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Matthias Rath MD believes that proline supplementation supports immune function before (prevention) and during (treatment) infection.

Discoveries

FORUM REVIEW ARTICLE

Role of Proline in Pathogen and Host Interactions

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Abstract

Significance: Proline metabolism has complex roles in a variety of biological processes, including cell signaling, stress protection, and energy production. Proline also contributes to the pathogenesis of various disease-causing organisms. Understanding the mechanisms of how pathogens utilize proline is important for developing new strategies against infectious diseases.
Recent Advances: The ability of pathogens to acquire amino acids is critical during infection. Besides protein biosynthesis, some amino acids, such as proline, serve as a carbon, nitrogen, or energy source in bacterial and protozoa pathogens. The role of proline during infection depends on the physiology of the host/pathogen interactions. Some pathogens rely on proline as a critical respiratory substrate, whereas others exploit proline for stress protection.
Critical Issues: Disruption of proline metabolism and uptake has been shown to significantly attenuate virulence of certain pathogens, whereas in other pathogens the importance of proline during infection is not known. Inhibiting proline metabolism and transport may be a useful therapeutic strategy against some pathogens. Developing specific inhibitors to avoid off-target effects in the host, however, will be challenging. Also, potential treatments that target proline metabolism should consider the impact on intracellular levels of Δ^1-pyrroline-5-carboxylate, a metabolite intermediate that can have opposing effects on pathogenesis.
Future Directions: Further characterization of how proline metabolism is regulated during infection would provide new insights into the role of proline in pathogenesis. Biochemical and structural characterization of proline metabolic enzymes from different pathogens could lead to new tools for exploring proline metabolism during infection and possibly new therapeutic compounds. *Antioxid. Redox Signal.* 30, 683–709.

Keywords: proline, pathogenesis, metabolism, PutA, stress

Introduction

THE ROLES OF PROLINE IN biology are complex and impact a wide range of cellular processes (Fig. 1). While proline is critical for protein synthesis and cell growth, it also has roles in osmoregulation (29, 53, 106, 155, 247), redox signaling (65, 134, 182, 256, 257), unfolded protein responses (129), protein stability (44, 97, 116, 129, 130, 178), cellular bioenergetics (35, 166, 181, 182, 226), and stress resistance (18, 111, 130, 182, 222, 258) (Fig. 1A). In addition, some organisms use proline directly for the biosynthesis of secondary metabolites with antibacterial or antifungal properties (Fig. 1B) (50, 79, 142, 148, 175, 197, 228).

Proline accumulation and its oxidative metabolism are major mechanisms by which proline affords protective benefits to organisms (130). The properties of proline as an organic osmolyte provide cellular protection against abiotic stress such as drought and osmotic shock (53, 222). The oxidative metabolism of proline enables cells to utilize proline as a carbon and nitrogen source, helping support growth and energy demands (2, 226, 233). Cells must appropriately manage proline levels to support protein biosynthesis and respond to stress and energy needs. Cellular processes such as import, catabolism, biosynthesis, and amino acid recycling coordinate proline flux for optimal growth and stress response. For further details concerning potential mechanisms of proline stress protection see reviews by Liang *et al.* (130) and Szabados and Savouré (222).

An interesting aspect of proline is its manipulation by pathogenic organisms in response to changes in the environment. There are a growing number of examples of proline facilitating the pathogenicity of different disease-causing

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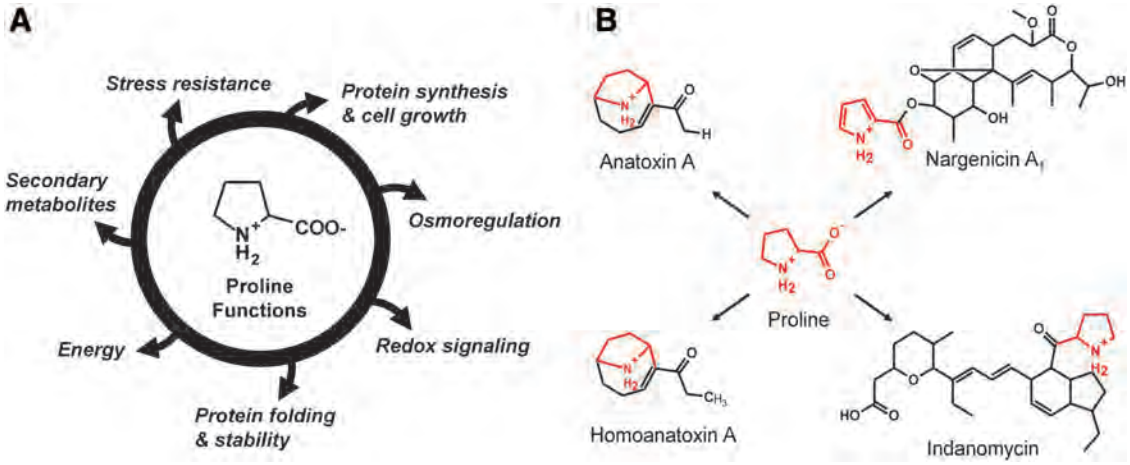

FIG. 1. Intracellular roles of proline. (A) Potential biological functions of proline. **(B)** Examples of proline being directly incorporated into the biosynthesis of antimicrobial compounds. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

organisms. Here, we review the role of proline metabolism in several bacterial and eukaryotic pathogens. Some of the bacterial pathogens highlighted in this review have high priority status for new antibiotics by the World Health Organization such as *Helicobacter pylori* (clarithromycin resistant), *Staphylococcus aureus* (methicillin resistant, vancomycin intermediate and resistant), and *Mycobacterium tuberculosis*. We also examine proline metabolism in entomopathogenic bacteria, which enable soil-dwelling nematodes to infect and multiply within insect larvae. Unfortunately, we were not able review pathogenesis in plants but interested readers are referred to some excellent reviews by Qamar *et al.* (185), Rizzi *et al.* (194), and Szabados and Savouré (222). From the literature it is evident that proline affords different benefits to pathogenic organisms according to the pathophysiological environment.

Proline Metabolism

Enzymes of proline metabolism

Proline is synthesized from glutamate in a series of steps (Fig. 2) (11, 54, 74). The enzyme γ-glutamyl kinase (GK; EC 2.7.2.11) converts glutamate to γ-glutamyl phosphate, which is next reduced to glutamate-γ-semialdehyde (GSA) by γ-glutamyl phosphate reductase (GPR; EC 1.2.1.41) (54, 74). GSA then cyclizes to form Δ^1-pyrroline-5-carboxylate (P5C). In higher organisms such as plants and animals, GK and GPR activities are combined into a bifunctional enzyme known as P5C synthetase (P5CS) (54, 74). In the next step, P5C is reduced to proline by the enzyme P5C reductase (P5CR; EC 1.5.1.2), which utilizes nicotinamide adenine dinucleotide (NADH) and/or nicotinamide adenine dinucleotide phosphate (NADPH) (45, 74). Proline biosynthesis from glutamate is subject to proline feedback inhibition of GK/P5CS thus limiting the intracellular pool of synthesized proline (74, 213, 223).

P5C can also be provided from ornithine *via* ornithine aminotransferase (OAT; EC 2.6.1.13), thereby linking proline metabolism to arginine (54, 74). In bacteria, such as *M. tuberculosis*, arginine serves as a key nitrogen and carbon source for ornithine, proline, and glutamate (91). In organisms that lack GK/GPR, such as *Trypanosoma brucei* and *S. aureus*, proline must be scavenged or synthesized *via* ornithine.

The catabolism of proline to glutamate relies on two enzymatic steps (Fig. 2) (2, 226). The first step involves proline dehydrogenase (PRODH; EC 1.5.99.8), a flavoenzyme that catalyzes the oxidation of proline to P5C with concomitant two-electron reduction of a noncovalently bound flavin adenine dinucleotide (FAD) cofactor (15, 110, 124, 136, 243). This is known as the PRODH reductive half-reaction. The PRODH oxidative half-reaction involves the transfer of electrons from reduced FAD to ubiquinone in the plasma membrane in prokaryotes and inner mitochondrial membrane in eukaryotes, thus linking proline catabolism with the respiratory chain (156, 158, 238). P5C undergoes a nonenzymatic hydrolysis to form GSA, which is then oxidized to glutamate by the nicotinamide adenine dinucleotide (NAD^+)-dependent enzyme P5C dehydrogenase (P5CDH; EC 1.5.1.12) (139, 177, 216). P5CDH is also known as L-glutamate-γ-semialdehyde dehydrogenase (GSALDH) (138). Glutamate dehydrogenase or amino acid transaminase converts glutamate to α-ketoglutarate, which feeds into the tricarboxylic acid (TCA) cycle (2, 35).

The overall conversion of proline to glutamate by PRODH and P5CDH is conserved across the domains of life, but there are significant differences in the arrangement of enzymes (Fig. 3) (226). In eukaryotes and gram-positive bacteria, the PRODH and P5CDH enzymes are expressed as two discrete monofunctional enzymes, whereas in gram-negative bacteria, the two enzymes are fused onto a single polypeptide called proline utilization A (PutA) (150, 226). Within the PutA family, there are PutAs that have an N-terminal ribbon-helix-helix (RHH) DNA binding domain known as trifunctional PutAs (9, 89, 120, 211, 226). Bacteria with bifunctional PutAs include *Helicobacter* spp., while those having trifunctional PutAs include *Escherichia coli* and *Salmonella typhimurium* (110, 226). Structural details concerning the classification of PutAs can be found in the work by Tanner and coworkers (109, 137, 138, 212, 226).

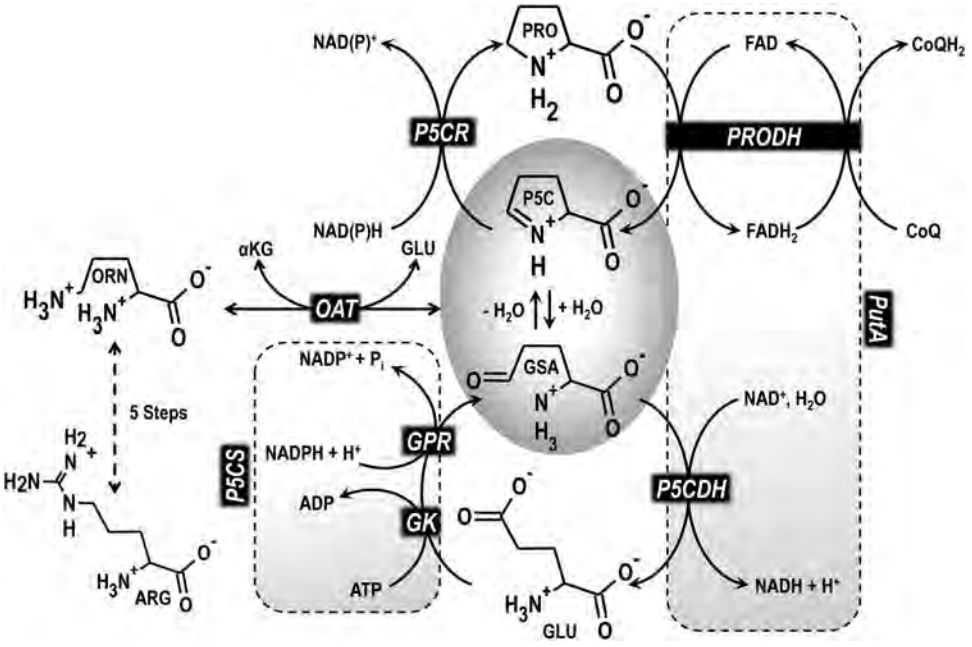

FIG. 2. Proline metabolic reactions. Proline biosynthesis begins with phosphorylation of glutamate by GK to form γ-glutamyl phosphate, which is then reduced to GSA by GPR. In higher organisms such as animals and plants, these two steps are catalyzed by the bifunctional enzyme P5CS. GSA cyclizes to P5C followed by NAD(P)H-dependent reduction to proline catalyzed by P5CR. P5C can also be generated from ornithine *via* OAT. Proline catabolism involves the two-step oxidation of proline to glutamate catalyzed by PRODH and P5CDH. In gram-negative bacteria, the PRODH and P5CDH activities are linked in the PutA bifunctional enzyme. PRODH is a flavin-dependent enzyme that couples the two-electron oxidation of proline to reduction of an electron acceptor in the membrane such as ubiquinone, and thus, proline catabolism feeds reducing equivalents into the respiratory chain. P5CDH pairs the oxidation of GSA to glutamate with reduction of NAD^+ to NADH. GK, γ-glutamyl kinase; GPR, γ-glutamyl phosphate reductase; GSA, glutamate-γ-semialdehyde; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide; NAD(P)H, reduced nicotinamide adenine dinucleotide (phosphate); OAT, ornithine aminotransferase; P5C, pyrroline-5-carboxylate; P5CDH, P5C dehydrogenase; P5CR, P5C reductase; P5CS, P5C synthetase; PRODH, proline dehydrogenase; PutA, proline utilization A.

