CHAPTER I

INTRODUCTION

1.1 L-Pipecolic acid

L-pipecolic acid, a non-proteingenic amino acid widely distributed in plants, animals, and microorganisms (Nazabadioko *et al.*, 1998, Fujii and Miyoshi, 1975 and Muramatsu *et al.*, 2006). It is an important precursor of many useful microbial secondary metabolites, such as selfotel, the anesthetic, peptide antibiotic and the *N*-methyl-D-aspartate antagonist (Lehmann *et al.*, 1988 and Boger *et al.*,1996), the anticancer agent (VX-710) (Germann *et al.*, 1997), the HIV protease inhibitor (palinavir) (Lamarre *et al.*, 1997), and the peperidine alkaloide (saframin) (Nazabadioko *et al.*, 1998), the immunosuppressant (rapamycin) (Paiva *et al.*, 1993 and Tanaka *et al.*, 1987), the antitumor agent (swainsonine), the peptide antibiotic (virginiamycin and Cyl-2) (Hirota *et al.*, 1973), and the anthelmintic agent (marcfortine and levobupivacaine) (Adger *et al.*, 1966). Structures of these substances are shown in Figure 1.1. In fact, the pipecolic acid-derived moieties often play important roles in the biological activities of some pharmaceutically important compounds. Elucidation of the origination of pipecolic acid thus serves as the first step toward generating novel analogs of these compounds for the structure-activity relationship studies.

1.2 L-Pipecolic acid pathway

Biosynthesis of pipecolic acid has been extensively investigated in animals and plants, mainly because of its close relationship with lysine metabolism (Broquist, 1991 and Gupta, and Spenser, 1969). The biosynthesis has two basic routes for converting lysine into pipecolic acid, distinguishable at the loss of a specific amino group of lysine. One route is through the loss of the α-amino group of lysine and the

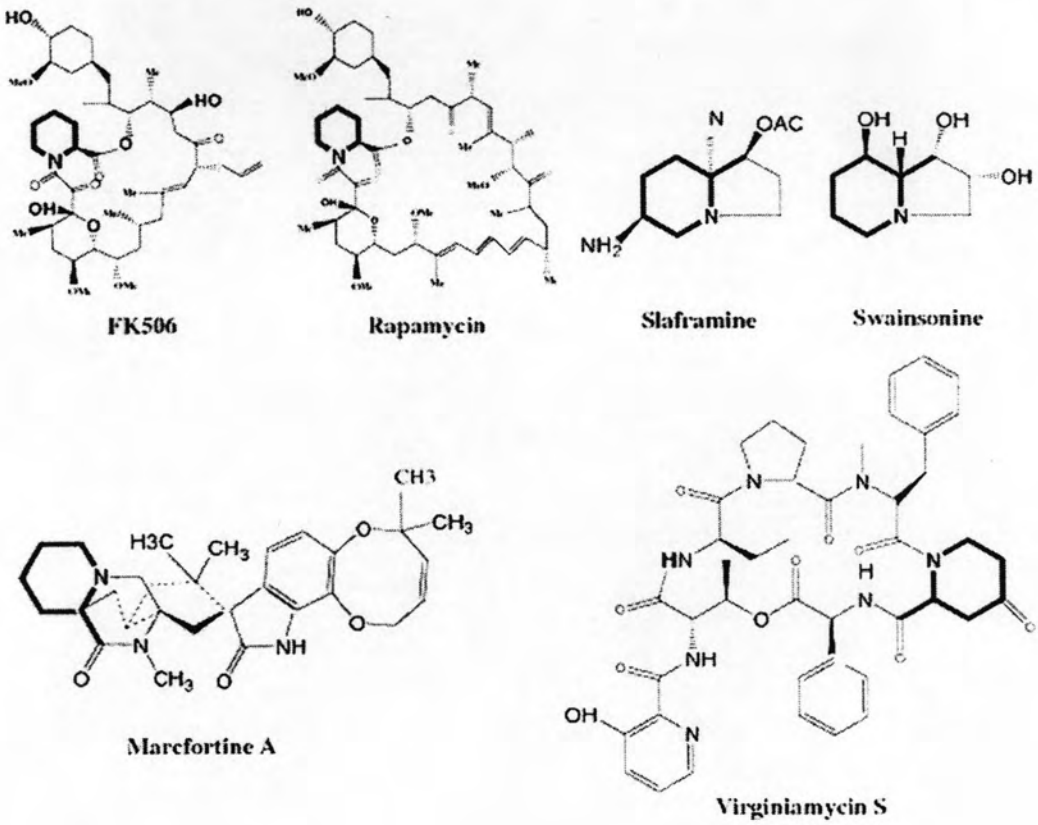


Figure 1.1 **Microbial secondary metabolites containing pipecolic acid derived moieties** (pipecolate moieties are shown in *bold-faced type* in the drawing)

