**L-Proline Catabolism in Gram-Positive Bacteria:**

**themes and variations**

**ABSTRACT**

The amino acid L-proline can be degraded to L-glutamate by many Gram-positive bacteria through a common pathway that uses the FAD-dependent activity L-proline dehydrogenase (PRODH) and the NAD+-dependent activity L-Δ1-pyrroline-5-carboxylate dehydrogenase (P5CDH), also called L-glutamate-γ-semialdehyde dehydrogenase (GSALDH).) The aim of this review is to summarize the processes of proline catabolism in the low G+C *Firmicutes* and the high G+C *Actinobacteria* and to illustrate the variations that commonly occur. In the low G+C clade, the two enzyme activities occur as single monofunctional proteins. In the high G+C clade, the two activities may occur either as monofunctional or bifunctional proteins. PRODH activity is a peripheral membrane-associated protein while P5CDH (GSALDH) activity may be found in the cytoplasm or associated with PRODH in the membrane fraction. The only major exception occurs in anaerobic *Clostridium* species which carry out cytoplasmic two-step Stickland reactions. Anfig important variation in proline catabolism among these microorganisms is in their regulation. Some bacteria in the low G+C clade such as *Bacillus* species have a specific regulatory protein that binds to DNA in the presence of L-proline and activates transcription. Other bacteria in this clade such as *Staphylococcus* species show no regulation of proline catabolism. Some bacteria in the high G+C clade such as *Mycobacterium* species also have a specific proline regulatory protein. In addition, L-proline catabolism may be affected by the global nitrogen and carbon regulators CodY and CcpA. However, CodY only appears to occur in the low G+C clade of Gram-positive bacteria. While carbon catabolite repression occurs in both clades, the protein CcpA is found in only some bacteria in the low G+C clade. Because a large amount of data is now available about various Gram-positive bacteria, further studies on the biochemistry, genetics, and physiology of L-proline degradation in these microorganisms may help explain the significance of these variations.

***Key Words:*** *Actinobacteria*, *Firmicutes*, Gram-positive bacteria, L-glutamate-γ-semialdehyde dehydrogenase,L-proline catabolism, L-proline dehydrogenase, L-Δ1-pyrroline-5-carboxylate dehydrogenase

1. **INTRODUCTION**

The amino acid L-proline plays several roles in the physiology of bacteria, plants, and animals. In addition to being incorporated into proteins and peptides, it can be used as a nitrogen and carbon source, an osmoprotectant, a mediator of redox signaling, a stabilizer of protein structure, a precursor of secondary metabolites, and an enhancer of resistance to a variety of stresses (Christgen & Becker, 2019; Patriarca et al., 2021; Szabodos & Savouré, 2010). In many organisms, L-proline can be synthesized endogenously from L-glutamate or L-ornithine, using L-Δ1-pyrroline-5-carboxylate as a key intermediate. In organisms that cannot synthesize L-proline, it can be taken up from the surrounding environment through several types of membrane transporters. Once accumulated in the cytoplasm, it may be chemically modified, degraded, or exported out of the cell. This review focuses on the processes of L-proline catabolism in the Gram-positive bacteria.

Bacteria are often divided into two major groups - the Gram-negative bacteria and the Gram-positive bacteria - based on the organization and staining of their cell walls. The Gram-positive microorganisms can be further divided into two major clades based on the G+C content of their DNA (Hogenhout & Loria, 2008). Both of these clades are well separated from the Gram-negative Proteobacteria and the Archaebacteria (Archaea). The *Firmicutes* or low G+C clade of bacteria includes microbes in the genera *Bacillus*, *Halobacillus*, *Lactobacillus, Clostridium*, *Staphylococcus*, and *Streptococcus*, as well as the cell-wall deficient *Mycoplasmas* (Ludwig et al., 2009; Oren & Garrity, 2021; Zhang & Lu, 2015). The DNAs in this group vary from 25% mol% G+C to 55 mol% G+C. The *Firmicutes* typically form cells that are simple rods or cocci. They may be aerobic, facultatively anaerobic, or anaerobic. Most are chemoheterotrophs that grow by respiration or fermentation but a few are chemoautotrophic or photosynthetic. Some are motile but many are nonmotile. Some form endospores as a stable resting stage but others do not. There are over 275 different species in this lineage. The *Actinobacteria* or high G+C clade includes bacteria in the genera *Actinomycetes*, *Micrococcus*, *Arthrobacter*, *Corynebacterium*, *Streptomyces*, and *Mycobacterium* (Barka et al., 2016; Chater, 2016; Gao & Gupta, 2012). The DNAs in this group vary from 55 mol% G+C to 75 mol% G+C. These microbes may grow as bacilli or cocci, or they may form straight or branching filaments or mycelia. Some species can form exospores or other stable resting forms. These bacteria may grow aerobically or anaerobically as chemoheterotrophs utilizing a wide range of organic compounds including amino acids. They are often found in terrestrial or aquatic ecosystems but some are commensals or pathogens associated with plants or animals.

1. **THE PATHWAY OF L-PROLINE CATABOLISM**

L-proline is most often degraded in these microorganisms by a catabolic pathway that is common to almost all organisms (Adams & Frank, 1980; Liu et al., 2017; Tanner, 2019). The pathway is shown in the following figure.

