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L-Proline catabolism by the high G + C Gram-positive bacterium *Paenarthrobacter aurescens* strain TC1

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Abstract *Paenarthrobacter aurescens* (formerly called *Arthrobacter aurescens*) strain TC1 is a high G + C Gram-positive aerobic bacterium that can degrade the herbicide atrazine. Analysis of its genome indicated strain TC1 has the potential to form a bifunctional PutA protein containing L-proline dehydrogenase and L-glutamate-γ-semialdehyde dehydrogenase (L-Δ^1-pyrroline-5-carboxylate dehydrogenase) activities. *P. aurescens* strain TC1 grew well in minimal media with L-Proline as a supplemental nutrient, the nitrogen source, or the sole carbon and nitrogen source. Multicellular myceloids induced by NaCl or citrate also grew on L-proline. The specific activity of L-proline dehydrogenase in whole cells was higher whenever L-proline was added to the medium. Both L-proline dehydrogenase and L-glutamate-γ-semialdehyde dehydrogenase (L-Δ^1-pyrroline-5-carboxylate dehydrogenase) activities were found primarily in a membrane fraction from exponential-phase cells. The two activities co-eluted from a Bio-Gel P-60 column after precipitation of proteins with ammonium sulfate and solubilization with 0.1% Tween 20. The PutA protein in the active fraction also oxidized 3,4-dehydro-DL-proline, but there was no activity with other L-proline analogues. 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 L-proline dehydrogenase and a concomitant increase in the intracellular concentration of L-proline. These results indicate that *P. aurescens* strain TC1 can use L-proline as a nutrient in a regulated fashion. Because this bacterium also showed the ability to degrade most of the other common amino acids, it can serve as a useful model for the control of amino acid catabolism in the high G + C *Actinobacteria*.

Keywords *Arthrobacter aurescens* · L-Glutamate-γ-semialdehyde dehydrogenase · *Paenarthrobacter aurescens* · L-Proline dehydrogenase · PutA protein · L-Δ^1-Pyrroline-5-carboxylate dehydrogenase

Introduction

The amino acid L-proline plays many different roles in bacterial physiology. In addition to being incorporated into proteins and peptides, it can be used as a carbon and nitrogen source, an osmoprotectant, a mediator of redox signaling, a stabilizer of protein structure, and an enhancer of resistance to a variety of stresses (Liang

All the experiments were designed and performed by Charles E. Deutch. He also created the figures and tables and wrote the manuscript.

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et al. 2013; Christgen and Becker 2018). L-Proline is degraded by a common catabolic pathway in all living organisms (Adams and Frank 1980; Liu et al. 2017). There is an initial FAD-dependent oxidation by L-proline dehydrogenase (PDH, EC 1.5.5.2, formerly 1.5.99.8) to form L-Δ^1-pyrroline-5-carboxylate (P5C). P5C is in spontaneous equilibrium with L-glutamate-γ-semialdehyde (GSA), which then undergoes a NAD^+-dependent oxidation by L-glutamate-γ-semialdehyde dehydrogenase (GSALDH, also called L-Δ^1-pyrroline-5-carboxylate dehydrogenase, P5CDH, EC 1.2.1.88, formerly 1.5.1.12) to form L-glutamate. The L-glutamate can be converted to 2-oxoglutarate (α-ketoglutarate) and further metabolized through the citric acid cycle or other metabolic pathways.

The two enzyme activities needed for L-proline degradation are organized in three basic ways (Tanner 2008). In organisms with monofunctional proteins, PDH activity is due to a membrane-associated enzyme commonly called PutA that transfers electrons to a quinone in the electron transport chain and GSALDH (P5CDH) activity is due to a cytoplasmic enzyme. In organisms with bifunctional proteins, PDH and GSALDH (P5CDH) activities are part of a single membrane-associated PutA enzyme. In organisms with trifunctional proteins, a PutA protein with both PDH and GSALDH (P5CDH) activities has an additional NH_2-terminal DNA-binding domain and acts as a transcriptional regulator of the genes encoding PutA and a major Na^+-dependent proline transporter called PutP. Cells often have additional proline transporters including an osmotically-inducible and activated H^+-dependent proline/betaine uptake protein called ProP and an osmotically-inducible ABC transporter called ProU (Wood 1988).

L-Proline catabolism has been previously studied in several Gram-positive bacteria including *Staphylococcus saprophyticus* (Deutch 2011), *Bacillus subtilis* (Moses et al. 2012), and *Mycobacterium tuberculosis* (Berney et al. 2012) and found to be due to two monofunctional proteins. On the other hand, *Corynebacterium glutamicum* has a bifunctional PutA protein that appears to contain both PDH and GSALDH (P5CDH) activities (Bott and Niebisch 2003). Recent detailed biochemical studies by Korasick et al. (2017) have indicated that the bifunctional PutA protein from *Corynebacterium freiburgense* has an additional C-terminal domain related to the aldehyde dehydrogenase superfamily which is essential for both PDH and GSALDH activities. While there has been an extensive study of L-proline uptake in *C. glutamicum* and its role in osmoregulation (Peter et al. 1997, 1998), less is known about the physiology of L-proline catabolism and the role of the PutA protein in various cellular processes in this and other high G + C Gram-positive bacteria.

Arthrobacter aurescens strain TC1 is a high G + C Gram-positive aerobe that 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 and now reassigned to the genus *Paenarthrobacter* as *Paenarthrobacter aurescens* (Busse 2016). 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 designated AAur_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). It also has genes homologous to *proW*, *proV*, *proY*, and *proZ* which appear to encode parts of ABC proline/glycine betaine transporters. On the other hand, there is no gene homologous to *opuE* that encodes an osmotically-inducible proline transporter in *B. subtilis* (Moses et al. 2012). *P. aurescens* strain TC1 has been considered a model system for the study of oxidative metabolism and survival mechanisms in arthrobacters and related aerobic soil bacteria (Mongodin et al. 2006). I have focused on the physiological role of the PutA protein in *P. aurescens* strain TC1 and now describe the functions and regulation of L-proline catabolism in this bacterium under different conditions.

Materials and methods

Bacteria and growth conditions

P. aurescens strain TC1 was acquired from Dr. Michael Sadowsky at the University of Minnesota and stored at − 80 °C in 20% (v/v) glycerol. Active stocks were grown at 30 °C on plates of tryptic soy

broth agar containing 0.5% (w/v) yeast extract (TSB/YE). The ability of the bacteria to degrade atrazine was periodically confirmed by streaking them on agar plates with atrazine as the sole nitrogen source. The morphology of the bacteria was regularly checked by phase-contrast light microscopy with a Nikon Alphaphot microscope and a 100× oil immersion objective.

