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Conversion of *Lactobacillus pentosus* D-Lactate Dehydrogenase to a D-Hydroxyisocaproate Dehydrogenase through a Single Amino Acid Replacement

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The single amino acid replacement of Tyr52 with Leu drastically increased the activity of *Lactobacillus pentosus* NAD-dependent D-lactate dehydrogenase toward larger aliphatic or aromatic 2-ketoacid substrates by 3 or 4 orders of magnitude and decreased the activity toward pyruvate by about 30-fold, converting the enzyme into a highly active D-2-hydroxyisocaproate dehydrogenase.

NAD-dependent L- and D-lactate dehydrogenases (L- and D-LDH, EC 1.1.1.27 and EC 1.1.1.28, respectively) convert pyruvate into L- and D-lactates, respectively, at the final step of anaerobic glycolysis, concomitantly oxidizing NADH into NAD^+ (16). Lactic acid bacteria possess at least one of the two types of LDHs, fermenting the corresponding stereoisomer of lactic acid (12). In spite of the similarity in their catalytic reactions, the two types of enzymes are evolutionally separate from each other, belonging to distinct protein superfamilies (4, 21, 33). D-LDHs share a common protein structure not only with various D-2-hydroxyacid dehydrogenases (2, 4, 10, 13, 14, 21, 25, 26, 29, 32, 33) but also other dehydrogenases such as formate (26) and L-alanine (3) dehydrogenases.

L- and D-LDHs are highly divergent enzymes in lactic acid bacteria, showing great variety in both their amino acid sequences and catalytic properties or substrate specificities (1, 2, 4, 7, 12, 21, 33). There is only 40 to 50% amino acid identity among known D-LDHs of different *Lactobacillus* species, which show significantly different kinetic parameters, such as k_{cat} and K_m for substrates (4, 7, 21, 33). Instead of or together with D-LDH, some lactobacilli such as *Lactobacillus casei* (17, 19, 25) and *L. delbrueckii* (5) have D-hydroxyisocaproate dehydrogenases (D-HicDHs), which exhibit high activity not toward pyruvate but 2-ketoacids with larger aliphatic or aromatic side chains at the C-3 position, while *L. confusus* has L-HicDH, an L-LDH-related enzyme (30). D-HicDHs show 40 to 50% amino acid identity with known *Lactobacillus* D-LDHs (5, 25, 33), which is comparable to the identity among the D-LDHs, suggesting that these two types of enzymes are particularly related evolutionally. Since optically active 2-hydroxyacids are valuable for the synthesis of useful compounds (17–19), D-HicDHs are promising enzymes for industrial application, although their actual physiological role remains uncertain.

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The substrate recognition by an enzyme has been extensively studied in the case of L-LDH, mostly through alteration of the substrate specificity by means of protein engineering (1, 8, 11, 15, 37, 38), but much less has been learnt about D-LDH or related enzymes. Recently, however, the three-dimensional structures of *L. pentosus* apo (32) and *L. bulgaricus* holo (27) D-LDHs, and the ternary complex of *L. casei* D-HicDH (10) were determined, implying their substrate recognition sites. Figure 1 shows the position of Leu51 in the substrate binding site of the *L. casei* D-HicDH ternary complex structure (10), together with those of Arg234, Glu263, and His294, which were indicated to be residues involved in the catalytic function of D-LDH by amino acid replacement studies (20, 22, 33–35). Since Leu51 is located very near the substrate C-3 position and is consistently replaced by conserved Tyr (Tyr52) in *Lactobacillus* D-LDHs (4, 21, 33), it is easily imaginable that the amino acid at this position defines the size or shape of the hydrophobic pocket for the C-3 groups of 2-ketoacid substrates (10). In this paper, we show how *L. pentosus* D-LDH is sufficiently converted into a D-HicDH through only 1 amino acid replacement of Tyr52 to Leu.