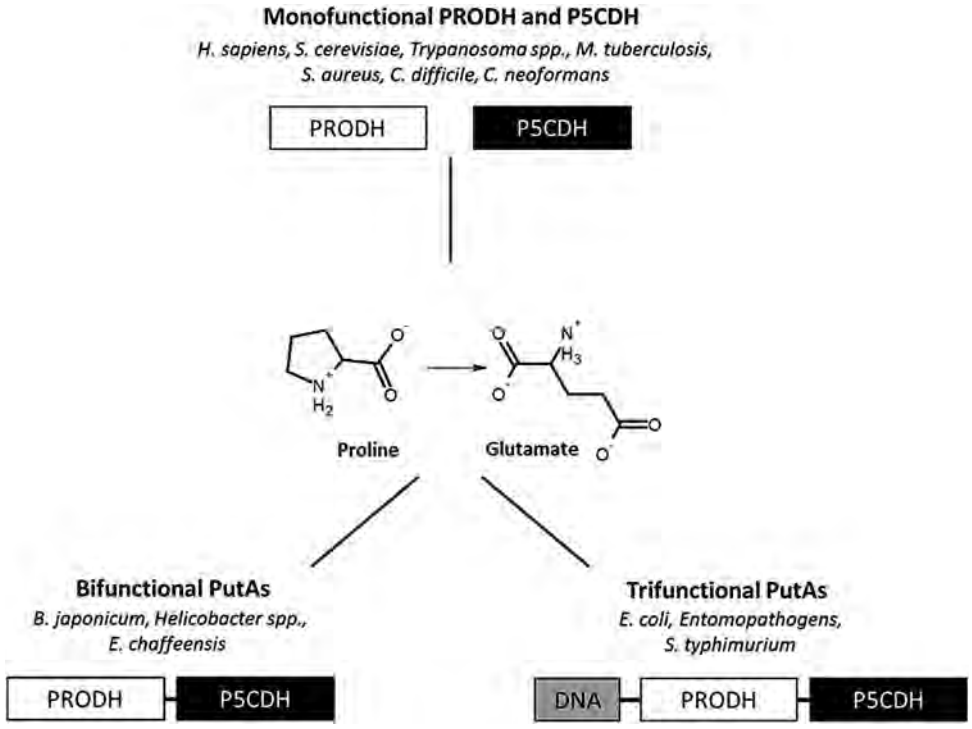

FIG. 3. Domain arrangement of proline catabolic enzymes in different organisms. The catabolism of proline to form glutamate is catalyzed by separate PRODH and P5CDH enzymes in eukaryotes and gram-positive bacteria such as *Trypanosoma* spp., *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Clostridium difficile*, and *Cryptococcus neoformans*. In gram-negative bacteria, PRODH and P5CDH are fused onto a single polypeptide called PutA. PutA enzymes can be classified as bifunctional, as in *Helicobacter* spp. and *Ehrlichia chaffeensis* or as trifunctional, depending on the presence of an N-terminal DNA binding domain such as in entomopathogenic bacteria, *Salmonella typhimurium*, and *Escherichia coli*.

Trifunctional PutAs function as a transcriptional repressor of the *put* regulon that encodes PutP, a high-affinity proline transporter, and PutA (150, 249, 263). The *putP* and *putA* genes are transcribed in opposite directions and are regulated by an intervening *put* control DNA region (Fig. 4) (87, 150, 264). Characterization of PutA from *E. coli* has shown that in the absence of proline, PutA binds to specific sites in the *put* control DNA region, thereby repressing transcription of the *put* genes (33, 83, 150, 263). In the presence of proline, PutA binds to the membrane rather than DNA, leading to upregulation of *put* gene expression (32, 172). The mechanism by which PutA regulates *put* gene expression is dependent on the redox state of the FAD cofactor. When the FAD cofactor is oxidized, that is, in the absence of proline, PutA binds *put* control DNA. In the presence of proline, the FAD cofactor is reduced, causing a conformational change in PutA that induces tight membrane binding, thereby switching PutA from a DNA binding protein to a membrane-bound proline

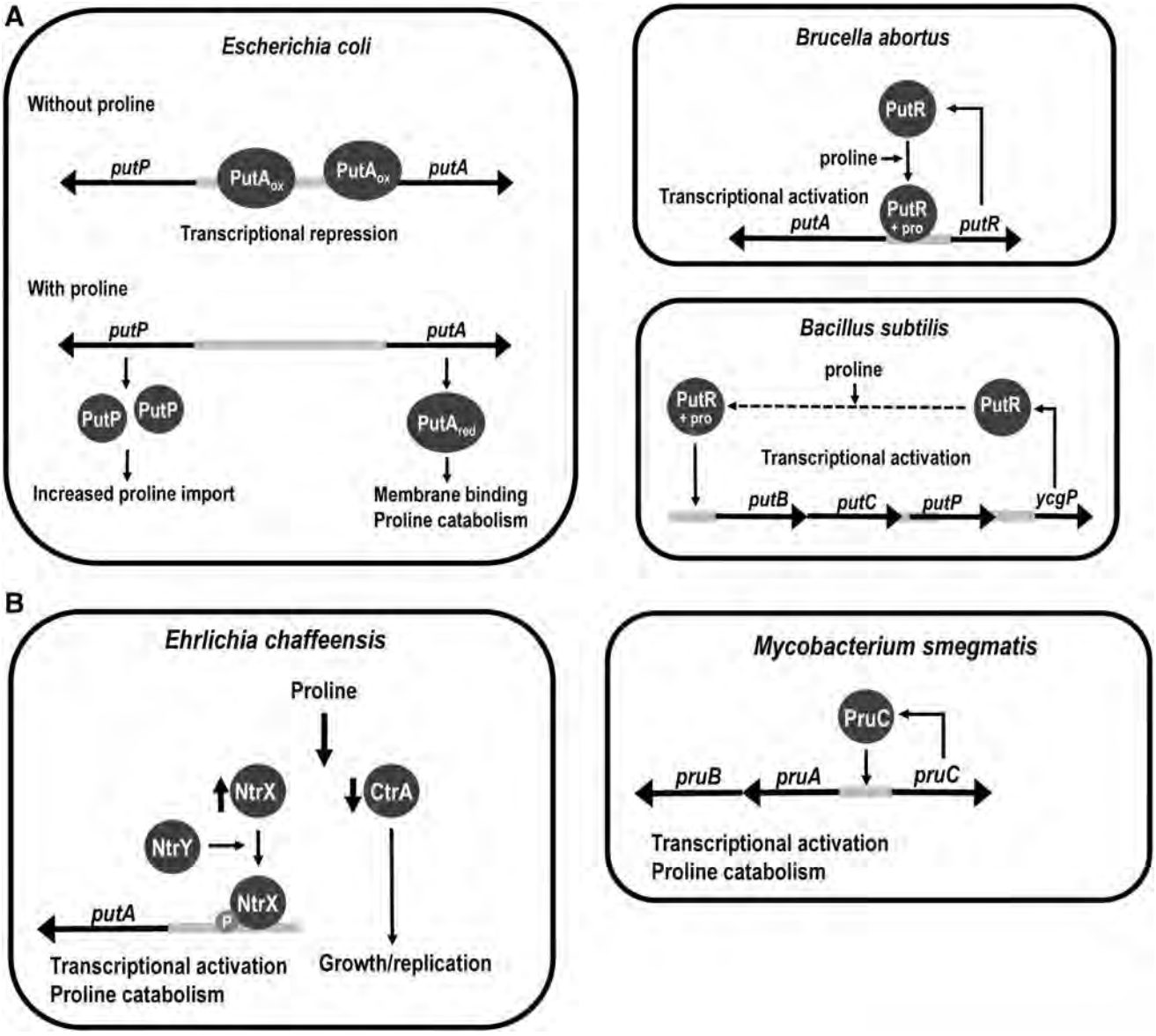

FIG. 4. Regulation of proline metabolism in different organisms. (A) *E. coli*: trifunctional PutA represses transcription of the *putA* and *putP* genes in the absence of proline. When proline becomes available, the FAD cofactor of PutA is reduced and PutA switches to a membrane-bound proline catabolic enzyme thereby relieving repression of the *put* genes. *Brucella abortus*: the PutR transcription factor (Lrp-type) is transcribed by the *putR* gene. PutR DNA binding is activated by proline leading to transcriptional activation of the *putA* gene. *Bacillus subtilis*: the PutR transcription factor (PucR-type) is transcribed by the *ycgP* gene. PutR DNA binding is activated by proline leading to transcriptional activation of *putB* (PRODH), *putC* (P5CDH), *and putP* genes. **(B)** *E. chaffeensis*: proline induces increased levels of the transcriptional regulator, NtrX, which on phosphorylation by NtrY binds to the *putA* promoter region and activates PutA expression. *Mycobacterium smegmatis*: PruC is transcribed divergently from the *pruAB* operon. Increased levels of PruC lead to higher expression of *pruA* (P5CDH) and *pruB* (PRODH). FAD, flavin adenine dinucleotide; Lrp, leucine-responsive regulatory protein.

catabolic enzyme (Fig. 4) (16, 217, 259, 260, 265, 267). To date, no evidence for PutA regulating other genes besides *putA* and *putP* has been reported.

Substrate channeling

An important metabolic aspect of PutA enzymes, regardless of being classified as bifunctional or trifunctional, is the channeling of the P5C intermediate between the PRODH and P5CDH active sites. Structural and kinetic data show evidence of substrate channeling for PutAs from five different gram-negative bacteria, such as *Bradyrhizobium japonicum* and *E. coli*, suggesting that substrate channeling is a conserved mechanism in the PutA family (10, 138, 157, 210, 211). Evidence for substrate channeling between separate monofunctional PRODH and P5CDH enzymes has been shown for PRODH and P5CDH from the gram-positive bacterium *Thermus thermophilus* (200). Whether PRODH and P5CDH enzymes in eukaryotes also channel P5C/GSA remains an open question. The benefit of channeling P5C in bacteria is largely to avoid futile proline-P5C cycling and to prevent the buildup of P5C/GSA, which has been shown to form unwanted adducts with other metabolites and exhibit toxic effects (11). These unwanted adducts include pyridoxal phosphate-P5C and acetoacetic acid-P5C, which have been found in human patients (237). P5C/GSA was recently classified as a ''Top 30'' damage-prone metabolite in a study analyzing the tendency of endogenous metabolites to undergo spontaneous chemical side reactions (125).

Proline and reactive oxygen species

Numerous studies have documented the role of proline catabolism in the protection of cells from oxidative stress and, somewhat counterintuitively, the generation of reactive oxygen species (ROS) (18, 110, 170, 182, 226). PRODH couples proline oxidation with reduction of the ubiquinone pool in the membrane, which can lead to ROS formation and impact intracellular signaling pathways. Proline protection against oxidative stress in mammalian cells was shown to rely on PRODH and downregulation of the forkhead transcription factor class O3 pathway *via* Akt (166). In *Caenorhabditis elegans*, PRODH was shown to upregulate the human homologue of Nrf-2, leading to increased antioxidant enzyme levels and life span of the worm (256).

In bacteria, proline oxidative metabolism by PutA has been shown to be important for oxidative stress protection *via* activation of the OxyR regulon (Fig. 5). Hydrogen peroxide formed by proline catabolism increases important antioxidant enzymes of the OxyR regulon such as catalase, thioredoxin, and glutaredoxin (257). Hydrogen peroxide was hypothesized to be a conserved metabolic by-product of proline catabolism due to proline oxidation being coupled with reduction of respiratory systems in different organisms (257). By increasing antioxidant defenses, proline may provide a preadaptive advantage, which in the context of pathogenesis would prolong cell survival, increase colonization efficiencies, and enhance virulence (134).

Regulation of proline catabolic genes

Proline utilization in bacteria is dependent on cellular energy status and the nutritional environment. Upregulation of proline catabolic genes relies not only on proline responsive transcription factors but also on global regulatory systems such as carbon catabolite repression (CCR) and nitrogen catabolite repression (NCR). Thus, the full utilization of proline requires the coordination of at least three different regulatory systems depending on the nutrient conditions.

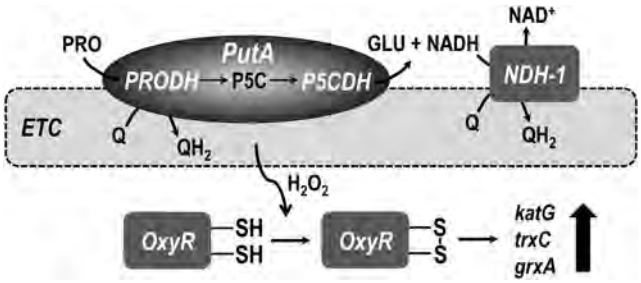

FIG. 5. Production of hydrogen peroxide from proline metabolism in *E. coli*. Oxidative proline metabolism by PutA leads to reduction of membrane-bound ubiquinone (Q) and formation of NADH, supplying electrons into the electron transport chain *via* NADH dehydrogenase (NDH-1). Hydrogen peroxide is generated as a by-product during respiratory chain activity to levels sufficient enough to activate the transcriptional regulator OxyR (-S-S-). OxyR subsequently upregulates expression of antioxidant genes such as thioredoxin (*trxC*), glutaredoxin (*grxA*), and catalase (*katG*).