Source: **Min, 2006**

incorporation of the ε-nitrogen into pipecolic acid (P-2-C pathway in the left branch of Figure 1.2). The alternative route is via the loss of ε-nitrogen and the incorporation of α-nitrogen into pipecolic acid (P-6-C pathway in the right branch of Figure 1.2). The important intermediates in these two pathways, Δ^1-piperideine-2-carboxylic acid (P-2-C) (Muramatsu *et al.*, 2006) and Δ^1-piperideine-6-carboxylic acid (P-6-C) (Fujii *et al.*, 2002), are isomers and exist in chemical equilibrium with their respective open-chain hydrated forms, α-keto-ε-amino-caproic acid and α-aminoadipic-δ-semialdehyde (Gupta, and Spenser, 1969).

1.2.1 P-2-C pathway

The P-2-C route for pipecolic acid synthesis has been studied in *Pseudomonas putida* as a part of D-lysine catabolic pathway. *Pseudomonas* strains use different routes to metabolize L-lysine and D-lysine for carbon and nitrogen sources (Chang and Adams 1971, 1974 and Fothergill and Guest 1977). Whereas L-lysine is degraded primarily through the daminovalerate pathway (Fothergill and Guest 1977), D-lysine is metabolized through the transamination of the α-amino group of lysine, resulting in the formation of P-2-C, which is reduced into pipecolic acid (Chang and Adams 1974 and Miller and Rodwell 1971). In the 1980s, Payton and Chang (1982) reported the purification and initial characterization of a reductase possessing such activity from *P. putida* ATCC 15070. At that time, information on the protein sequence or the nucleotide sequence of the gene encoding this enzyme was not available. In 2005, Muramatsu and his coworkers found that the product of a *P. putida* ATCC12633 gene, *dpk*A, which had previously been annotated as a malate/L-lactate dehydrogenase, was actually an NADPH-dependent P-2-C reductase. DpkA irreversibly catalysed the NADPH-dependent reduction of P-2-C to form L-pipecolic acid, as well as the reduction of Δ^1-pyrroline-2-carboxylate into L-proline. The dual functions of this enzyme have been demonstrated by *in vivo* genetic knockout experiment and *in vitro* biochemical assay using purified enzyme (Muramatsu *et al.*, 2005).

