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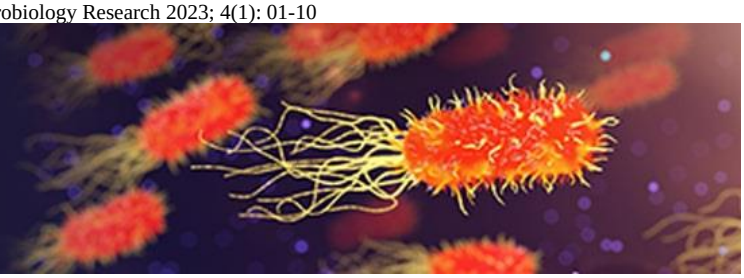

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Growth of *Escherichia coli* K-12 on L-proline in the absence of known proline transporters

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Abstract
Three transport systems have been implicated in the uptake of L-proline by the Gram-negative bacterium *Escherichia coli*. A triple mutant containing deletions of the *putP*, *proP*, and *proW* genes was constructed from strain BW25113 of the Keio collection. This mutant still grew slowly in a minimal salts medium with L-proline as the sole nitrogen source. Growth was dependent on the exogenous proline concentration but was not stimulated by 0.3 mol l^{-1} NaCl. It was reduced by addition of 5 mmol l^{-1} L-isoleucine by not by L-leucine, glycine betaine, γ- aminobutyric acid, or sarcosine. The triple mutant was partially inhibited by L-azetidine-2-carboxylate but not by 3,4-dehydro-DL-proline. Introduction of additional mutations in the *proY*, *brnQ*, or *gabP* genes had no effect. While the mechanism of proline uptake in this mutant is still unclear, it is likely that another membrane transporter can facilitate L-proline accumulation when the normal systems are unavailable.

Keywords: *Escherichia coli*, L-proline, mutant, nitrogen source, membrane transporter

1. Introduction
The amino acid L-proline plays several roles in the physiology of bacteria, plants, and animals. In addition to being incorporated into proteins and peptides, it can be used as a nitrogen and carbon source, an osmoprotectant, a mediator of redox signaling, a stabilizer of protein structure, a precursor of secondary metabolites, and an enhancer of resistance to a variety of stresses [1-3]. The Gram-negative bacterium *Escherichia coli* and the related microorganism *Salmonella enterica* (formerly *S. typhimurium*) can synthesize L-proline endogenously from L-glutamate [4]. The biosynthesis of L-proline is tightly controlled by feedback inhibition of the γ-glutamyl kinase reaction and the intracellular pool is normally very small except when the *proA* and *proB* genes are overexpressed or under conditions of osmotic stress [5-6].

L-proline also can be accumulated in the cytoplasm by active transport across the plasma membrane [7]. The PutP transporter is a Na^+-dependent symporter that has been considered the primary mechanism of L-proline accumulation for auxotrophic strains or during growth in media where proline is the sole nitrogen or carbon source [8-9]. The gene encoding PutP lies near the gene that encodes a trifunctional PutA protein needed for proline catabolism, but separated from it by a control region with multiple regulatory sites [10-11]. The PutA protein has an N-terminal DNA-binding domain that regulates transcription of both *putP* and *putA*, a L-proline dehydrogenase domain that catalyzes the FAD-dependent oxidation of L-proline to form L-Δ^1-pyrroline-5-carboxylate (P5C), and a L-glutamate-γ-semialdehyde dehydrogenase domain (also called P5C dehydrogenase) that catalyzes the NAD^+-dependent oxidation of P5C to form L-glutamate [12]. Expression of the *putA* gene is inducible by L-proline but sensitive to catabolite repression and the DNA-binding protein Integration Host Factor [13-14]. The ProP transporter is an H^+-dependent symporter that was initially identified using mutants defective in the PutP protein [15-16]. The *proP* gene is inducible by osmotic stress and regulated by growth stage [17]. The ProP transporter has a broad substrate specificity and can facilitate the uptake of glycine betaine, proline betaine, ectoine, and choline in addition to L-proline [18]. The protein is activated by hyperosmotic shifts and serves as the primary osmosensor in whole cells [19]. The ProU transporter is also inducible by osmotic stress and active in mutants with defects in PutP and ProP [9, 20]. ProU is an ATP-binding cassette (ABC) type of carrier composed of the ProW integral membrane protein, the ProY membrane-associated ATPase, and the ProX periplasmic binding protein [21]. The genes for these proteins form an operon and are strongly inducible by osmotic stress [22].