 **(EC 1.5.5.2) Δ1-pyrroline- (EC 1.2.1.88)**

 **5-carboxylate**

Fig 1 Pathway Of L-Proline Catabolism

There is an initial FAD-dependent oxidation by L-proline dehydrogenase (PRODH, EC 1.5.5.2, formerly called proline oxidase and designated 1.5.99.8) to form L-Δ1-pyrroline-5-carboxylate (P5C). Electrons are then transferred from FADH2 to a quinone in a membrane-associated electron transport chain. P5C is in spontaneous equilibrium with L-glutamate-γ-semialdehyde, which then undergoes a NAD+-dependent oxidation by L-Δ1-pyrroline-5-carboxylate dehydrogenase (P5CDH, EC 1.2.1.88, formerly 1.5.1.12). This enzyme is also called L-glutamate-γ-semialdehyde dehydrogenase (GSALDH). The product L-glutamate can be converted to 2-oxoglutarate (α-ketoglutarate) by several different enzymes including glutamate dehydrogenase and glutamate-pyruvate transaminase (Walker & van der Donk, 2016). The 2-oxoglutarate is then further metabolized through the citric acid cycle or used in other catabolic or anabolic pathways.

The two enzyme activities needed for L-proline degradation are organized in three basic ways (Tanner, 2008; Tanner, 2019). In organisms with monofunctional proteins, PRODH activity is due to a membrane-associated enzyme commonly called PutA that transfers electrons to a quinone in the electron transport chain. The P5CDH (GSALDH) activity is due to a separate cytoplasmic enzyme. In organisms with bifunctional proteins, the PRODH and P5CDH (GSALDH) activities are part of a single membrane-associated protein that is also called PutA. In some cases, there is an additional aldehyde dehydrogenase superfamily (ADHSF) domain at the carboxyl-terminal end of the protein. In organisms with trifunctional proteins, a PutA protein with both PRODH and P5CDH (GSALDH) activities has an additional amino-terminal DNA-binding domain. This domain can interact with specific DNA sequences and allows PutA to act as a transcriptional regulator of the genes encoding itself and a major Na+-dependent proline transporter called PutP. The PutA protein in these cases moves between the plasma membrane and the DNA depending on the oxidation state of the FAD cofactor (Wood, 1987; Zhang et al., 2004). Cells often have additional L-proline transporters including those that are osmotically-inducible or that are capable of facilitating the uptake of other solutes such as glycine betaine, proline betaine, ectoine, or choline.

The studies described in the next two sections indicate that proline catabolism in the low G+C and high G+C Gram-positive bacteria usually but not always involves monofunctional proteins with separate PRODH and P5CDH (GSALDH) activities. The synthesis of these enzymes is often induced by growing bacteria in the media containing L-proline, which allows the amino acid to be used as a carbon or nitrogen source. However, the extent of synthesis may be affected by the presence of other carbon or nitrogen sources such as D-glucose or ammonium sulfate. It may also be influenced by the external salt concentration.

1. **L-PROLINE CATABOLISM IN LOW G+C GRAM-POSITIVE BACTERIA**

**3.1 *Bacillus* species**

The genus *Bacillus* encompasses a large number of species that are generally described as Gram-positive endospore-forming chemoheterotrophic rods (Logan & De Vos, 2015; Zeigler & Perkins, 2021). They are usually motile and either aerobic or facultatively anaerobic. They are usually catalase-positive but may be oxidase-positive or oxidase-negative. The bacteria are usually able to grow in a minimal medium containing a simple carbon source such as D-glucose and a nitrogen source such as ammonium sulfate or an amino acid. The primary modes of metabolism are mixed-acid fermentation and respiration with either oxygen or nitrate as the terminal electron acceptor. Most species are not pathogenic to humans but many can synthesize important antibiotics. There is considerable debate about the classification of the many *Bacillus* species, and several investigators have proposed new taxonomic schemes for their grouping and identification (Gupta et al., 2020; Xu & Kovács, 2024).

De Hauwer et al. (1964) showed that *Bacillus subtilis* could form the two enzymes needed for the catabolism of L-proline, PRODH and P5CDH. Both activities were induced by the addition of L-proline to the medium. The P5CDH activity used for proline catabolism was biochemically different from the one used for the degradation of L-arginine. Laishley & Bernlohr (1968) demonstrated that both activities were also present in *Bacillus licheniformis*. In cells growing in a minimal medium with L-glutamate as the primary carbon and nitrogen source, the addition of 20 mmol L-1 L-proline resulted in a large increase in proline catabolism as measured manometrically by oxygen consumption. Proline oxidation also occurred in the presence of D-glucose but at half the rate, indicative of catabolite repression. Similar effects were obtained using a direct assay for P5CDH activity. These results were confirmed by Atkinson et al. (1990), who found that proline oxidase (proline dehydrogenase) activity in *Bacillus subtilis* as measured by the formation of L-Δ1-pyrroline-5-carboxylate was induced by the addition of L-proline to the medium but repressed by D-glucose and a mixture of 16 other amino acids. Although L-proline can stimulate germination of the endospores formed by *Bacillus* species, it has been shown that *B. megaterium* spores do not contain measurable levels of PRODH and P5CDH activity (Rossignol & Vary, 1979).

Proline catabolism in *B. subtilis* was studied in more detail by Moses et al. (2012). They described the structure of the *putBCP* gene cluster (formerly called *ycgMNO*) and its regulation. The gene *putB* encodes PRODH, *putC* encodes P5CDH, and *putP* encodes a proline transporter. The activity of PRODH as measured by a colorimetric assay for Δ1-pyrroline-5-carboxylate increased about five-fold in bacteria grown in the presence of 1 mmol L-1 L-proline prior to harvest. This did not occur in strains containing deletions in the *putB* gene and the growth yields in these strains in the presence of L-proline were reduced. The ability of the bacteria to use L-prolne as the sole carbon source was abolished in a *putBCP* deletion strain, although the bacteria could still grow with L-proline as the nitrogen source. This was shown to be due to the uptake of proline by the osmotically-inducible OpuE transporter. A double mutant with deletions in both *putP* and *opuE* could still take up radioactive 14C-proline and was partially sensitive to the proline analogues azetidine-2-carboxylate and 3,4-dehydroproline, indicating the presence of other proline transporters. Northern blot analysis indicated that the *putBCP* genes were transcribed as a unit (an operon), which was inducible by L-proline through an activator protein called PutR. In strains containing a fusion of *putB* with the gene *tre* encoding a phospho-α-(1,1)-glucosidase, measurement of expression of the operon by the hydrolysis of the chromogenic substrate p-nitrophenyl-α-D-glucopyranoside (α-PNPG) indicated that it was strongly induced by L-proline in the absence of added NaCl. In the presence of 0.6 mol L-1 NaCl, expression was also induced by dimethylproline and the proline analogues L-azetidine-2-carboxylate and 3,4-dehydro-L-proline. However, accumulation of intracellular L-proline during osmotic stress did not induce transcription as measured by Tre activity.