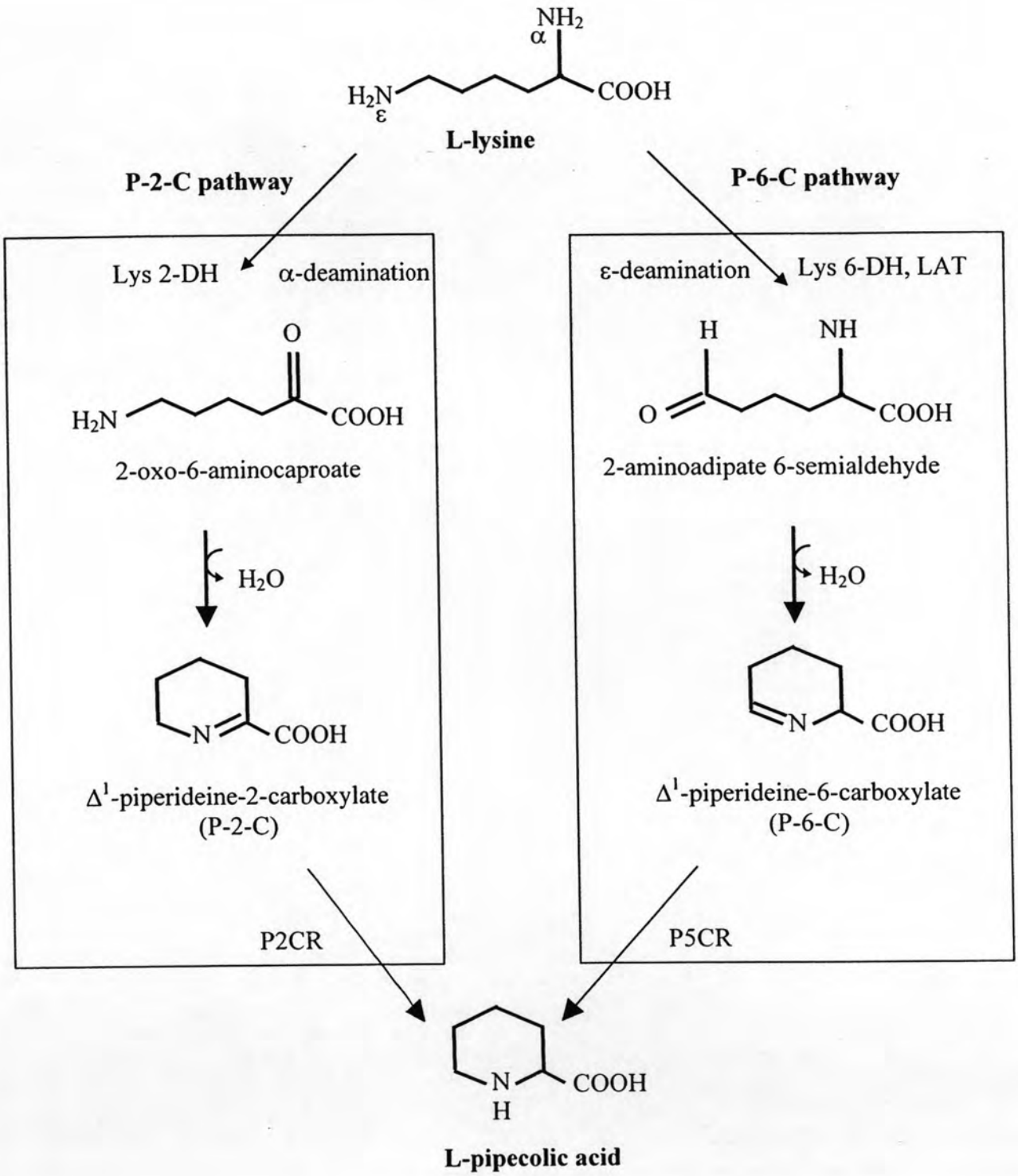


Figure 1.2 **Pipecolic acid biosynthesis in microorganisms**

Source: **modified from Min, 2006**

Sequence analysis revealed that this enzyme represents a novel subclass in an NAD(P)-dependent oxidoreductase superfamily, whose close homologs also found in *P. syringae* and *P. aeruginosa* (Muramatsu *et al.*, 2005). The specific enzyme for conversion of D-lysine to P-2-C has not been reported in *Pseudomonas*, although the D-amino acid aminotransferase from *Bacillus sphaericus* has been demonstrated to be capable of catalyzing the α-transamination of D-lysine with pyruvate to yield P-2-C (Yonaha *et al.*, 1975). A L-lysine α-oxidase catalyzing the oxidative deamination of the α-amino group of L-lysine has been isolated from the fungi, *Trichoderma viride* Y244-2 (Kusakabe *et al.*, 1980) and *T. harzianum Rifai* (Lukasheva and Berezov, 2002). The enzyme has been characterized as a flavoprotein with two identical subunits, each containing one molecule of FAD as the coenzyme. It exhibits a high stereospecificity, being absolutely inert toward D-lysine. In the reactions catalysed by this enzyme, the α-amino group of L-lysine is oxidized to yield α-keto-ε-aminocaproic acid, which is spontaneously converted into the dehydrated cyclic form, P-2-C (Kusakabe *et al.*, 1980 and Lukasheva and Berezov 2002)

It is noteworthy that an L-lysine α-aminotransferase has also been reported to catalyse the α-transamination reaction of L-lysine in *Streptomyces tendae* Tu 901, an actinomycete strain which produces peptidyl nucleoside antibiotic nikkomycin D (Bruntner and Bormann. 1998). Nikkomycin D does not contain any pipecolate moiety, but has a picolinic acid moiety which is also derived from L-lysine through a loss of α-amine (Jordan *et al.*, 1984). In the biosynthetic gene cluster of nikkomycin D, a gene has been identified to encode a protein (NikC), which belongs to a novel class of pyridoxamine or pyridoxal-phosphate-dependent dehydrases and aminotransferases. The function of NikC as L-lysine 2-aminotransferase has been clearly demonstrated by the *o*-aminobezaldehyde assay, which allows differentiation between the α-transamination and ε-transamination of lysine (Bruntner and Bormann. 1998).

1.2.2 P-6-C pathway

This pathway has been studied in fungi parasite *Rhizoctonia leguminicola*, because the synthesis of pipecolic acid via this route represents the initial steps in the production of two toxic octahydroindolzine alkaloids, slaframine, and swainsonine (Broquist, 1985). In the investigation of the origin of pipecolic acid in this fungus using cell-free enzyme systems, Wickwire and coworkers (1990a) have established a chain of reactions through which L-lysine was converted to saccharopine, which was in turn converted to P-6-C through oxidative cleavage. The latter was then readily reduced to pipecolic acid. A previously unrecognized flavin enzyme, saccharopine oxidase, has been identified in their study, which oxidatively cleaves saccharopine to yield P-6-C. Since saccharopine is a major metabolite in lysine degradation in *R. leguminicola*, saccharopine oxidase apparently functions to shunt saccharopine into secondary metabolism pathway to pipecolic acid for slaframine and swainsonine production (Wickwire, *et al.*, 1990b). It is interesting that in aerobic red yeast *R. glutinis*, the reverse reactions of this pathway are operative too, through which pipecolic acid is converted to lysine via P-6-C, α-aminoadipic semialdehyde, and saccharopine (Kinzel and Bhattacharjee, 1979 and Kurtz and Bhattacharjee, 1975). The direct formation of P-6-C from lysine through ε-transamination has also been observed in some microorganisms. In β-lactam producing actinomycetes, P-6-C was synthesized from lysine by L-lysine ε-aminotransferase (LAT, E.C.2.6.1.36) (Rius and Demain, 1997).