The mechanism of ProU regulation is complex and dependent on DNA supercoiling, potassium glutamate, and histone-like proteins [23]. The ProU system facilitates the uptake of several osmotically compatible solutes including glycine betaine and proline betaine [24-25]. However, recent studies indicate that the periplasmic binding protein ProX does not actually bind L-proline [26-27].

Although the PutP, ProP, and ProU transport systems have been extensively studied, several papers have indicated there may be other mechanisms involved in proline accumulation. Csonka [20] reported that a triple mutant of *S. typhimurium* (TL195) with defects in all three systems could still grow in minimal medium containing 1 mmol l^{-1} L-proline in the presence or absence of 0.3 mol l^{-1} NaCl. Grothe *et al*. [9] found that a similar triple mutant derived from *E. coli* CSH4 showed a measurable radioactive proline uptake which was about 10% of the control rate. Haardt *et al*. [25] showed that the triple mutant strain MKH13 derived from *E. coli* MC4100 grew slowly in minimal medium containing 0.8 mole l^{-1} NaCl in the absence of osmoprotectants. Barron *et al*. [26] observed that another mutant derived from *E. coli* MC4100 could take up proline in a *proU*$^{+}$ background, and speculated that even though the binding protein could not bind proline, there might be direct binding to the membrane protein ProW. Liao *et al*. [28] described a cryptic proline permease in *S. typhimurium* designated ProY that allowed growth with L-proline as a nitrogen source only if a mutation in different gene designated *proZ* was present or it was overexpressed from a multicopy plasmid. Accordingly, I have re-examined the role of these transporters in the accumulation and catabolism of L-proline using an isogenic set of deletion strains derived from *E. coli* BW25113 in the Keio collection [29]. I show that a new triple mutant lacking the *putP*, *proP*, and *proW* genes but containing a functional PutA protein can still grow in a minimal medium with L-proline as the sole nitrogen source and describe its unusual properties.

2. Materials and Methods

2.1 Bacterial strains

E. coli K-12 strain BW25113 [*Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^{-}, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*] and various derivatives of it from the Keio collection [29] including JW2653 (Δ*proW*::*kan*), JW4072 (Δ*proP*::*kan*), JW1001 (Δ*putP*::*kan*), JW0999 (Δ*putA*::*kan*), JW5055 (Δ*proY*::*kan*), JW0391 (Δ*brnQ*::*kan*), and JW2638 (Δ*gabP*::*kan*) were obtained from the Coli Genetic Stock Center at Yale University (New Haven, CT, USA) or purchased from Horizon Discovery (Cambridge, United Kingdom). Strain DH5α containing the plasmid pCP20 was provided by Dr. Janet M. Wood (University of Guelph, Canada).