**3.2 *Halobacillus* species**

The genus *Halobacillus* was first described in 1996 after the isolation of several new salt-tolerant bacterial species from the Great Salt Lake in Utah and the combination of these with *Sporosarcina halophila* (Claus et al., 1983; Spring et al., 1996). The bacteria are Gram-positive spore-forming cells which may be spherical, oval, or rod-shaped, sometimes with pointed ends (Spring, 2015). Many are motile and are capable of growth in 5% to 25% (0.5 mol L-1 to 2.0 mol L-1) NaCl. The bacteria are positive for both oxidase and catalase and often form orange-pigmented colonies. They are aerobic chemoheterotrophs that require chloride ions for growth (Roeßler & Műller, 1998; Saum et al., 2013). The bacteria can degrade polymers such as casein, gelatin, and starch. They metabolize simple carbohydrates such as glucose or fructose and degrade amino acids as nitrogen sources. The primary mode of metabolism is aerobic respiration using an electron transport chain containing a NADH dehydrogenase, succinate dehydrogenase, menaquinone, cytochrome b, cytochrome c, and several terminal oxidases (Pade et al., 2013).

Köcher et al. (2011) showed that *H. halophilus* could grow in a minimal medium with L-proline as the sole carbon and nitrogen source. Maximal growth occurred in the presence of 2.0 mol L-1 NaCl. The bacteria actively accumulated L-proline. After disruption of late exponential phase cells with a French Pressure Cell, PRODH activity could be measured by the reduction of the artificial electron acceptor dichlorophenolindophenol in the presence of L-proline, FAD, and the crude cell extract. Activity was reduced after growth in the presence of D-glucose. P5CDH activity could also be measured using the cell-free extract in the presence of L-Δ1-pyrroline-5-carboxylate by the reduction of NAD+ to NADH. However, activity was higher in the presence of NADP+ as the electron acceptor. This enzyme activity again was induced in the presence of L-proline but reduced when D-glucose was added.

Analysis of the *H. halophilus* genome indicated the presence of two genes for each of the degradative enzymes. The genes *pdh1* and *p5cdh1* were located in different parts of the genome while the genes *pdh2* and *p5cdh2* formed a cluster and were transcribed as unit. Both *pdh* genes encoded proteins of about the same size (305 or 307 amino acids), which showed high sequence identity and similarity to the PRODH from *Bacillus subtilis*. The two *p5cdh* genes also encoded proteins of about the same size and high sequence identity and similarity to the P5CDH from *B. subtilis*. The total PRODH and P5CDH activities were highest in the late exponential-phase of growth in medium with D-glucose as the carbon source when 3.0 mol L-1 NaCl was added to induce accumulation of L-proline as an osmoprotectant. The transcription of *pdh2* and *p5cdh2* was stimulated by 3.0 mol L-1 NaCl, but transcription of *pdh1* and *p5cdh1* was not affected by the salt concentration. The isoenzymes PRODH1 and P5CDH1 thus appear to be involved in L-proline degradation when it is the sole carbon or nitrogen source while the PRODH2 and P5CDH2 isoenzymes are used to degrade excess accumulated L-proline when other sources are exhausted.

**3.3 *Staphylococcus* species**

The genus *Staphylococcus* includes a very large number of species (Schleifer & Bell, 2015). The bacteria are spherical Gram-positive cells which are often found in pairs, tetrads, or small clusters. They are not motile and do not form spores or resting stages. The cell walls contain a peptidoglycan in which the diamino acid lysine is often crosslinked to D-alanine through short chains of glycine or other amino acid residues. The walls frequently contain teichoic acids but are not usually encapsulated. The DNA contains 30 mol% to 40 mol% G+C and is about 2-3 Mbp in size. Most staphylococci are moderately halophilic and can grow in media containing 5% to 10% NaCl. These microorganisms are facultative anaerobes that can grow by fermentation of sugars to form D- or L-lactic acid or by aerobic respiration using electron transport chains containing menaquinones and cytochromes a and b. They are positive for catalase but negative for oxidase activity. The host or niche range may be narrow or broad and many species are opportunistic pathogens of humans (Winstel et al., 2021).

*Staphylococcus* species are normally auxotrophic for L-proline and require an external source of this amino acid to grow in a defined medium (Gladstone, 1937; Emmett & Kloos, 1975). The genome of these bacteria lacks the genes for the most common pathway which leads from L-glutamate to Δ1-pyrroline-5-carboxylate to proline using the enzymes γ-glutamyl kinase and Δ1-pyrroline-5-carboxylate reductase. There are two alternative pathways (Townsend et al., 1996). One leads from L-arginine or L-ornithine to Δ1-pyrroline-5-carboxylate to L-proline and uses the enzymes arginase, ornithine aminotransferase, and Δ1-pyrroline-5-carboxylate reductase. The other leads from L-arginine to L-ornithine to L-proline and uses the enzymes arginase and ornithine cyclodeaminase. However, expression of the genes for these enzymes is repressed by the global regulatory protein CcpA (Li et al., 2010). Proline can be actively accumulated using several transport systems including the high affinity PutP system, the low affinity ProP system, the osmotically-regulated OpuC and OpuD systems, and a newly-recognized PutT system (Lehman et al., 2023; Schwan, 2022).