1.3 L-Pipecolic acid synthesis

LAT has been found in gram-negative bacterium *Flavobacterium lutescens* (Fujii *et al.*, 2000 and Soda *et al.*, 1968). Fujii and coworkers (2002a) combined LAT with pyrroline-5-carboxylate reductase (P5CR) for pipecolic acid production. They constructed the recombinant plasmid of the *lat* gene, encoding LAT, from *F. lutescens* and the *proC* gene, encoding P5CR from *Escherichia. coli.* This recombinant plasmid was transformed and expressed in recombinant *E. coli* JM109. This biotransformation system provided the pure L enantiomer of pipecolic acid, with ee-value (enantiomeric

excess, a measure for how much of one enantiomer is present compared to the other) was 100%. It is noteworthy that P5CR is present in almost all organisms, catalyzing the terminal step in proline biosynthesis conversion of Δ^1-pyrroline-5-carboxylate (P-5-C) into proline (Brandriss and Falvey, 1992, Delauney and Verma, 1990, Leisinger, 1987 and Smith *et al.*, 1980). P5CR also catalysed the reduction of P-6-C to L-pipecolic acid because the structural similarity of P-5-C with piperideine-6-carboxylate (P-6-C). It is possible that in the microorganisms that produce L-pipecolic acid via P-6-C pathway, the universally conserved P5CR is actually responsible, at least in part, for the reduction of P-6-C into L-pipecolic acid (Fujii *et al.*, 2002a). In their system, at most 3.9 g/L of L-pipecolic acid was synthesized in a 159 hours reaction and the production was increased to 16 g/L within 110 hours after increased lysine uptake of *E. coli* cell membrane.

1.4 Lysine dehydrogenases (LysDHs)

The alternative enzymes that can produce P-2-C or P-6-C which can be used for L-pipecolic acid production are L-lysine dehydrogenases. These enzymes catalyse the oxidative deamination of L-lysine in the presence of NAD^+; so far, two types of L-lysine dehydrogenases have been identified (Figure 1.3). The first type is lysine 2-dehydrogenase (Lys 2-DH, EC 1.4.1.15), which catalyses the oxidative deamination of the α-amino group of L-lysine (Burgi and Colombo, 1966). The second type of L-lysine dehydrogenase is lysine 6-dehydrogenase (Lys 6-DH, EC 1.4.1.18), which catalyses the oxidative deamination of the ε-amino group of L-lysine to form L-2-aminoadipate-6-semialdehyde, which in turn nonenzymatically cyclizes to form Δ^1-piperideine-6-carboxylate. Lys 6-DH is present in some microorganisms (Misono and Nagasaki, 1982, Misono and Nagasaki, 1983 and Hammer and Birnbaum, 1991). Among microbial Lys 6-DHs, the *A. tumefaciens* enzyme has been mainly studied (Misono and Nagasaki, 1983, Misono *et al.*, 1985, 1989 and 1990 and Hashimoto *et al.*, 1989b) and it is used for L-lysine determination (Hashimoto *et al.*, 1990a and Dempsey *et al.*, 1992) and P-6-C production (Fuente *et al.*, 1997). Recently, *Geobacillus stearothermophilus* was identified as a new lysine 6-dehydrogenase producer and the enzyme characteristic was already done (Heydari *et al.*, 2004).

