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Characterization of Psychrotrophic Alanine Racemase
and Evaluation of the Gene as an Indicator for
Detection of Bacteria

Division of Human Life and
Environmental Sciences
Graduate School of Human Culture
Nara Women's University

Yoko Okubo

1998
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GENERAL INTRODUCTION

Alanine racemase (EC 5.1.1.1) catalyzes racemization of L- and D-alanine, and provides the latter enantiomer for the construction of the peptidoglycan layer of bacterial cell walls. Since the enzyme is essential for the growth of bacteria, the enzyme has been studied as a target for antibacterial agents (Strominger et al., 1960; Manning et al., 1974; Wang et al., 1978; Atherton et al., 1986; Badet and Walsh, 1985). The enzymes from mesophiles, Bacillus subtilis var. aterrimus (Yonaha et al., 1975), Pseudomonas putida, (Adams et al., 1974), Streptococcus faecalis (Badet and Walsh, 1985), Staphylococcus aureus (Roze et al., 1966), Escherichia coli (Lambert et al., 1972), and Salmonella typhimurium (Esaki and Walsh, 1986; Wasserman et al., 1984), from a thermophile, Bacillus stearothermophilus (Inagaki et al., 1986), and a psychrotroph, Pseudomonas fluorescens (Yokoigawa et al., 1993) have been purified and characterized. Since the enzyme is believed to occur widely in bacteria, the enzyme is interesting from a standpoint of comparative biochemistry. However, psychrotrophic properties and thermolability of the enzymes from psychrotrophs are not examined in detail.

Psychrotrophic bacteria, which grow between 0°C and over 25°C (Herbert, 1986), often cause deterioration in foods stored at a low temperature (Raw et al., 1977), and accordingly have been studied to better understand food preservation (Faurbairn et al., 1986; Stepaniak et al., 1982). However, there is no effective method to suppress the growth of psychrotrophs in foods, except by thermal sterilization which changes the original taste of a food. In addition, little enzymological information on the growth of psychrotrophs has been reported, probably because of the
lability of enzymes from psychrotrophs. Characterization of psychrophilic enzymes essential for bacterial growth would provide useful information for growth-suppression of psychrotrophs. In chapter 1, the author describes the lability of alanine racemase from a psychrotroph.

The several kinds of alanine racemase genes from mesophiles and thermophiles including two isozyme genes, $dadB$ and $alr$ from *S. typhimurium*, were cloned, and their DNA and protein sequences were analyzed (Lobocka *et al.*, 1994; Tanizawa *et al.*, 1988; Ferrari *et al.*, 1985; Hols *et al.*, 1997; Galakatos *et al.*, 1986; Wasserman *et al.*, 1984). Some alanine racemase genes from *Haemophilus influenzae* (Fleischmann *et al.*, 1995), *Helicobacter pylori* (Tomb *et al.*, 1997), and *E. coli alr* (Blattner *et al.*, 1993) are also clarified by whole-genome sequencing. However, the enzyme genes from psychrotrophs have not been characterized. The amino acid sequences of alanine racemases show 9-10 conserved regions, including a highly conserved region around the active-site lysyl residue, distributed evenly in the genes (Tanizawa *et al.*, 1988). In addition, the molecular structure of the *B. stearothermophilus* enzyme is determined by X-ray crystallography (Shaw *et al.*, 1997), and theoretical methods to predict the tertiary structure of homologous alanine racemases are also developed (Wako and Kubota, 1991). Therefore, alanine racemase is suitable for study on comparative biochemistry of the enzyme from various bacteria to examine psychrotrophic properties and thermolability. In chapter 2, the author describes the cloning and sequencing of psychrotrophic alanine racemase from *Bacillus psychrosaccharolyticus*, the enzyme purification, and characterization.

Quality of foods is often reduced by contamination with bacteria.
The standard plate culture method is useful for detecting and quantifying bacteria. However, some bacteria such as thermophiles and psychrophiles are overlooked by the method. *B. stearothermophilus* and *B. psychrosaccharolyticus* are endospore-forming Gram-positive bacteria. These bacteria are non-pathogenic to human, and can't grow under normal storage conditions of foods. However, when foods are subjected to high or low temperature, these bacteria grow and cause deterioration in the foods as described in FDA Bacteriological Analytical Manual (1995b). Generally, the detection of psychrotrophs is time-consuming; the plate culture method at low temperature requires more than 7 days. However, few reports have been published on a specific and rapid method for detecting each of *B. stearothermophilus* and *B. psychrosaccharolyticus* in foods. Specific DNA probes for rapid detection of each bacterium are probably useful for quality control of foods. Alanine racemase is a typical prokaryotic enzyme, and is absent in food materials. Therefore, alanine racemase gene fragments containing the non-conserved regions of the genes from *B. stearothermophilus* and *B. psychrosaccharolyticus* may be useful probes for detecting the respective bacterium in foods. In chapter 3, the author describes the amplification of gene fragments of the *B. stearothermophilus* and *B. psychrosaccharolyticus* alanine racemases, and the specificity of each fragment as a probe for detecting the respective bacterium.

*E. coli* is an important indicator of fecal contamination in foods, the most common method for detecting and quantifying *E. coli* currently being the most probable number (MPN) method (Food and Drug administration, 1995a). However, this method is time-consuming and often inaccurate (Hsu et al., 1991). Considerable effort has been invested in the
development of accurate and rapid methods for detecting and identifying *E. coli*. Enzymatic methods based upon the activity of β-D-glucuronidase (Ratnam *et al.*, 1988; Frampton and Restaino, 1993) have been well studied. However, enterohaemorrhagic pathogens belonging to the *E. coli* O157:H7 strain do not possess this enzyme (Ratnam *et al.*, 1988). Although a hybridization method for detecting various *E. coli* strains with oligonucleotide probes specific for their 16S ribosomal RNA has been reported (Hsu *et al.*, 1991), the sensitivity of the method was insufficient with a the detection limit of $5 \times 10^5$ cells/ml. Therefore, selective enrichment of the *E. coli* culture was necessary. All *E. coli* strains are considered to have the alanine racemase gene, while the gene is probably absent in food materials. The gene fragment containing the non-conserved regions of the *E. coli* alanine racemase gene is considered to be a powerful probe for detecting *E. coli*. In chapter 4, the author describes the amplification of a fragment of the *E. coli* alanine racemase gene, and application of the fragment labeled with digoxigenin (DIG) to detect *E. coli* in foods. The gene fragment of alanine racemase could prove useful for detecting various *E. coli*.
CHAPTER 1

Lability of Alanine Racemase from a Psychrotroph

INTRODUCTION

Psychrotrophic bacteria, which grow between 0°C and over 25°C (Herbert et al., 1986) often cause deterioration in foods stored at a low temperature (Raw et al., 1977), and accordingly have been studied to better understand food preservation (Fairbairn et al., 1986; Stepaniak et al., 1982). However, there is no effective method to suppress the growth of psychrotrophs in foods, except by thermal sterilization which changes the original taste of a food. In addition, little enzymological information on the growth of psychrotrophs has been reported, probably because of the lability of enzymes from psychrotrophs.

Alanine racemase (EC 5.1.1.1) catalyzes the racemization of L- and D-alanine, and occurs widely in prokaryotes. This enzyme is essential to the metabolism of D-amino acids and for synthesizing the peptidoglycan of the bacterial cell wall by providing D-alanine. Accordingly, the enzyme has been studied as a target for antibacterial agents. (Strominger et al., 1960; Manning et al., 1974; Wang et al., 1978; Atherton et al., 1986; Badet et al., 1985). The enzymes from mesophilic bacteria {\( B. \) subtilis var. aterrimus (Yonaha et al., 1975), \( P. \) putida (Adams et al., 1974), \( S. \) faecalis (Badet et al., 1985), \( S. \) aureus (Roze et al., 1966), \( E. \) coli (Lambert et al., 1972), and \( S. \) typhimurium (Wasserman et al., 1983)} and a thermophilic bacterium {\( B. \) stearothermophilus (Inagaki et al., 1986)} have been purified and characterized. Yokoigawa et al. (1993) recently
purified and characterized alanine racemase (EC 5.1.1.1) from a typical psychrotroph, *P. fluorescens* TM5-2. This enzyme was similar to other alanine racemases in its basic enzymological properties, including the amino acid composition and N-terminal amino acid sequence. However, the enzyme showed high catalytic activity, even at 0°C.

The author have begun to study effective methods for inhibiting the growth of psychrotrophs by inactivating the thermolabile enzyme that is essential for their growth. The author reports here the lability of alanine racemase from *P. fluorescens* in comparison with the stability of the enzyme from a thermophile. The thermolabile enzyme was easily inactivated with organic solvents and denaturants.

**MATERIALS AND METHODS**

**Materials.**

Alanine racemase from *P. fluorescens* TM5-2 was purified to homogeneity as described by Yokoigawa et al. (1993) (the specific activity for L-alanine at 30°C was 1,440 units per mg of protein). Homogeneous thermostable alanine racemase (the specific activity for L-alanine at 37°C was 2,550 units per mg of protein) from a thermophile, *B. stearothermophilus* was kindly presented by Prof. Soda of Institute for Chemical Research at Kyoto University. D-Amino acid oxidase (EC 1.4.3.3) from pig kidney (0.09 unit per mg) was obtained from Sigma.
Enzyme and protein assays.

Assays were routinely done at 0 °C and 50 °C on enzymes from the psychrotroph and the thermophile, respectively. The standard reaction mixture contained 0.5 μM PLP, 40 mM sodium pyrophosphate buffer (pH 8.3), 250 mM L-alanine, and the enzyme in a final volume of 1 ml. The reaction was started by adding the enzyme, and after incubating for 10 min, the reaction was stopped by adding 0.5 ml of 2 N HCl. The D-alanine formed was measured with D-amino acid oxidase (Soda et al., 1968). One unit of the enzyme is defined as the amount of enzyme that catalyzed the formation of 1 μmol of D-alanine per min. The specific activity is expressed as units per mg of protein. Protein was measured by the method of Lowry et al. with bovine serum albumin as a standard.

Circular dichroic analysis.

Circular dichroic (CD) measurements were carried out with a Jasco J-600 recording spectropolarimeter at 25 °C with a 1-mm light path-length cell. The instrument was calibrated with (+)-10-camphorsulfonic acid, \( \Delta \varepsilon = +2.37 \text{ M}^{-1} \cdot \text{cm}^{-1} \) at 290.5 nm. To calculate the mean residue ellipticity \( [\theta] \), the mean residue weight was taken as 112.0 for the enzyme protein. The CD spectra were obtained at a protein concentration of 0.1 mg/ml under a nitrogen atmosphere. The \( \alpha \)-helix and \( \beta \)-structure contents of the native enzyme were calculated from the CD data by the least-squares method, using ellipticity values of 10 points in the region between 210 and 243 nm (Chen et al., 1974).
RESULTS AND DISCUSSION

Thermostability of alanine racemase.

The author compared the thermostability of alanine racemase from *P. fluorescens* TM5-2 with that of the enzyme from *B. stearothermophilus*. As shown in Fig. 1, the enzymes from the psychrotroph and thermophile were inactivated by incubating for 1 h at over 30 and 75 °C, respectively, the thermoinactivation process following first-order kinetics. The author can use, therefore, the first-order rate constant of thermoinactivation as a measure of the enzyme stability. The temperature dependence of the rate constant of thermoinactivation was then examined by Arrhenius plots (Fig. 2). The difference in thermostability was so great that it is practically impossible to compare the enzymes under the same experimental conditions. Therefore, the author compared the extrapolated data (Fig. 2, dashed line) corresponding to an intermediate temperature, e.g. 60 °C, as described by Mozhaev *et al.* (1988). The difference in thermostability (equal to the ratio of the rate constants for thermoinactivation of the thermolabile and thermostable enzymes) was about 1,000. Therefore, the author used the thermostable enzyme throughout this study to provide striking contrast to the thermolabile one in stability.

Effects of pH and denaturants on alanine racemase.

The enzyme from *P. fluorescens* was similar in pH stability to the enzyme from *B. stearothermophilus* (Fig. 3). Alanine racemase from mesophile (*B. subtilis* var. *a terrimus*) has been reported to be stable in a pH range from 8.3 to 10.5 (Yonaha *et al.*, 1975). These alanine racemases were similar to each other in pH stability irrespective of their
Fig. 1. Thermostability of alanine racemases from *P. fluorescens* (A) and *B. stearothermophilus* (B). Each enzyme (final concentration of 0.1 mg/ml) was incubated at various temperatures in a 10 mM potassium phosphate buffer (pH 7.5) containing 10 μM PLP and 0.01% 2-mercaptoethanol, and then the residual activities were immediately measured.
Fig. 2. Arrhenius plots of the first-order rate constants of thermoinactivation for the thermolabile and thermostable enzymes. A, thermostable enzyme.; B, thermolabile enzyme.

Fig. 3. Effect of pH on the thermolabile and thermostable enzymes. Each enzyme (final concentration of 0.1 mg/ml) was incubated at 0°C for 1 h at various pH values in a 200 mM citrate-phosphate buffer containing 10 μM PLP and 0.01% 2-mercaptoethanol, and then the residual activities were immediately measured.

○, thermolabile enzyme; ●, thermostable enzyme.
differing thermostability.

Although thermostable enzymes are well characterized and known to be generally resistant to denaturation by organic solvents and denaturants (Cho et al., 1988; Oshima et al., 1985), no thermolabile enzyme has been characterized in the resistance to these reagents. Therefore, the author examined the effects of denaturants and organic solvents on the enzyme (Fig. 4). The thermolabile enzyme lost about 50% of its initial activity after being incubated at 30°C for 5 min with sodium lauryl sulfate (0.08%), guanidine hydrochloride (1 M), urea (4 M), ethanol (45%), and dimethyl sulfoxide (50%), whereas the thermostable enzyme was resistant to those denaturants at the same concentrations (Fig. 4). This resistance of the enzymes from mesophiles has not previously been reported. The author observed that the enzymes from *B. subtilis* and *E. coli* in their cell-free extracts each lost about 5-25% of their initial activity under the same conditions with the five reagents at the respective concentration described here (unpublished data). Thus, the thermolabile enzyme was less resistant to denaturation by denaturants and organic solvents than the thermostable and mesophilic types.