The mechanisms by which proline upregulates expression of proline catabolic genes involve relief of transcriptional repression or activation of transcription depending on the organism. In gram-negative bacteria that contain trifunctional PutA, PutA is an autotranscriptional repressor of the *putA* and *putP* genes as described above (Fig. 4). From cell-based transcription assays in *E. coli*, it appears that PutA more strongly represses the *putA* gene than *putP* (262). Therefore, it is likely that *putP* expression is weakly repressed by PutA, allowing for rapid proline uptake and activation of *putA* gene expression when extracellular proline becomes available.

In organisms that lack PutA as an autogenous transcriptional repressor (*i.e.*, contain bifunctional PutA), transcription of proline catabolic genes is activated *via* proline-inducible transcription factors. In gram-negative *Rhodobacter capsulatus* (107), a transcriptional activator of the leucine-responsive regulatory protein (Lrp/AsnC) family called PutR was shown to activate expression of proline catabolic genes. PutR shares the conserved domains of the Lrp/AsnC family, which are an N-terminal helix-turn-helix DNA binding domain and a C-terminal α/β ligand binding domain (30, 227). PutR has also been demonstrated to activate proline catabolism in gram-negative pathogens, such as *Vibrio vulnificus* (123) and most recently the *putA* gene in *Brucella abortus* (37) (Fig. 4). In the pathogen *Pseudomonas aeruginosa PAO1*, PruR, a member of the AraC/XylS protein family, was found to be a transcriptional activator of *put* genes (161). An activator protein of proline catabolism in gram-positive *Bacillus subtilis* called PutR (*ycgP* gene) has also been described. PutR in *B. subtilis* is a PucR-like transcriptional regulator with a C-terminal helix-turn-helix DNA binding domain (17, 95, 155) (Fig. 4).

In yeast *Saccharomyces cerevisiae*, expression of proline utilization genes *PUT1* (PRODH), *PUT2* (P5CDH), and *PUT4* (proline-specific permease) is activated by a large transcription factor called Put3p (979 amino acids), which contains an N-terminal $Zn(II)_2Cys_6$ binuclear cluster DNA binding domain and a C-terminal proline activation domain (126, 208, 220). Proline binding to Put3p induces a conformational change leading to activation of Put3p-DNA binding and expression of the *PUT* genes (126, 208).

In addition to proline-specific regulation, proline catabolic genes are subject to global carbon and nitrogen regulatory mechanisms. The CCR in bacteria enables pathogens to exploit available carbon sources and also has an important role in the expression of virulence genes. In *E. coli*, CCR does not inhibit proline metabolism but is required for full induction. The cyclic AMP receptor protein (CRP) functions as an activator by increasing *putA* and *putP* promoter activity in poor nutrient environments (41, 163). CRP has two predicted DNA binding sites in the *put* control region of *E. coli* and has been shown to bind to *put* control DNA in response to cAMP (262). In *B. subtilis*, global CCR is mediated by the catabolite control protein A (CcpA) transcription factor, which under rich glucose conditions, represses genes necessary for metabolism of secondary carbon sources (80, 215). CcpA repression is relieved in glucose-deplete conditions thereby switching on catabolism of alternative carbon sources such as proline (80, 215). Proline catabolic genes in the pathogen *S. aureus* are also regulated by CcpA (90, 171).

Another global regulator of proline catabolism in gram-positive bacteria is the transcriptional regulator CodY, which is activated by branched-chain amino acids and GTP (215). CodY represses metabolism of poor nutrient sources and regulates virulence genes thereby helping cells adapt to changes in nutrients and the environment (215). CodY repression of proline catabolism is best understood in *B. subtilis* (12, 17, 31, 153) and is likely a relevant mechanism in other bacteria such as *S. aureus* (239). Belitsky showed that CodY repression of the proline utilization operon in *B. subtilis* involves displacement of the proline-specific activator (PutR) from the promoter region (17). As nutrient availability decreases, CodY becomes inactive leading to transcriptional activation of the proline utilization operon (17).

In addition to carbon, cells are faced with decisions of which nitrogen sources to utilize. Ammonium and glutamine are the first choices of nitrogen, whereas proline is of lower preference (215, 245, 246). Proline catabolism is generally responsive to nitrogen status *via* the global regulatory mechanism known as NCR (19). The *put* genes in *E. coli*, *Klebsiella aerogenes*, and *Pseudomonas putida* have been shown to be upregulated when cells are grown in limiting nitrogen conditions (41, 235, 251). The nitrogen assimilation control (NAC) regulator mediates the upregulation of *put* genes (81, 140). NAC, a LysR-type transcription factor, activates σ^{70}-dependent genes to generate ammonium and glutamate for glutamine synthesis (19). In *K. aerogenes*, *putP* expression is strongly activated by NAC, while *putA* expression seems to be activated indirectly by the resulting increase in proline uptake. The overall result of NAC-dependent regulation of *put* genes is the formation of glutamate for glutamine synthesis (19).

The regulation of proline catabolism by NCR has been studied in fungi as well. In *S. cerevisiae*, response to changes in nitrogen involves transcriptional regulators, GATA-type zinc finger proteins Gln3p and Gat1p (92) (Fig. 6). In nitrogen-poor medium, Gln3p activates expression of *PUT1* and *PUT4* (59, 61). Put4p is the yeast transporter specific for proline, but yeast also can import proline through the general amino acid permease (Gap1p) (92). Increased proline transport under nitrogen limitation leads to Put3p activation and further induction of the *PUT* genes (92). When yeast is grown on rich nitrogen medium, Ure2p interacts with Gln3p, which prevents nuclear localization of Gln3p and transcriptional activation of the *PUT* genes (114). Ure2p binding to Gln3p is dependent on TOR complex 1 phosphorylation of Gln3p, thus TORC1 has a primary role in the regulation of *PUT* genes by NCR (14, 126).

Proline transport and accumulation

E. coli and *Samonella enterica* utilize three different transport systems for manipulating intracellular proline levels. These uptake systems include the PutP high-affinity proline/sodium symporter, the ProP proton/osmolyte symporter, and the ABC-type transporter ProU (Fig. 7). PutP displays high specificity and affinity for proline, whereas ProP and ProU exhibit transport activity with a broad range of osmoprotectant compounds (5, 57, 100, 159, 257). ProU comprised membrane-bound ATPase (ProV) and permease (ProW) proteins, and a periplasmic binding protein ProX, which binds glycine betaine and proline betaine (84, 204, 248, 250). Studies of osmoregulatory mechanisms in *E. coli* have provided a foundation for understanding general prokaryotic osmotic stress responses (5, 51). In response to high external osmotic pressure (*e.g.*, increased NaCl), *E. coli* accumulate inorganic ions and organic solutes to help maintain cellular hydration and membrane turgor pressure (5, 247). Proline, glutamate, glycine betaine, and polyols such as trehalose are

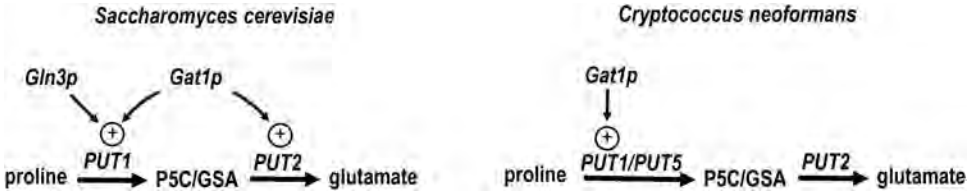

FIG. 6. Regulation of proline catabolic genes in fungi by nitrogen metabolite repression. The GATA transcription factors Gln3p and Gat1p activate proline catabolism in *Saccharomyces cerevisiae* when grown in poor nitrogen conditions. Gln3p activates *PUT1* (PRODH), while Gat1p activates both *PUT1* and *PUT2* (P5CDH). *C. neoformans* contains two PRODH genes, *PUT1* and *PUT5*. *PUT1* is activated by Gat1p, whereas *PUT5* and *PUT2* are not regulated by nitrogen metabolite repression.

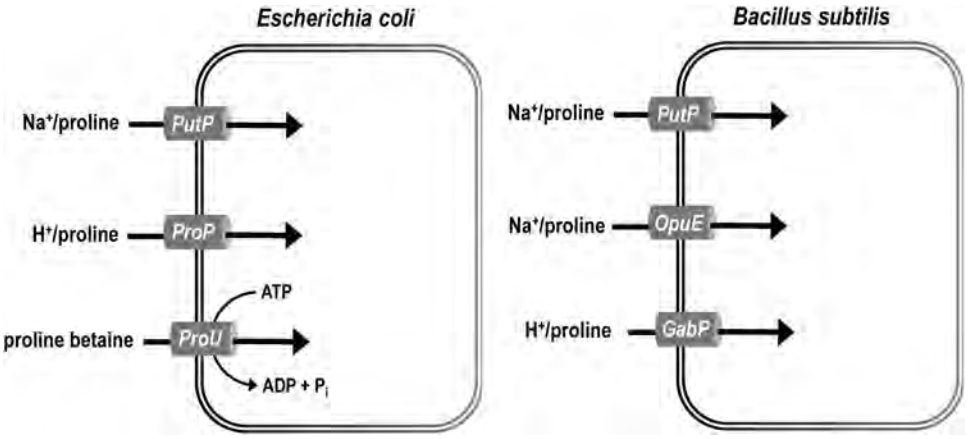

FIG. 7. Proline transporters in *E. coli* and *B. subtilis*. *E. coli* uptake systems are the high-affinity proline/sodium symporter PutP, the ProP proton/osmolyte symporter, and the ABC-type transporter ProU. *B. subtilis* contains a PutP homologue of *E. coli*, OpuE, which is a compatible solute transporter, and the γ-aminobutyrate transporter GabP. The PutP transporter is important for utilizing proline as a nutrient source. ProP and OpuE are the proline transporters most important for osmoregulation and protection against osmotic stress in *E. coli* and *B. subtilis*, respectively.

the major organic osmolytes or osmoprotectants (5, 53, 195). *E. coli* accumulate proline by importing it from the extracellular environment, as proline biosynthesis does not contribute to increased proline levels during osmotic stress (54). The osmolyte solutes transported by ProP and ProU broadly overlap and include proline, glycine betaine, proline betaine, and ectoine (141). ProP exhibits similar affinity for proline and glycine betaine (56), whereas ProU significantly favors glycine betaine over proline (86). Osmotic stress activates expression of the *proP* gene (40, 132, 149) and the *proU* operon, encoding the three components of the ProU transport system (*proVWX*) (5, 60, 104, 149). Wood and coworkers have shown that ProP is also regulated post-translationally (186), with ProP being nearly inactive at low osmolality but becoming maximally activated in a highly osmolality medium (56). The mechanism by which ProP is activated by increased osmolality has been elusive; however, Wood and coworkers recently proposed that the binding of cations (*e.g.*, potassium) to the C-terminal domain of ProP induces a conformational switch that increases transporter activity during osmotic stress (56, 58).

Proline is also a critical osmolyte in gram-positive bacteria such as *B. subtilis* with intracellular proline concentrations increasing up to 700 m*M* after osmotic shock (242). *B. subtilis* contains three main proline transporters: a PutP homologue, OpuE (254) of the Opu family of compatible solute transporters, and the γ-aminobutyrate transporter GabP (255) (Fig. 7). PutP and OpuE share 61% amino acid sequence identity and are high-affinity sodium/proline symporters (155). PutP is critical for use of proline as a nutrient source, while OpuE is important for using proline as an osmostress protectant (236). Bremer and coworkers have shown that OpuE is osmoregulated and responsible for increased proline transport in cells under osmotic stress (236, 254). In contrast to *E. coli*, *B. subtilis* also relies on proline biosynthesis to increase intracellular proline levels (28). Intriguingly, *B. subtilis* has an alternative route for proline biosynthesis involving paralogous enzymes for GK and P5CR (28). This second route is not subject to allosteric feedback inhibition by proline, and thus, *B. subtilis* is able to increase intracellular proline pools by proline import and biosynthesis (28).

Bacterial Pathogens

Escherichia *and* Salmonella

E. coli uropathogenic strains are a major cause of bladder infections and, less commonly, kidney infections or pyelonephritis (6, 94). Human urine is a good medium for *E. coli* bacterial growth with abundant carbon and nitrogen sources from amino acids, citrate, lactate, and ammonia. Identifying metabolic genes that are essential for infection has come from studies of *E. coli* uropathogens such as *E. coli* strain CFT073, which was originally isolated from a patient with pyelonephritis (152). Using different metabolic mutants of *E. coli* strain CFT073 and a mouse urinary tract infection model, Alteri and coworkers showed that mutants of gluconeogenesis and the TCA cycle were significantly defective in colonization, whereas other mutants of central metabolism, such as glycolysis and the pentose phosphate pathway, had no effect on colonization (6). Another study found that TCA and PPP are not critical for *E. coli* colonization of mouse intestine but that metabolism of gluconate by the Entner–Doudoroff pathway was most important (39). Whether proline oxidation is an important anaplerotic pathway for supplying carbon (*i.e.*, α-ketoglutarate) to the TCA cycle during urinary tract infection is not known.