Once in the cells, proline can be degraded by PRODH and P5CDH to form L-glutamate. The catabolic process was characterized in detail for *S. saprophyticus* (Deutch, 2011). Degradation of L-proline was initially demonstrated by the formation of small red colonies on proline-utilization test plates containing L-proline, a small amount of peptone, and 2,3,5-triphenyltetrazolium chloride. PRODH activity in *S. saprophyticus* was then measured in whole cells that had been frozen and thawed by reaction of the Δ1-pyrroline-5-carboxylate product with 2-aminobenzaldehyde to form a yellow complex. The specific activity was not affected by the addition of L-proline or NaCl to the growth medium but was reduced in the presence of D-glucose. The addition of L-proline at 10 mmol L-1 or 50 mmol L-1 to tryptic soy broth or Mueller Hinton broth did not increase the growth rate or yield of bacteria. Addition of 200 mmol L-1 was actually somewhat inhibitory. *S. saprophyticus* has complex growth requirements and it was not possible to determine if the bacteria could use L-proline as the sole carbon or nitrogen source.

PRODH activity was found to be localized in membrane fractions prepared after conversion of the bacteria to protoplasts and disruption in a French Pressure Cell. There was a low level of activity as measured by reaction of Δ1-pyrroline-5-carboxylate with *2-*aminobenzaldehyde, but much higher activity as demonstrated by reduction of *p*-iodotetrazolium violet in the presence of L-proline, FAD, and phenazine methosulfate. P5CDH activity, on the other hand, was found in a soluble fraction of *S. saprophyticus* prepared by disruption of the bacteria with glass beads and centrifugation at 105,00 x *g*. The activity was measured by the reduction of NAD+ to form NADH. The increase in absorbance at 340 nm was linear with time and the amount of extract. The enzyme showed simple Michaelis-Menten kinetics with an apparent *Km* for DL-Δ1-pyrroline-5-carboxylate of 1.43 mmol L-1 and an apparent *Km* for NAD+ of 0.167 mmol L-1.

**3.4 Lactic Acid Bacteria species**

The lactic acid bacteria are a large group of Gram-positive non-spore-forming microbes that produce lactic acid as a byproduct of their metabolism (Khalid, 2011). They may occur as rods or cocci and are usually nonmotile. They lack cytochromes and cannot use oxygen as a terminal electron acceptor although many are aerotolerant. They are catalase negative but often have multiple peroxidases. Most grow by fermentation of carbohydrates and can form L-lactate or D-lactate by a homofermentative or heterofermentative process. The major genera in this group are *Lactobacillus, Lactococcus, Streptococcus, Pediococcus, Leuconostoc*, and *Bifidobacteria*. Lactic acid bacteria are often associated with food production, but they are commonly found as commensal microorganisms in the mouth, intestine, or vagina of humans.

Most lactic acid bacteria can take up and degrade amino acids (Fernández & Zúñiga, 2006). Tammam et al. (2000) and Williams et al. (2001) showed that several species of *Lactobacillus* isolated from cheddar cheese could metabolize L-proline as measured by the reduction of a tetrazolium dye in metabolite test plates. This process required the presence of α-ketoglutaric acid (2-oxoglutarate), which was not metabolized or able to reduce the tetrazolium dye by itself. The product of proline catabolism and the enzymes involved were not identified, however. The source of the electrons for reduction of the tetrazolium dye in the metabolic test plates has not been determined. Tian et al. (2016) showed that addition of exogenous L-proline to cultures of *Lactobacillus-paracasei* improved growth and lactic acid formation. However, this appeared to be due to the amino acid’s ability to act as an osmoprotectant rather than its metabolism. Studies on other lactic acid bacteria such a *Lactococcus lactis* have indicated that L-proline stimulates growth but there is no evidence for its metabolism (Smid & Konings, 1990). This is not surprising since proline catabolism normally involves its oxidation by PRODH and transfer of electrons to a membrane-associated electron transport chain.

**3.5 *Clostridium* species**

The clostridia are a large group of Gram-positive bacteria that include the genus *Clostridium*, the genus *Veillonella*, the genus *Desullfotomaculum*, and the genus *Heliobacterium*. They have Gram-positive cell walls but often stain negatively in this process (Dűrre, 2014). They typically form rods but these may be bent or pleomorphic. They may be motile or nonmotile. They grow anaerobically, and while most are chemoheterotrophs, the heliobacteria are photoheterotrophs. The bacteria have complex growth requirements and normally require exogenous amino acids and vitamins (Feeney et al., 1944). Their metabolism involves fermentation of sugars, organic acids, and amino acids, leading the formation of H2, CO2, various acids, and solvents. Several species can form endospores when nutrients become limiting. Some like *C. tetani*, *C. botulinum*, and *C. difficile* are important human pathogens because they can form potent exotoxins.

Costilow & Cooper (1978) reported the purification of a soluble protein from *C. sporogenes* that had both proline dehydrogenase and Δ1-pyrroline-5-carboxylate reductase activities. This appeared to be the same enzyme normally used in the L-proline biosynthesis, but which was capable of carrying out a reversible reaction between proline and P5C. Monticello & Costilow (1981) later described the purification and characterization of this protein in more detail. The specific activity of the dehydrogenase reaction was much lower than that for the reductase reaction and decreased substantially after chromatography on hydroxyapatite or storage in the cold in solutions of low ionic strength. The proline dehydrogenase activity was sensitive to inhibition by low concentrations of L-glutamate but the reductase activity was not. There have been no further studies on this protein. It is possible that another protein in the *C. sporogenes* crude extract could bind to the enzyme with P5C reductase activity and reverse its directionality.