**Analysis of the denaturation process.**

To clarify the reason for the thermolabile enzyme being susceptible to denaturants and organic solvents, the denaturation process of the thermolabile enzyme was examined in respect of the dissociation of PLP and unfolding of the enzyme protein. The author used urea as a denaturant for this study, because the enzymes needed to be inactivated to various extents for a detailed analysis of the denaturation process, and only urea inactivated the thermolabile enzyme by pseudo-first-order
Fig. 4. Effects of denaturants and organic solvents on the thermolabile and thermostable enzymes.
Each enzyme (final concentration of 0.1 mg/ml) was incubated at 30°C for 5 min in a 10 mM potassium phosphate buffer (pH 7.5) containing 10 μM PLP, 0.01% 2-mercaptoethanol, and various concentrations of the denaturants and organic solvents, 10 μl of the solution then being immediately used for measurement of the residual activity in 1 ml of the standard reaction mixture. ○, thermolabile enzyme; ●, thermostable enzyme.
kinetics (Fig. 4). To avoid renaturation of the denatured enzymes, the enzyme solutions containing urea were directly used for the spectrophotometric measurements. When $K_m$ values of the denatured enzymes for L-alanine were measured, the standard reaction mixtures containing the respective concentration of urea were used.

The absorption spectra of the thermostable and thermolabile enzymes show maxima at 420 nm (Inagaki et al., 1986; Yokoigawa et al., 1993) in the visible region due to the aldimine linkage formed between the formyl group of PLP and the e-amino group of the active site. Therefore, the author examined spectrophotometrically the dissociation of PLP from the enzyme proteins during their denaturation with urea. As shown in Fig. 5, the absorbance at 420 nm decreased with increasing urea concentration. This indicates that the aldimine linkage was hydrolyzed to produce the aldehyde form of PLP by the addition of urea. No further appreciable change in the absorbance of the thermolabile and thermostable enzymes was observed above 3.5 M and 4.0 M urea, respectively. When $K_m$ values of the two enzymes for L-alanine were measured in the presence of urea at various concentrations, the $K_m$ values of the thermolabile and thermostable enzymes increased markedly at 3.5 M and 4 M urea, respectively (Fig. 6). Therefore, the increase in $K_m$ values and hydrolysis of the aldimine linkage occurred at the same urea concentration. Although the two enzymes were active after being treated with 4 M urea as already described, the PLP-dissociated enzymes (apoenzymes) may have been partially reconstituted with PLP by 100-fold dilution of the enzyme solutions containing urea with the standard reaction mixture (Fig. 4), because PLP is essential for the activities of the enzymes (Inagaki et al., 1986; Yokoigawa et al., 1993).
Fig. 5. Effect of urea on the absorbance of the enzymes at 420 nm. Each enzyme (final concentration of 0.1 mg/ml) was incubated with various concentrations of urea in a 10 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol at 30°C for 5 min, the absorbance at 420 nm of the enzyme solutions then being immediately measured. ○, thermolabile enzyme; ●, thermostable enzyme.

Fig. 6. Effect of urea on the $K_m$ values of the thermolabile and thermostable enzymes. Each enzyme (final concentration of 0.1 mg/ml) was incubated with various concentrations of urea in a 10 mM potassium phosphate buffer (pH 7.5) containing 10 μM PLP and 0.01% 2-mercaptoethanol at 30°C for 5 min, before being immediately used for measurement of the $K_m$ value. The $K_m$ values were measured with the standard reaction mixture containing urea at the respective concentration to avoid refolding of the enzyme protein during the assay. ○, thermolabile enzyme; ●, thermostable enzyme.
Figure 7 shows the CD spectrum of the thermolabile enzyme. The CD spectrum of the native enzyme was characterized by two negative minima around 210 and 222 nm, like the thermostable enzyme (Inagaki et al., 1986). The α-helix and β-structure contents of the native thermolabile enzyme were calculated to be about 25% and 37%, respectively. The unfolding of the thermolabile enzyme with urea was followed by a CD analysis, the mean residual ellipticity at 222 nm being measured at 20°C and plotted against the concentration of urea (Fig. 8). The ellipticity decreased with increasing concentration of urea. The transition midpoints of the thermolabile and thermostable enzymes, defined by the horizontal base lines and plateau, were at around 3.5 M and 5.5 M, respectively. No further appreciable change in the ellipticity of the thermolabile and thermostable enzymes was observed above 3.5 M and 5.5 M urea, respectively. These results suggest that the thermolabile and thermostable enzymes would be completely denatured by incubating with over 3.5 M and 5.5 M urea, respectively. Although Fig. 4 shows that both enzymes were active even after being treated with 6 M urea, the denatured enzymes may have been partially refolded and reconstituted with PLP during the measurement of residual activity.

Thus, the unfolding and hydrolysis of the aldimine linkage of the thermolabile enzyme occurred at the same urea concentration (3-3.5 M), whereas each of those changes to the thermostable enzyme occurred at different urea concentrations. Although this denaturation process is not parallel to the result with urea in Fig. 4, partial renaturation may have occurred under the assay conditions described in Fig. 4. This renaturation was unexpected because the thermostable enzyme denatured with guanidine hydrochloride has been reported not to be renatured below
Fig. 7. Circular dichroic spectrum of the thermolabile alanine racemase. The CD spectrum was taken in a 10 mM potassium phosphate buffer (pH 7.5) containing 10 μM PLP and 0.01% 2-mercaptoethanol at an enzyme concentration of 0.1 mg/ml.

Fig. 8. Effect of urea concentration on the mean residual ellipticity at 222 nm of the thermolabile and thermostable enzymes. Each enzyme (final concentration of 0.1 mg/ml) was incubated with various concentrations of urea in a 10 mM potassium phosphate buffer (pH 7.5) containing 10 μM PLP and 0.01% 2-mercaptoethanol at 30°C for 5 min, CD spectrum of the enzyme solution then being measured. ○, thermolabile enzyme; ●, thermostable enzyme.
pH 8.5 (Toyama et al., 1991). Both reversible and irreversible denaturation states may be present in alanine racemases.

These results suggest that the thermolabile enzyme is denatured by the treatment with urea through one detectable phase, and that the thermostable enzyme is denatured through two phases. Toyama et al. have reported that the thermostable enzyme was unfolded by treating with guanidine hydrochloride through two phases: phase 1 involved dissociation of the dimer into monomers and hydrolysis of the aldimine linkage; phase 2 involved unfolding of the monomer (Toyama et al., 1991). Subunit dissociation of the thermolabile enzyme may occur simultaneously with destruction of the secondary structure. This denaturation process of the thermolabile enzyme may be related to its susceptibility to denaturants; the dissociation of PLP from the enzyme protein may trigger the unfolding of the secondary structure.

The author have described here the lability of the thermolabile alanine racemase from a typical psychrotroph, *P. fluorescens*. The thermolabile enzyme was easily inactivated by heat treating at over 30°C and by incubating with organic solvents and denaturants. These treatments may effectively suppress the growth of psychrotrophs. This proposition is supported by her preliminary observations that the growth of the psychrotroph was markedly suppressed at over 30°C, and that about 80% and 5% of the psychrotroph and *E. coli* cells, respectively, died after incubating the cells at 25°C for 1 h in 0.85% NaCl containing 10% ethanol. These results obtained by analyzing the denaturation process also suggest that PLP plays an important role in maintaining the secondary structure of the thermolabile enzyme. Reagents such as hydroxyl amine, 3-methyl-2-benzothiazolinone hydrazone (MBTH), and aminoxyacetate that
dissociate PLP from the enzyme protein (Tanizawa et al., 1983) may also be effective for inhibiting growth of the psychrotroph.

**SUMMARY**

Suppression of the growth of psychrotrophs by inactivating an enzyme essential for their growth was studied by analyzing the lability of thermolabile alanine racemase from a typical psychrotroph, *P. fluorescens*, in comparison with the thermostable enzyme from *B. stearothermophilus*. The thermolabile enzyme lost about 50% of its initial activity after being incubated at 30°C for 5 min with sodium lauryl sulfate (0.08%), guanidine hydrochloride (1 M), urea (4 M), ethanol (45%), and dimethyl sulfoxide (50%), respectively, whereas the thermostable enzyme was resistant to these reagents at the same concentrations. Thus, the thermolabile enzyme was markedly less resistant to denaturation by denaturants and organic solvents than the thermostable type. The denaturation process for the thermolabile enzyme with urea was analyzed by circular dichroism, $K_m$ value for L-alanine, and the content of pyridoxal 5'-phosphate (PLP). The thermolabile enzyme was denatured in one transition phase occurring at a concentration of 3.5 M urea, whereas the thermostable type was denatured in two transition phases (dissociation of PLP with 4.0 M urea; unfolding with 5.5 M urea).
CHAPTER 2

Cloning and Sequencing of a Psychrotrophic Alanine Racemase Gene from *Bacillus psychrosaccharolyticus*, the Enzyme Purification, and Characterization

INTRODUCTION

Alanine racemase (EC 5.1.1.1) catalyzes racemization of L- and D-alanine, and provides the latter enantiomer for the construction of the peptidoglycan layer of bacterial cell walls. Accordingly, the enzyme is believed to occur widely in bacteria, and is interesting from a standpoint of comparative biochemistry. The enzymes from mesophiles, *B. subtilis* var. *aerrimus* (Yonaha *et al.*, 1975), *P. putida*, (Adams *et al.*, 1974), *Sr. faecalis* (Badet and Walsh, 1985), *S. aureus* (Roze *et al.*, 1966), *E. coli* (Lambert *et al.*, 1972), and *S. typhimurium* (Esaki and Walsh, 1986; Wasserman *et al.*, 1984), from a thermophile, *B. stearothermophilus* (Inagaki *et al.*, 1986), and from a psychrotroph, *P. fluorescens* (Yokoigawa *et al.*, 1993), have been purified and characterized. Generally, the intracellular content of the enzyme is markedly low, and many of these enzymes had to be purified more than 10,000-fold to homogeneity from wild bacterial strains. To obtain alanine racemases in large quantities, cloning and expression of the enzyme genes are essential for analysis of their function and structure.

The several kinds of alanine racemase genes from mesophiles and thermophiles including two isozyme genes, *dadB* and *alr* from *S. typhimurium*, were cloned, and their DNA and protein sequences were
analyzed (Lobocka et al., 1994; Tanizawa et al., 1988; Ferrari et al., 1985; Hols et al., 1997; Galakatos et al., 1986; Wasserman 1984). Some alanine racemase genes from *H. influenzae* (Fleischmann et al., 1995), *H. pylori* (Tomb et al., 1997), and *E. coli* air (Blattner et al., 1993) are also clarified by whole-genome sequencing. However, the enzyme genes from psychrophiles and psychrotrophs have not been characterized. The amino acid sequences predicted from the nucleotide sequences show 9-10 conserved regions, including a highly conserved region around the active-site lysyl residue, distributed evenly in the sequences (Tanizawa et al., 1988). Therefore, alanine racemase is suitable for study on comparative biochemistry of thermostability of the enzymes from various bacteria.

Alanine racemase also has been studied as a target for antibacterial drugs. In fact, some halogenated derivatives of D-alanine and phosphoalanine-containing dipeptides were found to act as anti-bacterials by blocking the racemization of L- to D-alanine (Strominger et al., 1960; Manning et al., 1974; Wang et al., 1978; Atherton et al., 1986; Badet and Walsh., 1985). However, the inhibition mechanism examined with an alanine analogue, (1-aminoethyl)-phosphonate (Ala-P), is different between the enzymes from Gram-positive and Gram-negative bacteria; the enzymes from Gram-positive bacteria are irreversibly inactivated by Ala-P, whereas the enzymes from Gram-negative bacteria were reversibly inhibited (Atherton et al., 1979; Badet et al., 1986). Development of alanine racemase-directed antibacterials effective to wide varieties of bacteria requires detailed studies on the chemistry and geometry of active sites of alanine racemases from various bacterial species.

Characterization of alanine racemases from bacteria of widely
diverged groups would provide useful information for comparative biochemistry, development of racemase-directed anti-bacterials, and the molecular evolution of the enzymes. The author here describes cloning and sequencing of a psychrotrophic alanine racemase gene from *B. psychrosaccharolyticus*, the enzyme purification, and characterization. The enzyme is extremely thermolabile and psychrotrophic.

**Materials and Methods**

*Microbe and culture conditions.*

*B. psychrosaccharolyticus* ATCC 23296 and *E. coli* XL1-Blue MRF' \[
\Delta(mcrA)183, \Delta(mcrCB-hsdSMR-mrr)173, \text{end}A1, \text{sup}E44, \text{thi}-1, \text{rec}A1, \text{gyrA96, rel}A1, \text{lac}, [\text{F'} \text{proAB, lac}^\text{I} \text{ZAM15}], \text{Tn}10(\text{tet'})\] were used as a donor strain of alanine racemase gene and the host strain for the gene cloned in λZapII (Stratagene, La Jolla, CA), respectively. *B. psychrosaccharolyticus* was aerobically cultivated at 23°C for 24 h in a medium containing 0.5% meat extract, 1% polypeptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.0). *E. coli* XL1-Blue MRF' was aerobically cultivated at 28°C for 18 h in LB medium (1% Tryptone, 0.5% yeast extract, 0.5% NaCl, 1 mM NaOH) containing 50μg/ml ampicillin.

*Preparation of a probe for gene cloning.*

A fragment of alanine racemase gene from *B. psychrosaccharolyticus* was prepared by the polymerase chain reaction (PCR) with genomic DNA of the bacterium as the template and degenerate oligonucleotide primers. The primers were designed from the consensus sequences of known alanine racemases around the active-site Lys residue.
and the Gly residue located about 250 amino acids downstream from the active site, between which the non-conserved regions are present. The sequences of the primers were 5’-AAAGCVRACGCTAYGGNCAYG as the sense primer and 5’-HRCCRTCSSCRTANCC as the antisense primer, in which the single-letter codes recommended by International Union of Biochemistry are used. Polymerase chain reaction (PCR) was performed with a PC-700 programmed temperature control system (Astec Co., Fukuoka, Japan). The cycle involved denaturation at 94°C for 2 min in the first run and for 20 s in all subsequent cycles (30 cycles), annealing at 54°C for 30 s, and extension at 72°C for 1.5 min. The standard reaction mixture contained 1 µg of genomic DNA, 9 mM Tris-HCl (pH 8.4), 1.5 mM MgCl₂, 250 pmol of each primer, 10 nmol of each dNTP (deoxyribonucleoside triphosphate), and 1.25 units of Taq DNA polymerase (Perkin-Elmer/Cetus) in a final volume of 50 ml. The resulting PCR product was electrophoresed in 2.5% NuSieve GTG agarose (FMC), stained with ethidium bromide, and photographed under ultraviolet light. The PCR product in the agarose gel was purified with the Easy Prep and PCR Product Prep kit (Pharmacia). The digoxigenin (DIG)-labeling of the PCR product was carried out by PCR under the conditions already described, except that genomic DNA and dNTP were replaced with 10 ng of the PCR products and the DIG DNA-labeling mixture, respectively and except that the annealing temperature was 48°C.