Besides adapting metabolically, pathogens also need to overcome osmotic challenges to survive and establish infection. Because proline is an osmolyte protectant, its role in osmotic stress protection has been extensively investigated in bacteria, including uropathogens (5, 51). Mutant strains of *S. typhimurium* that exhibit increased tolerance to osmotic stress were found to have elevated levels of proline (52). In *E. coli*, proline has been shown to be important for tolerance of high osmotic and urea stress environments such as urine. The importance of proline transport in *E. coli* pathogenesis has been examined by characterizing the growth and colonization of different *proP* and *proU* mutant uropathogenic strains (55). Deletion of *proP* and *proU* in the *E. coli* strain

HU734 resulted in slower growth in high-osmolality human urine but did not impact urinary tract colonization in mice (55). Studies of other bacteria showed that the ProU transporter is important for infection of moth larvae by *Shigella sonnei* (143). Also, ProP was concluded to be critical for survival of *S. enterica* serovar Typhimurium in desiccated environments such as stainless steel surfaces (75).

Entomopathogenic bacteria

Entomopathogenic bacteria and worms have complex and mutually beneficial relationships (78). Symbiotic relationships involve *Xenorhabdus* spp. with the Steinernematidae nematode family and *Photorhabdus* species with nematodes of the Heterorhabditidae family. Soil-dwelling nematodes use nutrient resources from insect hosts for reproduction and propagation (78). To successfully infect insect larvae, the worms utilize enterobacterial symbionts such as *Xenorhabdus nematophila* and *Photorhabdus luminescens* (78). *Xenorhabdus* and *Photorhabdus* spp. colonize the gut of nematodes in a symbiotic relationship, but transition into a pathogenic lifestyle once inside insect larvae. After being expelled from the worm, the bacteria secrete insecticidal enzymes and cytotoxins (*e.g.*, xenematide) that kill the insect larvae and synthesize antibiotic compounds to ward off competing microbes (22, 23, 50, 66, 119, 175). During the infection process, the bacteria also weaken the innate immune system of the host, thereby facilitating infection, enabling the bacteria to grow, and allowing the worms to reproduce inside the insect carcass (78, 176, 191). A summary of the mutualism between nematodes and *Xenorhabdus* and *Photorhabdus* spp is illustrated in Figure 8.

This biological cycle in the soil environment is a natural insecticide mechanism that has drawn interest for biocontrol of crop pests (117). Commercial applications of entomopathogenic bacteria and nematodes to soil have been used for natural pest control (69, 117). There are also efforts to identify the toxins and antibiotics produced by *Xenorhabdus* and *Photorhabdus* spp. in hopes of finding new molecules for treatment of areas that are often devastated by insects (117). The genome sequencing of *X. nematophila* and *Photorhabdus* spp. *(e.g., Photorhabdus temperata, P. luminescens, and Photorhabdus asymbiotica)* is greatly enhancing these efforts (66).

Insects have an abundant supply of proline in their hemolymph, which has raised curiosity as to the role of proline in the pathogenesis stage of *X. nematophila* and *Photorhabdus* spp. (50, 175). Crawford *et al.* investigated the metabolic changes in entomopathogenic bacteria that are associated with increased production of antimicrobial compounds such as stilbenes, nematophin, and the cyclodepsipeptide xenematide (50). Analysis of insect samples showed

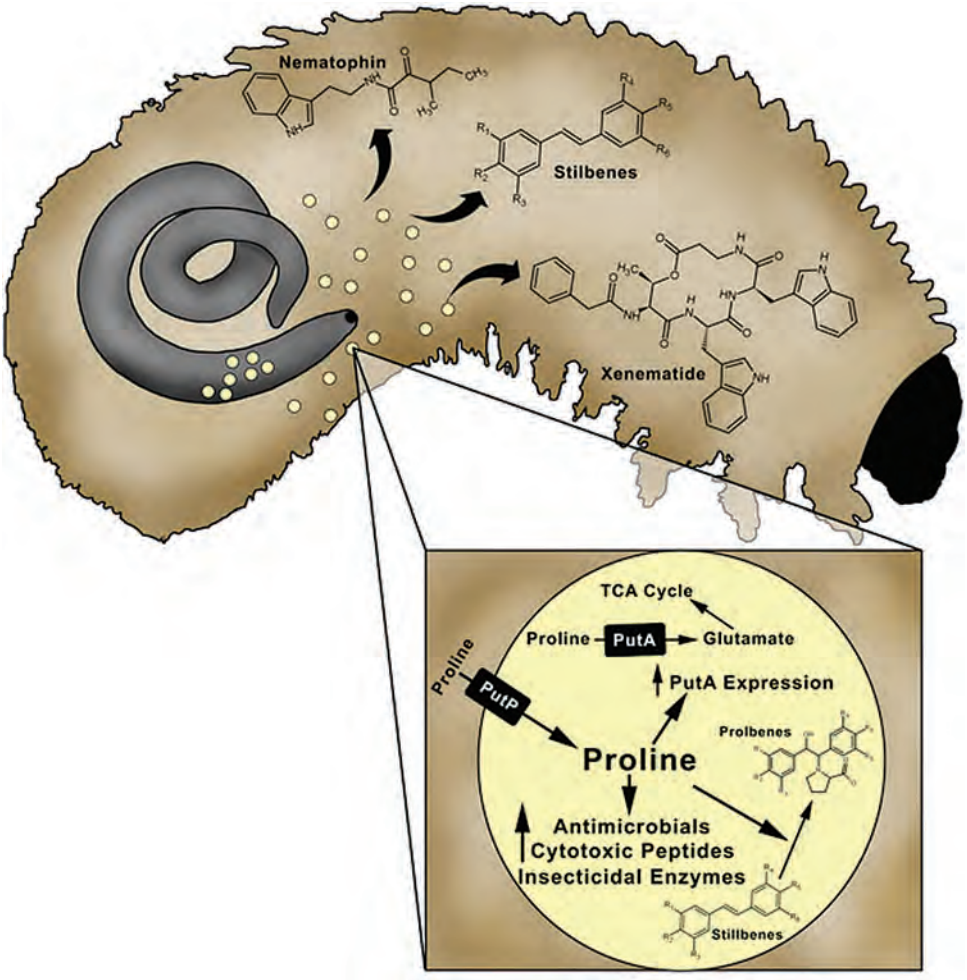

FIG. 8. Entomopathogenic bacteria and role of proline in insect larvae. After infection of insect larvae, nematodes harboring symbiotic entomopathogens expel the bacteria into the larval host. Cues from the new environment, including increased proline levels, cause the entomopathogens to undergo metabolic shifts, transitioning these bacteria from symbionts of the nematode to pathogens of the worm. This shift in metabolism leads to the generation of cytotoxic peptides, insecticidal enzymes, and antimicrobials that kill the larval host and bacterial competitors. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

that proline concentrations in the hemolymph range from 3 to 73 m*M*, indicating proline could be a metabolic signal that stimulates antibiotic production (50). Evidence for proline inducing metabolic changes was observed by treating cultures of *X. nematophila* and *P. luminescens* with proline (50). Bacterial cultures treated with insect hemolymph or proline ($\sim$5 m*M*) showed similar increases in the abundance of antimicrobial compounds (50). The role of proline was further tested by disrupting proline transport in *P. luminescens*, which encodes a homologue of the high-affinity PutP transporter from *E. coli*. In the Δ*putP* mutant strain of *P. luminescens*, proline did not induce a metabolic shift further supporting a key role for proline in the production of antibiotic compounds (50). Proline is also proposed to be part of stilbene detoxification pathway in the host bacterium in a reaction involving stilbene epoxidation and incorporation of L-proline generating less toxic compounds called prolbenes (Fig. 8) (175).

How proline activates antibiotic production in entomopathogenic bacteria is not clear. It may involve Lrp, a nutritional sensor, which could respond to proline and induce Lrp-dependent activation of virulence factors (36, 191). *X. nematophila* and *P. luminescens* also encode trifunctional PutAs, indicating that similar to *E. coli*, *putA* and *putP* gene expression would be subject to transcriptional autorepression in the absence of proline. The PutAs from *X. nematophila* (1326 residues, Accession No. CBJ89733) and *P. luminescens* (1326 residues, Accession No. CAE14250) have high-sequence identity (77%) with *E. coli* PutA (1320 residues, Accession No. AAB59985.1), including residues of the RHH DNA binding domain.

In both genomes of *X. nematophila and P. luminescens*, the *putA* and *putP* genes share a similar arrangement as that found in *E. coli* with the genes separated by an intervening *put* control region and the *putA* and *putP* genes transcribed in opposite directions. Previously, it was shown that GTTGCA is the consensus DNA binding motif for *E. coli* PutA (263), with five PutA binding sites in the *put* control DNA region (263). Analysis of the *put* control DNA sequences of *X. nematophila* (Ref Seq: NC_014228.1) and *P. luminescens* (Ref Seq: NC_005126.1) identified three consensus PutA DNA binding motifs. The fewer PutA binding sites in the *put* control region of *X. nematophila* and *P. luminescens* suggest that transcriptional repression by PutA may be less stringent than that of *E. coli*. It will be important to characterize the regulation of the *putA* and *putP* genes to gain further insights into the DNA binding role of PutA in these bacteria and the proline response mechanism that leads to upregulation of antibiotic production.

Proline is also predicted to serve as an important energy source for these bacteria due to its high abundance in the insect relative to glucose. Proline metabolism was shown to support respiratory activity, which was proposed to be important for the metabolic changes that favor antibiotic production (50). It would also be interesting to know if proline metabolism increases oxidative stress resistance by upregulation of antioxidant enzymes. Analysis of the genomes of *X. nematophila* and *P. luminescens* indicates the presence of an OxyR stress response regulator similar to *E. coli*. These genomes also encode thioredoxin and glutaredoxin proteins, but, interestingly, do not appear to have a *katG* homologue. A *katE* gene is predicted in *P. luminescens* and no catalase is annotated for *X. nematophila.* In *E. coli*, *katE* is not part of the OxyR stress response regulon but instead is upregulated during stationary phase. Activation of the OxyR regulon by proline metabolism in *X. nematophila* and *P. luminescens* could potentially facilitate evasion of the innate immune system and colonization of the insect host.

H. pylori

One of the major aspects of pathogenesis is the ability to metabolically adapt to the host or vector environment. An example of a bacterial pathogen that exploits proline as a carbon and energy source is *H. pylori*, which thrives in microenvironments enriched with proline (160, 162, 193). *H. pylori* is a microaerophilic gram-negative bacterium that colonizes the lower stomach, is the causative agent of peptic ulcer disease, and is associated with gastric inflammation and gastric cancer (77, 113, 179, 232). Together with L-serine, L-proline has been shown to be a preferred respiratory substrate of *Helicobacter* and has been found to be significantly elevated in the stomachs of patients infected with *H. pylori* (160). During infection, *H. pylori* secretes collagenase, which can breakdown collagen in the extracellular matrix of the stomach epithelium leading to increased proline levels (85, 105). In *Helicobacter* spp., PutA lacks the DNA binding domain of trifunctional PutAs, and thus, its central biological role is to enable the bacterium to utilize proline as a carbon source by coupling the formation of glutamate with reduction of the respiratory chain (110, 160, 167).

In *Helicobacter*, the *putA* and *putP* genes are arranged in a *putAP* operon. The *putAP* operon in *H. pylori* was shown by Pich *et al.* to be regulated by the ferric uptake regulator (Fur) (183). Fur represses the expression of iron uptake genes under iron-replete conditions thus preventing the toxic accumulation of metal in bacteria (183). Expression of *putA* was strongly repressed by Fe^{2+}-Fur indicating that PutA may contribute to pathogenesis by helping with reductive iron acquisition and stress adaptation (183). Other regulators of the *putAP* operon have not been identified. The genome of *H. pylori* does not appear to contain homologues for PutR and PruR, which are proline-specific regulators in other gram-negative pathogens as discussed in the Regulation of proline catabolic genes section (110).

Evidence for proline having an important role in *Helicobacter* infection is from animal studies using *H. pylori* mutant strains of *putA* and *putP*. In a signature tagged mutagenesis screen of 960 mutants of *H. pylori*, *putP* was identified along with 46 other genes to be essential for gastric colonization of a Mongolian gerbil oral infection model (105, 193). Similar to *E. coli*, PutP from *H. pylori* is an Na^+-L-proline symporter with K_m of $\sim$2 μM proline (193). *H. pylori* is also predicted to have homologues of *E. coli* ProP and ProU, however, these transporters were not identified as being essential for colonization (229). In another study, a *putA* mutant strain of *H. pylori* was unable to colonize mice. The *putA* mutant strain was defective in flagellar formation and motility, perhaps due to loss of proline respiratory activity, which resulted in lower virulence (162). However, the *H. pylori putA* mutant also did not import proline from the surrounding media (162). Because *Helicobacter putA* and *putP* genes are arranged in a *putAP* operon, the *putA* mutant may also have loss of *putP* expression resulting in a mutant strain deficient in proline import and catabolism (162).

