anthracis PCI 219; G. stearothermophilus, L. fermentum, L. salivarius, and butyrate are reportedly strong inhibitors of several short-chain carboxylates (acetate, propionate and butyrate) that inhibit other ALRs. In particular, this activation is in contrast

**Discussion**

To better understand the mechanism responsible for the high yield of d-alanine in *L. salivarius*, we characterized the ALR expressed by *L. salivarius*, which may be entirely responsible for its d-alanine production. We found that the fundamental properties of this enzyme, including its temperature and pH optima and kinetic parameters, are similar to those of ALRs from other sources such as *B. pseudofirmus* and *P. putida* (Ju et al. 2009; Liu et al. 2012). Nonetheless, the sequence homologies between *L. salivarius* ALR and the ALRs from other sources were not high, and the *L. salivarius* enzyme was activated by several short-chain carboxylates (acetate, propionate and butyrate) that inhibit other ALRs. In particular, this activation is in contrast *G. stearothermophilus*, which is clearly inhibited by both acetate and propionate (Morollo et al. 1999).

Six amino acid residues are responsible for the activity of *G. stearothermophilus* ALR (Morollo et al. 1999). Among the corresponding residues in *L. salivarius* ALR, five are conserved, but K129 of *G. stearothermophilus* is replaced by A131 in *L. salivarius*. Five are conserved, but K129 of *G. stearothermophilus* is replaced by A131 in *L. salivarius*. In addition, succinate, pyruvate, 2-oxoglutarate, oxaloacetate and aspartate also more strongly inhibited ALRA131K than ALR. In contrast, both propionate and butyrate strongly inhibited ALRA131K. In addition, succinate, pyruvate, 2-oxoglutarate, oxaloacetate and aspartate also more strongly inhibited ALRA131K than ALR.
therefore prepared and characterized an A131K variant of *L. salivarius* ALR. Interestingly the ALR$	ext{A}^{131K}$ variant enzyme exhibited much greater activity than the wild-type enzyme; moreover, the activation by acetate and propionate disappeared, replaced by a typical mix-type inhibition same as previous reports of *Bacillus* ALR (Kanodia et al. 2009). This suggests acetate and propionate may be involved in the control of ALR activity, and that A131 may be responsible for the regulation of d-alanine synthesis in *L. salivarius*.

In *G. stearothermophilus* ALR, K129 reportedly interacts with R136, which binds to carboxylate and PLP oxygen through formation of carbamate (Morollo et al. 1999). This interaction between the K129 and R136 residues through carbamylation has also been seen in *P. aeruginosa* ALR (LeMagueres et al. 2003), and the same interaction occurs in *Staphylococcus aureus* ALR, though a sulfate ion substitutes for the carbamylation (Scalleti et al. 2012). Similarly, an equivalent N129 residue of *B. anthracis* ALR interacts with R136 through a chloride ion (Couña-go et al.
From these reports, it appears that there is often an interaction between K129 or N129 and R136 mediated in various ways within ALR. However, the alanine side chain has no charge, so there is no interaction between A131 and R138 in *L. salivarius* ALR (which corresponds to R136 in *Bacillus* ALR). Consequently, R138 of *L. salivarius* ALR is probably poorly ordered and what would be the carboxyl group binding site is unstable. Acetate may stabilize *L. salivarius* ALR by binding to the carboxyl group binding site, and the activating effect of this structural stabilization may be more pronounced than acetate’s inhibitory effect so that the net effect on *L. salivarius* ALR activity is stimulation. Consistent with that idea is the dramatic increase in ALR activity and stability seen in the A131K variant (Fig. 2), as well as the activating and inhibitory effects of acetate on ALR activity (Fig. 4). These stabilizing effect of competitive inhibitors were found in other enzymes (Burton 1951; Alvaro et al. 1991). A more precise explanation of the structural basis of the activation and/or stabilization of *L. salivarius* ALR will require analysis of its crystal structure.

Zalan et al. reported the robust production of organic acids such as lactate and acetate by various *Lactobacillus* species (Zalán et al. 2010). In addition, propionate and butyrate are also produced by some *Lactobacillus* strains (Liong and Shah 2005; Pereira et al. 2003). Notably, when milk and Jerusalem artichoke juice are used for the growth medium, lactate production is depressed in many *Lactobacillus* strains, whereas acetate production is enhanced. As a result, the acetate concentration in cells from some *Lactobacillus* strains is higher than the lactate concentration, particularly under aerobic culture conditions (Bobillo and Marshall 1991; Cselovszky et al. 1992). This suggests that carboxylates such as acetate, propionate and butyrate might play regulatory roles affecting D-alanine production in *Lactobacillus* strains, through details of the control of ALR activity remain to be determined.