2.2 Growth conditions

Luria-Bertani (LB) and Minimal Medium A liquid and agar media with 0.5% (w/v) D-glucose or 0.5% (v/v) glycerol as the carbon source were prepared as described by Miller [30]. An ammonium-deficient version of Minimal Medium A was prepared by replacing the ammonium sulfate with sodium sulfate at the same concentration. To impose osmotic stress, solid NaCl was added to the Medium A before sterilization to give a final concentration of 0.3 mol l^{-1}. Where required, L-proline was added as the nitrogen source at 10 mmol l^{-1} unless otherwise indicated. Potential inhibitors of proline utilization were added either as solids or sterile solutions in water to give final concentrations of 5 to 10 mmol l^{-1}. Bacteria were grown at 37 °C in 125 ml Erlenmeyer or 300 ml nephelometer flasks containing less than 1/10 volume of medium in a shaker-incubator at 250 rpm. Culture turbidities were routinely followed in a Klett-Summerson colorimeter with a red (660 nm) filter. The morphology of the bacteria was regularly checked by phase-contrast light microscopy with a Nikon Alphaphot microscope and a 100X oil immersion objective. Antibiotic resistance was determined on LB agar plates containing either 25 μg ml^{-1} kanamycin sulfate or 100 μg ml^{-1} ampicillin. Sugar utilization phenotypes were confirmed on MacConkey agar plates (Difco MacConkey agar base containing 1% (w/v) lactose or 1% (w/v) L-arabinose). Proline-utilization (Put) indicator medium was prepared as described by Bochner and Savageau [61]. All growth experiments were done at least twice.

2.3 Genetic manipulations

Bacteriophage P1*vir* was used to introduce additional mutant genes containing kanamycin cassettes from other strains in the Keio collection into *E. coli* BW25113 and its derivatives by transduction [32]. Where desired, the kanamycin cassette then was removed using the plasmid pCP20. Plasmid pCP20 DNA was isolated from *E. coli* strain DH5α (pCP20) after growth overnight in LB containing 100 μg ml^{-1} ampicillin at 30 °C with aeration using a QIAprep Spin Miniprep kit (QIAGEN Inc., Valencia, CA, USA). Strains from the Keio collection to be transformed with pCP20 were grown in liquid LB at 37 °C for about two hr, harvested by centrifugation at 7,800 x *g* (10,000 rpm) in a SS-34 rotor in a Sorvall RC-5B Plus refrigerated centrifuge at 4 °C, washed four times with 10% (v/v) glycerol, and resuspended in 10% glycerol. The bacteria (50 μl) were combined with pCP20 DNA (two to five μl) and subjected to electroporation in one mm or two mm cuvettes in a Bio-Rad GenePulser Xcell instrument using the standard *E. coli* protocol (1800 V or 2500 V, 25 μF, 200 Ω). The cells were diluted with 1.0 ml of SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2.6\ H_2O$, 20 mmol l^{-1} $MgSO_4.6\ H_2O$, 20 mmol l^{-1} D-glucose), incubated at 30 °C for 120 min, and 100 μl portions spread on LB agar plates containing 100 μg ml^{-1} ampicillin. After incubation overnight, individual colonies were added to five ml of LB medium and incubated at 42 °C overnight. The culture was streaked onto LB agar plates and incubated at 37 °C. Individual colonies were then tested for loss of both kanamycin and ampicillin resistance and for retention of sugar utilization phenotypes.

2.4 PCR analysis

The presence or absence of intact *putP*, *proP*, *proW*, *proY*, *brnQ*, or *putA* genes in the original Keio collection strains or in the derivatives constructed here was confirmed by PCR analysis using genomic DNAs purified using the reagents in a Zymo Research Fungal/Bacterial DNA MiniPrep kit (The Epigenetics Company, Irvine, CA, USA). DNA concentrations were determined with a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Primer sequences were predicted using the Primer Quest software on the Integrated DNA Technologies (IDT, Coralville, IA, USA) web site and

synthesized by IDT. The primers used are listed in Table 1. 2X Master Mix (Promega Corporation, Madison, WI, USA or Zymo Research, Irvine, CA, USA) was combined with template DNAs and primers in 25 µl reaction mixtures, and amplification carried out under standard conditions (35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, polymerization at 72 °C for one min). PCR products were separated on 1.5% (w/v) agarose gels in TBE buffer (89 mmol l^{-1} Tris base, 89 mmol l^{-1} boric acid, 2 mmol l^{-1} EDTA) and stained with dilute ethidium bromide before imaging with a Bio-Rad VersaDoc (Bio-Rad Instruments, Hercules, CA, USA) or a Samsung A20 cell phone.