Recombinant DNA techniques.

Chromosomal DNA of B. psychrosaccharolyticus was isolated by a standard procedure (Current Protocols in Molecular Biology, 1994).

Plasmid DNA from E. coli and recombinant plasmids from transformants
were isolated with Easy Prep and Plasmid Prep Kit (Pharmacia). Digestion of DNA with a restriction endonuclease (EcoRI) was performed under the conditions recommended by the supplier. The digested DNA was electrophoresed on 0.5% agarose gels in 89 mM Tris-borate buffer containing 25 mM EDTA (pH 8.3), stained with ethidium bromide, and photographed under ultraviolet light. Ligation, packaging, and transfection were carried out with T4 DNA ligase, a Gigapack III Gold Packaging Extract and a Predigested λZapII/EcoRI/CIAP Cloning Kit (Stratagene, La Jolla, CA).

**Library construction and screening.**

Genomic DNA (10 µg) from *B. psychrosaccharolyticus* was partially digested with EcoRI (3 units), and resultant DNA fragments ranging from 2 to 10 kb were ligated into the EcoRI site of λZapII (2 µg) by T4 DNA ligase (2 units). *E. coli* XL1-Blue MRF' cells were infected with this recombinant phage, and plaques were screened for reactivity with the DIG-labeled alanine racemase gene fragment. Hybridization and chemiluminescent detection were carried out with a DIG luminescent detection kit (Boehringer Mannheim) and the nylon membrane (Hybond-N-, Amersham). Each membrane was incubated at 60°C for 2 h in a hybridization buffer containing the 75 mM citrate buffer (pH 7.0), 0.75 M NaCl, a 2% blocking reagent (Boehringer Mannheim), 0.1% lauryl sarcosine, 0.02% SDS, and 50% formamide. The membrane was transferred to a fresh hybridization buffer containing 50 pg/ml of the heat-denatured probe, and then incubated to hybridize it at 60°C for 16 h. Each membrane was subsequently rinsed twice at 65°C for 5 min with the 75 mM citrate buffer (pH 7.0) containing 0.75 M NaCl and 0.5% SDS, and
then washed with a 1.5 mM citrate buffer (pH 7.0) containing 1.0% SDS and 15 mM NaCl at 50°C for 30 min. The membrane was next incubated at 65°C for 30 min in a 0.1 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 0.05% Tween 20, and 3% bovine serum albumin, and then at 25°C for 30 min in a 0.1 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 0.05% Tween 20, and the conjugate of the anti-digoxigenin antibody and alkaline phosphatase (0.15 unit/ml). The membrane was washed twice at 25°C for 15 min with a 0.1 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and 0.05% Tween 20, and then equilibrated with 0.1 M Tris-HCl (pH 9.5) containing 0.1 M NaCl and 50 mM MgCl₂ for 5 min. The membrane was incubated with Lumigen PPD (0.1 mg/ml in 0.1 M Tris-HCl at pH 9.5 containing 0.1 M NaCl and 50 mM MgCl₂) at 25°C for 5 min in the dark. Each membrane was finally put into a folder (Photo Gene Development Folder, Gibco BRL), and the excess liquid droplets were removed from the membranes. The folder was overlaid with an X-ray film (type XAR, Eastman Kodak Co.), and incubated for 3 h in an X-ray film cassette (Fuji Film Co., Tokyo) to document the hybridization results. The positive phage was purified and the insert DNA in the λZapII vector was subcloned into pBluescript SK(-) phagemid according to the manufacture's manual.

**DNA sequencing.**

Nucleotide sequence analysis of DNA fragments in recombinant plasmids and PCR products was performed by the dideoxynucleotide chain termination method with a Dye Deoxy Terminator Cycle Sequencing kit and an ABI Prism 310 Genetic Analyzer (Perkin Elmer, Foster City, CA). In all instances, both strands of the DNA fragments were sequenced in their entirety.
Enzyme and protein assay.

Enzyme assays were routinely done at 0°C for 10 min. The standard reaction mixture contained 0.5 mM PLP, 40 mM Tris-HCl buffer (pH 8.3), 250 mM L-alanine, and enzyme in a final volume of 0.1 ml. The reaction was started by addition of the enzyme, and stopped by addition of 0.1 ml of 1 N HCl. After neutralization with 1 N NaOH, D-alanine formed was measured with D-amino acid oxidase (Soda, 1968). To assay L-alanine formed from D-alanine, L-alanine was replaced with D-alanine in the standard reaction mixture. L-Alanine formed was determined with L-alanine dehydrogenase (Tanizawa et al., 1986). Substrate specificity of alanine racemase was examined with a polarimeter SEPA-300 (Horiba, Kyoto, Japan). A unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 µmol of D- (or L-) alanine per min. The specific activity is expressed as units per mg of protein. Protein was measured with a Bio-Rad protein assay kit and bovine serum albumin as a standard.

Purification of alanine racemase.

E. coli SOLR carrying pYOK3 was cultivated aerobically at 28°C for 20 h in an Erlenmeyer flask with 3 L of LB medium containing 50 µg/ml of ampicillin. The cells were harvested by centrifugation, and washed with 0.85% NaCl. All the purification procedures were done at 0-5°C. Potassium phosphate buffer (10 mM, pH 7.0) containing 10% glycerol, 0.02% sodium azide, 0.01% 2-mercaptoethanol, 0.5 mM EDTA, 10 µM pyridoxal 5'-phosphate (PLP), and 0.1 mM phenylmethanesulfonfonyl fluoride was used as the standard buffer.

Step 1. Preparation of crude extract: The washed cells (15 g, wet
weight) were suspended in 15 ml of the standard buffer, and then disrupted by sonication at 0°C for 30 min. The intact cells and debris were removed by centrifugation.

**Step 2. Ammonium sulfate fractionation:** The supernatant solution was brought to 30% saturation with solid ammonium sulfate. The precipitate was removed by centrifugation, and ammonium sulfate was added to the supernatant solution to 70% saturation. The precipitate was collected by centrifugation, and dissolved in 10 ml of the standard buffer. The enzyme solution was dialyzed against the standard buffer.

**Step 3. Resource Q column chromatography:** The dialyzed solution was put on a Resource Q column (6 ml, Pharmacia) equilibrated with the standard buffer in an Äkta Purifier system (Pharmacia). After the column was washed with the same buffer, the enzyme was eluted with a linear gradient from 0 to 0.3 M NaCl in the standard buffer at a flow rate of 3 ml/min. The active fraction were combined and brought to 30% saturation with solid ammonium sulfate.

**Step 4. Phenyl-Sepharose column chromatography:** The enzyme solution was put on a Hiload 26/10 Phenyl Sepharose column (Pharmacia) equilibrated with the standard buffer containing ammonium sulfate at 30% saturation in an Äkta Purifier system. The column was washed with the same buffer, then the enzyme was eluted with a linear gradient from 30 to 0% saturated ammonium sulfate in the standard buffer. The active fractions were collected and dialyzed against the standard buffer.

**Step 5. Gel filtration chromatography:** The enzyme solution was put on a Hiload 16/60 Superdex 200pg column (Pharmacia) equilibrated with the standard buffer containing 0.15 M NaCl in an Äkta Purifier system. The elution was done with the standard buffer. The active fractions were
combined and dialyzed against the standard buffer.

**Step 6.** Mini Q column chromatography: The enzyme solution was put on a Mini Q column (PE 4.6/50, Pharmacia) equilibrated with the standard buffer. After the column was washed with the same buffer, the enzyme was eluted with a linear gradient from 0 to 0.3 M NaCl in the standard buffer at a flow rate of 0.5 ml/min. The active fraction were combined and stored at -80°C until use.

*N-terminal amino acid sequence.*

The purified enzyme (about 0.1 nmol) dialyzed against distilled water was treated by automated Edman degradation with an protein sequencer (Procise 494, Perkin Elmer, Foster City, CA). The PTH amino acid derivatives were separated and identified by an Procise PTH analyzer model 190 (Perkin Elmer, Foster City, CA).

**Measurement of molecular weight.**

The molecular weight of the native enzyme was measured by gel filtration as described above. Pig liver lactate dehydrogenase (Mr 142,000), bovine serum albumin (Mr 67,000), ovalbumin (Mr 43,000), chymotrypsinogen (Mr 25,000), and ribonuclease (Mr 13,700) were used as standards. The molecular weight of the subunit was estimated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis by the method of Laemmli (Laemmli., 1970). The standard proteins used for calibration were phosphorylase b (Mr 94,000), bovine serum albumin (Mr 67,000), ovalbumin (Mr 43,000), carbonic anhydrase (Mr 30,000), and soybean trypsin inhibitor (Mr 20,100).
Results

Cloning of the gene encoding the *B. psychrosaccharolyticus* alanine racemase.

A specifically amplified product with a size of about 760 bp was obtained by PCR with the *B. psychrosaccharolyticus* genomic DNA as a template and the consensus primers. The DNA fragment was confirmed to be an alanine racemase gene fragment by DNA sequencing; the conserved amino acid residues found in known alanine racemases are also found in the amino acid sequence predicted from the nucleotide sequence of the PCR product (data not shown). The gene fragment was then labeled with DIG as the probe for cloning of the gene encoding the *B. psychrosaccharolyticus* alanine racemase. The author screened about 32,000 recombinant bacteriophages from a *B. psychrosaccharolyticus* genomic library by plaque hybridization with this probe. One positive phage was purified and the insert DNA in the λZapII vector was subcloned into pBluescript SK(-) to obtain the recombinant plasmid pYOK3. The plasmid was purified and found to contain 3.3 kb of insert DNA as judged from agarose gel electrophoresis. After transformation of *E. coli* SOLR with the plasmid pYOK3, a transformant obtained was analyzed in the alanine racemase activity (Table I). When alanine racemase activity was measured at 30°C, the cell free extract of the transformant showed about 90 and 30-fold higher level of alanine racemase activity than those of *E. coli* SOLR and *B. psychrosaccharolyticus*, respectively. Even at 0°C, the transformant showed about 40-fold higher level of the activity than *B. psychrosaccharolyticus*, whereas *E. coli* SOLR did not show the detectable activity. These results suggest that the transformant produces a
psychrotrophic alanine racemase.

Table I. Activity of alanine racemase in cell-free extracts

<table>
<thead>
<tr>
<th>Strains</th>
<th>Specific Activity (U/mg)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>30 °C</td>
</tr>
<tr>
<td>A transformant</td>
<td>9.39</td>
</tr>
<tr>
<td><em>B. psychrosaccharolyticus</em></td>
<td>0.30</td>
</tr>
<tr>
<td><em>E. coli</em> SOLR</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Activity was measured at 30 or 0°C by the direction from L- to D-alanine.
DNA sequence of the psychrotrophic alanine racemase gene.

Figure 9 shows the strategy of the nucleotide sequencing of the 3.3 kb of the insert DNA. The position and direction of the alanine racemase gene in the inset DNA were examined by PCR with the consensus primers described above, and T3 and M13 -20 primers, the sequences of which are present in the multi-cloning site of pBluescript SK (-). Upstream of the alanine racemase gene there is an open reading frame, the sequence of which shows an excellent homology with the ydcC gene (function unknown) of *B. subtilis*. The entire continuous sequence of the alanine racemase gene is shown along with the translated protein sequence in Fig. 10. The gene has an open reading frame of 1149 nucleotides starting with the unusual initiation codon GTG and ending with termination codon TAA. The sequence around the initiation codon was also confirmed by DNA sequencing with BigDye Terminator Cycle Sequencing and DichloroRhodamine Terminator Cycle Sequencing kits (Perkin Elmer, Foster City, CA). The N-terminal amino acid sequence of the enzyme purified from the clone corresponded to the deduced amino acid sequence as described below. Seven bases upstream from the GTG initiation codon there is a 5-base sequence AACAG (-11 to -7) that is considerably complementary with the 3'-end of 16S rRNA from *Bacillus*. Upstream of the initiation codon there are sequences of TCGTTA (-67 to -62) and TATAAT (-46 to -41) that show high sequence-homology with the -35 and -10, respectively, regions of *Bacillus* promotors analyzed with the GenBank database. Downstream from the TAA stop codon there is a region (1225 to 1243) of dyad symmetry capable of forming a stem and loop structure.
Fig. 9. DNA sequencing scheme for the 3.3 kb insert DNA of pYOK3. The composition of pYOK3 is shown at the top. The arrows show the regions and direction sequenced by the dideoxy chain termination method.
Fig. 10. DNA sequence and translation of the alanine racemase gene. Coding region starts at position 1 and is terminated at position 1149. The lysyl residue involved in the binding of pyridoxal-P is indicated by an double underline. The -35 and -10 regions of the putative promoter and the proposed Shine-Dalgarno sequence (SD) for the ribosome binding site are underlined. In the 3'- flanking region from the coding sequence, a sequence capable of forming a stem and loop structure, which may be involved in transcription termination, is indicated by dashed underlines.
The G+C content of the coding region of alanine racemase is 41.7 mol %, which is lower than those of the enzymes from *B. subtilis* (47.9 mol %) (Ferrari *et al.*, 1985) and *B. stearothermophilus* (56.2 mol %) (Tanizawa *et al.*, 1988). In particular, the *B. psychrosaccharolyticus* gene shows a high preference for A and T residues at the third base (the wobble position) of the codons; the G+C contents at that position of the *B. psychrosaccharolyticus*, *B. subtilis*, and *B. stearothermophilus* genes are 32.9, 50.6, and 63.8%, respectively.

**Amino acid sequence and tertiary structure prediction of thermolabile alanine racemase.**

The alanine racemase gene encodes a protein of 383 amino acids. The molecular mass of the apoenzyme is estimated to be 42,519 Da. The complete amino acid sequences of alanine racemases from fourteen different sources [*B. psychrosaccharolyticus*, this paper; *B. subtilis*, Ferrari *et al.* (1985); *B. stearothermophilus*, Tanizawa *et al.* (1988) and Shaw *et al.* (1997); *H. influenzae*, Fleischmann *et al.* (1995); *Klebsiella aerogenes* *dadB*, accession no. AF016253; *Lactobacillus plantarum*, Hols *et al.* (1997); *S. typhimurium*, the *alr* gene, Galakatos *et al.* (1986); *S. typhimurium*, the *dadB* gene, Wasserman *et al.* (1984); *E. coli*, the *alr* gene, Blattner *et al.* (1993); *E. coli*, the *dadX* gene, Lobocka, *et al.* (1994); *H. pylori*, Tomb *et al.* (1997); *Mycobacterium leprae*, accession no. U00020; *Mycobacterium smegmatis*, accession no. U70872; *Synechocystis* sp., Kaneko *et al.*, 1995] are now available, and the fourteen sequences were linearly aligned essentially according to a mutation data scoring matrix (Dayhoff *et al.* 1983) as shown in Fig. 11. The amino acid sequence showed significant homology with those of other alanine
were shown to have an increased expression of the protein sequences of the alanine racemases from B. psychrosaccharolyticus, B. subtilis, B. eoli, and B. psychrophilus. The alignments were performed using the method of Dayhoff et al. (1983).