An oligodeoxynucleotide, 5′-C GGT GCC GAT GTA CTG CAG CAA AAG GAC TAT ACT GC-3, was purchased from Takara Shuzo in order to construct a mutant *L. pentosus* D-LDH (Y52L) in which Tyr52 was replaced with Leu. Site-directed mutagenesis was performed with a MUTA-GENE in vitro mutagenesis kit (Bio-Rad), according to the method described by Kunkel (23), and the DNA fragments were sequenced by the dideoxy chain terminator procedure (28) with a DNA sequencer model 4000L (LI-COR) to prove that only the mutation expected had occurred. The wild-type and Y52L mutant D-LDHs of *L. pentosus* JCM1558 (ATCC 8041), previously called *L. plantarum*, were produced in *Escherichia coli* MV1184, and the cultivation of *E. coli* cells harboring expression plasmids for the D-LDH genes was performed essentially according to the previously described procedure (34). Enzyme purification was performed with both the previous procedure and a slight modification of it, which involves hydrophobic column chromatography on Butyl Toyopearl 650 M gel

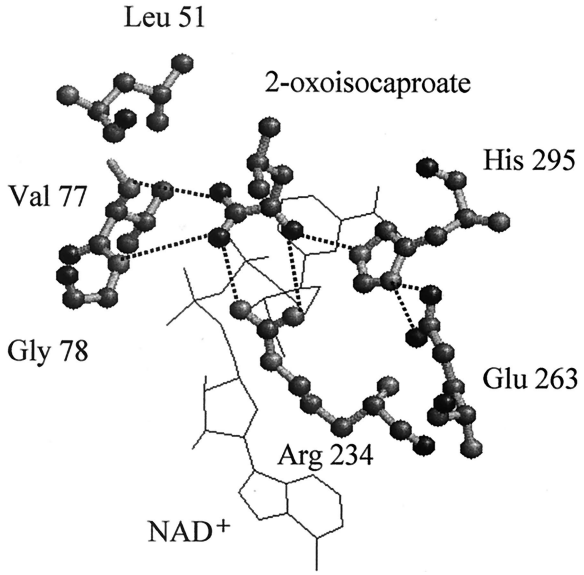

FIG. 1. The substrate binding site of *L. casei* D-HicDH. The figure was drawn by using RasMol, according to the substrate binding site of the *L. casei* D-HicDH ternary complex structure (10). The two bound ligands, NAD^+ and 2-ketoisocaproate, are shown as wire frame models. Leu51, Arg234, Glu263, and His294, which correspond to Tyr52, Arg235, Glu264, and His295 in D-LDHs, respectively, are indicated by ball-and-stick models.

(Tosoh, Tokyo, Japan) instead of affinity chromatography on a 5′-AMP Sepharose gel (Amersham-Pharmacia Biotech). In the modified procedure, the Butyl Toyopearl column is equilibrated with 25 mM Tris-HCl (pH 7.4) buffer containing ammonium sulfate (45% saturation) and is eluted with a linear gradient of ammonium sulfate, from 45 to 0% saturation, at room temperature with a BioLogic system (Bio-Rad). The enzyme sample obtained with the modified procedure contained no significant NADH or NAD^+ contamination as judged by spectroscopic analysis (data not shown) and gave a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method described by Laemmli (24) and exhibited essentially the same specific activity as that of the sample obtained by the previously described procedure (data not shown). During purification of the enzyme, protein concentrations were determined according to the method described by Bradford (6) with Bio-Rad Protein Assay protein reagent (Bio-Rad) by using bovine serum albumin as a standard protein. For kinetic and fluorescence analyses, the concentration of the purified enzyme was determined by using extinction coefficients at 280 nm of 27,045 and 25,855 $M^{-1}\ni 3\chi \cdot cm^{-1}$ for the coenzyme-free wild-type and Y52L enzymes, respectively, as determined from the amino acid composition and molecular weight of *L. pentosus* D-LDH (33). The enzyme activity toward various 2-ketoacids was assayed at 30°C in 100 mM sodium MES (2-[*N*-morpholino]ethanesulfonic acid) buffer (pH 5.5) containing 0.1 mM NADH and various concentrations of 2-ketoacids (sodium salts). One unit was defined as the conversion of 1 μmol of substrate per min. Kinetic parameters were calculated from plots of v/[S] versus [S], where [S] is substrate concentration.