Table 1: Primers used for PCR analysis of *E. coli* proline metabolism genes

Gene	Primers	Amplicon
putA	f 5'-GTG AGA TGT CGC CCG TTA TTA-3' r 5'-TTC AGC GAT GAG CGG AAT AG-3'	677 bp
putP	f 5'-GGC TTC TTT GGG ATT GCT TAC-3' r 5'-CTT TCT GGC TGG CAT GTT TAC-3'	244 bp
proP	f 5'-GCG GAC TCT TTC TTT GGT AGT-3' r 5'-GTC GCC CTG TTC CAG TTT AT-3'	470 bp
proW	f 5'-CCT GCT GTT CTG TAT CGT CAT-3' r 5'-CCA GAA TGG TCA GAC GGA TAA A-3'	221 bp
proY	f 5'-CCA GCT CTT TCT CGC GTT AT-3' r 5'-CGT TGC TCC ACA GGT TAT GA-3'	357 bp
brnQ	f 5'-GCG CAA TGG TGT TTG GTA TC-3' r 5'-CAT CAC CAC TGT TGG CAT TAA C-3'	656 bp

2.5 Radial streak assays for susceptibility to toxic analogues

Radial streak assays for analogue susceptibility were done as previously described [33-34]. A sterile six mm paper disk was placed in the center of a Minimal Medium A agar plate containing 0.5% (w/v) D-glucose or 0.5% (v/v) glycerol as the carbon source and 0 or 0.3 M NaCl. *E. coli* strains from overnight LB or Minimal Medium A cultures were streaked from the disk towards the edge for the plate. A test solution (usually 20 µl of 5 mg ml^{-1}) was added to the disk to give 0.1 mg of the compound of interest and zones of inhibition were measured after one to two days of incubation at 37 °C.

3. Results and Discussion

3.1 Construction of a new triple mutant defective in L-proline transport

To clarify the roles of the PutP, ProP, and ProU transporters in the uptake of L-proline in *E. coli*, the parental strain of the Keio collection (BW25113) and strains JW1001 (Δ*putP*::*kan*), JW4072 (Δ*proP*::*kan*), and JW2653 (Δ*proW*::*kan*) were obtained from the Coli Genetic Stock Center. A new double mutant and a new triple mutant were constructed using standard procedures [35]. The kanamycin cassette in the *proW* gene in *E. coli* strain JW2653 was removed and strain KAH4' (Δ*proW* Δ*proP*) was constructed by introducing the Δ*proP*::*kan* mutation from JW4072 into strain JW2653 and then removing the kanamycin cassette in the *proP* gene. *E. coli* strain CDC11-1 (Δ*proW* Δ*proP* Δ*putP*) was constructed by introducing the Δ*putP*::*kan* mutation from JW1001 into strain KAH4' and again removing the kanamycin cassette. At each step, the presence of the original Lac^- and Ara^- phenotpes of BW25113 was confirmed as was the presence or loss of the kanamycin and ampicillin resistance markers associated with the genetic manipulations. The deletion of the relevant gene in each new strain and the continued presence of the *putA* gene encoding L-proline dehydrogenase and L-glutamate-γ-semialdehyde dehydrogenase (P5C dehydrogenase) activities was confirmed by PCR analysis.

The properties of these strains are summarized in Table 2. The parental strain BW25113 formed red (Put^+) colonies on Put indicator medium in the absence and presence of 0.3 mol l^{-1} NaCl and was sensitive to both L-azetidine-2-carboxylate (A2C) and 3,4-dehydro-DL-proline (DHP) in the absence and presence of salt. The single mutants JW2653 and JW4072 with defects in *proW* and *proP* respectively also showed a Put^+ phenotype in the absence and presence of 0.3 mol l^{-1} NaCl due to the presence of the functional *putP* and *putA* alleles. The *putP* mutant JW1001 formed white (Put^-) colonies in the absence of NaCl but showed a Put^+ phenotype in the presence of NaCl due to the uptake of proline by the ProP and ProU transporters. The *putA* mutant JW0999 consistently gave a Put^- phenotype but was still sensitive to both proline analogues in the absence or presence of 0.3 mol l^{-1} NaCl. The double mutant KAH4' gave red Put^+ colonies in the absence of NaCl but white Put^- colonies in the presence of salt due to the defects in the ProP and ProU transporters and the limited expression of *putP*. The triple mutant CDC11-1 formed white Put^- colonies under all conditions and was resistant to both A2C and DHP in the absence or presence of NaCl. These phenotypic characteristics were consistent with previous studies.