The bacteria were routinely grown at 30 °C with aeration in 125 ml Erlenmeyer or 300 ml nephelometer flasks containing 10 ml of minimal liquid R medium (Strong et al. 2002; Anon., American Type Culture Collection 2016) supplemented with varying combinations of 0.2% (w/v) D-glucose, 10 mM $(NH_4)_2SO_4$, and 10 mM L-proline. Salt-induced myceloids were formed by growth in R medium to which different concentrations of NaCl were added. Citrate-induced myceloids were produced by growth in R medium containing 20 mM sodium citrate. For some experiments, glycine betaine or other L-amino acids were added to R medium as the nitrogen source or sole carbon and nitrogen source at concentrations of 10 mM or 30 mM as indicated below. Culture turbidities were routinely followed in a Klett-Summerson colorimeter with a red (660 nm) filter. Proline-utilization (Put) and betaine-utilization (But) indicator media were prepared as described by Bochner and Savageau (1977). Simmons citrate agar (Becton Dickenson BBL) was made according to the manufacturer's directions.

L-Proline degradation in whole cells

P. aurescens strain TC1 was grown in minimal R medium with 0.2% (w/v) D-glucose or 0.5% (v/v) glycerol as the carbon source in the presence of 10 mM ammonium sulfate or 10 mM L-proline to exponential phase (75–100 Klett $Units_{660\ nm}$). In some cases, the bacteria were grown in liquid TSB/YE medium with or without 10 mM L-proline. The bacteria were harvested by centrifugation at 4 °C at 10,000×*g* for 10 min. The bacteria were washed once with 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $MgSO_4{\cdot}7\ H_2O$, and resuspended in the same buffer to give a suspension with 100 Klett $Units_{660\ nm}$. The cell suspension was frozen and thawed once to promote permeation of substrates during the assay (Deutch 2011). A sample of the suspension was combined with an equal volume of 1.0 M L-proline and 1/10 volume of 0.05 M 2-aminobenzyaldehde (*o*-aminobenzaldehyde, Sigma Aldrich, St. Louis, MO, USA) in 20% (v/v) ethanol and shaken vigorously in a shaker-incubator at 30 °C. Three replicate 2.0 ml samples were removed periodically during a 180-min assay period and added to tubes containing 400 µl of 10% trichloroacetic acid. Each sample was filtered with vacuum through a Pall GN-6 membrane filter (25 mm, 0.45 µm pores) and the absorbance of the filtrate was determined in a Shimadzu UV–Visible spectrophotometer at 443 nm. Activities were calculated using an mM extinction coefficient of 2.71 for the P5C/2-aminobenzaldehyde complex. The average of the replicate samples was determined and varied by no more than 10%. For comparison of cultures, only the linear portion of a 180-min assay was used. All experiments with whole cells were done at least three times.

Preparation of cell extracts and partial purification of PutA

P. aurescens strain TC1 was grown to late exponential-phase in 500 ml portions of minimal R medium containing 0.2% (w/v) D-glucose, 10 mM ammonium sulfate, and 10 mM L-proline at 30 °C with aeration. The bacteria were harvested by centrifugation at 10,000×*g* for 10 min, washed once with 0.85% NaCl, and stored at − 20 °C as a frozen pellet. The cells were then thawed and resuspended in 20 ml of 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $MgSO_4{\cdot}7H_2O$. The bacteria were combined with 0.1 mm glass beads and disrupted by six one-min cycles in a Model 1107900 Bead Beater® (Biospec Products, Bartlesvillie, OK USA) while chilled in ice water. The beads and unbroken cells were removed by centrifugation for 10 min at 2000×*g* and then at 10,000×*g*.

To prepare a membrane fraction, the extract was transferred to polycarbonate tubes and centrifuged at 40,000 rpm (105,000×*g*) in a Ti70.1 rotor in a Beckman Optima L-90 ultracentrifuge for 2 h. The supernatant was removed and saved as the soluble fraction (S_{105}); the pellets were dissolved in a minimal amount of 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $MgSO_4{\cdot}7H_2O$ as the membrane fraction (P_{105}). To prepare a solubilized fraction, the proteins in the initial extract were precipitated with ammonium sulfate (80% saturation at 4 °C) after stirring on ice for 60 min. After centrifugation at

10,000×*g* for 10 min, the precipitated proteins were suspended in a minimal amount of 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $MgSO_4$·$7H_2O$. One-tenth volume of 1% (v/v) Tween 20 in 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $MgSO_4$·$7H_2O$ was added and the sample stirred on ice for 60 min. The sample was centrifuged at 10,000×*g* for 10 min and the supernatant fraction saved as the solubilized extract. The extract was applied to a 1.5 × 15 cm column of Bio-Gel P-60 beads equilibrated with 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $MgSO_4$·$7H_2O$ and the proteins eluted with the same buffer as a series of 200-drop fractions. The fractions were tested for both enzyme activities and for absorbance at 280 nm. Experiments with cell extracts and membrane fractions were done at least three times.

Enzyme assays with cell extracts and membrane fractions

L-Proline dehydrogenase (PDH) activity was measured as previously described (Deutch 2011). In one assay, 100 µl of 1.0 M L-proline, 100 µl of 0.05 M 2-aminobenzaldehyde, and 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $MgSO_4$·$7H_2O$ were combined with a fraction to give a total volume of 1000 µl. The change in absorbance at 443 nm was measured in a Shimadzu UV160U spectrophotometer for 5–10 min. Activities were calculated from the linear portion of each assay using a mM extinction coefficient of 2.71 for the P5C/2-aminobenzaldehyde complex. In another assay, 100 µl of 0.5 M Tris–HCl buffer, pH 7.5, 100 µl of 0.75% (v/v) Triton X-100, 10 µl of 1.0 M KCN, 10 µl of 2 mM FAD, 10 µl of 20 mM phenazine methosulfate, 100 µl of 1.0 M L-proline, 100 µl of 2 mM *p*-iodonitrotetrazolium violet (Sigma Aldrich, St. Louis, MO, USA), an enzyme fraction, and water were combined to give a total volume of 1000 µl. The change in absorbance at 500 nm was measured in a Shimadzu UV 160U spectrophotometer for 5–10 min. For assays of the soluble fraction, the KCN was omitted. Activities were calculated from the linear portion of each assay using a mM extinction coefficient for the reduced *p*-iodonitrotetrazolium of 11.5.