Fig. 11. Linear alignment of the protein sequences of alaline racemases from B. psychrosaccharolyticus, B. subtilis, B. steaorthermophilus, E. coli alr, E. coli dadX, H. influenzae, K. aerogenes dadB, L. plantarum, S. typhimurium alr, S. typhimurium dadB, H. pylori, M. leprae, M. smegmatis, and Synechocystis sp. The fourteen sequences were aligned by introducing gaps (hyphens) to maximize identities according to the method of Dayhoff et al. (1983). The residue numbers are given on the basic simplicity of the common basic sequences. Identical residues among the fourteen sequences (**) are shown below. The active-site lysyl residues are indicated by a sharp.
racemases reported, especially with that (57.0%) of the *B. stearothermophilus* enzyme. The conserved amino acid residues found in the sequences of known alanine racemases are also found in the reduced amino acid sequence. There are at least six conserved regions, and the active-site region is highly conserved. The other identical residues are not so much continuously arrayed.

The tertiary structure of the psychrotrophic enzyme was predicted by a theoretical method, the homology modeling reported by Wako and Kubota (1991) with the *B. stearothermophilus* enzyme as a standard, the tertiary structure of which was determined by X-ray crystallography (Shaw *et al.* 1997). Figure 12A shows the superimposition of the tertiary structures of the psychrotrophic enzyme upon that of the thermostable enzyme. The root mean square (rms) difference between the two conformations

\[
\text{rms} = \left\{ \sum_{i<j} (d_{ij} - d_{x,ij})^2 / m \right\}^{1/2}
\]

is calculated as 11.5 Å according to the method of Wako and Kubota (1991), where \(d_{ij}\) and \(d_{x,ij}\) are distances between residues \(i\) and \(j\) in optimized and X-ray conformations, respectively, and \(m\) is the number of pairs of residues. The tertiary structure of the mesophilic enzyme from *B. subtilis* was also superimposed upon that of the thermostable enzyme; the root mean square difference is calculated as 5.14 Å. (Fig. 12B) The thermostable enzyme is more homologous in the rms difference with the mesophilic enzyme than the psychrotrophic enzyme.
Fig. 12. The predicted tertiary structure of the psychrotrophic and mesophilic alanine racemases from *B. psychrosaccharolyticus* and *B. subtilis*, respectively. These were predicted by a theoretical method, the homology modeling reported by Wako and Kubota (1991) with the thermostable enzyme from *B. stearothermophilus* as a standard, the tertiary structure of which was determined by X-ray crystallography (Shaw *et al.* 1997).

A; The superimposition of the tertiary structures of the psychrotrophic enzyme upon that of the thermostable enzyme.

B; The superimposition of the tertiary structures of the mesophile enzyme upon that of the thermostable enzyme.
Purification of alanine racemase from E. coli SOLR carrying pYOK3.

Alanine Racemase was purified about 50-fold with an overall yield of 15% (Table II). The purified enzyme was homogeneous by polyacrylamide gel electrophoresis. Thermolabile enzymes from psychrotrophs are usually susceptible to proteolysis by contaminant psychrotrophs. The addition of sodium azide to the buffer was effective to protect the thermolabile alanine racemase from proteolytic inactivation. The enzyme was stable at 4°C for 1 week and at -80°C over 50 days in the standard buffer.

Table II. Purification of alanine racemase from E. coli SOLR carrying pYOK3

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (units)*</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1400</td>
<td>18700</td>
<td>13.0</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate (30-70%)</td>
<td>850</td>
<td>20000</td>
<td>21.0</td>
<td>107</td>
</tr>
<tr>
<td>Resource Q</td>
<td>77</td>
<td>11000</td>
<td>143</td>
<td>58</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>30</td>
<td>10200</td>
<td>340</td>
<td>55</td>
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<tr>
<td>Phenyl Sepharose</td>
<td>15</td>
<td>5500</td>
<td>370</td>
<td>29</td>
</tr>
<tr>
<td>Mini Q</td>
<td>4.3</td>
<td>2800</td>
<td>650</td>
<td>15</td>
</tr>
</tbody>
</table>

* Activity was measured at 0°C by the direction from L-to D-Alanine
Molecular weight and subunit structure.

The purified enzyme was found to have a molecular weight about 73,000 by gel filtration with Superdex 200 irrespective of the presence or absence of 2-mercaptoethanol. The molecular weight of the subunit was estimated as 43,800 by SDS polyacrylamide gel electrophoresis (Fig. 13). These show that the enzyme is composed of two subunits identical in molecular weight.

Fig. 13. Measurement of subunit molecular weight of alanine racemase from *B. psychrosaccharolyticus* by sodium dodecyl sulfate gel electrophoresis.
A; Standard protein, B; The enzyme.
Statistical analysis of amino acid composition and N-terminal amino acid sequence.

The deduced amino acid composition of the enzyme was statistically analyzed. Deviation function \( D = \left\{ \sum (X_{i1} - X_{2i})^2 \right\}^{1/2} \) analyzed by the method of Harris et al. (1969) is 0.036 and 0.067 against \( B. subtilis \) and \( B. stearothermophilus \), respectively.

The N-terminal amino acid sequence of the enzyme was analyzed by Edman degradation. Only one major PTH-amino acid was found in each cycle. The amino acid sequence from 1Met to 50Val was identical to that predicted from the nucleotide sequence.

Cofactor.

The absorption spectrum of the enzyme showed maxima at 280 and 420 nm with an A280/A420 ratio of 6.9 (Fig. 14). No appreciable spectral shifts occurred on varying the pH (6.0 to 9.0). The absorption peak at 420 nm is probably derived from an internal Schiff base formed between the formyl group of PLP and an ε-amino group of the active site by analogy with the bound cofactor of other PLP enzymes. The author measured PLP content of the enzyme by a fluorometric method (Adams, 1979), and calculated the content to be 2.0 mol/mol of the enzyme.

Reduction of the enzyme with sodium borohydride by dialysis method (Matsuo and Greenberg, 1959) affected the absorption spectrum (Fig. 14) and the activity. The reduced enzyme was catalytically inactive, and addition of PLP did not reverse the inactivation. The holoenzyme was resolved to the apoenzyme by dialysis for 24 h against 50 mM potassium phosphate buffer (pH 8.0) containing 10% glycerol and 10 mM \( \text{NH}_2\text{OH} \). The apoform does not absorb at 420 nm (Fig. 14) and is inactive. The
apoenzyme was also obtained by dialysis of the holoenzyme against 10 mM potassium phosphate buffer (pH 7.0) containing ammonium sulfate at 30% saturation, 10% glycerol, 0.02% sodium azide, 0.01% 2-mercaptoethanol, 0.5 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride at 4°C for 16 days. The activity was restored to 60% of the original activity by dialysis against the standard buffer at 4°C for 48 h. The activity measurement at 30°C of apoenzyme with various concentration of PLP gave the $K_m$ value (5.0 μM) for PLP.

Fig. 14. Absorption spectra of alanine racemase from *B. psychrosaccharolyticus*. Absorption spectra were taken in 10 mM potassium phosphate buffer (pH 8.0) at the enzyme concentration of 0.4 mg/ml: (a) holoenzyme; (b) NaBH₄-reduced enzyme; (c) apoenzyme.
Effects of temperature on the activity.

As shown in Fig. 15, the enzyme showed a high activity at low temperature. The maximum activity in the presence and absence of PLP was observed at 50 and 35°C, respectively. When the enzyme was incubated over 35°C for 1 h, the enzyme activity was quickly lost. The thermophilic enzyme from *B. stearothermophilus* is quite stable upon heat treatment at 70°C for 80 min (Inagaki *et al.* 1986), and the mesophilic enzyme is quite labile at more than 55°C (data not shown). The enzyme from the psychrotroph is quite thermolabile and psychrotrophic.

![Fig. 15. Effects of temperature on thermolabile enzymes. A: The enzyme activities were measured at various temperatures in the presence (a) and absence (b) of PLP. B: The enzyme solution (3 units/ml of 10 mM potassium phosphate buffer) was incubated at various temperatures for 1 h. The remaining activity was measured at 0°C.](image-url)
Physical and kinetic characterization.

The enzyme had its maximum reactivity in the pH range from 8 to 10 when examined in 40 mM pyrophosphate, Tricine-HCl, or potassium phosphate buffer at several pHs. The enzyme is stable in the pH range from 8 to 10. When the enzyme was assayed at various temperatures in the 10 mM potassium phosphate buffer (pH 8.3), the maximum activity was observed at 35°C. The temperature dependence of the $V_{\text{max}}$ was examined by Arrhenius plots, and the activation energy $E_a$ was 5.4 kcal/mol with calculated values of $\Delta H$, $\Delta G$, and $\Delta S = 5.7$ kcal/mol, 20.8 kcal/mol, and -49.1 cal/mol·deg., respectively. The $K_m$ for D-alanine and L-alanine are 12.2 and 17.9 mM, respectively at 30°C, and $V_{\text{max}}$ for racemization (D- to L-alanine and L- to D-alanine) is 1000 and 2000 units/mg, respectively. When these values were used, the calculated $K_{eq}$ for alanine racemization was 1.36, in good agreement with the theoretical value (1.0) for chemically symmetric reaction (L-alanine and D-alanine)(Briggs and Haldane, 1925). The $k_{\text{cat}}/K_m$ value was calculated to be ca. $1.6 \times 10^5$ M$^{-1}$s$^{-1}$.

Alanine is the exclusive substrate of the enzyme; L-lysine, L-arginine, L-glutamine, L-methionine, L-leucine, homoserine, L-asparagine, L-serine, L-cysteine, L-threonine, L-valine, L-glutamic acid, L-aspartic acid, L-proline, L-tyrosine, L-tryptophan, L-phenylalanine, L-histidine, and L-isoleucine were not racemized when we examined by polarimetry.

Discussion

Alanine racemase is a typical prokaryotic enzyme and is widely distributed in bacteria. Therefore, the enzyme is important and interesting from the standpoints of the development of antibacterials and
comparative biochemistry. To characterize psychrotrophic alanine racemase, the author cloned and expressed the enzyme gene from *B. psychrosaccharolyticus* into *E. coli* SOLR. A specifically amplified product obtained by PCR using consensus primers designed on the basis of the conserved sequences of known alanine racemases was used as a probe for the gene cloning. One positive transformant carrying the plasmid pYOK3 showed the high alanine racemase activity even at 0°C. The plasmid pYOK3 contains a single complete open reading frame (*alr*) preceded by a ribosome-binding site. The 5' region in front of the *alr* gene includes a 5'-truncated open reading frame, which is homologous with *ydcC* gene of *B. subtilis*. The *alr* gene consists of the 1149 base pairs with the unusual initiation codon GTG. The gene is similar in the size, but dissimilar in the initiation codon to other alanine racemase genes reported. Since a thermostable N-acylamino acid racemase gene from *Amycolatopsis* sp. has also the GTG initiation codon (Tokuyama and Hatano, 1995), the initiation codon is not considered to be characteristic to psychrotrophic enzyme genes. The GC content of the gene is lower than those of the genes from a thermophile, *Bacillus stearothermophilus*, and a mesophile, *Bacillus subtilis*. In particular, the *B. psychrosaccharolyticus* gene shows a low preference for G and C in the third position of the codons. The low GC content in the psychrotrophic enzyme gene, especially in the wobble position of each codon, may be one of the reason of the gene thermolability at high temperature.

The psychrotrophic enzyme is more thermolabile and psychrotrophic than the other two enzyme as described above. The distinctive thermolability and psychrotrophic properties presumably reflect the structural differences. The alanine racemase gene encodes a protein of
383 amino acids, and similar in the size to other alanine racemases. The conserved amino acid residues found in known alanine racemases are also found in the deduced amino acid sequence. The sequence of the psychrotrophic alanine racemase gene shows higher homology with that of the thermophilic enzyme from *B. stearothermophilus* than that of the mesophilic enzyme from *B. subtilis*. However, the deviation function between the amino acid compositions of the enzymes suggests that the structure of the psychrotrophic enzyme is more similar to that of the mesophilic enzyme than that of the thermophilic enzyme. Deviation functions higher than 0.05 are thought to be insignificant for showing a structural similarity between any two enzymes (Harris *et al.* 1969). Then, the author predicted the tertiary structures of the psychrotrophic and mesophilic enzymes by a homology modeling method with the thermophilic enzyme as a standard, the tertiary structure of which was determined by X-ray crystallography (Shaw *et al.* 1997). The predicted tertiary structure of the psychrotrophic enzyme is considered to be less homologous with that of the thermophilic enzyme than that of the mesophilic enzyme, as judged from the rms difference.

The psychrotrophic alanine racemase was purified to homogeneity and characterized. The absorption spectra and kinetic parameters of the psychrotrophic enzyme are similar to those of other well-characterized alanine racemases. A noteworthy difference was found in *K_m* value for PLP. The *K_m* value for PLP of the psychrotrophic enzyme is estimated to be 5.0 μM. However, the *K_m* values for PLP of the enzyme from *S. typhimurium alr* is reported to be 33 nM (Esaki and Walsh, 1986), although the *K_m* values for PLP of other alanine racemases are not reported or examined under incomparable conditions. The psychrotrophic enzyme
shows the maximum activity at 35°C in the absence of PLP, whereas in the presence of PLP the maximum reaction temperature is shifted to 50°C. The enzyme probably loses PLP during the racemization of the substrate because of the low affinity for PLP. Dissociation of PLP from the enzyme protein may be related to the change of the tertiary structure followed by the inactivation.

The enzyme is composed of two subunits identical in molecular mass, and is similar in this respect to the thermophilic enzyme from *B. stearothermophilus* (Inagaki et al., 1986) and to the psychrotrophic enzyme from *P. fluorescens* (Yokoigawa et al., 1993). In contrast, alanine racemases encoded by the *S. typhimurium dadB* (Wasserman et al., 1984) and *alr* (Esaki and Walsh, 1986), and *S. faecalis* (Badet and Walsh, 1985) shows a monomeric structure with a molecular mass of around 40,000.