The binding of NADH and oxamate to the enzymes was followed as the change in NADH fluorescence intensity (ΔF) essentially according to the method used for L-LDHs (31) with excitation and emission wavelengths of 340 and 460 nm, respectively, by using a JASCO FP-750 spectrofluorophotometer. ΔF was determined by comparing the fluorescence intensities of NADH in the presence and absence of the enzymes (15 μM) at 30°C in 50 mM sodium MES buffer (pH 6.0). Oxamate binding was determined in the presence of the enzymes (15 μM) and 0.1 mM NADH as the quenching of NADH fluorescence caused by various concentrations of sodium oxamate and was corrected by comparison with the nonspecific quenching in the absence of the enzymes. The dissociation constants for the enzymes with NADH and oxamate were determined according to the procedure for L-LDHs (9, 31) by curve fitting with KaleidaGraph.

TABLE 1. Kinetic parameters for various substrates for *L. pentosus* D-LDH

Substrate	Wild-type K_m (mM)	k_{cat} (s^{-1})	Y52L K_m (mM)	k_{cat} (s^{-1})
Pyruvate	0.12 ± 0.03	321 ± 12	1.8 ± 0.1	213 ± 4
Hydroxypyruvate	0.28 ± 0.03	257 ± 10	3.0 ± 0.1	407 ± 4
2-Ketobutyrate	8.1 ± 0.6	118 ± 4	0.38 ± 0.01	175 ± 2
2-Ketovalerate	17.0 ± 1.0	5.7 ± 0.2	0.15 ± 0.01	334 ± 6
2-Ketoisovalerate	27.0 ± 0.8	5.5 ± 0.1	11.7 ± 0.4	18.6 ± 0.4
2-Ketocaproate	20.9 ± 0.6	13.3 ± 0.2	0.056 ± 0.003	189 ± 3
2-Ketoisocaproate	31.0 ± 2.0	11.1 ± 0.1	0.12 ± 0.01	65.7 ± 0.5
Phenylpyruvate	0.8 ± 0.2	40 ± 2	0.067 ± 0.001	778 ± 34

Table 1 summarizes the K_m and k_{cat} values of the wild-type and Y52L mutant *L. pentosus* D-LDHs for various 2-ketoacid substrates, and Fig. 2 compares the catalytic efficiencies (k_{cat}/K_m) of these two types of enzyme and *L. casei* D-HicDH (19) for these substrates. Compared with the wild-type *L. pentosus*

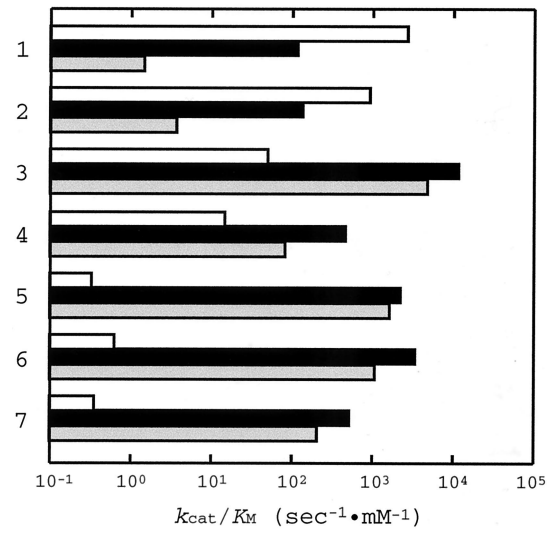

FIG. 2. Comparison of the substrate specificities of the wild-type and Y52L *L. pentosus* D-LDHs, and *L. casei* D-HicDH. The k_{cat}/K_m values for rows follow: 1, pyruvate; 2, hydroxypyruvate; 3, phenylpyruvate; 4, 2-ketobutyrate; 5, 2-ketovalerate; 6, 2-ketocaproate; and 7, 2-ketoisocaproate. They are logarithmically plotted for the wild-type (white boxes) and Y52L (black boxes) *L. pentosus* D-LDHs and *L. casei* D-HicDH (19) (gray boxes).