L-Glutamate-γ-semialdehyde dehydrogenase (L-Δ^1-pyrroline-5-carboxylate dehydrogenase, P5CDH) was also measured as previously described (Deutch 2011). DL-Pyrroline-5-carboxylate (P5C) was prepared as described by Williams and Frank (1975), eluted with 1 M HCl from a 1.5 × 15 cm column of Dowex-50, and quantified by reaction with 2-aminobenzaldehyde. Portions of the P5C solution were neutralized to pH 7 with NaOH just before use. Reaction mixtures contained 100 µl of 0.5 M potassium phosphate, pH 7.0 containing 10 mM $MgSO_4$·$7H_2O$, 100 µl of 0.75% (v/v) of Triton X-100, 100 µl of 20 mM NAD^+, 10 µl of 20 mM phenazine methosulfate, 100 µl of 2 mM *p*-iodonitrotetrazolium violet, neutralized P5C, water, and extract to give a total volume of 1000 µl. The change in absorbance at 500 nm was measured in a Shimadzu UV 160U spectrophotometer for 5–10 min. Activities were calculated from the linear portion of each assay using a mM extinction coefficient for the reduced *p*-iodonitrotetrazolium of 11.5.

Protein assays

Protein concentrations of cell suspensions, membrane fractions, and soluble extracts were determined by the bicinchoninic acid (BCA) method of Smith et al. (1985) using bovine serum albumin as the standard.

Measurement of intracellular proline concentrations

P. aurescens strain TC1 was grown at 30 °C with aeration to exponential phase in minimal R medium containing 0.2% (w/v) D-glucose, 10 mM ammonium sulfate, 10 mM L-proline, and varying concentrations of NaCl. Bacteria (9 ml) were harvested by centrifugation at 10,000×*g* for 10 min and the medium discarded. After resuspension in 9 ml of 0.85% (w/v) NaCl, 500 µl were set aside for a protein assay and the remaining 8.5 ml centrifuged again. The NaCl wash solution was discarded and the cell pellet stored at − 20 °C. To extract small molecules including L-proline, the cell pellets were thawed and suspended in 500 µl of 3% (w/v) 5-sulfosalicylic acid. After 20 min, each sample was transferred to a microcentrifuge tube and centrifuged at 10,000×*g* for 10 min. The supernatant was removed and used for determination of the proline concentration by the method of Bates et al. (1973). Briefly, different volumes were combined with 3% (w/v) 5-sulfosalicylic acid to give a total of 100 µl in a 2 ml microcentrifuge tube. The

acid ninhydrin reagent (200 µl) and glacial acetic acid (100 µl) were added to each tube and the samples heated at 90–95 °C for 60 min. After cooling to room temperature, 1200 µl of toluene were added and the sampled agitated on a Vortex mixer for 10 s. After centrifugation at 10,000×*g* for 1 min, the absorbance of the toluene phase was determined at 520 nm in a Shimadzu UV160U spectrophotometer. All assays were done in duplicate and the proline concentration determined from an L-proline standard curve. After determining the protein concentration of the corresponding cell suspension, the proline concentration was expressed in nmoles per mg cellular protein. All experiments were done at least four times.

Results and discussion

Predicted structure of the PutA protein from *P. aurescens* strain TC1

P. aurescens strain TC1 contains a gene (AAur_0671) that is predicted to encode a bifunctional PutA protein (AR2L2_PAEAT) of 1157 amino acids (molecular weight of 124,379). Residues 142–436 appeared to comprise a L-proline dehydrogenase (PDH) domain and residues 518–940 to comprise a L-glutamate-γ-semialdehyde dehydrogenase (GSALDH, L-Δ^1-pyrroline-5-carboxylate dehydrogenase, P5CDH) domain (Fig. 1). BLAST analysis of the predicted amino acid sequence revealed matches with > 60% identity to hundreds of other predicted proteins. An alignment of the *P. aurescens* strain TC1 sequence with the PutA proteins from *C. freiburgense* (A0A1X8XLF1), *C. glutamicum* (A0A160PL87) and *Arthrobacter globiformis* (H0QP87) showed identities of 43.1, 48.9 and 76.2%, respectively. In addition to the PDH and P5CDH domains, all the proteins contained a COOH-terminal domain (from about residues 900 to 1050) which Korasick et al. (2017) have identified as important for both activities. None of the proteins possessed a NH_2-terminal DNA binding domain that in *Escherichia coli* and other Gram-negative bacteria is involved in the regulation of transcription of the *putA* and *putP* genes. The predominant secondary structure in the predicted protein was α-helix (51.7%), with only limited segments of extended β-strand (10.6%). Modeling of the three-dimensional structure using the PutA protein from *C. freiburgense* as a template revealed a compact molecule with no transmembrane segments, consistent with a peripheral membrane-associated protein.

Growth of normal cells of *P. aurescens* strain TC1 on L-proline

P. aurescens strain TC1 is an aerobic prototroph that can be grown in a defined salts medium with simple carbon and nitrogen sources. To determine if strain TC1 can utilize L-proline as a nutrient, the bacteria first were grown with aeration at 30 °C in R minimal medium containing varying combinations of D-glucose, ammonium sulfate, and L-proline. The bacteria formed multicellular myceloids during early exponential phase, grew as bent rods during exponential phase, and divided into shorter cocci during stationary phase. The growth curves for bacteria in R medium supplemented with 0.2% (w/v) D-glucose and 10 mM ammonium sulfate and for those in R medium supplemented 0.2% (w/v) D-glucose, 10 mM ammonium sulfate, and 10 mM L-proline were similar (Fig. 2a). However, the growth rate in the proline-supplemented culture was slightly faster and the yield was somewhat higher. The growth rate of *P. aurescens* strain TC1 in R medium supplemented with 0.2% (w/v) D-glucose and 10 mM L-proline as the sole nitrogen source was slower but the yield of bacteria was the same. Strain TC1 also grew in R medium with 50 mM L-proline as the sole carbon and nitrogen source but the growth rate was reduced even further. When the bacteria were grown in R medium with varying concentrations of L-proline as the sole carbon and nitrogen source, the lengths of the lag phase and the growth rates were similar, but the yields of the cultures in stationary phase were proportional to the proline concentration up to a 20 mM (Fig. 2b).