The only well-supported process of proline catabolism in the clostridia occurs by the two-step Stickland reaction (Barker, 1981; Neumann-Schall et al., 2019; Stadtman & Elliott, 1957). In this process, one amino acid such as leucine, isoleucine, or alanine undergoes oxidative deamination or decarboxylation. Electrons are transferred to NAD+ and ATP is eventually formed to support growth and metabolism. The electrons then are transferred from NADH to a second amino acid such as glycine or proline which is reduced. In the case of proline metabolism in the clostridia, L-proline is first converted to D-proline by a proline racemase. The D-proline is then reduced by the selenium-containing enzyme D-proline reductase to form 5-aminovaleric acid. The energetics and the organization of the genes involved in this pathway were recently reviewed (Pavao et al., 2022), Proline has been found to increase the growth of *C. difficile* and transcription of the genes for proline reductase (Bouillaut et al., 2013). This was found to be dependent on an activator protein called PrdR, which is transcribed from a gene located just upstream of the genes needed for enzyme formation (Johnstone & Self, 2022). The protein also decreases transcription of the genes for the formation of a similar selenoprotein called glycine reductase.

1. **L-PROLINE CATABOLISM IN THE HIGH G+C GRAM-POSITIVE BACTERIA**

**4.1 *Streptomyces species***

The family Streptomycetaceae includes three genera, the most important of which is *Streptomyces* (Barka et al., 2015; Chater, 2016; Law et al., 2018). This genus includes over 150 species, which are strictly aerobic chemoheterotrophs that can form complex multicellular structures. The bacteria grow as a dense filamentous mycelium of vegetative hyphae. When nutrients become limiting, aerial hyphae are formed which can produce exospores.  *Streptomyces* species are often found in soil where they form antibiotics such as streptomycin, vancomycin, and tetracycline which inhibit the growth of other bacteria.

Kominek (1972) showed that 14C-L-proline could be degraded by a novobiocin-synthesizing strain of *Streptomyces niveus*, particularly in older cultures as macromolecular synthesis slowed and the antibiotic was formed. The percentage of utilization decreased from 100% in the presence of 1.5 mmol L-1 ammonium sulfate to 40% in the presence of 3.0 mmol L-1 ammonium sulfate to 0 in the presence of 6 mmol L-1 ammonium sulfate. There was a concurrent reduction in novobiocin synthesis. Shapiro & Vining (1985) found that *Streptomyces venezuelae* could grow and form chloramphenicol in a medium with L-proline as the sole nitrogen source. There was an increase in the rate at which the proline was utilized if ammonium was also present. Cheng et al. (2013) showed that the addition 100 g L-1 of L-proline to cultures of *Streptomyces lydicus* E9 resulted in an increase in streptolydigen accumulation and growth yield. A metabolomic analysis indicated that the intracellular concentration of glutamate increased 5.9-fold after 12 hr, indicating some of the exogenous proline was quickly taken up and degraded. More recently, Millan-Oropeza et al. (2017) studied the metabolism of *Streptomyces coelicolor* and *Streptomyces lividans* by a proteomic analysis. They found that when the bacteria were grown in a minimal medium with yeast extract and glucose, proteins associated with fermentation and glycolysis predominated in *S. lividans* while those associated with the citric acid cycle and gluconeogenesis predominated in *S. coelicolor*. The medium used was rich in amino acids. Proteins involved in the catabolism of amino acids including PRODH and P5CDH were more abundant in *S. coelicolor*. The concentration of L-proline in the medium decreased more rapidly in this organism.

Bascarán et al. (1989) measured the activity of PRODH in extracts of *Streptomyces clavuligerus* with a colorimetric assay based on the reaction of Δ1-pyrroline-5-carboxylate product with 2-aminobenzaldehyde to form a yellow complex. They found that the activity was high after growth in a medium with proline alone or with proline and ammonium as the nitrogen source. However, it was reduced when alanine or glutamate was the nitrogen source, This indicated that PRODH activity was inducible by proline but not repressed by ammonium in this species. Hood et al. (1992) found that PRODH (then called proline oxidase) and P5CDH activities could be detected in *Streptomyces coelicolor* strain J802 using an assay with 14C-L-proline. The proline oxidase activity was membrane bound while P5CDH activity was cytoplasmic. Formation of both activities was high when either ammonium or proline was the nitrogen source. However, P5CDH activity was reduced when the bacteria were grown in a medium with glutamate, glutamine, or arginine as the nitrogen source.

Smith et al. (1995) later described the purification of P5CDH from *Streptomyces coelicolor* A3(2) using an assay based on the reduction of NAD+. The specific activity of this enzyme was much higher after growth in a medium containing L-proline as the nitrogen source rather than ammonium. This increase in activity was inhibited by both rifampicin and chloramphenicol, indicating that it was due to gene transcription and mRNA translation and not simply activation of a preformed protein. SDS-polyacrylamide gel electrophoresis indicated that the denatured protein has a mass of 68 kDa. However, the native enzyme showed a mass of 305,000 by gel filtration chromatography and 265,000 by nondenaturing gel electrophoresis, suggesting it occurs as a tetramer in cells. L-proline did not affect the activity of the enzyme, but there was inhibition by NADH, which was competitive with both P5C (*Ki* of 770 µmol L-1) and NAD+ (*Ki* of 600 µmol L-1). There was only partial inhibition by L-glutamate (30% with 25 mmol L-1).