Further insights into the role of PutA in *Helicobacter* pathogenicity were reported with a *putA* knockout mutant strain of *Helicobacter hepaticus* (112). *H. hepaticus* is a mouse pathogen found in the liver of mice with high incidence of hepatitis and liver tumors. As an enterohepatic *Helicobacter* spp., *H. hepaticus* colonizes the lower bowel and progresses to the liver. Knockout of PutA in this murine pathogen did not impact colonization of the liver or cecum, however, inflammation of the liver during disease progression was significantly reduced relative to mice inoculated with the wild-type strain (112). Interestingly, the *putA* mutant strain exhibited lower catalase expression (*katA*) relative to wild-type *H. hepaticus*, indicating that proline oxidation *via* PutA upregulates *katA* gene expression and perhaps virulence. Virulence factors, such as catalase, are upregulated in *H. pylori* to combat oxidative stress from the host neutrophil response, resulting in persistent chronic inflammation (24, 101, 188). PutA metabolism of proline has been shown to increase oxidative stress resistance in *E. coli*, a potentially conserved feature of proline metabolism that should be further explored in the physiology and pathogenesis of *Helicobacter* spp. Altogether, these results indicate that proline metabolism plays an integral role in the colonization and pathogenesis of *Helicobacter* spp.

Ehrlichia chaffeensis

The zoonotic disease of human monocytic ehrlichiosis is caused by infection of white blood cells by the tick-borne bacterial pathogen *E. chaffeensis* (67). Human monocytic ehrlichiosis is a life-threatening disease, particularly in immunocompromised patients, and is characterized by lethargy, fever, and headache (67). Evidence for a role of proline in this disease was shown recently by Cheng *et al.*, in which *E. chaffeensis* infection of human monocyte THP-1 cells was decreased on inhibiting proline import by protamine treatment (42). *E. chaffeensis*, a gram-negative bacterium, has a considerably smaller genome (1.18 Mb) relative to *E. coli* (4.67 Mb) and is predicted to encode 883 proteins. Interestingly, the *E. chaffeenis* genome (Ref Seq: CP000236) has a *putA* gene, but lacks a *putP* homologue. It appears that *E. chaffeensis* primarily imports proline *via* a homologue of the ProP transporter from *E. coli*. A gene (Accession No. WP_006010458.1) within the *E. chaffeensis* genome, annotated as a member of the Major Facilitator Superfamily, has 33% amino acid sequence identity with the *E. coli* osmoregulatory proline/glycine betaine transporter ProP (Accession No. AAC44538.1, 86% query length). Additional analysis of the *E. chaffeensis* genome shows it lacks proline biosynthesis genes *proB* (GK) and *proA* (GPR) but has the *proC* gene, which encodes P5CR. Therefore, it appears *E. chaffeensis* is limited in proline biosynthesis and must rely on proline import and proline recycling *via* an X-Pro aminopeptidase, which is also annotated in the genome.

One of the mechanisms by which proline facilitates *E. chaffeensis* infection is proposed to involve the utilization of proline as a carbon and energy source (42). The PutA protein from *E. chaffeenis* (1044 amino acids, Accession No. ABD44729.1) shares 47% amino acid sequence identity with *E. coli* PutA (78% query length) and was shown to complement a *putA* mutant *E. coli* strain, confirming that *E. chaffeenis* PutA is functional in proline catabolism (42). *E. chaffeensis* PutA lacks the N-terminal RHH DNA binding of *E. coli* PutA and is predicted to be a bifunctional PutA (212), and therefore, it is unlikely to have a DNA binding function. The PutA catalyzed oxidation of proline to glutamate followed by conversion of glutamate into α-ketoglutarate by NAD^+-dependent glutamate dehydrogenase would funnel carbon into the TCA cycle and support ATP production. The direct coupling of PutA/PRODH activity with the respiratory chain of *E. chaffeensis* would also provide energy. *E. chaffeensis* was shown to be deficient in glutamate import, thus supporting a critical role for proline catabolism in supplying intracellular glutamate (42). Glutamine was also shown to enhance *E. chaffeensis* growth and infection. In addition, expression of glutamine synthetase (GlnA) was shown to have a similar temporal expression pattern and transcriptional regulation as PutA, highlighting an important metabolic link between proline and glutamine (42).

PutA expression is controlled by the two-component regulatory system of nitrogen metabolic regulators NtrY and NtrX (42, 115). NtrY contains an N-terminal sensor domain and C-terminal histidine kinase domain, while NtrX contains an N-terminal phosphorylation domain and C-terminal helix-turn-helix DNA binding domain (115). Cheng *et al.* showed that NtrX binds to the promoter region of the *E. chaffeensis putA* gene, with DNA binding affinity significantly increased on phosphorylation of NtrX. These results indicate that *putA* gene expression is activated by the NtrY/X system (42).

Another potential mechanism by which proline could facilitate infection involves enhanced developmental cycling of *E. chaffeensis*, which, during infection, involves cycling between small- and large-sized cell bodies (43). The smaller sized aggregates are the more infectious form that enters and exits host cells. On entering a host cell, *E. chaffeensis* develops into the larger aggregate replicative form. Once reaching stationary phase inside the host, *E. chaffeensis* transitions back into the smaller sized form and exits the host cell, thereby initiating a new round of infection (43). A key regulatory protein in this process is CtrA, which is highly expressed at stationary phase, downregulates DNA replication, and upregulates genes important for the small-sized aggregate form (42, 43). Interestingly, proline was observed to accelerate the degradation of CtrA in host cells (42). Thus, proline may be a nutrient signal that facilitates the transition of *E. chaffeensis* to the larger sized replicative form by helping downregulate the CtrA regulon, thereby promoting the developmental cycling of *E. chaffeensis* during infection.

Figure 4 summarizes the potential effects of proline on CtrA and the regulation of the *putA* gene, which involves NtrY phosphorylation of NtrX. Exactly how proline enhances CtrA degradation and NtrX phosphorylation by NtrY is not known. Further studies on proline metabolism and regulation in *E. chaffeensis* seem to be an important area to pursue.

S. aureus

S. aureus is a gram-positive bacterial pathogen that often resides in the nose without signs of illness. However, untreated infection with methicillin-resistant *S. aureus* can cause a range of illnesses, including potentially life-threatening conditions such as pneumonia and sepsis (206). In *S. aureus*, proline has an important role in osmoregulation (207). Intracellular levels of proline, along with other osmolytes such

as glycine betaine, increase when *S. aureus* is grown in a high osmotic environment (4, 82, 207). The rise in intracellular proline levels is mediated by at least two transporters: a high-affinity transport system, encoded by the gene *putP*, and a low-affinity transporter homologous to *E. coli* ProP (207). The expression of *putP* increases in tandem with increased osmolarity and decreased intracellular proline levels, indicating that proline import is required for osmotic stress protection (206). In addition, Bae and Miller demonstrated that the low-affinity transport system ProP is activated under periods of high osmolarity, resulting in increased proline accumulation that helps *S. aureus* tolerate periods of osmotic stress (13).

Proline import has been shown to be critical for infection. A transposon mutation in the *putP* gene resulted in decreased survival of *S. aureus* in animal models (205). Accordingly, expression of *putP* was shown to increase by over 100-fold on *S. aureus* infection in various mice organs, further demonstrating the importance of rapid proline import during infection (206). Interestingly, the expression of *proP* also increased, but this increase occurred at a time significantly later than the initial infection (241). These observations have led to a model in which PutP is proposed to be critical for early stages of infection, whereas ProP predominates at later stages of infection after tissue damage has occurred (207).

In addition to proline uptake, *S. aureus* can also synthesize proline. However, as it lacks *proB* (GK) and *proA* (GPR) genes, *S. aureus* does not synthesize proline from glutamate (231). Instead, this pathogen synthesizes proline from arginine *via* ornithine and OAT to generate GSA/P5C, which is then reduced to proline by P5CR (*proC* gene) (Fig. 2) (231). Thus, arginine uptake will lead to increases in intracellular proline (231).

Proline catabolism also supports the growth of *S. aureus* as demonstrated recently by Fey and coworkers (90). *S. aureus* PRODH (333 amino acid residues) is encoded by a gene annotated as *putA* (WP_001196354.1) even though it lacks the P5CDH domain. A second gene called *rocA* (Accession No. AMV81009) encodes P5CDH. Deletion of *putA* results in a growth defect in media lacking glucose (90). NMR metabolomics using ^{13}C-labeled proline showed proline is oxidized principally to glutamate with subsequent conversion to α-ketoglutarate by glutamate dehydrogenase thus supplying carbon to the TCA cycle and helping generate ATP (90). These results led Fey and coworkers to propose that proline catabolism is important for the pathophysiology of *S. aureus* during abscess formation, in which significant tissue remodeling occurs and proline is released from the breakdown of collagen (90). Glucose can become severely limited during infection, whereas amino acids, such as proline, are more abundant and can be utilized as important sources of carbon and energy for survival (90).

Recent immunological studies of *S. aureus* and *Streptococcus pneumoniae* discovered P5CDH on the outer surface of the bacteria (131). Whether P5CDH may have another role in virulence unrelated to proline catabolism is unclear, but the association of P5CDH on the bacterial surface has implicated it as a potential component of vaccines against *S. aureus* (131).

M. tuberculosis

The ability of *M. tuberculosis* to survive in a highly stressed host environment is a characteristic that makes it such a dangerous human pathogen. By entering a dormant state and undergoing metabolic alterations, *M. tuberculosis* is able to hide within the human host asymptomatically for long periods of time before reactivating (199). Proline metabolic genes are important for the growth of *M. tuberculosis* and also appear to have an important role apart from proline utilization as a carbon and energy source (20, 21, 118, 201). *M. tuberculosis* encodes separate proline catabolic enzymes PRODH and P5CDH, and proline biosynthesis enzymes GK, GPR, and P5CR (118, 209). PRODH couples the oxidation of proline to reduction of menaquinone in the respiratory chain thereby facilitating energy production while P5CDH forms glutamate to supply α-ketoglutarate for the TCA cycle (20, 21, 118, 209).

Insights into the regulation of proline metabolism were gained by Berney *et al.* who showed that the *pruC* gene (MSMEG_5120), which is divergently transcribed from the *pruAB* operon, is a transcriptional activator of the *pruA* and *pruB* genes (Fig. 4) (21). PruC is a PucR-type regulatory protein that binds directly to the *pruAB* promoter and is also membrane associated. Interestingly, PruC activation of *pruAB* expression was shown to occur independently of exogenous proline, indicating that other regulatory mechanisms are involved (21).

How *Mycobacterium* regulates proline metabolism in response to nitrogen stress or limiting nitrogen is also important to understand as amino acids are critical for cells to survive in nitrogen-deplete environments. Using global transcriptional analysis, expression levels of genes encoding PRODH, P5CDH, and OAT were found to be significantly induced by nitrogen limitation in *Mycobacterium smegmatis* consistent with proline being converted to glutamate (244). Using a continuous cell culture system for *M. smegmatis*, however, Petridis *et al.* found that these same proline metabolic genes were downregulated under nitrogen limitation (180). A study on *M. tuberculosis* by Williams *et al.* reported that OAT expression is significantly upregulated by limiting nitrogen (245). It will be important to continue investigating nitrogen limitation in *M. tuberculosis* to better understand the metabolic adaptations that enable utilization of different amino acids such as aspartate, which may be an essential nitrogen source for *M. tuberculosis* infection (70).

Proline catabolism and biosynthesis were identified as essential pathways for optimal growth of *Mycobacterium* from a large library of transposon insertion mutants (201). Null mutations of the *pruA* (P5CDH) and *pruB* (PRODH) genes, which are transcribed from the *pruAB* operon in saprophytic *M. smegmatis*, confirmed that PRODH and P5CDH are essential for growth on proline, which is primarily utilized as a growth substrate under low glucose conditions (20, 201). In a transposon site hybridization (TraSH) study of *M. tuberculosis*, proline metabolic genes were not identified as being essential for survival in macrophages (190). However, a *proC* (PYCR) mutant of *M. tuberculosis*, which is auxotrophic for proline, was shown to have severely diminished survival in mouse macrophages and to be avirulent in a mouse infection model (214). The potential of the *proC* mutant as a vaccine candidate for *M. tuberculosis* was thus suggested (214). Consistent with these findings, a recent study using a saturating transposon mutagenesis approach of the *M. tuberculosis* genome reported the *proC* gene to be essential for *in vitro* growth (62).