P. aurescens strain TC1 grew well on R medium agar plates containing 0.2% D-glucose and 10 mM ammonium sulfate or on R medium agar plates containing 50 mM L-proline as the sole carbon and nitrogen source. While the colonies were similar in size, texture, and margins, those on the proline medium were noticeably more yellow in colour. Liquid cultures containing L-proline as the sole carbon and nitrogen source also turned yellow in stationary phase. The pigment from *P. aurescens* strain TC1 was extracted with methanol after growth for 5–7 days in liquid medium with L-proline as the sole carbon and

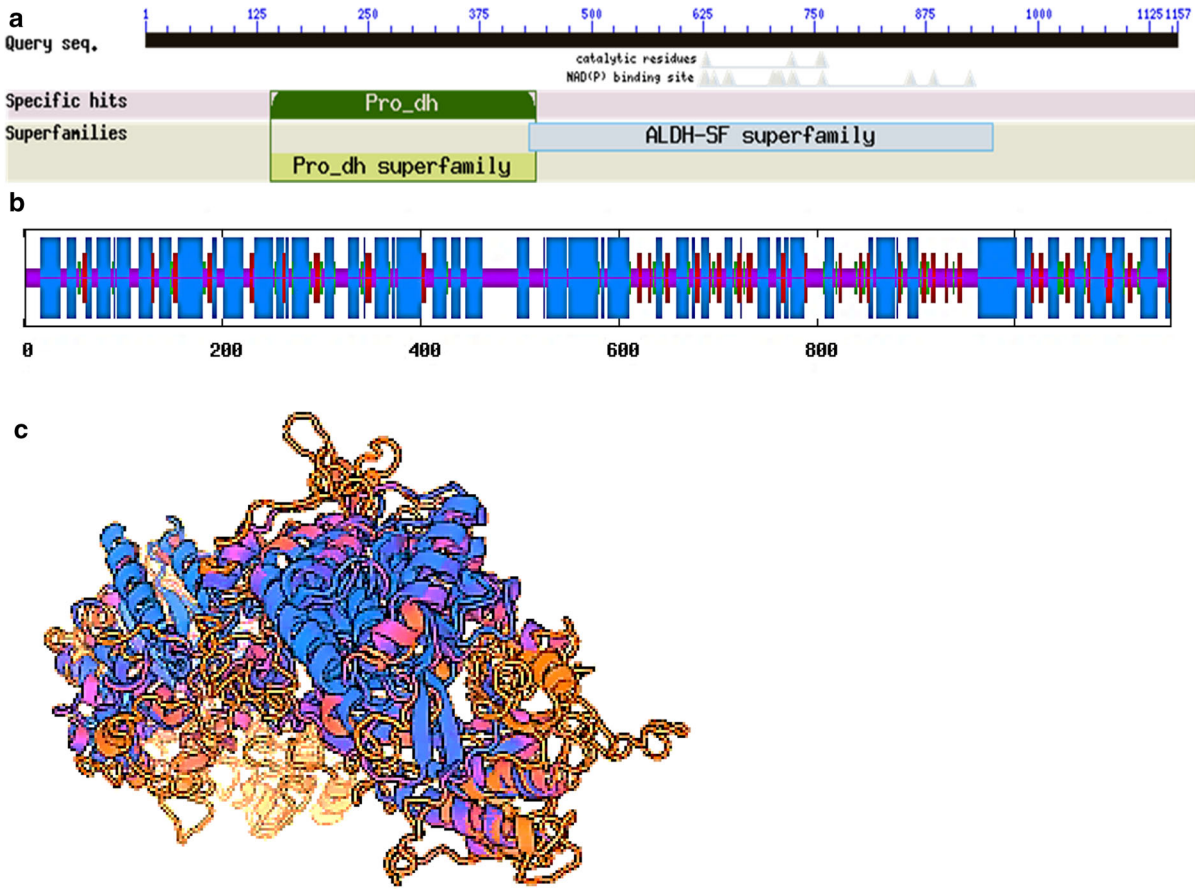
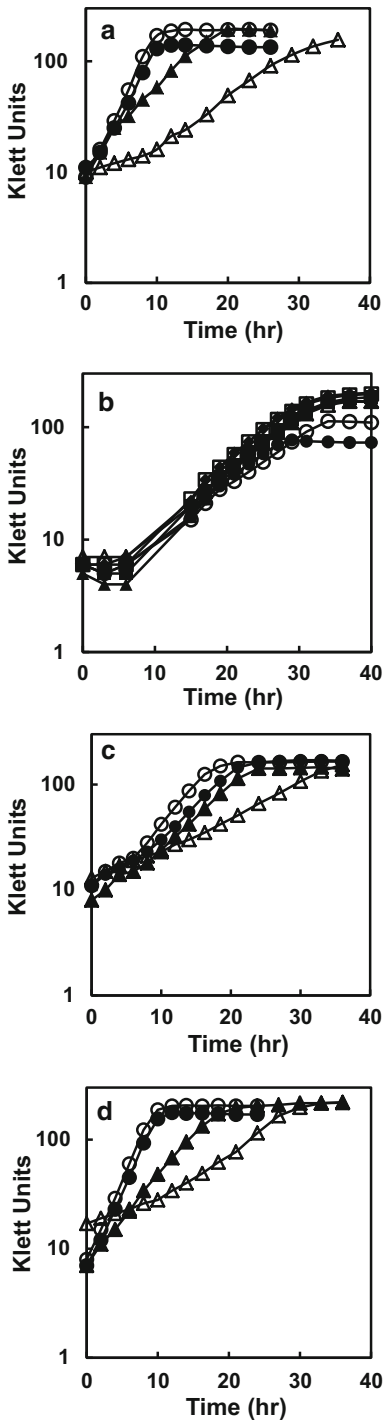


Fig. 1 Predicted structure of the 1157 amino acid PutA protein from *P. aurescens* strain TC1. Panel **a** shows the domain structure of the protein as visualized by the Conserved Domain function in the Protein database at the National Center for Biological Information (NCBI) site. Pro_dh superfamily corresponds to the L-proline dehydrogenase activity; ALDH-SF superfamily corresponds to the L-glutamate-γ-semialdehyde dehydrogenase (L-Δ^1-pyrroline-5-carboxylate dehydrogenase) activity. The triangles indicate residues believed to form the NAD^+ binding site. Panel **b** shows the predicted secondary structures of the protein as determined by Network Protein Sequence Analysis (Combet et al. 2000) at the Pôle Rhone-Alpes de Bioinformatique. The taller blue bars indicate regions of α-helix; the shorter red bars indicate regions of extended β-strand. Panel **c** shows the predicted tertiary structure of the protein as determined by the Swiss-Model program in the ExPASy portal at the Swiss Institute of Bioinformatics using the crystal structure of the PutA protein from *C. freiburgense* (5us5.1.A) as a template. (Colour figure online)

nitrogen source. The bright yellow solution showed absorption maxima at 465 nm, 437 nm, and 412 nm. Similar spectra were found for the C50 carotenoid purified from *Arthrobacter arilaitensis* samples associated with smear-ripened cheese (Monnet et al. 2010; Sutthiwong and Dufossé 2014). Although L-proline has been shown to be a direct precursor of prodigiosin, the red pigment found in *Serratia marcescens* and other bacteria (Williamson et al. 2006), the metabolic connection between proline degradation and carotenoid synthesis is less clear. *P. aurescens* strain TC1 has the genes needed for terpenoid and carotenoid biosynthesis from citric acid cycle or other metabolic intermediates that can be derived from proline (Mongodin et al. 2006).