* 1. ***Arthrobacter* and *Paenarthrobacter species***

The genus *Arthrobacter* has historically been included in the suborder Micrococcinae and is characterized by a dimorphic life cycle in which exponential phase cells occur as rods and stationary phase cells occur as cocci (Busse et al., 2015). Some of these bacteria form multicellular myceloids with rudimentary branches when subjected to osmotic stress or nutrient limitation (Germida & Casida, 1980; Deutch & Perera, 1992). Over 90 different species have been isolated from a variety of habitats including soil, fresh water, and sea water. Although most arthrobacters are not pathogenic, a few human pathogens including isolates of *A. cumminsii* and *A. albus* have now been identified. The bacteria have a typical Gram-positive cell wall as seen by electron microscopy but tend to decolorize easily and are often reported to be Gram-variable (Beveridge, 1990). They usually contain lysine rather than diaminopimelic acid in their peptidoglycan, which may be connected to D-alanine by a peptide bridge. The taxonomy of the genus *Arthrobacter* was revised in 2016 and several species including *A. aurescens* were transferred to a new genus *Paenarthrobacter* on the basis of their lipid profiles (Busse, 2016).

The only microbe in this group whose proline metabolism has been studied in detail is *Arthrobacter aurescens* strain TC1, now called *Paenarthrobacter aurescens* strain TC1. This high G+C Gram-positive aerobe was isolated from atrazine-contaminated agricultural soil in South Dakota USA (Strong et al., 2002). It has been characterized in detail biochemically with respect to atrazine degradation. The genome of *P. aurescens* strain TC1 has been completely sequenced and consists of a circular chromosome and two large circular plasmids (Mongodin et al., 2006). The main chromosome contains a gene designatedAAur\_0671 that is predicted to encode a bifunctional PutA protein of 1157 amino acids (A1R2L2\_PAEAT). Although *P. aurescens* strain TC1 does not contain a gene homologous to *putP* that encodes the major Na+/proline transporter in other bacteria, there is a gene homologous to *proP* that appears to encode a proline/glycine betaine transporter (A1R1I8\_PAEAT)*.* This microbe also has genes homologous to *proX*, *proW, and proV* which appear to encode parts of possible ABC proline/glycine betaine transporters. However, there is no gene homologous to *opuE* that encodes an osmotically-inducible proline transporter in *Bacillus subtilis* (Moses et al., 2012).

*P. aurescens* strain TC1 was shown to grow well in minimal media with L-proline as a supplemental nutrient, the nitrogen source, or the sole carbon and nitrogen source (Deutch, 2019). Multicellular myceloids induced by NaCl or citrate also grew on L-proline. The specific activity of PRODH in whole cells as measured by reaction of Δ1-pyrroline-5-carboxylate with 2-aminobenzaldehyde was higher whenever L-proline was added to the medium. Both PRODH and P5CDH activities were found primarily in a membrane fraction from exponential-phase cells. PRODH was measured both by reaction of Δ1-pyrroline-5-carboxylate with 2-aminobenzaldehyde and by reduction of p-iodonitrotetrazolium violet in the presence of L-proline, Triton X-100, KCN, FAD, and phenazine methosulfate. P5CDH activity was measured in a similar way in the presence of DL-pyrroline-5-carboxylate, Triton X-100, NAD+, and phenazine methosulfate. The two activities eluted together from a Bio-Gel P-60 column after precipitation of proteins with ammonium sulfate and solubilization with 0.1% Tween 20. This was consistent with the presence of a bifunctional PutA protein, although the activity of P5CDH was consistently higher than that of PRODH. The PutA protein in the active fraction also oxidized 3,4-dehydro-DL-proline, but there was no activity with other L-proline analogues. The oxidation of both L-proline and 3,4-dehydro-DL-proline was inhibited by L-lactate, a known inhibitor of the PutA protein in *Escherichia coli* (Scarpulla & Soffer, 1978). When *P. aurescens* strain TC1 was grown in minimal media containing increasing concentrations of NaCl, there was a progressive decrease in the specific activity of PRODH in whole cells and a concomitant increase in the intracellular concentration of L-proline.

* 1. ***Corynebacterium* species**

The corynebacteria are a group of high G+C Gram-positive bacteria which typically form short rod-shaped cells arranged in a V (Eggeling & Bott, 2021), They may be curved or club-like and commonly form palisades of cells lined up side by side. They are catalase positive but do not form spores. The bacteria may grow by aerobic or anaerobic respiration or by fermentation. The various species vary in their G+C content and in their genome complexity (Oliveira et al., 2017). Some such as *Corynebacterium diphtheriae* are important human or animal pathogens and form multiple exotoxins (Bernard, 2012). Others like *C. glutamicum* are important industrially because they form and excrete large amounts of amino acids such as L-glutamate (Lee et al., 2016).

Several studies have indicated that *Corynebacteria* species can take up and degrade L-proline. Sabart et al. (1986) reported that a virulent strain of the plant pathogen *C. fascians* which could infect pea seedings was able to grow in a minimal medium with L-proline or agmatine as the nitrogen source. Avirulent mutants could not. Bott & Niebisch (2003) reported that *C. glutamicum* could grow slowly with L-proline as the sole carbon and nitrogen source and suggested that this was due to a bifunctional PutA protein homologous to that from *E. coli*. However, no data were provided. Mounier et al. (2007) showed that *C. casei* and *C. variabile* isolated from surface-ripened cheese could consume L-proline from a semisynthetic medium during growth. They also showed that the addition of L-proline at the end of the exponential growth phase resulted in an additional increase in turbidity. Other studies indicated that *C. glutamicum* forms an active transport system for L-proline called PutP as well as transporters for glycine betaine and ectoine (Peter et al., 1998). The PutP system remained active in the presence of high salt concentrations and so was thought to contribute to osmotic stability. Coudert & Vandecasteele (1975) described the properties of an L-amino acid oxidase that was isolated from *Corynebacterium* A-20 after growth in a minimal medium with 1% glucose as the carbon source and 10 mmol L-1 L-lysine as the nitrogen source. The activity of the enzyme with L-proline as the substrate as measured by oxygen uptake was only about 10% of that seen with L-lysine or several other amino acid substrates. This enzyme has not been characterized further.