Perhaps related to the essential role of *proC* is the need of proline for the biosynthesis of the Pro-Glu (PE)/Pro-Pro-Glu (PPE) proteins. These abundant proteins (168 in *M. tuberculosis*) are found on the bacterial cell surface and have a highly conserved N-terminal domain that contains the PE/PPE motif and a variable C-terminal domain that provides a diverse suite of functions (27). PE/PPE proteins are critical for *M. tuberculosis* virulence and have multiple roles in pathogenesis, including protection against genotoxic stress and regulation of cytokine production in host cells (27, 164).

Another key role for proline metabolism in *Mycobacterium* was shown to involve protection against methylglyoxal, an electrophilic and potentially toxic metabolic intermediate that is formed nonenzymatically from metabolites such as dihydroxyacetone phosphate (Fig. 9) (21). Interestingly, only the first step of proline catabolism, the conversion of proline to P5C, was shown to be necessary for this protection (21). The intermediate of the proline utilization pathway, P5C, is capable of reacting with methylglyoxal to form 2-acetyl-1-pyrroline, which is nontoxic (96). Thus, detoxification of methylglyoxal was proposed to occur directly with P5C, resulting in formation of 2-acetyl-1-pyrroline (Fig. 9) (21). The importance of proline catabolism in protecting against methylglyoxal stress during *M. tuberculosis* is not yet known (21). However, increased methylglyoxal concentrations have been reported in macrophages infected with *M. tuberculosis* indicating that proline catabolism, or specifically P5C generation, could serve a protective function by preventing DNA damage and other unwanted modifications by this reactive carbonyl species (187). Whether *Mycobaterium* PRODH and P5CDH are promising drug targets will require further investigation. Characterization of the *M. tuberculosis* P5CDH revealed a larger substrate binding site relative to other P5CDH enzymes, suggesting the potential to design a unique molecule that would specifically inhibit proline catabolism in *Mycobacteria* (118).

Finally, the significance of osmoregulation in *Mycobacterium* during growth in macrophages has been tested. An ABC transporter ProXVWZ, predicted to transport glycine betaine, choline, and proline, was shown to be important for the ability of *M. tuberculosis* to colonize macrophages. ProXVWZ, however, accumulated mainly glycine betaine instead of proline during growth in macrophages (184).

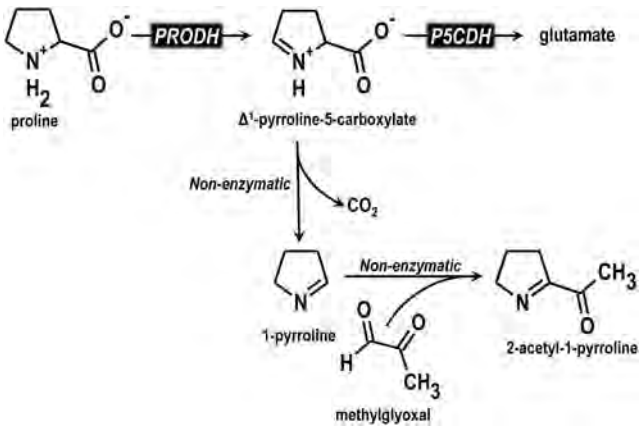

FIG. 9. Role of proline in *M. tuberculosis*. The intermediate of proline metabolism, P5C, is proposed to undergo nonenzymatic decarboxylation to generate 1-pyrroline. Methylglyoxal reacts with 1-pyrroline to form 2-acetyl-1-pyrroline thus reducing potential cellular damage caused by the buildup of methylglyoxal.

Clostridium difficile

A recent study by the Centers for Disease Control and Prevention estimated that nearly 500,000 *Clostridium difficile* infections occur in a single year, leading directly to about 15,000 deaths (1). *C. difficile* is a gram-positive nosocomial pathogen that forms spores and grows anaerobically in the human gut causing diarrhea, inflammation of the colon, and colitis. *C. difficile* was first isolated from the microflora of newborn infants (88). Certain bacteria from the genus *Clostridium* use an interesting metabolic pathway for growing on amino acids. The process, called the Strickland reaction or fermentation, was first described in nonpathogenic *Clostridium sporogenes* and involves the coupling of electron donor amino acids that are oxidatively deaminated with electron acceptor amino acids that are reductively deaminated (25, 169, 218). For example, oxidation of alanine involves deamination and decarboxylation to acetic acid and ammonia coupled to either reductive deamination of glycine to acetyl-phosphate for substrate-level production of ATP or reduction of proline to δ-aminovaleric acid, which is coupled to transmembrane proton translocation (135). In different *Clostridium* spp., the Strickland reaction has been shown to involve leucine, valine, isoleucine, and alanine as donors and glycine, proline, and hydroxyproline as electron acceptors. In *C. difficile,* proline is an essential amino acid for growth, along with cysteine, isoleucine, leucine, tryptophan, and valine (103).

The key enzyme catalyzing the reduction of proline in the Strickland pathway is D-proline reductase (PR), a selenoenzyme that comprised subunits PrdA (45 kDa) and PrdB (27 kDa) with a molecular mass of 280 kDa indicating a higher oligomeric structure (99, 102). PR is specific for D-proline as it does not use L-proline or D- and L-4-hydroxyproline as substrates (8). The catalytic mechanism of PR has been proposed to involve a selenocysteine residue functioning as an active site nucleophile, attacking the α-carbon of D-proline resulting in cleavage of the N-C bond (Fig. 10) (76, 102). Before nucleophilic attack by the selenocysteine, the N-C bond is likely activated by adduct formation between the proline amine and a pyruvoyl group in the active site (102). The selenocysteine substrate adduct would then be resolved by formation of a mixed selenide/sulfide bond with an active site cysteine, releasing δ-aminovalerate (102). The PrdC thiol oxidoreductase, which contains a C-terminal CxxU motif, would then reduce the selenide/sulfide bond of PR to complete the catalytic cycle (76). The physiological electron donor for PrdC is not known but may involve NADH (76).

Colonization of the gut by *C. difficile* involves secretion of glycosylating toxins and production of L-proline aminopeptidase to release *trans*-4-hydroxy-L-proline and L-proline from host collagen (73, 93). Recently, a *trans*-4-hydroxy-L-proline dehydratase (*t*4L-HypD) was identified in the human microbiome from *C. difficile* (127). The enzyme *t*4L-HypD is a member of the glycine radical enzyme superfamily and catalyzes the dehydration of *trans*-4-hydroxy-L-proline to

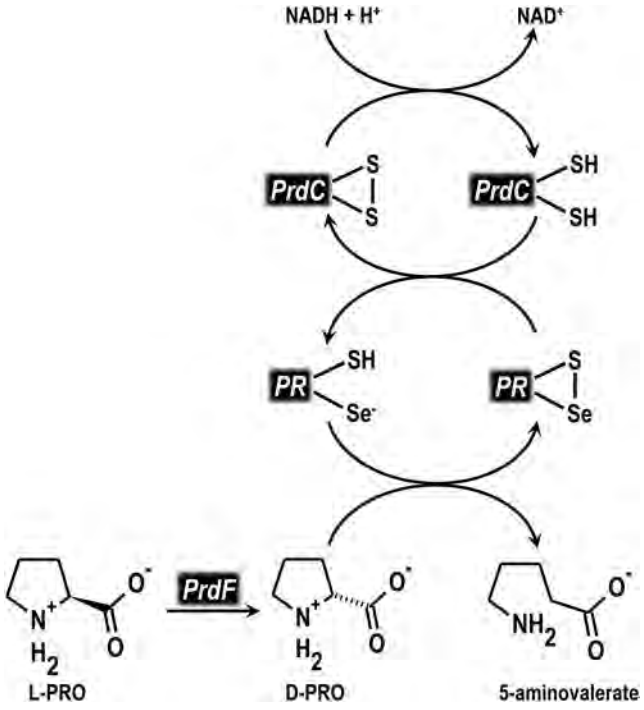

FIG. 10. Reduction of D-proline in *C. difficile*. L-proline is first converted into D-proline by proline racemase. PR then catalyzes the two-electron reduction of D-proline to 5-aminovalerate. The catalytic cycle of PR is completed by the thiol oxidoreductase PrdC, which has been proposed to capture reducing equivalents from NADH. PR, D-proline reductase.

P5C (127). Balskus and coworkers showed that *C. difficile* enzymes *t*4L-HypD and P5CR coordinate the conversion of *trans*-4-hydroxy-L-proline to L-proline, thus providing a mechanism for the bacterium to acquire proline from host and dietary proteins in the gut (127).

Increased levels of proline lead to upregulation of PR in *C. difficile* to promote growth. Bouillaut and coworkers identified PrdR as the protein responsible for activating transcription of the proline reductase operon that encodes PR subunits (*prdA*, *prdB*, and *prdD*), proline racemase (*prdF*), and the PrdC thiol oxidoreductase protein (*prdC*) (25). Therefore, the increased bioavailability of proline during infection leads to upregulation of the proline reductase operon.

The importance of proline during *C. difficile* infection was examined by Wu and Hurdle using a hamster infection model and a Δ*prdF* mutant strain made by insertional mutagenesis (253). Because proline racemase is essential for generating D-proline from L-proline, disruption of *prdF* is expected to block utilization of proline as a Strickland acceptor amino acid. Wu and Hurdle observed no significant difference in the survival between hamsters infected with wild-type *C. difficile* JIR8094 and the Δ*prdF* mutant strain (253). These results indicate that proline as a Strickland acceptor amino acid may not be critical for *C. difficile* pathogenesis. Nevertheless, additional work is needed to fully assess the role of proline metabolism during *C. difficile* infection, such as testing the impact of PR directly by depleting a PR subunit gene.

Eukaryotic Human Pathogens

Trypanosomatidae family

Trypanosomes are kinetoplastid parasites with life cycles that alternate between infection in humans and insects (196, 234) (Fig. 11). In humans, they are responsible for a number of diseases, including Chagas disease, caused by *Trypanosoma cruzi*, African trypanosomiasis, also known as African sleeping sickness, caused by *T. brucei*, and leishmaniasis, caused by various members of the *Leishmania* genus (196). These parasites adapt to fluctuations in nutrients as they move between human host and insect vector with glucose consumed primarily in mammalian blood and proline utilized as a carbon source in the insect (72, 145, 146, 173, 196, 221, 234).

The oxidative conversion of proline to glutamate is catalyzed by PRODH and P5CDH in the mitochondrion of trypanosomes (35, 145, 173). Glutamate is subsequently converted to α-ketoglutarate by glutamate dehydrogenase or L-alanine amino acid transferase (35, 145, 173). The flux of α-ketoglutarate into the TCA cycle and formation of key TCA intermediates such as of succinate and malate is required for proline-dependent growth of trypanosomes in the absence of glucose (35, 145, 173). Knockdown of succinate dehydrogenase in proline growth medium was shown to be lethal in *T. brucei* (48). High glucose levels attenuate proline catabolism, indicating that proline is not the preferred carbon source in glucose-rich environments (48). Recently, transcription levels of PRODH (Tb927.7.210) were found to be regulated by circadian rhythms in the bloodstream form of *T. brucei* along with other metabolic genes that are associated with ATP production (192).

Evidence for the importance of proline as a carbon and energy source during parasite life cycles and infection has been shown in *Trpanosoma* spp. (68, 72, 145, 173, 221, 234). When the production of ATP from proline is inhibited in *T. cruzi*, the ability of this parasite to invade host cells is severely diminished (145, 146). Furthermore, Martins *et al.* found that proline plays an important role in parasitic translocation across a gastric mucin layer, a process that is vital for host infection by *T. cruzi* (146). In addition, proline was shown to have a critical role in the differentiation of *T. cruzi* (38, 203, 230). The differentiation of this parasite between various infective host and replicative vector forms is crucial to disease development and the spreading of disease across a population (196) (Fig. 11A). By using a host cell that requires proline, Tonelli *et al.* were able to prevent the differentiation of *T. cruzi* amastigotes (mammalian host form) into trypomastigotes (insect vector form) (230). By adding excess L-proline to the media, differentiation was restored (230).