The enzyme from *B. psychrosaccharolyticus* shows a high catalytic activity even at 0°C. The thermostable enzyme from *B. stearothermophilus* is catalytically inert at 0°C. Although the activity of the enzyme from mesophilic bacteria at 0°C has not been reported, we observed that the cell free extracts of *B. subtilis* and *E. coli* showed no alanine racemase activity at 0°C. Thus, the enzyme from *B. psychrosaccharolyticus* is psychrotrophic. This is the first report for characterization of a psychrotrophic alanine racemase and its gene.

**SUMMARY**

A gene of thermolabile alanine racemase from a psychrotroph, *B. psychrosaccharolyticus*, was cloned by plaque hybridization using an alanine racemase gene fragment as a probe, which was amplified from the
genomic DNA by polymerase chain reaction using consensus primers designed on the basis of the conserved sequences of known alanine racemases. The gene analyzed by DNA sequencing has an open reading frame of the 1149 base pairs starting with the unusual initiation codon GTG and ending with termination codon TAA. The gene encodes a protein of 383 amino acid residues: the molecular weight of the apoenzyme is estimated as 42519. The GC content of the gene (42 mol %) is lower than those of the genes from a thermophile (56 mol %), B. stearothermophilus, and a mesophile (48 mol %), B. subtilis. The GC content at the third positions of the gene of the psychrotroph is markedly lower than those at the third positions of the other two genes. Alanine racemase produced in a clone carrying the plasmid pYOK3 (6.3 kbp) was purified to homogeneity from the cell extract. The sequence of the N-terminus of the protein corresponded to the deduced amino acid sequence. The enzyme has the apparent molecular weight of about 73,000 on gel filtration and consists of two subunits identical in molecular weight (about 43,000) in agreement with the gene structure. The enzyme contains two mol of pyridoxal 5'-phosphate per mol as a coenzyme, and is specific to alanine. The enzyme is extremely labile over 35°C, and shows the high catalytic activity even at 0°C; it is thermolabile and psychrotrophic. Although the amino acid sequence of the enzyme from the psychrophroph showed higher homology with that of the thermophilic enzyme from B. stearothermophilus (57%) than that of the mesophilic enzyme from B. subtilis (54%), the tertiary structure of the psychrotroph enzyme predicted by homology modeling is more homologous with that of the mesophilic enzyme than that of the thermophilic enzyme.
CHAPTER 3

Evaluation of Alanine Racemase Gene Fragments as Probes for Detecting each of *Bacillus stearothermophilus* and *Bacillus psychrosaccharolyticus* in Foods

INTRODUCTION

Quality of foods is often reduced by contamination with bacteria. The standard plate culture method is useful for detecting and quantifying bacteria. However, some bacteria such as thermophiles and psychrophiles are overlooked by the method. *B. stearothermophilus* is an endospore-forming gram positive thermophile. Since the endospores of the bacterium are reported to be still viable after 2.0 min at 132°C in a steam autoclave (Vesley *et al.*, 1992), the bacterium is sometimes found in cow’s milk and canned foods. The bacterium is non-pathogenic to human, and can't grow under normal storage conditions of foods. However, when foods are subjected to high temperature, the bacterium grows and causes deterioration in the foods as described in FDA Bacteriological Analytical Manual (1995b). The absence of the bacterium is important especially in canned provisions warmed by automatic vending machines. *B. psychrosaccharolyticus* is an endospore-forming gram positive psychrotroph. The bacterium is also non-pathogenic to human, but sometimes causes deterioration in foods stored at a low temperature. Generally, the detection method of psychrotroph is time-consuming; the plate culture method at low temperature requires more than 7 days.
However, few reports have been published on a specific and rapid method for detecting each of *B. stearothermophilus* and *B. psychrosaccharolyticus* in foods. Specific DNA probes for rapid detection of each bacterium may be useful for quality control of foods.

Alanine racemase (EC 5.1.1.1) catalyzes racemization of L- and D-alanine, and occurs widely in bacteria. The enzyme is essential for biosynthesis of the peptidoglycan of bacterial cell wall by providing D-alanine, and therefore is a typical prokaryotic enzyme. All strains of *B. stearothermophilus* and *B. psychrosaccharolyticus* are considered to have the enzyme gene, while the gene is probably absent in food materials. Several nucleotide sequences of alanine racemase genes have been analyzed (Wasserman *et al.*, 1984; Ferrari *et al.*, 1985; Galakatos *et al.*, 1986; Tanizawa *et al.*, 1988; Blattner *et al.*, 1993; Lobocka *et al.*, 1994; Fleischmann *et al.*, 1995). The sequences show 9-10 conserved regions distributed evenly in the genes (Tanizawa *et al.*, 1988). However, the other regions are not conserved. Therefore, alanine racemase gene fragments containing the non-conserved regions of the genes from *B. stearothermophilus* and *B. psychrosaccharolyticus* may be useful probes for detecting the respective bacterium.

The author describes here the amplification of gene fragments of the *B. stearothermophilus* and *B. psychrosaccharolyticus* alanine racemases, and the specificity of each fragment as a probe for detecting the respective bacterium. An alanine racemase gene may be a useful index for detecting *B. stearothermophilus* and *B. psychrosaccharolyticus* in foods.
Materials and Methods

Materials.

A DIG DNA-labeling mixture and a DIG luminescence-detection kit were obtained from Boehringer Mannheim, while nylon membranes (Hybond N+) was obtained from Amersham. Sperm DNAs from salmon and herring were obtained from Sigma and Boehringer Mannheim, respectively. All other chemicals were of analytical grade.

Microbes and culture conditions.

Microbes were obtained from Institute for Fermentation, Osaka (IFO), Japan, Institute for Applied Microbiology, Tokyo University (IAM), Japan, Northern Regional Research Center (NRRL), Peoria, Ill., and American Type Culture Collection (ATCC). Bacteria used were listed in Table III. Psychrotrophs (B. psychrosaccharolyticus ATCC 23296, Bacillus psychrophilus ATCC 23304) were aerobically cultivated at 22°C for 16 h in a medium containing 0.5% meat extract, 1% peptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.0). A thermophile (B. stearothermophilus IFO 12550) was aerobically cultivated at 55°C in a medium containing 1.5% peptone, 0.1% glycerol, 0.01% yeast extract, 0.01% meat extract, 0.5% NaCl, 0.2% K2HPO4, 0.1% KH2PO4, and 0.01% MgSO4 (pH 7.2). Aspergillus niger IFO 4343 and bakers' yeast (Toyo Jozo Co. Ltd., Tokyo, Japan) were cultivated at 28°C for 24 h in YPD medium (0.5% yeast extract, 0.5% peptone, and 2% glucose). All other bacteria were cultivated at 30°C for 18 h in LB medium (1% Tryptone, 0.5% yeast extract, 0.5% NaCl, and 1 mM NaOH, pH 7.0).
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<td>35</td>
<td><em>Escherichia coli</em> IFO 3301</td>
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Primers and templates for PCR.

Primers were designed from the two conserved sequences of known alanine racemases around the active site Lys residue and the His residue located at about 130 amino acids downstream of the active site, between which the non-conserved regions are present (Fig. 16). Three types of primers were designed in due consideration of the codon usage of the genes and of the number of inosine introduced. These primers were synthesized and purified by Funakoshi Co. Ltd., Tokyo, Japan. Genomic DNAs from bacteria, yeasts, and fungi were prepared as described by Wilson (1992), Treco (1992), and Hynes et al. (1983), respectively, and used as the PCR templates.

Amplification of alanine racemase gene fragments and sequence analysis.

Routinely, 25 cycles of PCR were performed in a Program Temp Control System PC-700 (Astec Co., Fukuoka, Japan). The cycle is carried out as described in Chapter 2. The resulting PCR products were electrophoresed in 3.5% NuSieve GTG agarose (FMC Bioproducts, Rockland, ME), stained with ethidium bromide, and photographed under ultraviolet light. The product in the gel was purified with Easy Prep and PCR Product Prep Kit (Pharmacia). Direct sequencing of the both strands of the product purified was performed by the dideoxynucleotide chain termination method with Dye Deoxy Terminator Cycle Sequencing Kit and ABI 373A DNA Sequencer (Applied Biosystems) using the primers A3 and C3 described in Fig. 16.
(A)

A1: 5'-aaigciaaicgcaigaigg C1: 3'-gtraarcgikbicgict
A2: 5'-aargcnaaygcntayggncga C2: 3'-gtraarcgnkbnecnict
A3: 5'-aaagcnaagcycyayggnca C3: 3'-gtaaaagkibicgsect

(B) Lys41   His168
---aaagcgaacgcctatgggacatgg---cattttgctgctggat---B.stearothermophilus
---aaagcgaacgctacgcccagctag---cattttgctgacgctggat---B.subtilis
---aaagcgaacgcctatgggctag---cattttgctgctggctggat---E.coli alr
---aaagcgaacgcctacgcccatacg---cattttgctgacgctggat---E.coli dadX
---aaagcgaacgcctatgggctag---cattttgctgacgctggat---H.influenzae
---aaagcgaacgcctatgggctag---cattttgctgacgctggat---S.typhimurium alr
---aaagcgaacgcctacgcccag---cattttgctgctgcgctggat---S.typhimurium dadB

Fig. 16. Primers used for amplification of a gene fragment of alanine racemase (A) and the conserved sequences of the enzyme genes around $^{41}$Lys and $^{168}$His residues (B). Symbols are single-letter codes recommended by Nomenclature Committee of International Union of Biochemistry (1985), and I is inosine.
Labeling of the gene fragment.

The digoxigenin-labeled gene fragments of alanine racemases were prepared as described in Chapter 2.

Preparation of samples for hybridization.

Solid food samples were aseptically homogenized at 0°C for 10 min with an equal volume of 0.85% NaCl solution. The homogenized foods and liquid foods were incubated at 25°C for 10 min with an equal volume of a lysis solution (0.2 N NaOH and 1.0% SDS). The debris was removed by centrifugation, and each supernatant solution was immediately used as a sample for the hybridization. Bacteria used for analysis of specificity of each probe were grown to the late logarithmic phase, and harvested by centrifugation. The cells were suspended in 0.85% NaCl solution to give the absorbance 1.0 at 660 nm, then treated with the lysis solution under the same conditions. The cell lysates were immediately used as samples for the hybridization analysis.

Hybridization and detection.

The samples for DNA hybridization (1 μl) were spotted on to a Hybond-N+ membrane. The membrane was placed for 20 min on Whatman 3MM paper wet with 0.4 N NaOH, then washed with 75 mM citrate buffer containing 0.75 M NaCl (pH 7.0). Hybridization and chemiluminescent detection were carried out with DIG Luminescent Detection Kit as described in Chapter 2, except for the hybridization for 2 h.
Results

Amplification of an alanine racemase gene fragment.

To obtain an alanine racemase gene fragment containing the non-conserved regions of the gene from *B. psychrosaccharolyticus*, consensus primers (Fig. 16A) were designed on the basis of the two conserved sequences (Fig. 16B) of alanine racemase genes reported (Wasserman *et al.*, 1984; Ferrari *et al.*, 1985; Galakatos *et al.*, 1986; Tanizawa *et al.*, 1988; Blattner *et al.*, 1993; Lobocka *et al.*, 1994; Fleischmann *et al.*, 1995). With these primers and genomic DNA from *B. psychrosaccharolyticus* as a template, the author examined the conditions for specific amplification of the gene fragment. The size of the amplified product is predicted to be about 390 bp as judged from the distance between the two regions used for design of primers. When the primers A3 and C3 were used in the PCR, a DNA fragment with a size of about 390 bp was specifically amplified (Fig. 17). However, the PCR with other pairs of primers yielded no product or nonspecifically amplified products. When genomic DNA from *B. stearothermophilus* was used as the template, about 390 bp of DNA fragment was also specifically amplified under the same conditions with primers A3 and C3 (data not shown).
Fig. 17. Effect of primer species on the specific amplification of a gene fragment of alanine racemase from *B. psychrosaccharolyticus*. A, φX174/HaeIII digest; B, PCR with A1 and C1; C, PCR with A2 and C1; D, PCR with A1 and C2; E, PCR with A2 and C2; and F, PCR with A3 and C3.
Sequence of PCR products.

To confirm the PCR products as alanine racemase gene fragments, the nucleotide sequences of the amplified products were determined by direct sequencing. The products from *B. psychrosaccharolyticus* and *B. stearothermophilus* were composed of 395 and 398 nucleotides, respectively. The amino acid sequence of the *B. stearothermophilus* alanine racemase was recently corrected by Shaw *et al.*, (1997); the exact sequence of the gene is not reported. Therefore, the amino acid sequences predicted from the nucleotide sequences of the PCR products were compared to those of the corresponding peptides of other alanine racemases reported. As shown in Fig. 18, the conserved amino acid residues found in alanine racemases reported were also present in the peptide sequence translated from the PCR product from *B. psychrosaccharolyticus*. The amino acid sequence translated from the PCR product from *B. stearothermophilus* was identical to that between $^{41}$Lys and $^{165}$His of the *B. stearothermophilus* alanine racemase (Tanizawa *et al.*, 1988; Shaw *et al.*, 1997).

Specificity of amplified products as a probe.

Each amplified product from *B. psychrosaccharolyticus* and *B. stearothermophilus* was labeled with digoxigenin, and then analyzed as a probe for detecting the respective bacterium. A total of 69 strains (23 genera and 52 species) of bacteria (Table III) were used for analysis of the specificity of probes. Cell lysates of these bacteria were spotted on to Hybond N+ membranes for DNA hybridization. As shown in Fig. 19, each probe showed a positive signal only to the respective bacterium. When a mixed bacterial population containing all of the strains except for
Fig. 18.  Alignment of amino acid sequences translated from the nucleotide sequences of the PCR products and the corresponding sequences of seven alanine racemases from *B. subtilis* (Ferrari et al., 1985), *B. stearothermophilus* (Tanizawa et al., 1988), *E. coli* (Lobocka et al., 1994; Blattner et al., 1993), *H. influenzae* (Fleischmann et al., 1995), and *S. typhimurium* (Wasserman et al., 1984, Galakatos et al., 1986). Asterisks show the identical residues among the seven sequences of the alanine racemases reported.
the target-strain for detection was used as the sample for the hybridization, each probe did not react with the respective bacterium-free sample (Fig. 19; sample no. 70).

Detection of *B. stearothermophilus* and *B. psychrosaccharolyticus* in food samples.