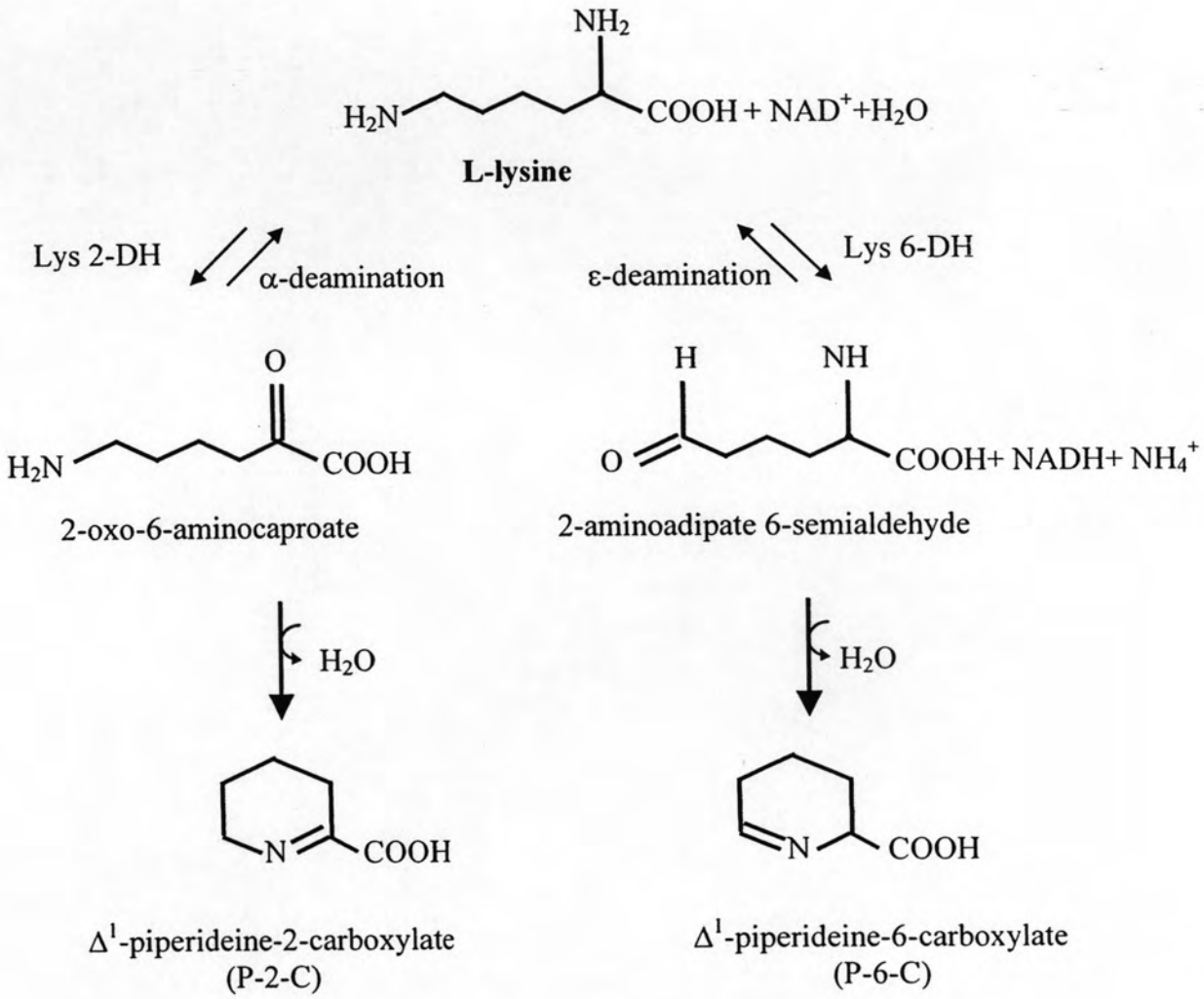


Figure 1.3 Reaction scheme for two types of lysine dehydrogenase

Source: Misono and Nagasaki, 1982

1.4.1 Screening and isolation of Lys 6-DH

The NAD^+-dependent Lys 6-DH was first discovered from plant-pathogenic bacterium, *Agrobacterium tumefaciens* (Misono and Nagasaki 1982). Consequently, Misono and colleagues screened the enzyme activity among microorganisms from soil samples and culture collections. It was found that the enzyme activity was very narrowly distributed only in *Agrobacterium tumefaciens* (Misono and Nagasaki 1982), *Alcaligenes faecalis*, *Bacillus sphaericus*, *Klebsiella pneumoniae*, and *Pseudomonas fragi* (Misono and Nagasaki 1983). The others microorganisms, *Bos taurus* sp., *Agrobacterium tumefaciens*, *Micrococcus sp.*, *Proteus morganii*, *Brevibacterium ammoniagenes*, *Hafnia alvei*, *Corynebacterium pseudodiphtheriticum*, *Arthrobacter atrocyaneus* and different yeasts did not show activities of Lys 6-DH. During 1990 to 2003, it has only one report that Lys 6-DH activity was found in *Candida albicans* (Hammer *et al.*, 2004).

Recently, Heydari and and colleagues (2004) had screened several strains of thermophilic bacteria and hyperthermophilic *archaea* from culture collections, including *Pyrococcus furiosus* DSM 3638, *Pyrococcus horikoshii* OT-3, *Thermococcus litoralis* DSM 5473, *Thermococcus profundus* DSM 9503, *Thermococcus peptonophilus* DSM 10343, *Pyrobaculum islandicum* DSM 4184, *Aeropyrum pernix* JCM 9820, *Sulfolobus tokodaii* JCM 10545, *G. stearothermophilius* DSM 297, DSM 456, DSM 458, DSM 494, IFO 12550, and UTB 1103, *Bacillus sphaericus* DSM 461 and DSM 462, and *Thermus* sp. strain UTB 1104. They found that only *Geobacillus stearothermophilus*, screened from a Japanese hot spring, had Lys 6-DH activity.