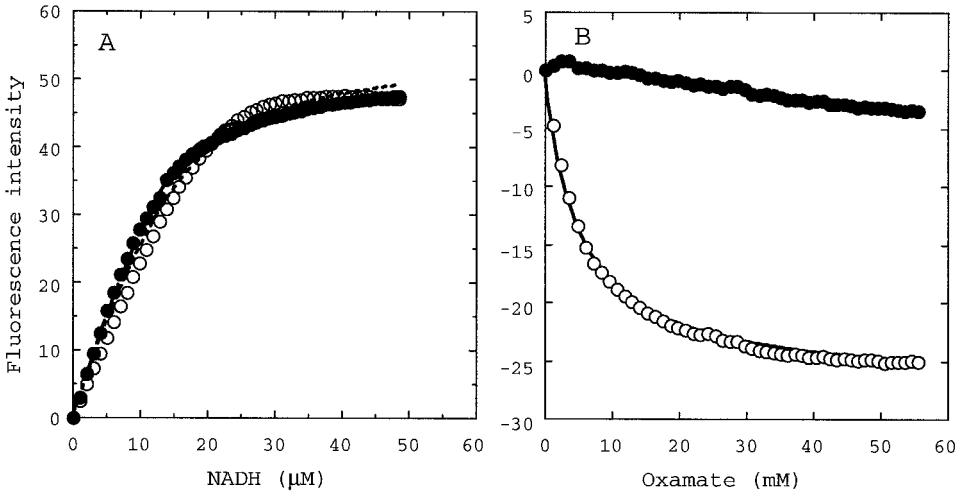

FIG. 3. Change in NADH fluorescence intensity showing NADH binding (A) and oxamate binding (B) to the wild-type (open circles) and Y52L (closed circles) *L. pentosus* D-LDHs. The lines in panel A indicate the calculated curves obtained with the equation of Stinson and Holbrook (31) by using maximal ΔF of 53.8 and 49.0 and K_d values of 3.4 and 1.4 μM for the wild-type and Y52L enzymes, respectively. The line for oxamate binding to the wild-type enzyme (B) indicates the curve using a maximal fluorescence change of −27.5 and a K_d of 5 mM.

D-LDH, the Y52L enzyme exhibited slightly reduced catalytic activity toward pyruvate and hydroxypyruvate, which are favorable substrates for the wild-type enzyme, by about 23- and 7-fold in terms of k_{cat}/K_m, respectively. These reductions in k_{cat}/K_m were mostly due to the increased K_m values (15- and 10-fold higher, respectively), while the k_{cat} values for these two substrates were not markedly affected by this amino acid replacement (Table 1). In contrast to the cases of these two substrates, the Y52L enzyme exhibited drastically increased catalytic activity toward 2-ketovalerate, 2-ketocaproate, and 2-ketoisocaproate by 3 or 4 orders of magnitude in k_{cat}/K_m. In these cases, the increases in activity resulted from both the reduction in K_m (6- to 50-fold) and the increases in k_{cat} (more than 100-fold) (Table 1). The Tyr52-to-Leu replacement also increased the catalytic activity of the enzyme toward 2-ketobutyrate and phenylpyruvate by 30- and 240-fold in k_{cat}/K_m, respectively, by both reducing K_m and increasing k_{cat}. Consequently, the Y52L mutant enzyme exhibited comparable levels of activity toward these five substrates to the activity that the wild-type enzyme showed toward pyruvate or hydroxypyruvate (Fig. 2A and B).