Table 2. Phenotypic characteristics of *E. coli* strains

Strain	Genotype	Put medium	Put medium + NaCl	Carbon Source	Added NaCl	A2C inhibition	DHP inhibition
BW25113	$proW^+$ $proP^+$ $putP^+$ $putA^+$	Red	Red	Glucose	0	14	13
				Glucose	0.3 M	19	12
				Glycerol	0	12	13
				Glycerol	0.3 M	21	13
JW2653	$proW^-$ $proP^+$ $putP^+$ $putA^+$	Red	Red	Glucose	0	14	12
				Glucose	0.3 M	20	11
				Glycerol	0	11	11
				Glycerol	0.3 M	19	11
JW4072	$proW^+$ $proP^-$ $putP^+$ $putA^+$	Red	Red	Glucose	0	12	9
				Glucose	0.3 M	17	6
				Glycerol	0	8	12
				Glycerol	0.3 M	19	13
JW1001	$proW^+$ $proP^+$ $putP^-$ $putA^+$	White	Red	Glucose	0	0	3
				Glucose	0.3 M	22	12
				Glycerol	0	0	0

				Glycerol	0.3 M	20	12
JW0999	*proW*$^{+}$ *proP*$^{+}$ *putP*$^{+}$ *putA*$^{-}$	White	White	Glucose Glucose Glycerol Glycerol	0 0.3 M 0 0.3 M	15 20 15 20	16 16 20 20
KAH4'	*proW*$^{-}$ *proP*$^{-}$ *putP*$^{+}$ *putA*$^{+}$	Red	White	Glucose Glucose Glycerol Glycerol	0 0.3 M 0 0.3 M	16 12 13 8	10 0 11 10
CDC11-1	*proW*$^{-}$ *proP*$^{-}$ *putP*$^{-}$ *putA*$^{+}$	White	White	Glucose Glucose Glycerol Glycerol	0 0.3 M 0 0.3 M	0 0 0 0	0 0 0 0

3.2 Growth of *E. coli* strains with L-proline as the sole nitrogen source

To assess the functional effects of the mutations in these strains, the bacteria were grown in minimal Medium A lacking ammonium sulfate and containing 10 mmol l^{-1} L-proline as the sole nitrogen source (Fig. 1). Glycerol (0.5% v/v) was used as the primary carbon source to minimize the potential effects of catabolite repression. Panel A shows the data for bacteria grown in the absence of osmotic stress and panel B shows the data for bacteria grown in the presence of 0.3 mol l^{-1} NaCl. The parental strain BW25113 grew well in both media and the mutant strain JW0999 lacking *putA* showed no growth. The mutant JW1001 lacking the primary PutP transporter grew slower in medium without NaCl but much better in medium containing salt, apparently due to expression of the ProP and ProU transporters. In medium lacking NaCl, the mutants with single defects in ProP and ProU or the double mutant lacking both ProP and ProU were similar and showed some growth that was apparently dependent on the PutP transporter. In medium containing 0.3 mol l^{-1} NaCl, both of the strains with single defects in ProP and ProU showed some growth. The growth of the ProP^{+} ProW^{-} strain was better than that of the proP^{-} proW^{+} strain, which was consistent with previous studies on the importance of the ProP carrier and the fact that the ProU binding protein does not actually bind proline. The proP^{-} proW^{-} double mutant had very poor growth. The most interesting observation was that the PutP^{-} ProP^{-} ProW^{-} triple mutant CDC11-1 showed consistently observable growth in the absence or presence of NaCl. The rate and extent of growth was similar in both cases, suggesting L-proline uptake in this strain was not dependent on an osmotically-inducible transport system.