Utilization of L-proline by other bacteria has been demonstrated on Put-indicator agar plates containing L-proline, peptone, and 2,3,5-triphenyltetrazolium chloride (Bochner and Savageau 1977). *P. aurescens* strain TC1 formed small dark red colonies on Put-indicator plates, although it took about 7 days for the colonies to be fully developed. Because the uptake of L-proline by strain TC1 appears to be mediated by transport systems that also facilitate the uptake of



◀**Fig. 2** Growth of *P. aurescens* strain TC1 in the presence of L-proline. Panel **a**: Growth of *P. aurescens* TC1 at 30 °C with aeration in minimal R medium containing 0.2% (w/v) D-glucose and 10 mM $(NH_4)_2SO_4$ (filled circle), 0.2% D-glucose (w/v), 10 mM $(NH_4)_2SO_4$, and 10 mM L-proline (circle), 0.2% D-glucose (w/v) and 10 mM L-proline (filled triangle), or 50 mM L-proline (triangle). Panel **b**: Growth of *P. aurescens* TC1 at 30 °C with aeration in minimal R medium containing 5 mM L-proline (filled circle), 10 mM L-proline (circle), 20 mM L-proline (filled triangle), 30 mM L-proline (triangle), 50 mM L-proline (filled square), 70 mM L-proline (square), or 100 mM L-proline (diamond). Panel **c**: Growth of *P. aurescens* TC1 at 30 °C with aeration in minimal R medium with 0.4 M NaCl containing 0.2% (w/v) D-glucose and 10 mM $(NH_4)_2SO_4$ (filled circle), 0.2% D-glucose (w/v), 10 mM $(NH_4)_2SO_4$, and 10 mM L-proline (circle), 0.2% D-glucose (w/v) and 10 mM L-proline (filled triangle), or 50 mM L-proline (triangle). Panel **d**: Growth of *P. aurescens* TC1 at 30 °C with aeration in minimal R medium with 20 mM sodium citrate containing 0.2% (w/v) D-glucose and 10 mM $(NH_4)_2SO_4$ (filled circle), 0.2% D-glucose (w/v), 10 mM $(NH_4)_2SO_4$, and 10 mM L-proline (circle), 0.2% D-glucose (w/v) and 10 mM L-proline (filled triangle), or 50 mM l^{-1} L-proline (triangle). Turbidities were measured in Klett-Summerson colorimeter with a red (660 nm) filter

glycine betaine, the bacteria were also tested for this phenotype on Put-indicator plates that had been spread with 100 µl of 1.0 M of glycine betaine. While the bacteria still formed red colonies, the intensity of the colour was reduced. Control experiments indicated that strain TC1 could use glycine betaine as the sole nitrogen source. However, the bacteria grew poorly on But-indicator plates containing glycine betaine and a tetrazolium dye and formed only very small pink-coloured colonies.

Growth of myceloids of *P. aurescens* strain TC1 on L-proline

One of the distinctive features of arthrobacters is their ability to grow as multicellular myceloids in exponential and stationary phase when they subjected to osmotic stress or to vitamin or metal ion-deficiency (Deutch and Perera 1992; Chan et al. 1973; Germida and Casida 1980). These myceloids differ from the normal bent rods in their metabolism and other physiological properties (Malwane and Deutch 1999). Recent studies have indicated that myceloid formation is due to a reduction in OtsA activity, a protein also used in trehalose formation (Chen et al. 2017). *P. aurescens* strain TC1 formed well-developed myceloids in minimal R medium containing NaCl concentrations above 0.2 M. The growth curves for these myceloids in R medium containing 0.4 M NaCl supplemented with 0.2% D-glucose and 10 mM ammonium sulfate or in R medium containing 0.4 M

NaCl supplemented with 0.2% D-glucose, 10 mM ammonium sulfate, and 10 mM L-proline were similar (Fig. 2c). The bacteria also grew well in R medium containing 0.4 M NaCl supplemented with 0.2% D-glucose and 10 mM L-proline as the sole nitrogen source or in R medium containing 0.4 M NaCl supplemented with 50 mM L-proline as the sole carbon and nitrogen source.

P. aurescens strain TC1 formed somewhat larger myceloids in R medium containing 20 mM sodium citrate which causes calcium deficiency. The growth curves for these myceloids in R medium with 0.2% D-glucose and L-proline as a supplemental nutrient or as the sole nitrogen source were again like those seen with regular cells (Fig. 2d). The citrate-induced myceloids also grew in the presence of L-proline as the sole carbon and nitrogen source but often formed larger aggregates that were visible to the human eye. These myceloids broke up into single cells and smaller fragments in stationary phase. Control experiments indicated that *P. aurescens* strain TC1 could grow with citrate as the sole carbon source and could cause Simmons citrate indicator medium to change colour from blue to green.

L-Proline degradation in whole cells of *P. aurescens* strain TC1

L-Proline dehydrogenase (PDH) activity in whole cells of *P. aurescens* strain TC1 could be detected by reaction of L-Δ^1-pyrroline-5-carboxylate with 2-aminobenzaldehyde to give a yellow-coloured product (Dendinger and Brill 1970; Deutch 2011). The rate of the reaction in *P. aurescens* strain TC1 that had been frozen and thawed to allow permeation of substrates was linear for 60–180 min depending on the level of activity and was faster in exponential-phase cells than in stationary-phase cells (data not shown). To determine the effect of growth conditions on the formation of PDH, the specific activity was determined after growth of strain TC1 in various media (Fig. 3). The specific activity in nmol min^{-1} (mg protein)$^{-1}$ was higher whenever L-proline was added to the growth medium. The presence of ammonium sulfate increased both the growth rate and the specific activity of PDH but the use of D-glucose or glycerol as the primary carbon source made little difference. The specific activity was highest in medium containing L-proline as the sole carbon and nitrogen source. The activity was much lower in complex medium such as the tryptic soy broth/yeast extract medium used for maintaining bacterial cultures. These results are consistent with previous studies of other Gram-negative or Gram-positive bacteria which have indicated that PDH activity is inducible by L-proline. While expression of the *putA* gene in *E. coli* and other enteric bacteria is controlled by a transcriptional repressor domain that is part of the PutA protein (Zhou et al. 2008), expression in the Gram-positive bacterium *B. subtilis* is regulated by a separate transcriptional activator called PrcR (Moses et al. 2012; Huang et al. 2011). This protein has also been called PutR and expression of the *putA* gene is also affected by a repressor called CodY (Belitsky 2011). An analysis of the genome of *P. aurescens* strain TC1 indicates that it encodes a homologue of PrcR or PutA (A1R960_PAEAT) but not one of CodY.