The author examined the specificity of the probes for detecting each of *B. stearothermophilus* and *B. psychrosaccharolyticus* in food samples, which were naturally contaminated with $10^2$-to-$10^6$ bacterial cells per g of foods as judged by the standard plate culture method (data not shown). These food samples inoculated with *B. stearothermophilus* IFO 12550 or *B. psychrosaccharolyticus* ATCC 23296 ($10^5$ cells/g of food) were analyzed by the hybridization method. As shown in Fig. 20A and 20C, all the inoculated food samples showed positive signals with a similar intensity. Therefore, the detection method is probably not interfered with various components in the food samples surveyed. When respective uninoculated food samples were analyzed by the method, no positive signal was obtained (Fig. 20B and 20D).
Fig. 19. Specificities of the probes for detecting each of *B. psychrosaccharolyticus* (A) and *B. stearothermophilus* (B). Samples for DNA hybridization were prepared by mixing each cell suspension (the absorbance 1.0 at 660 nm) with an equal volume of a lysis solution (0.2 N NaOH and 1% SDS). Mixed bacterial populations were prepared by mixing the above cell suspensions. The strain no. panel (C) shows the place at which cell lysate of each bacterium (Table 1) was spotted. The strain no. 70 is the cell lysate of a mixed bacterial population containing all of the strains except for *B. psychrosaccharolyticus* (A; no. 70) or *B. stearothermophilus* (B; no. 70).
Fig. 20. Detection of *B. psychrosaccharolyticus* and *B. stearothermophilus* in food samples.

A, food samples inoculated with *B. psychrosaccharolyticus*; B and D, food samples not inoculated; C, food samples inoculated with *B. stearothermophilus*. A and B, hybridization with the probe for *B. psychrosaccharolyticus*; C and D, hybridization with the probe for *B. stearothermophilus*. Inoculation of the cells was $10^5$ cells/g of each food sample. Sample nos. panel shows the place at which each sample was spotted. Food samples: 1, beef; 2, pork; 3, chicken; 4, cow's milk; 5, canned fruits; 6, canned coffee; 7, canned curry soup; 8, carrot; 9, potato; 10, radish; 11, soybean; 12, tomato; 13, egg; 14, natural cheese; 15, yogurt; 16, worcester sauce; 17, ketchup; 18, soy sauce; 19, boiled rice; 20, bread. Sample no. 21 is the control strain (A and B, *B. psychrosaccharolyticus*; C and D, *B. stearothermophilus*).
Discussion

Specific DNA probes are essential for DNA hybridization to detect bacteria in foods, which contain a wide variety of components. The author evaluated gene fragments of a typical prokaryotic enzyme, alanine racemase, as probes for detecting each of *B. stearothermophilus* and *B. psychrosaccharolyticus* in foods. These bacteria sometimes cause deterioration in foods stored at high and low, respectively, temperature, whereas few report has been published on the specific and rapid detection methods of these bacteria. To obtain alanine racemase gene fragments containing the non-conserved region from these bacteria, the author designed several degenerate primers on the basis of the conserved sequences of the enzymes reported. Although the enzymes have 9-10 conserved regions distributed evenly in their entire sequences (Tanizawa *et al.*, 1988), the author chose the regions around the \(^{41}\text{Lys}\) and \(^{168}\text{His}\) residues for the design of the primers, between which the non-conserved regions are present, because of the length of continuous sequence of conserved residues and the minimization of the degeneracy. Polymerase chain reaction with a combination of primers (A3 and C3) designed in due consideration of the codon usage of alanine racemase genes was found to produce a specifically amplified product from each genomic DNA of *B. psychrosaccharolyticus* and *B. stearothermophilus*. Primer A1 containing large numbers of inosine molecules and primer C2 with high degeneracy were unsuitable for specific amplification of the gene fragment.

Each PCR product was confirmed as a gene fragment of alanine racemase by DNA sequencing and translation of the nucleotide sequences obtained. The conserved amino acid residues present in the enzymes
reported were also found in the translated sequences of the PCR products from *B. stearothermophilus* and *B. psychrosaccharolyticus*. These results suggest that the PCR products are alanine racemase gene fragments.

Two types of alanine racemases are reported to be present in *S. typhimurium* (Wasserman *et al.*, 1984; Esaki and Walsh 1986) and *E. coli* (Lobocka *et al.*, 1994; Blattner *et al.*, 1993). If two isozyme genes are present in the bacteria examined, two amplified products from the two genes may be produced by the PCR method. This PCR method produced one gene fragment of alanine racemase from the respective bacterium as judged from the DNA sequencing. *B. stearothermophilus* and *B. psychrosaccharolyticus* may have only one type of the enzyme gene.

The author analyzed the specificity of the amplified gene fragment as a probe for detecting the respective bacterium. These fragments show 41-58% of sequence homology with gene fragments of other alanine racemases, when analyzed by the Blastn program with the GenBank DNA database. Each fragment was labeled with digoxigenin and analyzed. Each probe showed a positive signal only to the respective bacterium by the dot blot hybridization. Caccone *et al.*, (1988) reported that a 1.7% mismatch of base pairs in hybrid DNA decreased its $T_m$ value by 1°C. Therefore, these probes having 41-58% homologies with other alanine racemase genes reported appear to be specific to the respective bacterium at the level of organisms tested.

*B. stearothermophilus* or *B. psychrosaccharolyticus* mixed with various food samples could be specifically detected by this method, while food samples uninoculated with the bacteria did not show positive signals. Alanine racemase is a typical bacterial enzyme, and is believed to be absent in other organisms, except for one fungus (Hoffmann *et al.*, 1994).
A gene fragment of alanine racemase thus appears to be useful for detecting each of *B. stearothermophilus* and *B. psychrosaccharolyticus* in a wide variety of foods.

Amplified products with the size of about 390 bp were also obtained by the PCR with primers A3 and C3 from other bacteria such as *B. subtilis*, *Enterobacter cloacae*, *E. coli*, *P. fluorescens*, *Serratia marcescens*, *S. aureus*, and *Bacillus psychrophilus*, whereas no PCR product was obtained from genomic DNA of eukaryotes (bakers' yeast and *Aspergillus niger*) and sperm DNA from herring and salmon (data not shown). Although the amplification from cDNA of these eukaryotes was not examined, these results consist with the general acceptance that alanine racemase is a typical prokaryotic enzyme. These primers (A3 and C3) may be useful for amplifying the gene fragment of alanine racemase from various bacteria at the level of the organisms tested.

The author here described the amplification of gene fragments of alanine racemases and the specificity of each amplified product as a probe. Alanine racemase gene fragments containing the non-conserved regions of the gene may be useful for detecting each of *B. stearothermophilus* and *B. psychrosaccharolyticus* in a wide variety of foods.

**SUMMARY**

Alanine racemase gene fragments containing non-conserved regions of the gene were evaluated as probes for detecting each of *B. stearothermophilus* and *B. psychrosaccharolyticus* in foods. The gene fragments were amplified from genomic DNA of the respective bacterium by polymerase chain reaction with degenerate oligonucleotide primers, and labeled with digoxigenin as probes for detecting each bacterium. Foods
and bacteria were each treated at 25°C for 10 min in 0.1 N NaOH containing 0.5% SDS, before being directly spotted on to nylon membranes for DNA hybridization. When the specificities of the probes were analyzed with a total of 69 strains (23 genera and 52 species) of bacteria, each probe was specific to the respective bacterium. Various foods inoculated with each of *B. stearothermophilus* and *B. psychosaccharolyticus* showed positive signals by the hybridization with the respective probe, whereas no uninoculated foods showed any signal. An alanine racemase gene may be a useful index for detecting these bacteria.
CHAPTER 4

Evaluation of an Alanine Racemase Gene as an Indicator for the Detection of Various *Escherichia coli*

Section 1. An Alanine Racemase Gene As a New Index For Detecting *Escherichia Coli* In Foods

INTRODUCTION

*E. coli* is an important indicator of fecal contamination in foods, the most common method for detecting and quantifying *E. coli* currently being the most probable number (MPN) method (Food and Drug Administration, 1995a). This method identifies *E. coli* on the basis of its ability to ferment lactose with the evolution of gas. Since various non-*E. coli* species also ferment lactose, the MPN method needs an enriched culture of *E. coli*. Thus, the method requires four to seven days for completion. In addition, the method is often inaccurate (the false result rate is about 23%) (Hsu *et al.*, 1991). Therefore, a more accurate and rapid method for detecting *E. coli* is needed.

Considerable effort has been invested in the development of accurate and rapid methods for detecting and identifying *E. coli*. Enzymatic methods based upon the activity of β-D-glucuronidase (Ratnam *et al.*, 1988; Frampton *et al.*, 1993) have been well studied, and a simple method for detecting *E. coli* on membrane filters based upon the enzyme activity has also been reported (Shadix *et al.*, 1993). However, enterohaemorrhagic pathogens belonging to the *E. coli* O157:H7 strain do not possess this
enzyme (Ratnam et al., 1988), although 94-97% of E. coli strains have shown the enzyme activity (Frampton et al., 1993). In addition, some Shigella, Salmonella and Yersinia species also produce the enzyme, and therefore yield false positive results (Feng et al., 1982; Kilian et al., 1979; Kampfer et al., 1991).

Numerous rapid and simple DNA-hybridization methods with DNA probes based on gene-encoding virulence factors have been reported for detecting pathogenic E. coli (Rademaker et al., 1992; Szewzyk et al., 1994; Tamanai-Shacoori et al., 1994). However, few reports have been published on the detection of various E. coli strains including both pathogenic and non-pathogenic types. Although a hybridization method for detecting various E. coli strains with oligonucleotide probes specific for their 16S ribosomal RNA has been reported (Hsu et al., 1991), the sensitivity of the method was insufficient with a detection limit of 5 \times 10^5 cells/ml. Therefore, selective enrichment of the E. coli culture was necessary (Hsu et al., 1991). In addition, the amount of ribosomal RNA in E. coli cells is dependent on the number of ribosomes and, therefore, on their physiological state. Until now, little information has been reported on other RNA and DNA probes for detecting various E. coli strains.

Alanine racemase (EC 5.1.1.1) is believed to be distributed only in bacteria, with exception of one fungus (Hoffmann et al., 1994), and provides D-alanine that is essential for synthesis of the peptidoglycan of the bacterial cell wall. Therefore, all E. coli strains are considered to have this enzyme gene, while the gene is probably absent in food materials. Several genes of the enzyme have been cloned, and their DNA sequences analyzed (Ferrari et al., 1985; Galakatos et al., 1986; Inagaki et al., 1986; Tanizawa et al., 1988; Wasserman et al., 1984). The sequences show
9-10 conserved regions distributed evenly in the genes (Tanizawa et al., 1988). However, the other regions are not conserved. In consequence, a gene fragment containing the non-conserved regions of the \textit{E. coli} alanine racemase gene is considered to be a powerful probe for detecting \textit{E. coli}.

The author describes here the amplification of a fragment of the \textit{E. coli} alanine racemase gene, and application of the fragment labeled with digoxigenin (DIG) to detect \textit{E. coli} in foods. The gene fragment of alanine racemase could prove useful for detecting \textit{E. coli}.

\textbf{MATERIALS AND METHODS}

\textit{Materials}.

A DIG DNA-labeling mixture and DIG luminescent detection kit were obtained from Boehringer Mannheim, while the nylon membrane (Hybond-N+) was from Amersham. All other chemicals were of analytical grade.

\textit{Primers}.

A gene fragment containing the non-conserved regions of alanine racemase was prepared by the polymerase chain reaction (PCR) with genomic DNA of \textit{E. coli} JM109 as the template and degenerate oligonucleotide primers. The primers were designed from the consensus sequences (Tanizawa et al., 1988) of known alanine racemases around the active-site Lys residue and the His residue located about 130 amino acids downstream from the active site, between which the non-conserved regions are present. The sequences of the primers were 5'-
GCGGAATTCAARGCNAAYGCNTAYGGNCA as the sense primer and 5'-TCCAAGCTTTTCIGCIBKIGCRAARTG as the antisense primer, in which I is inosine and the other symbols are the single-letter codes recommended by International Union of Biochemistry (Nomenclature Committee of International Union of Biochemistry, 1985). An EcoRI or HindIII restriction site was added to the 5' ends of each primer to allow cloning and subsequent sequencing. Three additional bases were added to the 5' ends to facilitate digestion with the restriction enzymes after the amplified products had been isolated. Inosine in the antisense primer was used to minimize degeneracy, no amplified product being obtained without the use of inosine. These primers were synthesized and purified by Funakoshi Co., Tokyo.

**PCR.**

PCR (40 cycles) was performed as described in Chapter 2. The resulting PCR product was electrophoresed in 3.5% NuSieve GTG agarose (FMC), stained with ethidium bromide, and photographed under ultraviolet light.

**DNA sequencing.**

The PCR product in the agarose gel was purified with the Easy Prep and PCR Product Prep kit (Pharmacia), digested with EcoRI and HindIII, and ligated into the linearized pUC18 plasmid vector. The resulting ligated mixture was used directly for transforming *E. coli* JM109. Plasmid DNA was prepared from one of the transformants, and double-strand sequencing of the plasmid obtained (pEC389) was performed by the dideoxynucleotide chain termination method with the Dye Deoxy
Terminator Cycle Sequencing kit and ABI 373A DNA sequencer (Applied Biosystems).

**Probe.**

The digoxigenin-labeled gene fragment of alanine racemase was prepared as described in Chapter 2, except that genomic DNA and dNTP were replaced with 10 ng of pEC389 and the DIG DNA-labeling mixture, respectively.

**Bacteria and media.**

Pathogenic strains of *E. coli* and *Shigella sonnei* were kindly presented by Nara Prefectural Institute of Hygiene, Japan. The other strains were obtained from Institute for Fermentation, Osaka (IFO), Institute for Applied Microbiology, Tokyo University (IAM), Northern Regional Research Center, Peoria, Ill. (NRRL), and American Type Culture Collection (ATCC). The *E. coli* and non-*E. coli* species used in this study are listed in Tables IV and V, respectively. *E. coli* IFO 14605 was cultured at 28°C in an LB medium (1% Tryptone, 0.5% NaCl, and 0.5% yeast extract at pH 7.0) supplemented with 0.1 μg/ml of N-acetylglucosamine; *E. coli* IFO 14360 was cultured at 28°C in an LB medium supplemented with 40 μg/ml of ampicillin; and *S. typhimurium* IFO 14209 was cultured at 37°C in a medium containing 0.3% meat extract, 0.5% peptone, and 0.5% NaCl at pH 6.8. All the other bacteria were cultured at 28°C in the LB medium.
Table IV.  List of *E. coli* strains

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<td></td>
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<td>Xanthomonas campestris IAM 1671</td>
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Preparation of samples for hybridization.