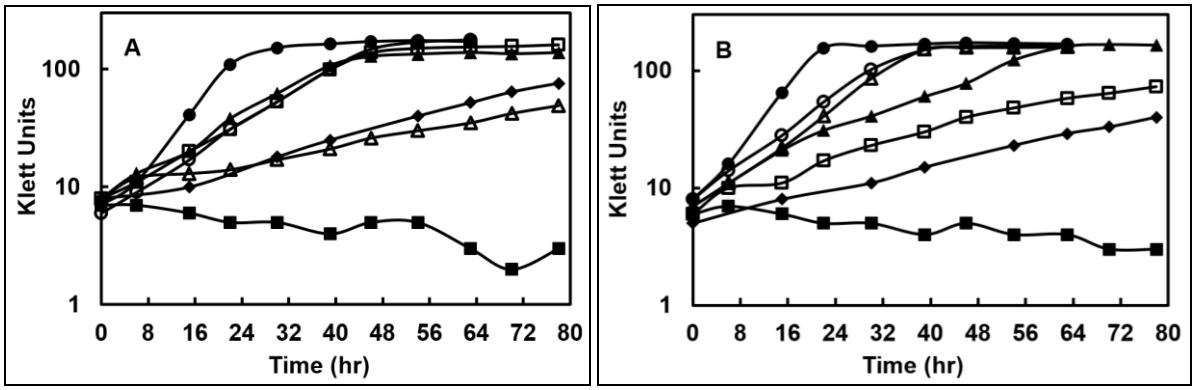


Fig 1: Growth of *E. coli* strains in Medium A without ammonium sulfate containing 0.5% (w/v) glycerol and 10 mmol l^{-1} L-proline at 37 °C. Panel A shows the growth curves in plain Medium A and Panel B shows the growth curves in Medium A containing 0.3 mol l^{-1} NaCl. The strains were BW25113 (●), JW1001 (Δ*putP* ○), JW4072 (Δ*proP* ▲), JW2653 (Δ*proW* △), JW0999 (Δ*putA* ■), KAH4' (Δ*proP* Δ*proW* □), and CDC11-1 (Δ*putP*Δ*putP*Δ*putP* ◆)

3.3 Growth of *E. coli* strain CDC11-1 with L-proline as the nitrogen source

The ability of the triple mutant strain CDC11-1 to grow with L-proline as the sole nitrogen source in Medium A without ammonium sulfate was characterized in detail. The L-proline concentration was varied from 0 to 50 mmol l^{-1} in cultures containing 0.5% (v/v) glycerol as the primary carbon source (Fig. 2 panel A). There was no growth in the absence of added proline, indicating that there was no contaminating nitrogen source in the medium. As the L-proline concentration was increased, the growth rate and the yield of cells increased proportionately. The final yield in stationary phase was the same for concentrations greater than 20 mmol l^{-1}. L-Proline is a charged zwitterionic amino acid at pH 7 and cannot freely cross phospholipid bilayers, although some permeation of more nonpolar amino acids into or out of liposomes may occur [36]. Previous studies showed that active L-proline transport could occur in artificial proteoliposomes that were reconstituted with purified PutP [37] or ProP [38] proteins. More recent studies indicated that facilitated diffusion of L-proline could also occur with the synthetic carrier calyx[4]pyrrole in liposomes and living HeLa cells [39]. The apparent saturation kinetics for L-proline utilization by the triple mutant CDC11-1 suggested that another membrane transporter might be involved.