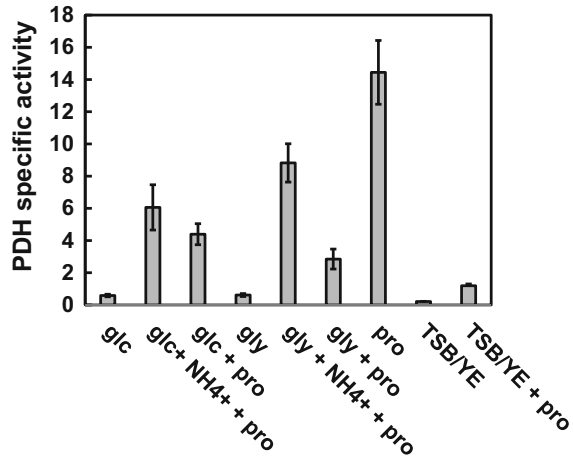


Fig. 3 Specific activities [nmoles min^{-1} (mg protein)$^{-1}$] of L-proline dehydrogenase in whole cells of *P. aurescens* after growth to exponential phase in various media. The results show the means and standard deviations of three to six separate experiments with each medium

Because proline uptake in *P. aurescens* strain TC1 appears to be mediated by membrane transporters that also facilitate the uptake of glycine betaine, the possibility that glycine betaine could induce L-proline dehydrogenase formation was tested. The specific activity of PDH in whole cells after growth in R medium containing 0.2% (w/v), 10 mM ammonium sulfate, and 10 mM glycine betaine was similar to that in bacteria grown in R medium containing 0.2% (w/v) and 10 mM ammonium sulfate and much less than that found in bacteria after growth in medium containing

10 mM L-proline. To determine if other compounds might induce L-proline dehydrogenase formation, the bacteria were tested for PDH activity after growth in R medium containing 0.2% (w/v) D-glucose, 10 mM ammonium sulfate, and 1 mM D-proline, L-thiazolidine-4-carboxylate (thiaproline), L-thiazolidine-2-carboxylate (beta-thiaproline), L-glutamate, L-arginine, or L-valine. L-Thiazolidine-4-Carboxylate can act as an inducer of proline catabolism in *E. coli* (Deutch 1992), L-glutamate is the biosynthetic precursor of L-proline in most organisms (Adams and Frank 1980), L-arginine and L-proline metabolism are intimately linked in *S. aureus* (Nuxoll et al. 2012), and L-valine can act as an inducer of PDH in *Agrobacterium tumefaciens* (Cho and Winans 1996). None of these compounds increased the PDH activity in whole cells of *P. aurescens* strain TC1 above the uninduced level.

Co-localization of L-proline dehydrogenase and L-glutamate-γ-semialdehyde dehydrogenase (L-Δ^1-pyrroline-5-carboxylate dehydrogenase) activities

The PutA protein of *P. aurescens* strain TC1 was predicted to contain both L-proline dehydrogenase and L-glutamate-γ-semialdehyde dehydrogenase (L-Δ^1-pyrroline-5-carboxylate dehydrogenase) activities. To confirm this prediction and to determine its cellular localization, membrane and soluble fractions of strain TC1 were prepared from exponential-phase cells grown in minimal R medium containing 0.2% (w/v) D-glucose, 10 mM ammonium sulfate, and 10 mM L-proline. Each enzyme activity was measured with spectrophotometric assays and the specific activities determined (Table 1). There was some variation in the specific activities from experiment to experiment depending on the efficiency of cell disruption, but the results were consistent among five independent preparations. There was low PDH activity in the membrane fraction as measured by the reaction of L-Δ^1-pyrroline-5-carboxylate with 2-aminobenzaldehyde but this activity was not detectable in the soluble fraction. The specific activity of PDH in the membrane fraction as measured by the proline-dependent reduction of *p*-iodonitrotetrazolium (INT) was much higher. The specific activity of L-glutamate-γ-semialdehyde dehydrogenase (L-Δ^1-pyrroline-5-carboxylate dehydrogenase) as measured by the P5C-dependent reduction of *p*-iodonitrotetrazolium was about half that of L-proline dehydrogenase. There was some tetrazolium-dependent activity (12–15%) of each enzyme in the soluble fraction.

To demonstrate that the L-proline dehydrogenase and L-glutamate-γ-semialdehyde dehydrogenase (L-Δ^1-pyrroline-5-carboxylate dehydrogenase) activities in the membrane fraction from *P. aurescens* strain TC1 were due to the same protein, the proteins in the P_{105} fraction were solubilized in several ways. Treatment of the membrane fraction with a CHAPS–EDTA extraction buffer (Brown and Wood 1992), with 1.0 M NaCl in 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $MgSO_4 \cdot 7H_2O$, with 1% CHAPS in 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $MgSO_4 \cdot 7H_2O$, or with 1% Triton X-100 in 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $MgSO_4 \cdot 7H_2O$ did not result in the extraction of the L-proline dehydrogenase activity as measured by *p*-iodonitrotetrazolium reduction in the resulting soluble fraction. However, both L-proline dehydrogenase and L-glutamate-γ-semialdehyde dehydrogenase (L-Δ^1-pyrroline-5-carboxylate dehydrogenase) activities could be extracted from the membrane fraction with 1% Tween 20 in 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $MgSO_4 \cdot 7H_2O$. The specific activities were 25.20 and 56.51 nmol min^{-1} (mg protein)$^{-1}$ respectively for the preparation shown in Table 1. Other preparations gave generally similar results, but ratio of the two activities varied and both rapidly decreased during storage at 4 °C.

Table 1 Distribution of proline catabolic activities in extracts of *P. aurescens* strain TC1

Fraction	Proline dehydrogenase (nmoles min^{-1} mg^{-1})		P5C dehydrogenase (nmoles min^{-1} mg^{-1})
	2-AB assay	INT assay	INT assay
Membrane (P_{105})	2.72	63.88	34.98
Soluble (S_{105})	–	7.55	5.45

To confirm the co-localization of the two enzyme activities, the proteins in the initial Bead Beater® extract of *P. aurescens* strain TC1 were precipitated with ammonium sulfate and then solubilized with 0.1% Tween 20. The proteins in the Tween 20 extract were then separated on a column of BioGel P-60 beads (Fig. 4). The L-proline dehydrogenase and L-glutamate-γ-semialdehyde dehydrogenase (L-Δ^1-pyrroline-5-carboxylate dehydrogenase) activities both eluted in the same initial fractions that corresponded to the void volume of the column. Interestingly, the activity of L-Δ^1-pyrroline-5-carboxylate dehydrogenase was consistently higher than that of L-proline dehydrogenase. These activities were well separated from other proteins in the extract which showed absorbance at 280 nm, but SDS-PAGE analysis indicated that the active fraction still contained other proteins of various sizes. Korasick et al. (2017) prepared a solubilized and purified form of the PutA protein from *C. freiburgense* by chemically synthesizing the gene with an 8X His tag, expressing the protein in *E. coli*, and purifying the protein on a Ni-affinity and other chromatography columns. They found that the K_m for P5C with the L-glutamate-γ-semialdehyde dehydrogenase activity was much lower than that for L-proline with the L-proline dehydrogenase activity and the K_{cat}/K_m ratio for the GSALDH (P5CDH) activity was much higher.