1.4.2 Purification and characterization of Lys 6-DH

Since the enzyme was first found in *Agrobacterium tumefaciens* in 1982, (Misono and Nagasaki 1982) only two Lys 6-DHs from *Agrobacterium tumefaciens* and *Geobacillus stearothermophilus* were identified and characterized. The properties of Lys 6-DHs from both sources are summarized in Table 1.1. The Lys 6-DHs exhibit

a narrow range of subunit molecular masses between 39 and 43 kDa. The enzyme from *Agrobacterium tumefaciens* was shown to be dimer in the absence of L-lysine and could be a tetramer when incubated with L-lysine while the *Geobacillus stearothermophilus* enzyme was hexamer. The enzyme from *Agrobacterium tumefaciens* and *Geobacillus stearothermophilus* showed maximal activity for oxidation deamination at pH between 9.7 and 10.0, respectively. The high activity of these two enzymes at rather high pH was similar to those of other amino acid dehydrogenases (Ohshima *et al.*, 1985).

Moreover, it was noteworthy that the *G. stearothermophilus*, a thermophilic bacterium, enzyme which was much more thermostable than Lys 6-DH from *A. tumefaciens* which is a mesophile. The optimum temperature of *G. stearothermophilus* enzyme was 70 °C while Lys 6-DH from *A. tumefaciens* was 35°C. The optimum temperature of *Agrobacterium tumefaciens* enzyme could be increase up to 50 °C upon the incubation with L-lysine. Lys 6-DHs from both sources have no broad substrate specificities. They only use L-lysine as a substrate. They was relatively selective for L-lysine as the electron donor, and either NAD^+ or $NADP^+$ and some NAD^+ analogs: deamino-NAD^+ and 3-acetylpyridine-NAD^+ could serve as the electron acceptor but $NADP^+$ and some NAD^+ analogs gave lower activity than NAD^+.

1.4.3 Cloning and expression of Lys 6-DH

Recently, about 17 different amino acid dehydrogenases have been identified in various organisms (Kawarabayasi *et al.*, 1998, Ohshima and Soda, 2000 and Ohshima and Soda, 1989). In contrast to the abundant information available about glutamate, alanine, leucine, and phenylalanine dehydrogenases (Ohshima and Soda, 2000), information about the structure and function of lysine dehydrogenase is rather limited due to the instability of the enzyme (Heydari *et al.*, 2004). In particular, only one group of Heydari and coworkers (2004) reported on the primary structure of lysine dehydrogenases. They isolated putative *lys 6-dh* gene fragment from chromosomal DNA of *G. stearothermophilus* by using colony and Southern blot hybridizations with 5′-ATGAARGTIYTIGTIYTIGGIGCIGGIYIATGGGIAARGA RGC-3′ probe which

Table 1.1 Properties of lysine 6-dehydrogenase from various sources

Properties	*Agrobacterium tumefaciens*	*Geobacillus stearothermophilus*
Specific activity of final preparation (U/mg protein)	4.54	6.2
Molecular mass of native enzyme (kDa)		
- gel filtration	70,000	-
- deduced amino acid sequence	-	42,239
Molecular mass of subunit	39,000	-
Number of subunit	2	-
pH optimum for oxidative deamination	9.7	10.0
pH stability	5.0 - 7.5	6.0 - 9.0
Optimum temperature (°C)	-	70
Thermostability (% remaining activity after incubation at 30 °C for 10 minutes)	40	60
Apparent K_m (mM) for		
- L-Lysine	1.5	0.73
- NAD^+	0.059	0.088
- NADH	-	0.48
- 3-acetylpyridine-NAD+	3.13	-
- Deamino-NAD+	1.85	-

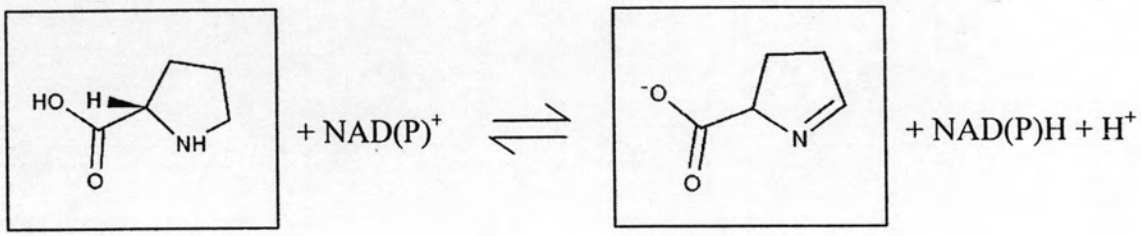
Note: - = no data

Source: *Agrobacterium tumefaciens* ((Misono and Nagasaki 1982), *Geobacillus stearothermophilus* (Heydari *et al.*, 2004)