The Y52L enzyme exhibited less enhanced activity toward 2-ketoisovalerate than the other substrates with a large hydrophobic side chain, with only an approximately 2.5-fold-smaller K_m and a 3.5-fold-increased k_{cat} compared to the wild-type enzyme (Table 1). In addition, benzoylformate or D-mandelate was also inert for this mutant enzyme, as in the case of the wild-type enzyme (data not shown), suggesting that the Tyr-to-Leu replacement is less effective for substrates with a C-3-branched side chain. These results may not be so strange, since there is no known *Lactobacillus* D-HicDH that exhibits high activity toward 2-ketoisovalerate or benzoylformate. It is known that benzoylformate is a favorable substrate for NAD-dependent D-mandelate dehydrogenases (D-ManDH) that have been purified from *L. curvatus* (18) and *Enterococcus faecalis* (36). However, much less information has been obtained about the structures of these D-ManDHs than has been obtained about D-LDHs or D-HicDHs.

Comparison of the k_{cat}/K_m values showed that the *L. pentosus* Y52L D-LDH and *L. casei* D-HicDH (19) exhibit quite similar trends in their substrate specificities, although the former enzyme exhibits still markedly higher activity toward pyruvate and hydroxypyruvate than the latter one (Fig. 1B and C). This comparison clearly indicates that *L. pentosus* D-LDH was sufficiently converted into a D-HicDH through only 1 amino acid replacement of Tyr52 for Leu, demonstrating that only the difference in the amino acid residue at position 52 distinguishes the enzyme functions of *L. pentosus* D-LDH and *L. casei* D-HicDH, although these two enzymes have only 46% identical amino acid residues (33).

For both the wild-type and Y52L mutant enzymes, the fluorescence intensity of NADH markedly increased when NADH was bound into the enzymes, giving the same apparent dissociation constant (K_d) level of 3.4 ± 0.3 and 1.4 ± 0.1 μM, respectively (Fig. 3A). On the other hand, the fluorescence of the enzyme-bound NADH was markedly quenched on the addition of oxamate, a pyruvate analogue, in the case of the wild-type enzyme (Fig. 3B), giving an apparent oxamate K_d of about 5 mM, which is in good agreement with the reported competitive inhibitor constant (K_i) of oxamate (5 mM) at the same pH (35). In the case of the Y52L enzyme, in contrast, the NADH fluorescence was not markedly quenched by oxamate up to 60 mM. These results indicate that the Tyr52-to-Leu replacement markedly affects substrate binding but not coenzyme binding.

It is known that some lactobacilli possess both D-LDH and D-HicDH (5), although it is uncertain whether *L. pentosus* JCM1558 cells has D-HicDH besides D-LDH or not, since no, if any, marked D-HicDH activity was detected in the cell extract (data not shown). Our results demonstrate that *Lactobacillus* D-LDH and D-HicDH were readily converted into each other, through a small structural change such as a single amino

acid replacement. From the aspect of protein engineering, the results strongly suggest that various enzyme functions can be introduced into the framework of D-LDHs, or related enzymes, by means of only a few amino acid replacements, as already demonstrated for L-LDH (8, 37).