Solid food samples were prepared as described in Chapter 3. When the specificity of the probe was examined, various bacterial cells were treated with the alkaline-SDS solution under the same conditions. The cell lysates were immediately used as samples for the hybridization analysis.

Detection and quantification of E. coli in the death phase.

E. coli IFO 3301 was aerobically cultivated at 28°C in 100 ml of the LB medium, and 3-ml samples were taken at various cultivation times (48, 60, 72, 84 and 96 h). The number of living cells of E. coli in each sample was analyzed by the plate culture method with an eosin-methylene blue agar medium (EMB agar; Nissui Co., Tokyo). The absorbance at 660 nm of each sample was measured after being appropriately diluted with 0.85% NaCl. For the hybridization analysis, each sample was incubated at 25°C for 10 min with an equal amount of the alkaline-SDS solution as already described.

Hybridization and detection.

Hybridization and chemiluminescent detection were carried out with a DIG luminescent-detection kit. Each sample (1-2 µl) was spotted on a Hybond-N+ membrane. The membrane was placed on Whatman 3MM paper wetted with 0.4 N NaOH for 20 min, and then washed at 25°C for 5 min in a 75 mM citrate buffer containing 0.75 M NaCl (pH 7.0) while agitating. Hybridization and chemiluminescent detection were carried out with DIG luminescent detection kit as described in Chapter 2.
Results

Preparation of the probe.

To obtain a gene fragment of alanine racemase from *E. coli*, the author performed PCR with genomic DNA from *E. coli* and the degenerate oligonucleotide primers already described. As shown in Fig. 21, a DNA fragment with a size of about 390 bp was specifically amplified. The amplified product was ligated into pUC18, and then the ligation mixture was directly used for the transformation of *E. coli* JM109. Plasmid pEC389 was obtained from one of the transformants and used for double-stranded DNA sequencing of the inserted DNA fragment. The inserted DNA was composed of 389 nucleotides, and the sequence was identical to that between positions 103 and 491 (position 1 corresponds to the first position of the initiation codon) of *E. coli* catabolic alanine racemase gene *dadX* (Lobocka et al., 1994). The amino acid sequence predicted from the nucleotide sequence was compared with those of the corresponding peptides of other alanine racemases from *B. subtilis* (Ferrari et al., 1985), *B. stearothermophilus* (Tanizawa et al., 1988), *S. typhimurium* (Galakatos., 1986; Wasserman., 1984), and *H. influenzae* (Fleischmann., 1995), (Fig. 22). Although the peptide sequences have conserved regions from 41 Lys to 48 Gly, from 69 Leu to 87 Leu, from 135 Gly to 140 Gly, and from 168 His to 172 Ala, the other 77% of the residues were non-conserved. Therefore, the PCR product was labeled with digoxigenin as already described, and then used as the probe for detecting *E. coli*. The apparent size of the probe was increased to 460 bp by labeling (Fig. 21).
Fig. 21. Agarose gel electrophoresis of the amplified product (A) and the digoxigenin-labeled gene fragment (D). Lane M: size marker, φX174/HaeIII digest.
Fig. 22. Comparison of the peptide sequence predicted from the nucleotide sequence of the probe with alanine racemases in the region from \(^{41}\)Lys to \(^{112}\)Ala.

The five sequences were aligned by introducing gaps (hyphens) to maximize the identity. Identical residues among the five sequences are marked with an asterisk. Residue numbers are given as reported by Tanizawa et al. (1988).
Specificity of the probe.

The author examined the specificity of the digoxigenin-labeled gene fragment of alanine racemase for detecting *E. coli*. A total of 48 strains of *E. coli*, including enteropathogenic, enteroinvasive, enterohemorrhagic and enterotoxigenic strains (Table IV), and a total of 59 strains of non-*E. coli* species (Table V) were used for the hybridization analysis. All strains of *E. coli*, including the pathogenic ones, showed positive signals (Fig. 23A), whereas all the non-*E. coli* species, except *Shigella sonnei*, were negative when tested by this method (Fig. 23B).

Quantification of *E. coli*.

The cell suspension of *E. coli IFO 3301* (optical density of 1.0 at 660 nm, corresponding to $1.6 \times 10^9$ cells/ml) was serially diluted in 0.85% NaCl and treated with alkaline-SDS as already described. The solutions obtained were analyzed by the hybridization method. Figure 24 shows the relationship between the cell number and the signal intensity on the X-ray films. The intensity at 460 nm increased with increasing number of *E. coli* cells in the range from 1 to $10^6$ cells per spot.

Detection and quantification of *E. coli* in the death phase.

To examine whether *E. coli* cells in the death phase could be precisely enumerated by the hybridization method, the author analyzed the number of *E. coli IFO 3301* cells in the death phase by the hybridization method, by monitoring the absorbance at 660 nm, and by the plate culture method (Fig. 25A). On the basis of these data, the author examined the correlation between the hybridization method and the plate culture method: as shown in Fig. 25B, the signal intensity by the hybridization method was
linearly correlated to the number of living cells determined by the plate culture method.

Fig. 23. Reactivity of the probe with cell lysates of *E. coli* (A) and non-*E. coli* species (B). The strain no. panel under each photograph shows the places at which cell lysate of each bacterium (Tables IV and V) was spotted.
Fig. 24. Densitometric quantification of *E. coli* (IFO 3301) cells. Vertical bars show the standard error, and points are the means from three measurements.

Fig. 25. Detection and quantification of *E. coli* in the death phase. A: growth of *E. coli* IFO 3301 analyzed by the hybridization method, by monitoring absorbance at 660 nm, and by the plate culture method. B: correlation between the hybridization method and the plate culture method for quantifying *E. coli*.
Detection of *E. coli* in food samples.

A variety of food samples inoculated with *E. coli* IFO 3301 \(10^5\) cells/g of food) were analyzed by the hybridization assay. As shown in Fig. 26A, all the inoculated food samples showed positive signals with a similar intensity. Therefore, the detection method was probably not interfered with various components in the food samples surveyed. When respective uninoculated food samples were analyzed by the detection method, no positive signal was obtained (Fig. 26B).

![Fig. 26. Detection of *E. coli* in food samples. A, food samples inoculated with *E. coli* IFO 3301; B, food samples not inoculated. Inoculation of the cells was at \(10^5\) cells/g of each food sample. Food samples: a1, beef; a2, boiled rice; a3, radish; a4, croquette; a5, miso; b1, pork; b2, bread; b3, soybean; b4, curry soup; b5, sake; c1, chicken; c2, bean jam; c3, frozen vegetable; c4, ketchup; d1, milk; d2, potato; d3, canned fruits; d4, worcester sauce; e1, egg; e2, carrot; e3, konbu; e4, soy sauce.](image-url)
Discussion

To evaluate the *E. coli* alanine racemase gene as a new index for detecting *E. coli*, the author amplified a fragment of the gene containing the non-conserved regions from genomic DNA of *E. coli*. The amplified product was confirmed to be a fragment of the *E. coli* catabolic alanine racemase gene (\textit{dadX}) by DNA sequencing. Although two alanine racemase genes \{\textit{dadX} (Lobocka \textit{et al.}, 1994) and \textit{alr} (Blattner \textit{et al.}, 1993), differing in their structure and function\} are present in *E. coli*, the amplified product was confirmed to be a fragment of the *E. coli* \textit{dadX} gene. This gene fragment was labeled with DIG, and used as a probe for detecting *E. coli*.

The specificity of the DIG-labeled probe was analyzed with 48 strains of *E. coli* and 59 strains of non-*E. coli* species (22 genera, 42 species, and 59 strains). All the *E. coli* strains, including such pathogenic strains as *E. coli* O157, showed a positive signal. Although *S. sonnei* showed a false positive signal, the other non-*E. coli* species, including *S. typhimurium*, showed a negative signal by the hybridization procedure. When the nucleotide sequence of the probe was analyzed by the Blastn program with the GenBank DNA database, *S. typhimurium* \textit{dadB} showed the highest sequence homology (79\%) with that of the probe. Caccone \textit{et al.} have reported (Caccone \textit{et al.}, 1988) that a 1.7\% mismatch of base pairs in hybrid DNA decreased its $T_m$ value by 1°C. Therefore, hybrid DNA of \textit{dadB} and the probe with a 21\% mismatch of base pairs was significantly thermolabile compared with hybrid DNA of the probe and *E. coli* \textit{dadX} under these stringent conditions for hybridization.

The false-positive result rate for this hybridization method was
calculated to be 1.7%, while no false-negative signal was obtained. Hsu et al. have examined the specificity of oligonucleotide probes designed from 16S ribosomal RNA of *E. coli* toward 233 strains of *E. coli* and 214 strains (26 genera and 97 species) of non-*E. coli* species, and identified the false-negative and false-positive result rates of 1.2% and 17%, respectively (Hsu et al., 1991). Although the genera and species of bacteria used in our study are not identical to those used in their study, the gene fragment of alanine racemase from *E. coli* appears to be specific to *E. coli* at the level of the organisms tested.

The lower detection limit for this hybridization method (1-10 cells/spot) shows that it is more sensitive than the method by Hsu et al. (1991) (2 x 10^5 cells/ml). This difference in sensitivity between the two methods may be due to the number of molecules of the labeling compounds introduced into the probe. The Hsu et al. oligonucleotide probe has been reported to be labeled with only two molecules of fluorescein isothiocyanate. However, on the basis of the sizes of these labeled (about 460 bp) and unlabeled (about 390 bp) gene fragments as measured by agarose gel electrophoresis, about 100 molecules of DIG (mol. wt. of about 390) were probably introduced into this probe.

When measuring the number of *E. coli* cells in the death phase, this hybridization method showed good correlation with the plate culture method. The alanine racemase gene from the dead and lysed cells may have been digested by intracellular nucleases, so that this hybridization method may only detect the gene from the living cells.

The MPN method and the other hybridization method reported (Hsu et al., 1991) both need an enriched culture of *E. coli*. Therefore, these methods are time-consuming. Although the enzymatic detection method
for *E. coli* is rapid in use, false-positive and false-negative results still require consideration (Ratnam *et al.*, 1988). This method for detecting *E. coli* required two days to complete. However, when the hybridization time was reduced by 2 h by using a high concentration of the probe (500 pg/ml), the detection and quantification of *E. coli* could be completed within less than 1 day.

*E. coli* mixed with various food samples could be specifically detected by this method, while food samples not inoculated with *E. coli* did not show positive signals. Alanine racemase is a bacterial enzyme, and is believed to be absent in other organisms, except for one fungus (Hoffmann *et al.*, 1994). A gene fragment of alanine racemase thus appears to be suitable for detecting *E. coli* in a wide variety of foods.

The author designed the primers from the consensus sequences (Tanizawa *et al.*, 1988) of known alanine racemases. Therefore, PCR with these primers can be expected to amplify an alanine racemase gene fragment from not only *E. coli*, but also from other bacteria. Alanine racemase gene fragments from other enteric bacteria such as *E. cloacae* were also amplified by PCR under similar conditions (data not shown). The general application of primers for the amplification of an alanine racemase gene fragment from various bacteria is now under investigation.

The author have described here a new index, an alanine racemase gene, to detect *E. coli* in foods. The detection of *E. coli* with the gene fragment containing the non-conserved region of the alanine racemase gene is specific to *E. coli*. This gene fragment of alanine racemase is thus likely to be useful as a probe for detecting *E. coli* in foods.
SUMMARY

A gene of alanine racemase, a typical prokaryotic enzyme, was evaluated as a new index for detecting *E. coli* in foods. An alanine racemase gene fragment containing a non-conserved sequence of the gene was amplified from genomic DNA of *E. coli* by a polymerase chain reaction, and then labeled with digoxigenin as a probe for detecting *E. coli*. Food samples and bacteria were each treated at 25°C for 10 min in 0.1 N NaOH containing 0.5% SDS, before being directly spotted on to nylon membranes for DNA hybridization. The probe was specific for *E. coli*; all 48 strains of *E. coli* examined, including such pathogenic strains as *E. coli* O157:H7, showed positive signals, whereas all 59 strains of non-*E. coli* species, except for one strain (*S. sonnei*), did not show a signal. Various foods inoculated with *E. coli* K-12 showed positive signals, whereas no uninoculated foods showed any signal. Quantification of *E. coli* cells in the death phase by the hybridization method showed good correlation with that by the plate culture method. The alanine racemase gene could prove useful as an index for detecting *E. coli* in foods.
Section 2. Reactivity of the Gene Fragment with Various E. coli O157:H7 Isolates

INTRODUCTION

E. coli is an important indicator of fecal contamination of foods. The most common method for detecting E. coli is the most probable number (MPN) method (Food and Drug Administration., 1995a). However, this method is time-consuming and often inaccurate (Hsu et al., 1991). In addition, the growth of some E. coli isolates, including the serotype O157:H7, is inhibited by bile salts No. 3 (Difco, Detroit) of the EC broth (2% peptone, 0.5% lactose, 0.15% bile salts No. 3, 0.4% K₂HPO₄, 0.15% KH₂PO₄ and 0.5% NaCl, pH 6.9) at 45.5°C (Okrend et al., 1990).

Although a simple enzymatic method for detection of E. coli based upon β-glucuronidase activity is widely used, the method is reported not to detect E. coli O157:H7 strains, because most of these strains do not possess the enzyme (Ratnam et al., 1988). E. coli O157:H7 is a human pathogen causing hemorrhagic colitis and hemolytic uremic syndrome. Therefore, this strain should be detectable by E. coli detection tests.

Alanine racemase (EC 5.1.1.1) provides D-alanine that is essential for the synthesis of peptidoglycan of the bacterial cell walls, and therefore is a typical prokaryotic enzyme. In section 1, the author reported that an E. coli alanine racemase gene (dadX) is a useful indicator for the detection of E. coli in foods; an alanine racemase gene fragment labeled with digoxigenin (DIG) is a useful probe for the detection of E. coli (Yokoigawa et al., 1996). However, various subtypes of E. coli O157:H7 isolates have been reported. With respect to the production of
verocytotoxins associated with human disease, two subtypes of \( E. coli \) O157:H7 isolates have been reported (Heuvelink et al., 1995). One produces both types of verocytotoxin, verocytotoxin 1 (VT1, Shiga-like toxin I) and verocytotoxin 2 (VT2, Shiga-like toxin II). The other produces only VT2. Subtypes of \( E. coli \) O157:H7 isolates have also been reported based on genomic profile analysis (Meng et al., 1995), plasmid profile analysis (Ostroff et al., 1989), multilocus enzyme electrophoresis (Whittam et al., 1988), antimicrobial susceptibility tests (Swerdlow et al., 1992), and bacteriophage typing (Ahmed et al., 1987). Thus, \( E. coli \) O157:H7 isolates are genetically diverse. The level of genetic diversity in \( E. coli \) is reported to vary depended on genes (Selander et al., 1980). If many polymorphic nucleotides are present in the alanine racemase genes of \( E. coli \) O157:H7 isolates, this DNA hybridization method which involves the use of an alanine racemase gene fragment as the probe for the detection of \( E. coli \) may overlook some \( E. coli \) O157:H7 isolates. In this section, the author describes the isolation of \( E. coli \) O157:H7 strains, their VT productivity, and hybridization of their DNA with an \( E. coli \) alanine racemase gene fragment.