was synthesized based on the N-terminal amino acid sequence of the enzyme (MKVLVLGAGLMGKEAARDLVQSQDV). After ligation of the putative gene fragment with linearized pUC18 and transformed into *E. coli* JM109. They reported that *lys 6-dh* gene consisted of 1,155-bp open reading frame encoding for 385 amino acids residues and the specific activity of the crude *E. coli* clone extract was about 800 fold higher than the activity of the *G. stearothermophilus* extract. The similarity of the amino acid sequence of *G. stearothermophilus* Lys 6-DH was sought among the sequences in the GenBank databases by using the BLAST server. The similarity was found to be 65% identity to that of the *Oceanobacillus iheyensis* HTE831 hypothetical protein (Lu *et al.*, 2002 and Takami *et al.*, 2002); somewhat lower level of similarity was found to a *P. horikoshii* hypothetical protein (33% identity) (Kawarabayasi *et al.*1998), a *Thermoplasma acidophilum* hypothetical protein (31% identity) (Ruepp *et al.* 2000), and *A. tumefaciens* strain C58 putitive dehydrogenase (30% identity) (Goodner *et al.*, 2001 and Wood *et al.*, 2001).

1.5 Pyrroline-5-carboxylate reductase (P5CR, EC 1.5.1.2)

The second enzyme that involves in the second step of L-pipecolic production via P-6-C pathway is pyrroline-5-carboxylate reductase (P5CR) which belongs to the P5CR family. P5CR catalyses the NADPH-dependent conversion of pyrroline-5-carboxylate (P-5-C) to L-proline in proline biosynthesis pathway from L-glutamate (Deutch *et al.*, 1982, Hayzer *et al.*, 1980 and Rossi *et al.*, 1977). It is found in almost all organisms (Leisinger *et al.*, 1987 and Smith *et al.*, 1980). Pathways of proline biosynthesis are well established for several organisms (Adams and Frank, 1980, Leisinger, 1996, Brandriss and Falvey, 1992, Dougherty *et al.*, 1992 and Delauney *et al.*, 1993)



L-proline

Δ^1-pyrroline-5-carboxylate reductase (P5CR)

Figure 1.4 Reaction scheme for Δ^1-pyrroline-5-carboxylate reduxtase (P5CR)

Soure: **Hayzer *et al.*, 1980**

To date, a large number of P5CR enzymes from different sources have been studied. They showed considerable diversity with respect to reaction rate with the nicotinamide coenzymes (NADPH or NADH), molecular mass and affinity to substrate, as well as a product inhibition. It was proposed that maybe this diversity reflects the different role the enzyme plays in regulation and metabolism (Merrill *et al.*, 1989). In some organisms, the activity of P5CR is osmoregulated concomitantly with proline accumulation (Taylor, C. B. 1996, Hanson *et al.*, 1982 and Hare and Cress, 1997). For higher plants like soybean, the mRNA level of P5CR increased after osmotic stress, and transgenic plants expressing P5CR were more stress-resistant to heat and drought (Delauney and Verma, 1990). In insects like *Drosophila melanogaster*, the transcription of P5RC is increased in response to cold stress (Misener *et al.*, 2001). In cultured cells, P5C and the proline cycle activate the pentose phosphate shunt and increase nucleotide synthesis by using the salvage and *de novo* pathways (Phang, 1985, Phang *et al.*, 1982 and Kohl *et al.*, 1988).

P5CR has been shown to play a role in the regulation of *de novo* purine biosynthesis through the generation of $NADP^+$, which is required for the synthesis of the purine precursor ribose 5-phosphate (Yeh *et al.*,1981). In human erythrocytes, the level of P5CR activity is comparable with the activity levels of major erythrocyte enzymes. This is consistent with the interpretation that the function of the enzyme in human erythrocytes may be to generate an oxidizing potential in the form of $NADP^+$ (Yeh *et al.*,1981). In some mammalian cells, the inter-conversion of proline and P5C provides a metabolic shuttle of redox equivalents between the cytosol and mitochondria. P5C can be transported into cells as a source of oxidizing potential where its reduction to proline generates $NADP^+$ (Hagedorn, 1986 and Hagedorn *et al.*, 1986). The process is mediated by significant amounts of P5CR found in the cytosol and some membrane-containing fractions. The proline can then be transported into the mitochondria, in which proline oxidase mediates its conversion back to P5C with the concomitant production of NADPH and ATP, thereby completing a proline cycle. In this process, proline mediates the generation of reactive oxygen species that have been linked to apoptosis (Donald *et al.*, 2001 and Polyak *et al.*, 1997).