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REFERENCES

1. **Arai, K., T. Kamata, H. Uchikoba, S. Fushinobu, H. Matsuzawa, and H. Taguchi.** 2001. Some *Lactobacillus* L-lactate dehydrogenases exhibit comparable catalytic activities for pyruvate and oxaloacetate. J. Bacteriol. **183:**397–400.
2. **Arthur, M., C. Molinas, S. Dutka-Malen, and P. Courvalin.** 1991. Structural relationship between the vancomycin resistance protein VanH and 2-hydroxycarboxylic acid dehydrogenases. Gene **103:**133–134.
3. **Baker, P. J., Y. Sawa, H. Shibata, S. E. Sedelnikova, and D. W. Rice.** 1998. Analysis of the structure and substrate binding of *Phormidium lapideum* alanine dehydrogenase. Nat. Struct. Biol. **5:**561–567.
4. **Bernard, N., T. Ferain, D. Garmyn, P. Hols, and J. Delcour.** 1991. Cloning of the D-lactate dehydrogenase gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* by complementation in *Escherichia coli.* FEBS Lett. **290:**61–64.
5. **Bernard, N., K. Johnsen, T. Ferain, D. Garmyn, P. Hols, J. J. Holbrook, and J. Delcour.** 1994. NAD+-dependent D-2-hydroxyisocaproate dehydrogenase of *Lactobacillus delbrueckii* subsp. *bulgaricus.* Gene cloning and enzyme characterization. Eur. J. Biochem. **224:**439–446.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Bugg, T. D., G. D. Wright, S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry **30:**10408–10415.
8. **Clarke, A. R., T. Atkinson, and J. J. Holbrook.** 1989. From analysis to synthesis: new ligand binding sites on the lactate dehydrogenase framework. Trends Biochem. Sci. **14:**101–105, 145–148.
9. **Clarke, A. R., D. B. Wigley, D. A. Barstow, W. N. Chia, T. Atkinson, and J. J. Holbrook.** 1987. A single amino acid substitution deregulates a bacterial lactate dehydrogenase and stabilizes its tetrameric structure. Biochim. Biophys. Acta **913:**72–80.
10. **Dengler, U., K. Niefind, M. Kieβ, and D. Schomburg.** 1997. Crystal structure of a ternary complex of D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei*, NAD+ and 2-oxoisocaproate at 1.9 Å resolution. J. Mol. Biol. **267:**640–660.
11. **El Hawrani, A. S., R. B. Sessions, K. M. Moreton, and J. J. Holbrook.** 1996. Guided evolution of enzyme with new substrate specificities. J. Mol. Biol. **264:**97–110.
12. **Garvie, E. I.** 1980. Bacterial lactate dehydrogenases. Microbiol. Rev. **44:**106–139.
13. **Goldberg, J. D., T. Yoshida, and P. Brick.** 1994. Crystal structure of an NAD-dependent D-glycerate dehydrogenase at 2.4 Å resolution. J. Mol. Biol. **236:**1123–1140.
14. **Grant, G. A.** 1989. A new family of 2-hydroxyacid dehydrogenase. Biochem. Biophys. Res. Commun. **165:**1371–1374.
15. **Hogan, J. K., C. A. Pittle, J. B. Jones, and M. Gold.** 1995. Improved specificity toward substrates with positively charged side chains by site-directed mutagenesis of the L-lactate dehydrogenase of *Bacillus stearothermophilus.* Biochemistry **34:**4225–4230.
16. **Holbrook, J. J., A. Liljas, S. J. Steindel, and M. G. Rossmann.** 1975. Lactate dehydrogenase, p. 191–292. *In* P. D. Boyer (ed.), The enzymes, 3rd ed., vol. 11. Academic Press, New York, N.Y.
17. **Hummel, W., H. Schütte, and M.-R. Kula.** 1985. D-2-Hydroxyisocaproate dehydrogenase from *Lactobacillus casei.* A new enzyme suitable for stereospecific reduction of 2-ketocarboxylic acids. Appl. Microbiol. Biotechnol. **21:**7–15.
18. **Hummel, W., H. Schütte, and M.-R. Kula.** 1988. D-(-)-Mandelic acid dehydrogenase from *Lactobacillus curvatus.* Appl. Microbiol. Biotechnol. **28:**433–439.
19. **Kallwass, H. K. W.** 1992. Potential of R-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei* for stereospecific reductions. Enzyme Microb. Technol. **14:**28–35.