Interestingly and further highlighting the importance of proline in differentiation, both intracellular proline levels and levels of proline catabolic enzymes in *T. cruzi* vary as the pathogen's life stage changes. The amastigote form has the highest levels of free proline, whereas PRODH activity is highest in the intracellular epimastigote form and P5CDH activity is elevated in the metacyclic trypomastigote and bloodstream trypomastigote forms (145, 173, 230). Proline racemases also appear to be involved in parasite differentiation. Coutinho *et al.* have observed that treatment of *T. cruzi* trypomastigotes with proline racemase antibodies leads to decreased invasion of host cells (49). *T. cruzi* proline racemase has also been shown to initiate B cell mitogenic

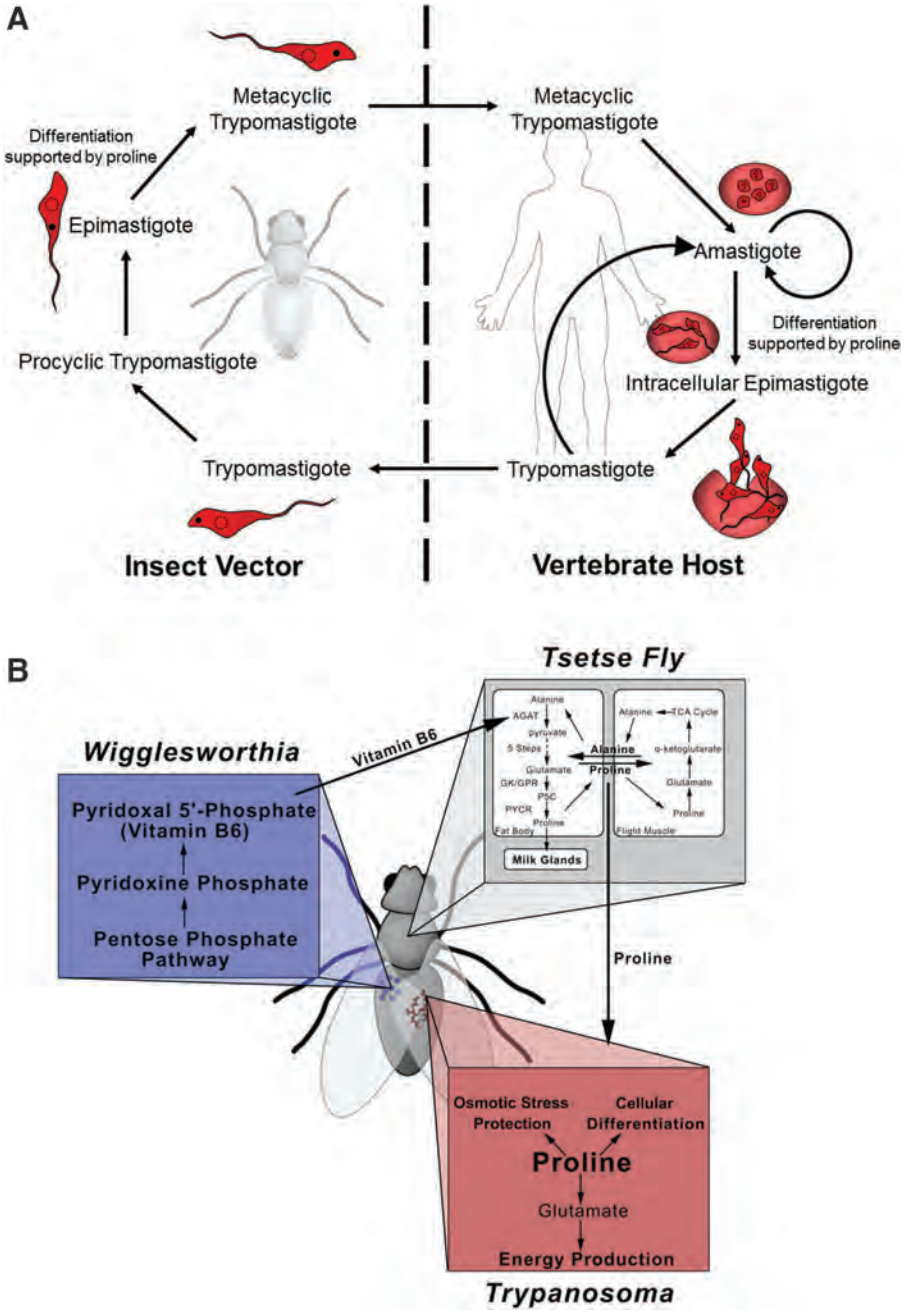

FIG. 11. Relationship between proline, trypanosomes, and the insect host. (A) Trypanosomes exhibit a complex life cycle, cycling between a vertebrate host and an insect vector. On infection of a vertebrate host, metacyclic trypomastigotes will differentiate into the intracellular amastigote form, a form characterized by no visible flagella. The amastigotes will then reproduce within host cells before differentiating into intracellular epimastigotes, followed by differentiation into trypomastigotes and subsequent bursting of the host cell. The trypomastigotes are then free to infect new cells or be passed into the insect vector. In the insect vector, trypomastigotes differentiate into procyclic trypomastigotes capable of reproduction. The procyclic trypomastigotes will then differentiate into epimastigotes, followed by differentiation into the infective metacyclic trypomastigote form. The trypomastigote and epimastigote forms are primarily distinguished by the location of the kinetoplast (*solid*, *black circle*) relative to the nucleus (*dashed circle*). Proline and its catabolism have been implicated in the differentiation of trypanosomes from the epimastigote to the metacyclic trypomastigote forms and from the amastigote to the intracellular epimastigote forms. **(B)** In the tsetse fly, proline is utilized as an energy source by flight muscles or during lactation. Alanine generated from proline is then transported to fat bodies for proline regeneration. This conversion of alanine back to proline relies on pyridoxal 5′-phosphate (vitamin B_6) generated by the symbiont *Wigglesworthia*. Trypanosomes actively uptake proline from the insect host for utilization in numerous processes, including energy production, cellular differentiation, and osmotic stress protection. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

activity, but this effect does not appear to be dependent on its enzymatic activity (189).

Trypanosomes have active transport systems for obtaining proline from the environment. In *Leishmania major*, proline has been shown to be actively transported in promastigote (insect vector residing) and amastigote (mammalian host residing) forms (29). Because proline is abundant in the hemolymph of most insects, including the tsetse fly, trypanosomes can readily acquire proline from the insect host and are not dependent on proline biosynthesis (29). This is significant as not all trypanosomatids are able to synthesize proline from glutamate (29). Trypanosomatids all have P5CR but exhibit genetic differences in GK and GPR. For example, *Leishmania* spp. encode GK and GPR and *T. cruzi* encodes a putative P5CS-like protein (TCSYLVIO_005298) with predicted GK and GPR domains; however, *T. brucei* lacks both GK and GPR. Trypanosomatids also lack OAT, and thus, proline can only be synthesized *via* GK/GPR.

Proline, alanine, and glutamate are the major amino acids used for osmoregulation by trypanosomes (29, 98). Under hypotonic stress, *T. cruzi* has been shown to release large amounts of proline and alanine (29, 198). In addition, increased transport and accumulation of proline in *T. cruzi* have been shown to increase resistance to trypanocidal drugs such as nifurtimox and benznidazole (202). Overexpression of a D, L-proline transporter led to elevated intracellular proline levels and increased resistance to trypanocidal drugs (202). The increased import of proline into the cell also increased resistance to hydrogen peroxide and nitric oxide, suggesting that proline plays a part in multiple stress protection pathways (202).

In the case of trypanosomes, adapting to utilize proline as an energy source involves the addition of a third symbiotic relationship to the already intricate association between the pathogen and insect (Fig. 11B). Proline is a major source of energy during flight in many insects, including the insect vectors of different parasites (35). Tsetse flies, the insect vector of *T. brucei*, store high amounts of proline in the hemolymph (>100 m*M*) for ATP production during energetically strenuous activities such as flight and lactation (34, 35, 151). Tsetse flies infected with *T. brucei* exhibit decreased proline levels in the hemolymph and a concurrent decrease in fecundity indicating an energetic burden due to draining of proline resources (151).

The high levels of proline in the tsetse fly are produced from alanine in an eight-step pathway with the first step catalyzed by alanine-glyoxylate transaminase (AGAT), a pyridoxal 5′-phosphate (PLP)-dependent enzyme (151). Intriguingly, the tsetse fly relies on an obligate symbiont, *Wigglesworthia glossinidia*, to provide PLP (vitamin B_6) for AGAT activity (151). *W. glossinidia*, a gram-negative bacterium with a genome size of $\sim$0.7 Mb (3), also provides vitamins necessary for the fertility of female tsetse flies (174). Interestingly, *W. glossinidia* encodes a trifunctional PutA but lacks the corresponding *E. coli* PutP homologue and is missing genes for proline biosynthesis (3). The delicate ecosystem whereupon the pathogen and vector are competing for proline as a shared energy source is reliant on a third symbiont that lacks several proline metabolic genes (Fig. 11B) (151).

Cryptococcus

The fungus *Cryptococcus*, namely the species *Cryptococcus neoformans* and *Cryptococcus gattii*, is the causative agent of cryptococcosis in humans (168). Cryptococcosis occurs in immunocompromised individuals due to *C. neoformans* exposure, whereas *C. gattii* infects individuals with noncompromised immune systems (168). The spreading of cryptococcal infection into the central nervous system leads to meningoencephalitis. In the pathogenesis of *Cryptococcus*, the fungus adapts to changes in the nutritional environment and combats oxidative stress defenses of the human immune system. Studies of how proline metabolism contributes to the virulence and pathogenicity of *Cryptococcus* have implicated P5CDH as having an essential role (122).

Similar to *S. cerevisiae*, *Cryptococcus* contains *PUT1* and *PUT2* genes that encode for PRODH and P5CDH, respectively (122). Intriguingly, *C. neoformans* encodes a second PRODH gene called *PUT5*. The biological significance of the PRODH gene duplication is not apparent but suggests that proline catabolism is important for survival. Interestingly, *PUT5* was shown to encode the more active PRODH as deletion of *PUT1* did not impact the ability of *C. neoformans* to utilize proline as a nitrogen and carbon source (122). The *PUT1*, *PUT5*, and *PUT2* transcript levels are upregulated by proline (122). *PUT1* is activated by the GATA transcription factor, Gat1p, which regulates genes responsible for nitrogen utilization in *C. neoformans* (108, 121) (Fig. 6). Unexpectedly, *PUT5* and *PUT2* expression was found not to be subject to nitrogen metabolite repression, unlike yeast, in which Gat1p activates expression of the *PUT1* and *PUT2* genes in response to limiting nitrogen availability (121) (Fig. 6). Thus, the regulation of proline catabolism in *C. neoformans* is dissimilar to that in yeast and will require more investigation, especially in the context of infection.

The role of proline metabolism in the expression of virulence factors and infection was examined by characterizing mutants of *PUT1*, *PUT5*, and *PUT2 in the C. neoformans* strain H99 (122). PRODH genes *PUT1* and *PUT5* were concluded not to be necessary for expression of cryptococcal virulence factors such as antiphagocytic polysaccharide capsule and melanin, or for infection, as determined using a mouse inhalation infection model (122). In contrast, deletion of *PUT2* resulted in significantly lower levels of virulence factors, and using a mouse inhalation infection model, the *C. neoformans Δput2* mutant strain was shown to be avirulent with no killing of the infected mice (122). Pathogen virulence was restored on complementation with *PUT2*, confirming the importance of P5CDH (122). It is suggested that increased superoxide generated from the mitochondria in the *Δput2* mutant relative to wild-type *C. neoformans* results in increased susceptibility to apoptosis or cell death, possibly explaining why P5CDH is critical in *C. neoformans* virulence (122). Accumulation of P5C has previously been implicated in causing oxidative stress in yeast, suggesting a similar mechanism may be occurring in *C. neoformans* (165). Higher levels of P5C have been proposed to impact the transfer of reducing equivalents across the mitochondrial membrane *via* the proline-P5C pathway, thus imposing an oxidative stress burden on the mitochondria (165).

Summary

Proline is crucial for the growth of all the aforementioned pathogens under nutritional stressed conditions that are encountered during infection. In some pathogens, proline is also

important for osmoregulation (*e.g.*, *E. coli*, *Trpanosoma* spp., *S. aureus*) and stress resistance *(M. tuberculosis*, *entomopathogens*). Infection models using mutant strains of proline import and metabolism have provided insights into the importance of proline during pathogenesis of *E. coli* (Δ*proP*, Δ*proU*), *Helicobacter* spp. (Δ*putA* and Δ*putP*), *S. aureus* (Δ*putP*), *M. tuberculosis* (Δ*proC*) *C. difficile* (Δ*prdF*), and *C. neoformans* (*Δput2* mutant). A summary of the functions of proline in these different pathogens is provided in Table 1.

Future Directions

Physiology of host/pathogen interactions

Understanding the mechanisms by which pathogens acquire nutrients during infection and survive host innate immunity systems will continue to be an important area of future research (261). Amino acids such as proline are key sources of nitrogen and carbon for pathogens. Some pathogens, such as *T. brucei*, must import amino acids from the host, including proline (144, 147), whereas others, such as *M. tuberculosis*, are self-reliant in producing amino acids, including synthesizing their own proline (46, 261). The capacity of a host to limit nutrient availability, which includes amino acids such as proline, is an important defense mechanism (261). The significant attenuation in virulence of the *M. tuberculosis proC* strain, which is auxotrophic for proline, demonstrates this defense strategy (214).

An intriguing area of research is to explore how pathogens are able to avoid amino acid starvation and in some cases increase host production or transport of amino acids into the host cell (261). In addition, understanding the alternative sources of carbon and nitrogen that pathogens utilize is critical. Although not highlighted in this review, proline has been proposed in *Campylobacter jejuni* to be a secondary carbon/nitrogen source used after aspartate, asparagine, and serine pools have been drained (252). Interestingly in *C. jejuni*, a significant upregulation of *putA* and *putP* expression at stationary phase was observed to coincide with a large increase in proline utilization (252). Whether proline metabolism is important for *C. jejuni* survival in the host will need to be determined.