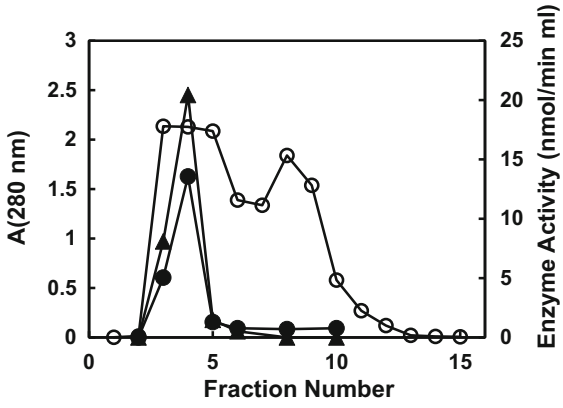


Fig. 4 Elution of proteins in the Tween 20 supernatant of a *P. aurescens* TC1 extract from a Bio-Gel P-60 column. The proteins were eluted with 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM $MgSO_4$.7 H_20. The fractions were tested for absorbance at 280 nm (circle), L-proline dehydrogenase activity (filled circle), and L-glutamate-γ-semialdehyde dehydrogenase (L-P5C dehydrogenase activity filled triangle)

Oxidation of L-proline analogues by *P. aurescens* strain TC1

PutA proteins from other bacteria have been shown to degrade various analogues of L-proline including L-thiazolidine-4-carboxylate (T4C, thiaproline, Deutch 1992) and 3,4-dehydroproline (DHP, Wood 1981). To determine if the PutA protein from *P. aurescens* strain TC1 can degrade these or other related compounds, the ammonium sulfate precipitate from an extract of strain TC1 was tested for oxidative activity in the presence of *p*-iodonitrotetrazolium as an electron acceptor (Table 2). There was low activity with 10 mM L-proline but higher activity with 100 mM L-proline. There was very high activity with 3,4-dehydro-DL-proline or L-thiazolidine-2-carboxylate (beta-thiaproline) as substrates. There was some activity with L-thiazoline-4-carboxylate as the substrate, but because of a high spontaneous reaction, this activity could only be measured in the absence of phenazine methosulfate. There was no activity with D-proline, L-azetidine-2-carboxylate, tetrahydro-2-furoic acid, L-pipecolic acid, *cis*-4-hydroxy-L-proline, *trans*-4-hydroxy-L-proline, pyroglutamic acid, piperazine-2-carboxlylate, or 2-oxo-4-thiazoline-4-carboxylic acid as substrates. Similar results were obtained with the proteins in the active fraction from the BioGel P-60 column after treatment of the ammonium sulfate fraction with 0.1% Tween 20.

L-Lactate is known to be a potent active site inhibitor of the PutA protein from *E. coli* (Scarpulla and Soffer 1978; Zhang et al. 2004). There was small amount of activity with this compound as a substrate in the tetrazolium reduction assay using the ammonium sulfate precipitate from the initial extract of strain TC1 (Table 2). When 100 mM of L-lactate was added to reaction mixtures containing 10 mM of L-proline or 10 mM 3,4-dehyro-DL-proline as the primary substrate, there was a marked reduction of activity. However, when L-lactate was added to the mixture containing thiazoline-4-carboxylate (T4C) or thiazolidine-2-carboxylate (T2C) as the substrate, the activity remained the same or increased slightly, suggesting that oxidation of these substrates was due to other proteins in the membrane or solubilized protein fraction. Previous studies have indicated that T4C can be oxidized by P5C reductase (Deutch et al. 2001) and 3.4-dehydro-D-proline by D-amino acid dehydrogenase (Deutch 2004). Analysis of the genome of *P.*

Table 2 Effect of L-lactate on the oxidation of L-proline and its analogues by proteins in the ammonium sulfate fraction from *P. aurescens* strain TC1[a]

Substrate	Conc (mM)	Activity w/o L-lactate (nmol min^{-1} ml^{-1})	Activity + L-lactate[b] (nmol min^{-1} ml^{-1})
L-Lactate	100	–	6.5
L-Proline	10	27.8	11.3
L-Proline	100	68.7	36.5
3,4-Dehydro-DL-proline	10	313	62.6
L-Thiazolidine-4-carboxylate[c]	10	23.0	23.9
L-Thiazoline-2-carboxylate	10	621	763

[a]The protein concentration of the ammonium sulfate fraction was 9.01 mg/ml

[b]Where added as an inhibitor, the L-lactate concentration was present at 100 mM

[c]Because of a high spontaneous rate of reaction, the activity with L-thiazolidine-4-carboxylate was determined in the absence of phenazine methosulfate

aurescens strain TC1 indicates that this bacterium has genes encoding each of these activities.

Because several proline analogues were oxidized by the fraction from *P. aurescens* strain TC1 with L-proline dehydrogenase and L-glutamate-γ-semialdehyde dehydrogenase (L-Δ^1-pyrroline-5-carboxylate dehydrogenase) activities, these compounds were tested as potential nitrogen sources in R minimal medium with D-glucose as the primary carbon source. Although the bacteria grew well with L-proline as the sole nitrogen source, they could not grow with 3,4-dehydro-DL-proline, thiazolidine-2-carboxylate, or thiazolidine-4-carboxylate as nitrogen sources. However, the bacteria did grow well with the dipeptides L-Ala-L-Pro or L-Leu-L-Pro as the nitrogen sources. Control experiments indicated that strain TC1 grew well with L-alanine as the sole nitrogen source, but L-leucine was a poor nitrogen source (see Table 3 below).