![Fig. 4](image_url) **Fig. 4** Kinetics of acetate inhibition of ALR and ALR<sup>A131K</sup>. a, b ALR and ALR<sup>A131K</sup> were assayed for 5 min at 30 °C and pH 7.0 (MES buffer) using various concentrations of L-alanine and acetate as the substrate and inhibitor, respectively. Shown are double reciprocal plots of the initial velocity of ALR (a) or ALR<sup>A131K</sup> (b) against L-alanine concentrations at several concentrations of acetate (n = 3 for each enzyme). Open circles, without acetate; filled circles, 1.0 mM acetate; open squares, 10.0 mM acetate; filled squares, 20.0 mM acetate; open triangles, 40.0 mM acetate.

![Fig. 5](image_url) **Fig. 5** Effect of various carboxylates on ALR and ALR<sup>A131K</sup> activities. ALR and ALR<sup>A131K</sup> were assayed for 5 min at 30 °C and pH 7.0 (MES buffer) using 20 mM L-alanine as the substrate in the presence of the indicated carboxylates (1 mM). The specific activities of ALR and ALR<sup>A131K</sup> at 100% were 171 ± 0.559 and 527 ± 5.17 μmol/min mg, respectively (n = 3). Open bars, ALR activities; filled bars, ALR<sup>A131K</sup> activities.
Abbreviations
ALR: alanine racemase; ALRB: alanine racemase A131K variant; PLP: pyridoxal 5'-phosphate; UPLC: ultra-performance liquid chromatography.

Authors’ contributions
HS, KJ and T Ohshima designed the study. JY performed a part of plasmid constructions and protein purifications. YS performed a part of UPLC analyses. JK performed main experiments and wrote the manuscript. T Ohmori performed UPLC analyses for additional data. All authors read and approved the final manuscript.

Author details
1 Microbial Genetics Division, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan. 2 Applied Molecular Microbiology and Biomass Chemistry Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan. 3 Department of Biomedical Engineering, Faculty of Engineering, Osaka Institute of Technology, Osaka 567-8585, Japan. 4 Functional Genomics of Extremophiles, Faculty of Agriculture, Graduate School, Kyushu University, Fukuoka 812-8581, Japan. 5 Present Address: Department of Biomedical Engineering, Faculty of Engineering, Osaka Institute of Technology, 5-16-1 Ohmiya, Osaka 535-8585, Japan.

Acknowledgements
This work was supported by a grant for Promotion of Basic Research Activities for Innovative Bioscience from the Bio-oriented Technology Research Advancement Institution (BRAIN, Tokyo) and in part Kobayashi International Scholarship Foundation (Tokyo).

Competing interests
The authors declare that they have no competing interests.

Received: 18 December 2014 Accepted: 24 December 2014
Published online: 24 October 2015