Strain CDC11-1 was also grown in similar cultures with 10 mmol l^{-1} L-proline and different carbon sources (Fig. 2 panel B). The growth curves with 0.5% (v/v) glycerol, 50 mmol l^{-1} succinate, and 50 mmol l^{-1} L-lactate were very similar. Growth with 0.5% D-glucose was much slower, which was consistent with previous studies showing that expression of the *putA* gene and L-proline dehydrogenase activity in both *E. coli* and *S. enterica* is sensitive to

catabolite repression [13, 33, 40]. This depends on the Catabolite Activator Protein and the concentration of cAMP [41]. Growth of the bacteria with 50 mmol l^{-1} L-proline alone was slightly better than with 10 $mmol^{-1}$ L-proline and glycerol, succinate, or L-lactate. This was consistent with the result shown in Fig. 2 panel A.

The uptake of L-proline by the three known proline transporters can be inhibited by potentially toxic proline analogues including L-azetidine-2-carboxylate (A2C) and 3,4-dehydro-DL-proline (DHP) [42]. To determne if any of these compounds can affect the growth of strain CDC-11-1, the bacteria were cultured in Medium A lacking ammonium sulfate with 0.5% glycerol, 10 mmol l^{-1} L-proline, and 5 mmol l^{-1} concentrations of speciifc analogues. D-proline, L-thiazolidine-4-carboxylate, L-thiazolidine-2-carboxylate, 3,4-dehydro-DL-proline, cis-4-hydroxyproline, trans-4-hydroxyproline, pyroglutamic acid, piperazine-2-carboxylate, and 4-oxo-azetidine-2-carboxylate had no effect. Pipecolic acid and pyrrole-2-carboxylate acid were slightly inhibitory but the most pronounced effect was a reduction in growth by A2C (Fig. 2 panel C). The growth rate and the yield decreased proportionally as the A2C concentration increased from 1 to 5 mmol l^{-1}.

The primary alternative substrate for the ProP and ProU proline transporters is the quaternary amine glycine betaine, which is used most often as an osmoprotectant [43]. To determine if this compound affects the growth of *E. coli* strain CDC11-1, the bacteria were grown in minimal medium without ammonium sulfate with 0.5% (v/v) glycerol, 10 mmol l^{-1} L-proline, and different concentrations of glycine betaine from 1 to 5 mmol l^{-1} (Fig. 2 panel D). There was no inhibition of growth at any of these concentrations. Increasing the glycine betaine concentration to 10 or 20 mmol l^{-1} also had no effect. There was also no growth of strain CDC 11-1 on 5 mmol l^{-1} of glycine betaine alone. While some bacteria do form a glycine betaine monooxygenase that facilitates its degradation, *E. coli* is not one of them [44].