More recently, Korasick et al. (2017) described a detailed analysis of proline degradation in *C. freiburgense* based on the crystal structure of its PutA protein. In this bacterium, proline catabolism is due to a bifunctional protein with PRODH and P5CDH activities. The *Km* for L-proline was found to be 145 mmol L-1, while that for P5C was 54 µmol L-1. There is an additional aldehyde dehydrogenase superfamily domain at the carboxyl-terminal end of this protein, the deletion of which dramatically reduced both catalytic activities. This PutA protein exhibited several novel structural features and normally formed dimers in the presence of the proline analogue L-tetrahydro-2-furoic acid and NAD+. Zhang et al. (2020) subsequently reported that deletion of the gene encoding PutA in *C. glutamicum* could greatly increase the formation of L-proline by glucose-grown cells.

**4.4 *Mycobacterium* species**

The mycobacteria are a group of high G+C Gram-positive bacteria whose cell envelope is modified by the presence of an outer membrane and the presence of long chain mycolic acids linked to a layer of arabinogalactans (Rastogi et al., 2001; Rigouts & Cogneau, 2021). As a result the cell wall is very hydrophobic and only effectively stained using the acid-fast procedure. While many mycobacteria are free living saprophytes, some such as *Mycobacterium tuberculosis* and *Mycobacterium leprae* are important human pathogens that cause tuberculosis and leprosy, respectively. They are aerobic or microaerophilic nonmotile non-spore-forming microorganisms that most often occur as rod-shaped or curved cells. The mycobacteria are chemoheterotrophs that grow by respiration in both extracellular and intracellular environments (Cook et al., 2013), The mycobacteria have often been divided into two major groups (slow growers and fast growers) based on their relative rates of growth and differentiated on the basis of a variety of phenotypic characteristics. Although some have proposed dividing the mycobacteria into different phyla, most scientists still consider them a single group based on nucleotide sequencing (Meehan et al., 2021; Tortoli et al., 2017).

Smith et al. (2001) showed that an auxotrophic mutant of *Mycobacterium tuberculosis* could grow in axenic cultures in a defined medium with L-proline as a supplement or in the lung tissues of mice. However, it could not grow as an intracellular form in macrophages. The mutant did show good survival during starvation for 15 weeks (Parish, 2003). Agapova et al. (2019) reported more recently that L-proline could be taken up by *Mycobacterium tuberculosis* and could increase the intracellular pool but was poorly used as a nitrogen source. These studies indicated that transport systems for this amino acid must exist but did not address the issue of its catabolism.

By contrast, Berney et al. (2012) showed that L-proline could be used as carbon and nitrogen source by *Mycobacterium smegmatis*. Some species of the genus *Mycobacterium* have recently been renamed to *Mycolicibacterium*, so that *M. smegmatis* is now called *Mycolicibacterium smegmati*s in some papers. Proline utilization depended on two genes designated *pruA* and *pruB*, which encoded the monofunctional enzymes P5CDH and PRODH, respectively. The genes were expressed in a polycistronic mRNA. Proline catabolism also depended on a high affinity sodium/proline symporter called PutP. The *putP* gene encoding this protein was transcribed in the opposite direction. Transcrription of *pruA*, *pruB*, and *putP* was controlled by a membrane-associated DNA-binding activator protein called PruC and the presence of L-proline. Mutants with deletions in *pruA*, *pruB*, and *pruC* could not grow with proline as the sole carbon source although those with mutations in *putP* showed continued slow growth indicating the presence of other proline transporters. The gene cluster associated with the proline catabolism was also found in *M. tuberculosis*, *M. bovis*, *M. avium*, *M. avium paratuberculosis*, and *M. vanbaalenii*. The mutant with a deletion in *pruC* showed an increased expression of a large set of genes involved in SOS-response, and several DNA gyrase and DNA polymerase genes. It was also implicated in the detoxification of methyl glyoxal, suggesting the protein has more general regulatory activity.

Serrano & Blanchard (2013) extended these studies by cloning the *pruB* gene encoding PRODH from *Mycobacterium tuberculosis* and expressing it first in *E. coli* to verify its sequence and then in *M. smegmatis* to overproduce the protein. The amino acid sequences of *M. tuberculosis* and *M. smegmatis* had a high degree of similarity but very limited homology to the PutA sequence from *E. coli*. The kinetic properties of the enzyme were determined using an assay based on the reduction of the electron acceptor 2,6-dichlorophenolindophenol in the presence of L-proline. The *Km* for L-proline was 5.7 mmol L-1. The enzyme was most active over a broad range of alkaline pHs. Studies with defined mutants indicated that a lysine residue at position 110 and a tyrosine residue at position 203 were critical for activity. They proposed a model for the enzyme’s mechanism based on the removal of a proton from the imino group of proline by a deprotonated lysine-110 residue, followed by a transfer of a hydride ion to FAD.

Lagautriere et al. (2014) amplified the *pruA* and *pruB* genes from *Mycobacterium tuberculosis* and expressed them in *M. smegmatis*. The PruA protein with a His6 tag containing the P5CDH activity was purified from *M. smegmatis* cells by a combination of metal affinity chromatography and size exclusion chromatography. using an assay based on the reduction of NAD+. The isolated protein was crystallized alone and in the presence of NAD+. A selenomethionine substituted protein was also crystallized. The structural features of the proteins were defined and a model consisting of catalytic domain and a NAD+-binding domain was developed. The amino acid sequence was found to be most homologous to other monofunctional P5CDH proteins. Analysis of the PruA crystals indicated that the protein forms a highly stable dimer. A model for NAD+ binding and the catalytic reaction was proposed. The PruB protein containing PRODH activity was also purified in this project using the same general methods. A coupled reaction leading to the proline-dependent formation of glutamate and an increase in A340 was observed over a period of 18 hr when the two proteins were combined in a ratio of 1 PruA:10 PruB.