**MATERIALS AND METHODS**

Antisera against various \( E. coli \) O and H antigens were obtained from Denka Seiken Co., Tokyo. Primers for the amplification of a fragment of VT1 or VT2 genes were purchased from Takara, Kyoto. An \( E. coli \) verocytotoxin detection kit was purchased from Denka Seiken Co., Tokyo. The DIG-labeled fragment of an \( E. coli \) alanine racemase gene (\( badX \)) was prepared by the polymerase chain reaction (PCR) as described in Chapter 2.
A DIG luminescent detection kit was obtained from Boehringer Mannheim, while the nylon membrane (Hybond N+) was from Amersham. All other chemicals were of analytical grade.

*E. coli* O157:H7 strains were isolated from diverse geographic locations in Nara Prefecture, Japan, between 1994 and 1996. *E. coli* K12 and *S. typhimurium* were obtained from Institute for Fermentation, Osaka (IFO), and used as positive and negative control strains, respectively, in the DNA hybridization experiment.

The verocytotoxin productivity of each isolate was determined by a latex agglutination test with the *E. coli* verocytotoxin detection kit. Each isolate was aerobically cultivated at 37°C for 20 h in CAYE medium containing 2% Casamino acids, 0.6% yeast extract, 0.25% NaCl, 0.87% K₂HPO₄, 50 ppm MgSO₄, 5 ppm MnCl₂ and 5 ppm FeCl₃ (pH 8.5). After centrifugation at 5,000 x g for 30 min, the supernatant solution was used for analysis of verocytotoxins.

The verocytotoxin genes of each isolate were analyzed by the PCR. Each isolate was aerobically cultivated at 37°C for 18 h in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 0.1% glucose, pH 7.2). After centrifugation at 5,000 x g for 15 min, precipitated cells were suspended in an appropriate volume of distilled water. The suspensions were boiled for 5 min, and 2 μl aliquots of the suspensions were used as templates for the amplification of fragments of the verocytotoxin genes. The PCR (25 cycles) was performed using a PC-700 programmed temperature control system (Astec Co., Fukuoka). Each PCR cycle involved denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The standard reaction mixture contained 2.5 units of *Taq* DNA polymerase (Perkin Elmer), 20 nmol of each dNTP
(deoxyribonucleoside triphosphate), 50 mM KCl, 10 mM Tris-HCl (pH 8.5), 1.5 mM MgCl₂, 0.01% gelatin, 10 pmol of each primer and template DNA in a final volume of 100 ml. The resulting PCR products were analyzed by electrophoresis on 3.5% NuSieve GTG agarose gels (FMC BioProducts, Rockland).

RESULTS AND DISCUSSION

After the isolation of *E. coli* strains that cannot ferment sorbitol (March *et al.*, 1986), their serotypes were determined using antisera against various *E. coli* O and H antigens. A total of 100 *E. coli* O157:H7 strains were isolated. These included ten isolates from the feces of beef cattles, one isolate from a leg of beef, and 89 isolates from the feces of human patients with diarrhea (Table VI). Although many of the strains from the feces of human patients were isolated during the same month, this was not due to mass food poisoning, but to sporadic food poisoning. When the author analyzed the DNA of several of the strains isolated in July 1996 by pulsed-field gel electrophoresis, each isolate showed a distinct genomic profile (Fig. 27). This result suggests that the strains isolated during the same month were genetically diverse. The author used all of the isolates for further analyses.

The isolates were analyzed by the PCR using primers specific to the VT1 or VT2 gene and by a latex agglutination test using the *E. coli* verocytotoxin detection kit. As shown in Table VI, four isolates (nos. 61-63, and 74) produced only VT2, the gene for which was detected by the PCR. Although neither VT1 nor VT2 was detected in one isolate (no. 1) under the conditions used, the VT2 gene was detected in this isolate. The
Table VI. List of *E. coli* O157:H7

<table>
<thead>
<tr>
<th>No.</th>
<th>Source</th>
<th>Date isolated</th>
<th>Vero-cytotoxin</th>
<th>No. Occurrence</th>
<th>Date isolated</th>
<th>Vero-cytotoxin</th>
</tr>
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<tr>
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<td>A leg of beef</td>
<td>Sep., 1996</td>
<td>nd(VT2)*</td>
<td>51 Feces from human patient</td>
<td>Sep., 1994</td>
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<td>Jun., 1996</td>
<td>VT2</td>
</tr>
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<td>Feces from beef cattle</td>
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<td>Jun., 1996</td>
<td>VT2</td>
</tr>
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<td>VT2</td>
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<td>Jul., 1996</td>
<td>VT1, VT2</td>
</tr>
</tbody>
</table>

* Although neither VT1 nor VT2 was detected in the isolate, the VT2 gene was detected.
Fig. 27. Pulsed-field gel electrophoresis of the DNA of several *E. coli* O157:H7 strains isolated during the same month (July, 1996). *XbaI*-digested genomic DNAs from several *E. coli* O157:H7 isolates were prepared by the method of Meng et al. (Meng *et al.*, 1995). Electrophoresis was performed using a contour-clamped homogeneous electric field device (CHEF DRIII; Nippon Bio-Rad Laboratories, Osaka) under conditions suitable for *E. coli* as recommended by the manufacturer. Lane A contains size markers (Lambda concatemer ladder; Promega, Madison). Lanes B, C, D, and E correspond to strain nos. 97, 98, 99 and 100, respectively.
amounts of VT2 produced by isolate no. 1 may be below the detection limit of the *E. coli* verocytotoxin detection kit (1 ng/ml). It is unclear at present whether the isolate from the leg of beef is pathogenic to humans. The other 94 strains produced both VT1 and VT2, and the genes for these toxins were also detected. These isolates were classified into two types of *E. coli* O157:H7 strains based on the types of verocytotoxin produced. Heuvelink *et al.* (Heuvelink *et al.*, 1995) reported that 85% of *E. coli* O157 strains isolated in the Netherlands, Belgium and Germany between 1989 and 1993 produced only VT2, and 15% produced both VT1 and VT2. The ratio of VT2-producing to VT1/VT2-producing *E. coli* O157:H7 isolates may depend on the place and time at which strains are isolated.

To determine whether a DIG-labeled gene fragment of an *E. coli* alanine racemase can be used to detect all of the *E. coli* O157:H7 isolates, the author investigated the hybridization of the probe with DNA isolated from the *E. coli* O157:H7 isolates using a dot blot hybridization method. *S. typhimurium* was used as a negative control because the *S. typhimurium* alanine racemase gene (*dadB*) is highly homologous to the *E. coli* alanine racemase gene (*dadX*) as determined using the Blastn program and the GenBank DNA database. As shown in Fig. 28, all isolates exhibited positive signals, although the signal intensity varied depending on the strains. The negative control did not exhibit a signal. The differences in signal intensities between various *E. coli* O157:H7 isolates may be due to nucleotide polymorphisms between the alanine racemase genes of the isolates. Generally, the number of nucleotide polymorphisms varies depending on genes (Selander *et al.*, 1980); the proline permease gene (1467 bp) and glyceraldehyde-3-phosphate dehydrogenase gene (924 bp) from 13 strains of *E. coli* have 216 and 12,
Fig. 28. Reactivity of the probe with cell lysates of E. coli O157:H7 isolates. The isolates were aerobically cultivated at 37°C for 16 h in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.0). The cells were collected by centrifugation and suspended in a lysis solution (1% NaCl, 1% Tween 20, and 1 mM EDTA-2Na) to give an absorbance of 1.0 at 660 nm. The cell suspensions were incubated at 100°C for 10 min, and then 1 μl of each cell lysate was directly spotted onto nylon membranes for DNA hybridization. Hybridization and chemiluminescent detection were carried out using a DIG luminescent detection kit as described previously (Yokoigawa et al., 1996). The strain no. panel under the photograph shows the place at which each lysate (Table 1) was spotted. The positive control (PC) was E. coli K12 (IFO 3301), and the negative control (NC) was S. typhimurium IFO 14209.
respectively, polymorphic nucleotides (Nelson et al., 1992; Nelson et al., 1991). Since the *S. typhimurium* *dadB* gene having 79% homology with this probe was not detected, some *E. coli* alanine racemase genes having less than 79% homology with this probe may not be detected by this hybridization method. However, this probe appears to detect alanine racemase genes from various *E. coli* O157:H7 isolates irrespective of the nucleotide polymorphisms of the genes at the level of the isolates tested.

The author have described here the isolation of *E. coli* O157:H7 strains, their VT productivity, and the hybridization of their DNA with an *E. coli* alanine racemase gene fragment. The gene fragment labeled with DIG was shown to hybridize with DNAs from various *E. coli* O157:H7 isolates. This probe for the detection of *E. coli* is considered to detect *E. coli* O157:H7 isolates which differ in the types of verocytotoxin they produce.

**SUMMARY**

The author determined whether various *E. coli* O157:H7 isolates could be detected using a probe, an alanine racemase gene (*dadX*) fragment, specific to *E. coli*. One hundred strains of *E. coli* O157:H7 were isolated from fecal samples of healthy cattle and human patients with diarrhea and from a leg of beef. Most isolates (95 strains) produced both verocytotoxin 1 (VT1, Shiga-like toxin I) and verocytotoxin 2 (VT2, Shiga-like toxin II), whereas four isolates produced only VT2. Although neither VT1 nor VT2 was detected in one isolate under the conditions used, the VT2 gene was detected. When all isolates were analyzed by a DNA hybridization method involving use of the probe, positive signals were obtained from all isolates. The DNA hybridization method used for detection of *E. coli* is considered to detect various *E. coli* O157:H7 isolates
which differ in the types of verocytotoxin they produce.
CONCLUSION

In this thesis, the author characterized psychrotrophic alanine racemases and evaluated the gene as an indicator for detection of bacteria.

In chapter 1, the author have described the lability of the thermolabile alanine racemase from a typical psychrotroph, *P. fluorescens*. The thermolabile enzyme was easily inactivated by heat-treating at over 30°C and by incubating with low concentration of organic solvents and denaturants. These treatments also suppressed the growth of the psychrotroph. Analysis of the denaturation process of the enzyme suggest that PLP plays an important role in maintaining the secondary structure of the thermolabile enzyme.

In chapter 2, the author cloned and expressed the psychrotrophic alanine racemase gene from *B. psychrosaccharolyticus* into *E. coli* SOLR. The plasmid pYOK3 purified from the transformant contains a single complete open reading frame (*alr*) preceded by a ribosome-binding site. The 5' region in front of the *alr* gene includes a 5'-truncated open reading frame, which is homologous with *ydcC* gene of *B. subtilis*. The *alr* gene consists of the 1149 base pairs with the unusual initiation codon GTG. The GC content of the gene is lower than those of the genes from a thermophile, *Bacillus stearothermophilus*, and a mesophile, *Bacillus subtilis*. In particular, the *B. psychrosaccharolyticus* gene shows a low preference for G and C in the third position of the codons. The predicted tertiary structure of the psychrotrophic enzyme is considered to be less homologous with that of the thermophilic enzyme than that of the mesophilic enzyme, as judged from the rms difference.

The author purified to homogeneity the alanine racemase from the
cell extract of a clone carrying the plasmid pYOK3 (6.3kbp) and characterized. The enzyme from \textit{B. psychrosaccharolyticus} shows a high catalytic activity even at 0°C. The thermostable enzyme from \textit{B. stearothermophilus} is catalytically inert at 0°C. Although the activity of the enzyme from mesophilic bacteria at 0°C has not been reported, the author observed that the cell free extracts of \textit{B. subtilis} and \textit{E. coli} showed no alanine racemase activity at 0°C. Thus, the enzyme from \textit{B. psychrosaccharolyticus} is psychrotrophic. The absorption spectra and kinetic parameters of the psychrotrophic enzyme are similar to those of other well-characterized alanine racemases. A noteworthy difference was found in $K_m$ value for PLP. The enzyme probably loses PLP during the racemization of the substrate at high temperature because of the low affinity for PLP. Dissociation of PLP from the enzyme protein may be related to the change of the tertiary structure followed by the inactivation.

In chapter 3, the author evaluated gene fragments of a typical prokaryotic enzyme, alanine racemase, as probes for detecting each of \textit{B. stearothermophilus} and \textit{B. psychrosaccharolyticus} in foods. Each fragment was labeled with digoxigenin and used as probes. Each probe showed a positive signal only to the respective bacterium by the dot blot hybridization. \textit{B. stearothermophilus} or \textit{B. psychrosaccharolyticus} mixed with various food samples could be specifically detected by this method, while food samples uninoculated with the bacteria did not show positive signals. A gene fragment of alanine racemase thus appears to be useful for detecting each of \textit{B. stearothermophilus} and \textit{B. psychrosaccharolyticus} in a wide variety of foods.

In chapter 4, the author have evaluated an alanine racemase gene as an indicator to detect \textit{E. coli} in foods. The author amplified a fragment of
the gene containing the non-conserved regions from genomic DNA of *E. coli* and examined the specificity of the DIG-labeled fragment for detection of *E. coli*. The probe was specific for *E. coli*; all 48 strains of *E. coli* showed positive signals, whereas all 59 strains of non-*E. coli* species (22 genera, 42 species, and 59 strains), except for *Shigella sonnei*, did not show a positive signal. *E. coli* mixed with various food samples could be also specifically detected by this method, while food samples not inoculated with *E. coli* did not show positive signals. The gene fragment of alanine racemase thus appears to be suitable for detecting *E. coli* in a wide variety of foods.

The author have also described the isolation of various *E. coli* O157:H7 strains, their VT productivity, and the hybridization of their DNA with the *E. coli* alanine racemase gene fragment. The gene fragment labeled with DIG was shown to hybridize specifically with DNAs from various *E. coli* O157:H7 isolates. The gene fragment of alanine racemase is a useful probe for detecting various *E. coli* strains in foods.
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