Other factors of pathogenesis that need to be further investigated include examining the effect of proline in the host diet and competition between commensal and pathogenic bacteria. Momose and colleagues have shown that nonpathogenic, proline-consuming strains of *E. coli* were able to suppress the growth of pathogenic *E. coli* O157:H7 (154). This suppression was reversed through the addition of proline to the growth media, implying that competition for proline consumption between pathogenic and nonpathogenic strains of *E. coli* may lead to poor growth and increased elimination of the pathogenic *E. coli* (154). Thus, dietary proline is a potential environmental factor that should be considered when examining the host microbiota and pathogen colonization (7). This seems especially relevant to the colonization and carcinogenesis of *H. pylori*, which exhibits considerable pathogenic variability depending on the genetic makeup of the particular strain and human host, and environmental factors such as diet (7).

Also, evidence was reported for the entomopathogen *P. asymbiotica* being able to infect human cells (47). Different strain isolates of *P. asymbiotica* from humans have been found, which are able to induce apoptosis in human macrophage cell lines (47). These findings highlight the need to understand more about the biology of entomopathogenic bacteria and the role of proline metabolism to combat these new emerging human pathogens. Clearly, additional investigations are needed to fully define the mechanisms and magnitude by which proline enhances virulence of different pathogenic organisms.

Drug design and targets

Gaining a better understanding of how proline metabolism is involved in the infection and survival of pathogens will be important for improving treatments of infectious diseases.

Targeting unique aspects of proline utilization in prokaryotic and eukaryotic pathogens may enhance selectivity of antibiotics and avoid toxic effects in the host. Structural studies by Tanner's group of PutAs and monofunctional PRODH enzymes strongly indicate that the active site structure and overall fold are similar between bacterial and human enzymes (133, 243). It should be noted, however, that a structure of a eukaryotic PRODH, including human, has not been reported yet. Likewise, bacterial and human P5CDH (or ALDH4A1) share a common fold (133, 216) and are part of the aldehyde dehydrogenase superfamily.

The conservation of active site residues in PRODH and P5CDH enzymes was examined by performing a sequence alignment of the PRODH, P5CDH, and PutA enzymes from the pathogens highlighted in this review. The multiple sequence alignments of the PRODH and P5CDH active sites are shown in Figure 12. For PRODH and P5CDH, key residues in both active sites are identical or highly conserved between pathogenic and human enzymes, denoted in Figure 12 by red arrows. For example, in PRODH, two tandem Arg residues (R556 and R556 in PutA from *E. coli*), which are required for proline binding in bacterial PutA and PRODH enzymes, are identical in human PRODH (133).

In the P5CDH active site, an Arg/Lys-Cys-Ser-Ala motif on the catalytic loop (R916, C917, S918, and A919 in PutA from *E. coli*) is conserved between pathogenic PutA/P5CDHs and human P5CDH. The Cys residue on this loop is the catalytic nucleophile in the aldehyde reaction and the adjacent Arg (Lys347 in human P5CDH) and Ser residues form water-mediated and direct hydrogen bonds, respectively, to the α-carboxylate of glutamate (216). Thus, it is likely that compounds that target only the PRODH and P5CDH catalytic sites will not specifically inhibit pathogenic enzymes. Illustrating this is that L-tetrahydro-2-furoic acid, a proline analog (124, 266), has been shown to inhibit PutA (158) and human PRODH enzymes (224), and proline metabolism in cell cultures (71, 166, 257). To design specific inhibitors of proline metabolism in pathogenic organisms, properties that are unique to pathogenic enzymes will need to be found by future structural and kinetic studies. For a complete analysis of PRODH and P5CDH structural biology, readers are referred to the review by Tanner in this Forum Issue (225).

A more promising approach, at least for gram-negative pathogens, may be to develop allosteric inhibitors that target the channeling step in PutAs. These types of inhibitors would selectively block the use of proline as a carbon or nitrogen source in gram-negative bacterial pathogens. This approach

Table 1. Summary of Proline Metabolism in Different Pathogens

Pathogen	*Disease*	*Proline biosynthesis*	*Proline catabolic enzymes*	*Proline uptake*	*Primary functions*	*References*
Ehrlichia chaffeensis	Monocytic ehrlichiosis	P5CR	Bifunctional PutA (PRODH/P5CDH)	H^+-symporter (ProP)	Carbon/nitrogen/energy	(42, 67)
Entomopathogens Xenorhabdus nematophila Photorhabdus luminescens	Symbiotic relationship with worms	GK, GPR P5CR	Trifunctional PutA (PRODH/P5CDH)	Na^+-symporter (PutP) H^+-symporter (ProP)	Carbon/nitrogen/energy Osmoregulation Stress resistance	(50, 78, 175)
Escherichia coli	Urinary tract infection	GK, GPR P5CR	Trifunctional PutA (PRODH/P5CDH)	Na^+-symporter (PutP) H^+-symporter (ProP)	Carbon/nitrogen/energy Osmoregulation Stress resistance	(152, 227, 248, 249)
Clostridium difficile	Diarrhea, colitis	P5CR	Proline reductase	ABC transporter permease	Energy	(88, 102, 135)
Cryptococcus neoformans	Cryptococcosis	GK, GPR P5CR	PRODH, P5CDH	Amino acid permease	Carbon/nitrogen/energy	(108, 121, 122, 168)
Helicobacter spp.	Peptic ulcers, gastric cancer	P5CR	Bifunctional PutA (PRODH/P5CDH)	Na^+-symporter (PutP) H^+-symporter (ProP)	Carbon/nitrogen/energy Stress resistance	(105, 110, 112, 160, 162, 193)
Mycobacterium tuberculosis	Tuberculosis	GK, GPR P5CR	PRODH, P5CDH	Na^+-symporter (PutP)	Carbon/nitrogen/energy Stress resistance	(20, 21, 118, 199, 201, 209)
Staphylococcus aureus	Wide range of infections; skin, blood, endocarditis, osteomyelitis	P5CR	PRODH, P5CDH	Na^+-symporter (PutP) H^+-symporter	Carbon/nitrogen/energy Osmoregulation Stress resistance	(90, 206, 207, 232)
Trypanosoma spp. *Leishmania* spp.	Chagas disease; African sleeping sickness; leishmaniasis	GK, GPR (varies with species) P5CR	PRODH, P5CDH	H^+/ATP-dependent transporter Proline/alanine transporter Proline permease (amino acid/auxin permease)	Carbon/nitrogen/energy Osmoregulation Stress resistance Cell differentiation	(29, 35, 48, 145, 146, 173, 196, 202, 210)

GK, γ-glutamyl kinase; GPR, γ-glutamyl phosphate reductase; P5CDH, Δ^1-pyrroline-5-carboxylate dehydrogenase; P5CR, Δ^1-pyrroline-5-carboxylate reductase; PRODH, proline dehydrogenase; PutA, proline utilization A.

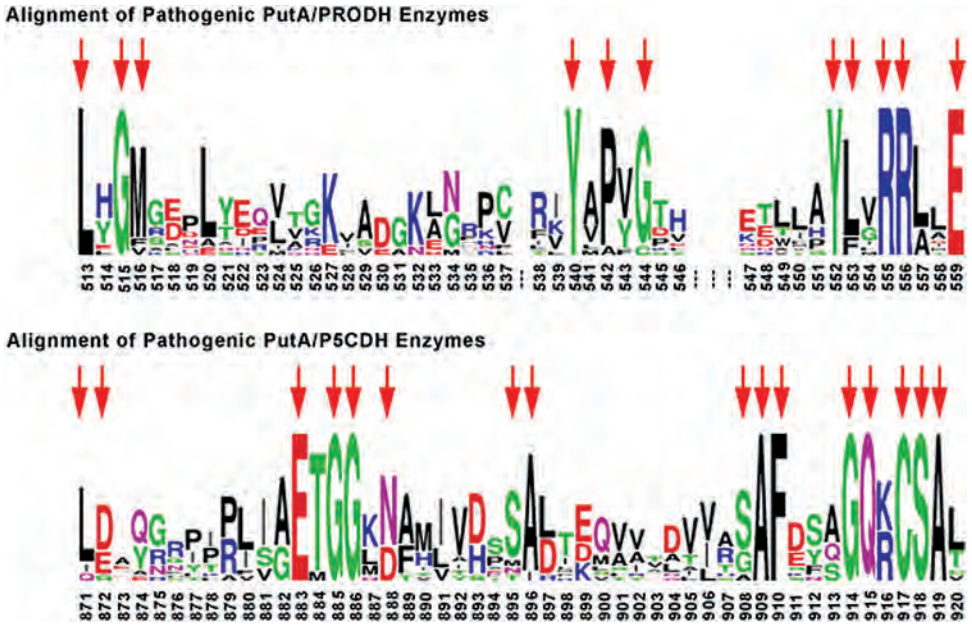

FIG. 12. Results from multiple sequence alignment of PRODH, P5CDH, and PutA enzymes from pathogenic organisms. Alignment was computed using T-Coffee computing alignment server and the resulting alignment visualized using WebLogo software. The resulting WebLogo images were altered so that residue numbering is according to PutA from *E. coli*. Shown are the alignments of the PRODH and P5CDH catalytic sites. The GenBank accession numbers of the aligned PutA sequences are *E. coli* PutA (AAB9985), *Helicobacter pylori* PutA (WP_001169408.1), *E. chaffeensis* PutA (WP_011452748.1), *Pseudomonas putida* PutA (WP_043207299.1), *Samonella enterica* PutA (WP_050954140.1), *Photorhabdus luminescens* PutA (WP_036807552.1), *Xenorhabdus nematophila* PutA (WP_010848062.1), *M. tuberculosis* PRODH (WP_031710787.1) and P5CDH (EFO75557.1), *Trypanosoma cruzi* PRODH (ADZ31479.1) and P5CDH (CDG15436.1), *Trypanosoma brucei* PRODH (XP_845645.1) and P5CDH (XP_822573.1), *C. neoformans* PRODH (XP_571179.1) and P5CDH (XP_572420.1), and *S. aureus* PRODH (WP_031824363.1) and P5CDH (AGU62685.1). *Red arrows* indicate residues that are identical or highly conserved in human PRODH (AAD24775.1) and P5CDH (P30038.3) enzymes. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

would be similar to the allosteric inhibition recently reported for tryptophan synthase in *M. tuberculosis*. A small-molecule inhibitor was discovered to bind to the substrate channel in tryptophan synthase and kill *M. tuberculosis* (240). Additional work is needed to establish if substrate channeling is present in pathogenic organisms that contain monofunctional PRODH and P5CDH enzymes. If so, perhaps small molecules that block PRODH/P5CDH functional interactions would prevent proline utilization and lead to toxic accumulation of P5C in the pathogenic organism.

Continued studies of proline transport are also needed to guide future drug design. Recently, Jung and colleagues identified residues that are critical for external gate closure of PutP, potentially providing new insights into how the proline transport cycle could be specifically inhibited in bacteria (26). Specific inhibitors of proline transport in trypanosomes would potentially impede the infectious life cycle of these parasites by limiting growth and osmotic stress protection. Another strategy to prevent proline utilization in pathogens would be to use RNA interference or novel antisense synthetic oligomers to silence proline metabolic gene expression (219). Also, the possibility of using proline analogs as antimicrobials needs to be further pursued (57). Deutch and colleagues have shown the viability of this approach using the proline analog, L-selenazolidine-4-carboxylic acid (or, L-selenaproline), which they found inhibits the growth of the uropathogenic *E. coli* strain CFT073 (63, 64). Selenazolidine compounds, such as L-selenaproline, have been shown not to have toxic effects in mice when supplemented in the diet and were suggested to be viable compounds for cancer therapy and delivery of selenium (128). The potential use of L-selenaproline as an antimicrobial compound against urinary tract infection thus seems worthwhile to pursue.

Acknowledgment

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Abbreviations Used

AGAT = alanine-glyoxylate transaminase
CcpA = catabolite control protein A
CCR = carbon catabolite repression
CRP = cyclic AMP receptor protein
FAD = flavin adenine dinucleotide
Fur = ferric uptake regulator
GK = γ-glutamyl kinase
GPR = γ-glutamyl phosphate reductase
GSA = glutamate-γ-semialdehyde
Lrp = leucine-responsive regulatory protein
NAC = nitrogen assimilation control
NAD^+ = nicotinamide adenine dinucleotide
NADH = nicotinamide adenine dinucleotide
NCR = nitrogen catabolite repression
OAT = ornithine aminotransferase
P5C = Δ^1-pyrroline-5-carboxylate
P5CDH = Δ^1-pyrroline-5-carboxylate dehydrogenase
P5CR = Δ^1-pyrroline-5-carboxylate reductase
P5CS = Δ^1-pyrroline-5-carboxylate synthetase
PE = Pro-Glu
PLP = pyridoxal 5′-phosphate
PPE = Pro-Pro-Glu
PR = D-proline reductase
PRODH = proline dehydrogenase
PutA = proline utilization A
RHH = ribbon-helix-helix
ROS = reactive oxygen species
TCA = tricarboxylic acid