Reduction in L-proline catabolism in strain TC1 during osmotic stress

L-Proline is commonly accumulated by bacteria and other organisms as an osmoprotectant during growth in high-salt media (Wood 2011, 2015). *P. aurescens* strain TC1 was found to grow in R medium containing concentrations of NaCl up to 0.8 M. When strain TC1 was grown in R medium containing increasing concentrations of NaCl, 0.2% (w/v) D-glucose, 10 mM ammonium sulfate, and 10 mM L-proline, there was a progressive decrease in the specific activity of L-proline dehydrogenase as measured in whole cells (Fig. 5a). Under similar conditions, there was an increase in the intracellular concentration of L-proline (Fig. 5b). The concentration gradually increased and was highest in the myceloids seen in 0.4–0.8 M NaCl. Similar reductions in PDH activity have been observed in *E. coli* (Deutch et al. 1989) and related to proline accumulation in some plants (Miller et al. 2005).

Catabolism of other amino acids by *P. aurescens* strain TC1

Because *P. aurescens* strain TC1 could grow well with L-proline as a nutrient, the bacteria also were tested for their ability to grow with the other common amino acids as the sole nitrogen source. The bacteria grew well in R medium with 0.2% D-glucose (w/v) as the primary carbon source and 10 mM concentrations of 18 of the common amino acids (Table 3). There was no growth with L-cysteine or L-methionine. There was growth on L-isoleucine and L-leucine but at a much slower rate. Of the amino acids that were degraded by *P. aurescens* strain TC1 as nitrogen sources, all except L-isoleucine, L-leucine, and L-valine could also serve as the sole carbon and nitrogen source at a concentration of 30 mM. *P. aurescens* strain TC1 thus has great flexibility in its use of simple amino acids as nutrients.

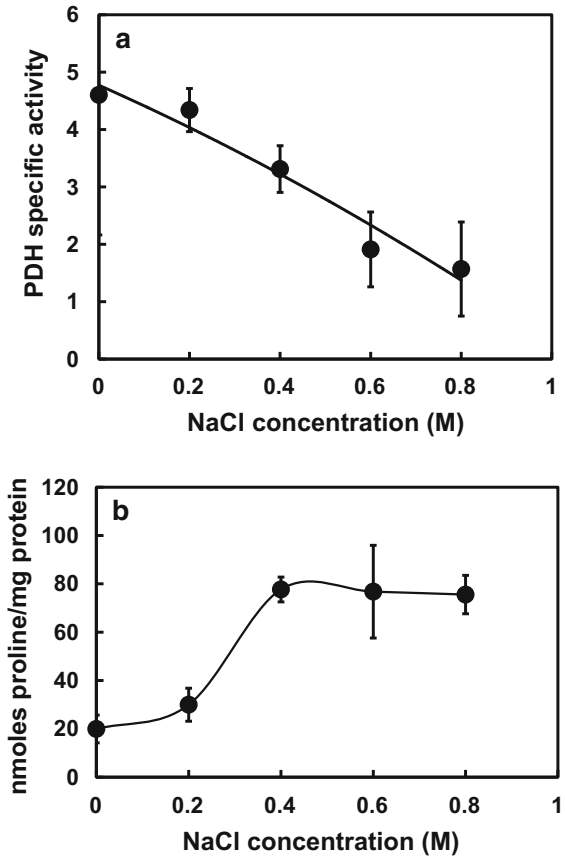


Fig. 5 Effect of increasing concentrations of NaCl on the intracellular proline concentration and specific activity of L-proline dehydrogenase in *P. aurescens* strain TC1. Panel **a** shows the specific activity of L-proline dehydrogenase as measured in whole cells after growth to exponential phase in R medium with 0.2% (w/v) D-glucose, 10 mM ammonium sulfate, 10 mM L-proline, and various concentrations of NaCl. Panel **b** shows the intracellular proline concentrations in nmoles of proline per mg cellular protein in cultures after growth to exponential phase in R medium with 0.2% (w/v) D-glucose, 10 mM ammonium sulfate, 10 mM L-proline, and various concentrations of NaCl. Both panels show the means and standard derivations of three to five separate experiments

Conclusions

The *Actinobacteria* are a large complex lineage of microorganisms within the Domain Bacteria (Ventura et al. 2007). These organisms vary in morphology, development, metabolism, and ecology, and include both pathogenic and nonpathogenic bacteria. *Paenarthrobacter* is a newly-created genus that includes several species previously included in the genus *Arthrobacter* (Busse 2016). *P. aurescens* strain TC1 has a completely-sequenced genome and so can serve as a model of various metabolic and physiological processes in this high G + C lineage. The results reported here indicate that this organism can grow efficiently with L-proline as a sole nitrogen or carbon and nitrogen source by degrading it with a bifunctional membrane-associated PutA protein. Formation of this protein is inducible by L-proline but reduced during osmotic stress. Among the questions that we would like to explore further with this system are (1) what is the role of the ProP homologue and various ABC transporters in the uptake of L-proline? (2) what is the mechanism by which expression of the gene encoding PutA controlled by the PutR (PrcR) homologue predicted to be found in strain TC1? (3) what is the process by which expression of the PutA protein is reduced during osmotic stress? (4) Can these results be extended to other high G + C *Actinobacteria*? (5) In what ways do these studies of L-proline catabolism apply to the degradation of other L-amino acids? Hodgson (2000) reviewed the metabolism of amino acids in streptomycetes some time ago, but this area of microbial metabolism in the high G + C bacteria has been largely unexplored. A major limitation in studying arthrobacters has been the absence of good genetic systems for manipulating their genomes. Zhang et al. (2011) recently described conditions for transformation of these bacteria by electroporation and shown they can be applied to *A. aurescens*. We intend to apply them to the further study of L-proline catabolism in *P. aurescens* strain TC1.

Table 3 Utilization of L-amino acids by *P. aurescens* strain TC1

Amino acid	Used as nitrogen source[a]	Used as carbon/nitrogen source[b]
L-Proline	+	+
L-Alanine	+	+
L-Arginine	+	+
L-Aspartate	+	+
L-Asparagine	+	+
L-Cysteine	0	nt
L-Glutamate	+	+
L-Glutamine	+	+
Glycine	+	+
L-Histidine	+	+
L-Isoleucine	+	0
L-Leucine	+	0
L-Lysine	+	+
L-Methionine	0	nt
L-Phenylalanine	+	+
L-Serine	+	+
L-Threonine	+	+
L-Tryptophan	+	+
L-Tyrosine	+	+
L-Valine	+	0

[a]L-amino acids were tested for use as nitrogen sources at a concentration of 10 mM in R medium containing 0.2% (w/v) D-glucose as the carbon source. Cultures were grown at 30 °C for a maximum of 3 days

[b]L-amino acids were tested for use as sole carbon and nitrogen sources at concentration of 30 mM in R medium. Cultures were grown at 30 °C for a maximum of 3 days

+ growth; 0 no growth; *nt* not tested

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Compliance with ethical standards

Conflicts of interest The author declares that he has no conflicts of interest in publishing this work.

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Human and animal rights statement No humans or animals were used in this project.

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