20. **Kochhar, S., N. Chuard, and H. Hottinger.** 1992. Glutamate-264 modulates the pH dependence of the NAD+-dependent D-lactate dehydrogenase, J. Biol. Chem. **267:**20298–20301.
21. **Kochhar, S., P. E. Hunziker, P. Leong-Morgenthaler, and H. Hottinger.** 1992. Primary structure, physicochemical properties, and chemical modification of NAD+-dependent D-lactate dehydrogenase. Evidence for the presence of Arg-235, His-303, Tyr-101, and Trp-19 at or near the active site. J. Biol. Chem. **267:**8499–8513.
22. **Kochhar, S., V. S. Lamzin, A. Razeto, M. Delley, H. Hottinger, and J. E. Germond.** 2000. Roles of his205, his296, his303 and Asp259 in catalysis by NAD+-specific D-lactate dehydrogenase. Eur. J. Biochem. **267:**1633–1639.
23. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–49229.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
25. **Lerch, H. P., H. Blocker, H. K. W. Kallwass, J. Hoppe, H. Tsai, and J. Collins.** 1989. Cloning, sequencing and expression in *Escherichia coli* of the D-2-hydroxyisocaproate dehydrogenase gene of *Lactobacillus casei.* Gene **78:**47–57.
26. **Popov, V. O., and V. S. Lamzin.** 1994. NAD+-dependent formate dehydrogenase. Biochem. J. **301:**625–643.
27. **Razeto, A., S. Kochhar, H. Hottinger, M. Dauter, K. S. Wilson, and V. S. Lamzin.** 2002. Domain closure, substrate specificity and catalysis of D-lactate dehydrogenase from *Lactobacillus bulgaricus.* J. Mol. Biol. **318:**109–119.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **742:**5463–5467.
29. **Schuller, D. J., G. A. Grant, and L. J. Banaszak.** 1995. Allosteric ligand site in the Vmax-type cooperative enzyme phosphoglycerate dehydrogenase. Nat. Struct. Biol. **2:**68–76.
30. **Schütte, H., W. Hummel, and M.-R. Kula.** 1984. L-2-Hydroxyisocaproate dehydrogenase. A new enzyme from *Lactobacillus confusus* for the stereospecific reduction of 2-ketocarboxylic acid. Appl. Microbiol. Biotechnol. **19:**167–176.
31. **Stinson, R. A., and J. J. Holbrook.** 1973. Equilibrium binding of nicotinamide nucleotides to lactate dehydrogenases. Biochem. J. **131:**719–728.
32. **Stoll, V. S., M. S. Kimber, and E. F. Pai.** 1996. Insights into substrate binding by D-2-ketoacid dehydrogenases from the structure of *Lactobacillus pentosus* D-lactate dehydrogenase. Structure **4:**437–447.
33. **Taguchi, H., and T. Ohta.** 1991. D-Lactate dehydrogenase is a member of the D-isomer-specific 2-hydroxyacid dehydrogenase family. Cloning, sequencing, and expression in *Escherichia coli* of the D-lactate dehydrogenase gene of *Lactobacillus plantarum.* J. Biol. Chem. **266:**12588–12594.
34. **Taguchi, H., and T. Ohta.** 1993. Histidine 296 is essential for the catalysis in *Lactobacillus plantarum* D-lactate dehydrogenase. J. Biol. Chem. **268:**18030–18034.
35. **Taguchi, H., T. Ohta, and H. Matsuzawa.** 1997. Involvement of Glu-264 and Arg-235 in the essential interaction between the catalytic imidazole and substrate for D-lactate dehydrogenase. J. Biochem. **122:**802–809.
36. **Tamura, Y., A. Ohkubo, S. Iwai, Y. Wada, T. Shinoda, K. Arai, S. Mineki, M. Iida, and H. Taguchi.** 2002. Two forms of NAD-dependent D-mandelate dehydrogenase in *Enterococcus faecalis* IAM 10071. Appl. Environ. Microbiol. **68:**947–951.
37. **Wilks, H. M., K. W. Hart, R. Feeney, C. R. Dunn, H. Muirhead, W. N. Chia, D. A. Barstow, T. Atkinson, A. R. Clarke, and J. J. Holbrook.** 1988. A specific, highly active malate dehydrogenase by redesign of a lactate dehydrogenase framework. Science **242:**1541–1544.
38. **Wilks, H. M., D. J. Halsall, T. Atkinson, W. N. Chia, A. R. Clarke, and J. J. Holbrook.** 1990. Designs for a broad substrate specificity ketoacid dehydrogenase. Biochemistry **29:**8587–8591.