References
Alvaro G, Fernandez-Lafuente R, Blanco RM, Guisán JM (1991) Stabilizing effect of penicillin G sulfoxide, a competitive inhibitor of penicillin G acylase: its practical applications. Enzyme Microb Technol 13:210–214. doi:10.1016/0141-0229(91)90130-3
Bobbilo M, Marshall VM (1991) Effect of salt and culture aeration on lactate and acetate production by Lactobacillus plantarum. Food Microbiol 8:153–160. doi:10.1016/0740-0020(91)90008-P
Burton K (1951) The stabilization of o-amino-acid oxidase by flavin-adenine dinucleotide, substrates and competitive inhibitors. Biochem J 48:458–467
Cougha R, Davileva M, Strych U, Hill R, Krause KL (2009) Biochemical and structural characterization of alanine racemase from Bacillus anthracis (Ams). BMC Struct Biol 9:5. doi:10.1186/1472-6807-9-5
Cselovszky J, Wolf G, Hammes WP (1992) Production of formate, acetate, and succinate by anaerobic fermentation of Lactobacillus pentosus in the presence of citrate. Appl Microbiol Biotechnol 37:94–97. doi:10.1007/BF00174210
Erbe T, Brückner H (2000) Chromatographic determination of amino acid enantiomers in beers and raw materials used for their manufacture. J Chromatogr A 881:81–91. doi:10.1016/S0021-9673(00)00525-7
Hejncjort JV (2001) Recent advances in the formation of the bacterial peptidoglycan monomer unit. Nat Prod Rep 18:503–519. doi:10.1039/A004532A
Hochbaum A, Kolodkin-Gal I, Foulston L, Kolter R, Rizenberg J, Lesicki R (2011) Inhibitory effects of o-amino acids on Staphylococcus aureus biofilm development. J Bacteriol 193:5616–5622. doi:10.1128/JB.05534-11
Inagaki K, Tanizawa K, Bader B, Walsh CT, Tanaka H, Soda K (1986) Thermostable alanine racemase from Bacillus steatorrhophilus: molecular cloning of the gene, enzyme purification, and characterization. Biochemistry 25:3268–3274. doi:10.1021/bi00359a028
Johnston MM, Diven WF (1969) Studies on amino acid racemases. I. Partial purification and properties of the alanine racemase from Lactobacillus fermentum. J Biol Chem 244:5414–5420
Ju J, Xu S, Wen J, Li G, Ohnishi K, Xue Y, Ma Y (2009) Characterization of endogenous pyridoxal 5'-phosphate-dependent alanine racemase from Bacillus pseudofirmus OF4. J Bacterio Bioeng 107:225–229. doi:10.1016/j.jbibe.2008.11.005
Kanda-Nambu K, Yasuda Y, Tochikubo K (2000) Isozymic nature of spore coat-associated alanine racemase of Bacillus subtilis. Amino Acids 18:375–387. doi:10.1007/s0072600170076
Kanodia S, Agarwal S, Singh P, Agarwal S, Singh P, Bhatnagar R (2009) Biochemical characterization of alanine racemase: a spore protein produced by Bacillus anthracis. BMC Rep 42:47–52
Kobayashi J, Shimmizu Y, Mutaguchi Y, Doi K, Ohshima T (2013) Characterization of o-amino-acid aminotransferase from Lactobacillus salivarius. J Mol Catal B Enzym 94:15–22. doi:10.1016/j.molcatb.2013.04.013
Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kötler R, Losick R (2010) o-Amino acids trigger biofilm disassembly. Science 328:627–630. doi:10.1126/science.1188626
Kullman JP, Chen X, Armstrong DW (1999) Evaluation of the enantio-meric composition of amino acids in tobacco. Chirality 11:669–673. doi:10.1002/(SICI)1525-636X(199911)11:9<669::AID-CHIR10>3.0.CO;2-8
LeMagueres P, Im H, Dvorak A, Strych U, Benedikt M, Krause KL (2003) Crystal structure at 1.45 Å resolution of alanine racemase from a pathogenic bacterium, Pseudomonas aerugi-nosa, contains both internal and external aldime forms. Biochemistry 42:14752–14761. doi:10.1021/bi030166v
Liong MT, Shah NP (2005) Production of organic acids from fermentation of mannotriol, fructooligosaccharide and inulin by a cholesterol removing Lactobacillus acidophilus strain. J Appl Microbiol 99:783–793. doi:10.1111/j.1365-2672.2005.02677.x
Liu JL, Liu QJ, Shy WW (2012) Expression, purification, and characterization of alanine racemase from Pseudomonas putida YZ-26. World J Microbiol Biotechnol 28:267–274. doi:10.1007/s11274-011-0816-1
Morollo AA, Petsko GA, Ringe D (1999) Structure of a micaclis complex analogue: propionate binds in the substrate carbamate site of alanine racemase. Biochem 38:3293–3301. doi:10.1016/S0006-291X(98)022792
Mutaguchi Y, Dhomtory A, Akane H, Doi K, Ohshima T (2013) Distribution of o-amino acids in vinegars and involvement of lactic acid bacteria in the production of o-amino acids. Springerplus 2:691. doi:10.1186/2193-1801-2:691
Pätzold R, Scheiber A, Brückner H (2005) Gas chromatographic quantification of free o-amino acids in higher vertebrates. Biomed Chromatogr 19:466–473. doi:10.1002/bmc.515
Pereira DI, McCartney AL, Gibson GR (2003) An in vitro study of the probiotic potential of a bile-salt-hydrolyzing bacteria in the substrate carboxylate site of alanine racemase. Biochem 38:3293–3301. doi:10.1016/S0006-291X(98)022792
Rosti G, Takashima K, Adams E (1969) Coenzyme content of purified alanine racemase from Pseudomonas. Biochim Biophys Acta 19:466–473. doi:10.1002/bmc.515
Scaletti ER, Luckner SR, Krause KL (2012) Structural features and kinetic characterization of alanine racemase from Pseudomonas aerugi-nosa (Mu50). Acta Crystallogr D Biol Crystallogr 68:822–92. doi:10.1107/S090944491050682
Shaw JP, Petsko GA, Ringe D (1997) Determination of the structure of alanine racemase from Bacillus steatorrhophilus at 1.9-A resolution. Biochem 36:1329–1342. doi:10.1021/bi016856c
Tanizawa K, Ohshima A, Scheidegger A, Inagaki K, Tanaka H, Soda K (1988) Thermolabile alanine racemase from Bacillus steatorrhophilus: DNA and protein sequence determination and secondary structure prediction. Biochemistry 27:1311–1316. doi:10.1021/bi00400a033
Watanabe A, Yoshimura T, Mikami B, Hayashi H, Kagamiyama H, Esaki N (2002) Reaction mechanism of alanine racemase from Bacillus steatorrhophilus: X-ray crystallographic studies of the enzyme bound with N-(4'-phosphopyrididyloxy) alanine. J Biol Chem 277:19166–19172. doi:10.1074/jbc.M201615200
Zalán Z, Husdáček J, Stětina J, Chumchalová J, Halász A (2010) Production of organic acids by Lactobacillus strains in three different media. Eur Food Res Technol 230:395–404. doi:10.1007/s00217-009-1179-9