1. **DISCUSSION**

These studies indicate that almost all Gram-positive bacteria degrade L-proline by the standard pathway which leads from L-proline to L-Δ1-pyrroline-5-carboxylate to L-glutamate-γ-semialdehyde to L-glutamate. In the low G+C clade, the two enzyme activities required for this process – PRODH and P5CDH (GSALDH) – always seem to occur as single monofunctional proteins. In the high G+C clade, the two activities occur either as monofunctional proteins in the case of *Streptomyces* and *Mycobacterium* species or as bifunctional proteins in the case of *Arthrobacter/Paenarthrobacter* and *Corynebacterium* species. Where it has been characterized biochemically, PRODH is found as a peripheral membrane-associated protein that can donate electrons to a quinone in the electron transport chain. There is no evidence for a strictly cytoplasmic soluble version of this protein with this catalytic activity. P5CDH (GSALDH) may be found in the cytoplasm or associated with PRODH in the membrane fraction. The only major exception occurs in anaerobic *Clostridium* species which carry out the two-step Stickland reactions.

It is not surprising that the bacteria in the two clades of Gram-positive bacteria share a common pathway for proline catabolism but vary in specific details. It is thought that the basic biosynthetic and catabolic pathways were developed early in the cellular life (Lazcano & Miller, 1999; Scossa & Fernie, 2020). They then evolved within the various phylogenetic lineages now found to occur within the domains Archaea, Bacteria, and Eukarya (Stackebrandt, 2009). Several new models for the evolution of prokaryotes have been proposed (Gupta, 2000; Lake et al., 2009; Skophammer et al. 2007; Coleman et al., 2021), but all of them suggest an early separation between the low G+C Gram-positive *Firmicutes* and the high G+C Gram-positive *Actinobacteria*. There are now many differences between the bacteria in these two groups including their patterns of cell growth and division (simple binary fission or formation of filaments and mycelia) and the types of resting cells they may form (endospores or exospores). Other differences include nucleotide inserts in specific genes including those for RNA polymerase subunit C, small ribosomal protein S12, dihydroorotate dehydrogenase, and uroporphyrinogen decarboxylase (Skophammer et al., 2007).

An important variation among the proline catabolic enzymes is in their regulation. In some bacteria within the low G+C clade such as *Bacillus* species, there is a specific regulatory protein (PutR) that binds to DNA in the presence of L-proline and activates transcription (Moses et al., 2012). The gene for this protein is located adjacent to and downstream of the *putBCP* cluster but is not itself autoregulated by L-proline. The PutR protein binds to a specific inverted-repeat sequence near the promoter for *putB*. PutR is a novel protein that shows no obvious significant sequence similarity to other transcriptional activators (Huang et al., 2011). In other bacteria in this clade such as *Staphylococcus* species, there appears to be no regulation of proline catabolism. The catabolism of L-proline in the high G+C Gram-positive bacteria does appear to be regulated in some cases. In *Arthrobacter* and *Paenarthrobacter*, formation of the catabolic enzymes is increased in medium containing L-proline (Deutch, 2019), but no specific regulatory protein has been identified. In other *Mycobacterium* *smegmatis*, there is a specific regulatory protein (PruC) that controls proline catabolism (Berney et al., 2012). Another key point of variation involves the global regulatory proteins CodY and CcpA. CodY only appears to occur in the low G+C clade of Gram-positive bacteria. Carbon catabolite repression occurs in both clades but the protein CcpA is involved only in some bacteria in the low G+C clade. One area of regulation that has not been well studied involves the effects of osmotic stress on proline catabolism. L-proline is often accumulated during growth in medium containing high concentrations of NaCl as an osmotic protectant. In *E. coli*, this can be associated with the induction of new transport systems and a repression of proline catabolism (Culham et al., 1994, Deutch et al., 1989). However, the mechanism(s) by which this occur in the Gram-positive bacteria are not understood. Further studies on the regulation of proline degradation in both the low G+C clade of Gram-positive bacteria and the high G+C clade of Gram-positive bacteria therefore would be useful.

Christgen & Becker (2016) have summarized the importance of the proline degradation pathway in host/pathogen interactions and suggested ways in which new drugs might be designed to treat infections in humans. Khalid et al. (2022) have emphasized the importance of L-proline metabolism in the responses of plants to abiotic stresses. Because some Gram-positive bacteria can be human or plant pathogens, studies on proline catabolism in these bacteria contribute to our understanding of other biologically important processes.

1. **CONCLUSIONS**

The degradation of L-proline by the common catabolic pathway that uses the FAD-dependent activity L-proline dehydrogenase (PRODH) and the NAD+-dependent activity L-Δ1-pyrroline-5-carboxylate dehydrogenase (P5CDH, GSALDH) frequently occurs within the Gram-positive bacteria, However, there are important variations within this group. These variations include the presence of monofunctional enzymes in the low G+C Firmicutes and either monofunctional or bifunctional enzymes in the high G+C Actinobacteria. While the formation of these enzymes is regulated at the level of transcription by some bacteria in each group, it is unregulated in others. Regulation may occur either by gene-specific proteins like PutR or PruC or by global regulators like CodY or CcpA. Detailed biochemical analyses have been done for only a few species in each group (*Bacillus subtilis, Staphylococcus saprophyticus, Streptomyces coelicolor, Paenarthrobacter aurescens, Mycobacterium tuberculosis*). Because a large amount of sequence data is now available about various Gram-positive bacteria, further studies on the genetics, biochemistry, and physiology of L-proline degradation in other species would help explain the significance of the observed variations.

**DISCLAIMER (ARTIFICIAL INTELLIGENCE)**

The author hereby declares that no generative artificial intelligence (AI) tools such as Scalable Language Models (Chat GPT, COPILOT, etc.) or text-to-image generators were utilized in authoring or editing this paper.

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