1.6 Objectives of this research

Our research group screened for Lys 6-DH producing bacteria strain K-1 from soil samples in Japan. An isolate giving a high activity of Lys 6-DH was bacteria strain K-1. Since this enzyme also gave P-6-C from L-lysine which used for L-pipecolic acid production. The Lys 6-DH should be replaced LAT (Rius and Demain. 1997) to produce L-pipecolic acid by co-orperative with P5CR. But the low yield of Lys 6-DH from bacteria strain K-1 triggered us to use recombinant DNA technology to obtain a sufficient amount of the Lys 6-DH in order to study the enzyme characteristics and application for convert L-lysine to L-pipecolic acid by co-orperative with P5CR in *E. coli.* BL21 (DE3) (Figure 1.5).

The objectives of this research

1. Identification of Lys 6-DH producer by biochemical and 16S rRNA.
2. Cloning and expression of the *lys 6-dh* gene from *A. denitrificans* on pET-17b.
3. Purificaion and characterization of Lys 6-DH from recombianant clone.
4. Cloning and expression of the *p5cr* gene from *Bacillus cereus* on pET-17b.
5. Heterologous gene cloning and expression of the *lys 6-dh* and *p5cr* genes on pET-17b in *E. coli* BL21(DE3).
6. L-pipecolic acid production from L-lysine by Lys 6-DH and P5CR using organic solvent treated cells.

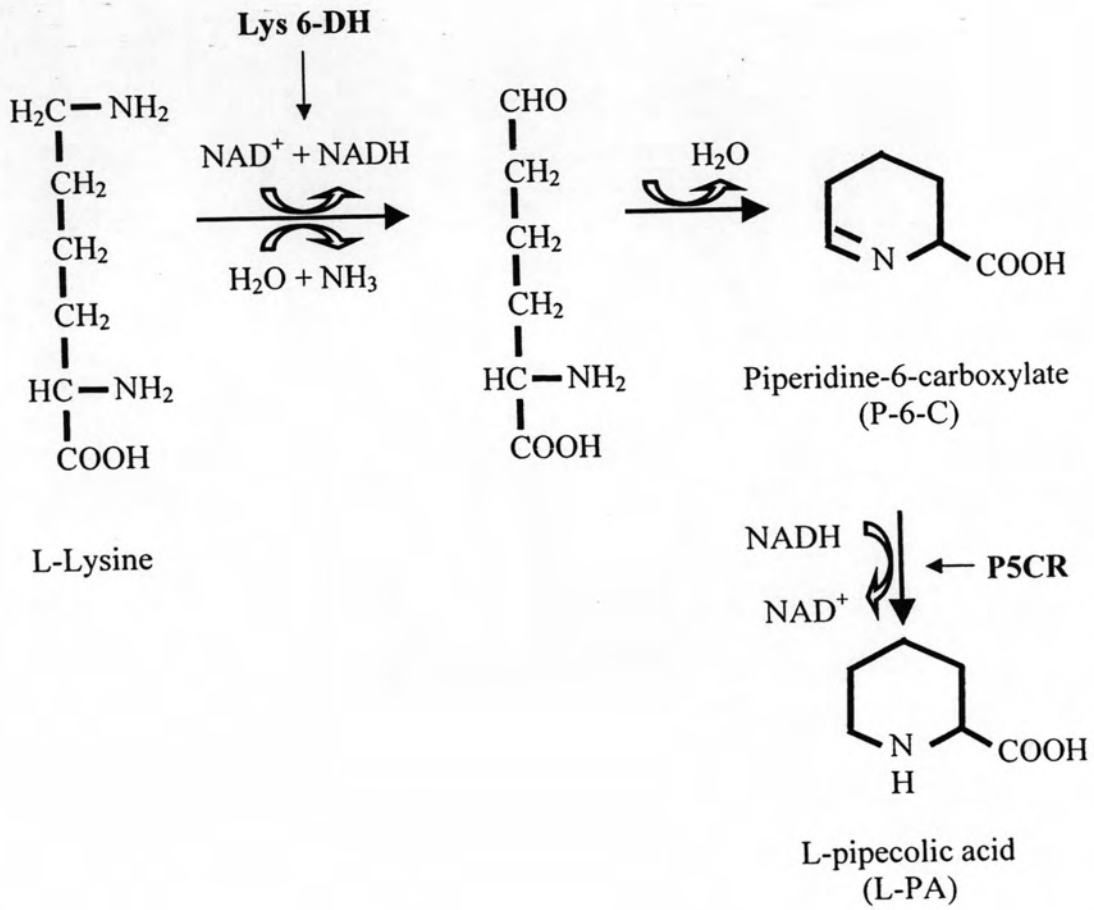


Figure 1.5 **Reaction scheme of L-pipecolic acid biosynthesis by Lys 6-DH and P5CR**