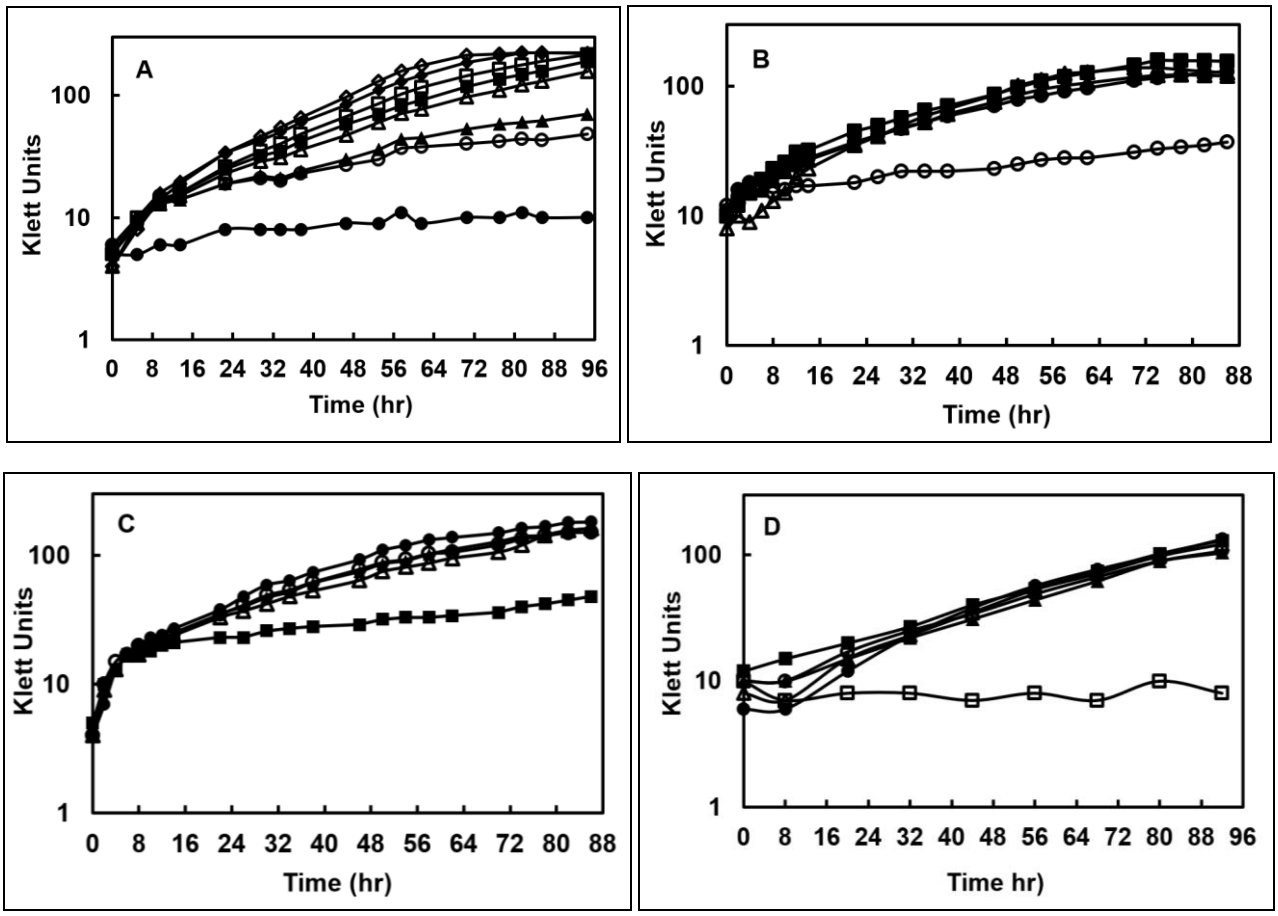


Fig 2: Growth of *E. coli* strain CDC11-1 (Δ*putP*Δ*putP*Δ*putP*) in Medium A without ammonium sulfate at 37 °C. Panel A shows the growth of bacteria in medium containing 0.5% (w/v) glycerol and 0 (●) , 1 (○) , 2 (▲), 5 (△), 10 (■), 20 (□), 30 (◆) or 50 (◇) mmol l^{-1} L-proline. Panel B shows the growth of the bacteria in medium supplemented with 10 mmol l-^{1} L-proline and 0.5% (v/v) glycerol (●), 0.5% D-glucose (○), 50 mmol l^{-1} succinate (▲), or 50 mmole l^{-1} L-lactate (△), or with 50 mmol l^{-1} L-proline alone (■). Panel C shows the growth of bacteria in medium containing 0.5% (v/v) glycerol, 10 mmol l-^{1} L-proline, and 0 (●), 1 (○), 2 (▲), 3 (△), or 5 (■) mmol l^{-1} azetidine-2-carboxylate (A2C). Panel D shows the growth of bacteria in medium containing 0.5% (v/v) glycerol, 10 mmol l-^{1} L-proline, and 0 (●), 1 (○), 2 (▲), 3 (△), or 5 (■) mmol l^{-1} glycine betaine or 5 mmol l^{-1} glycine betaine as the sole nitrogen source (□).

3.4 Effect of other amino acids on the growth of *E. coli* strain CDC11-1

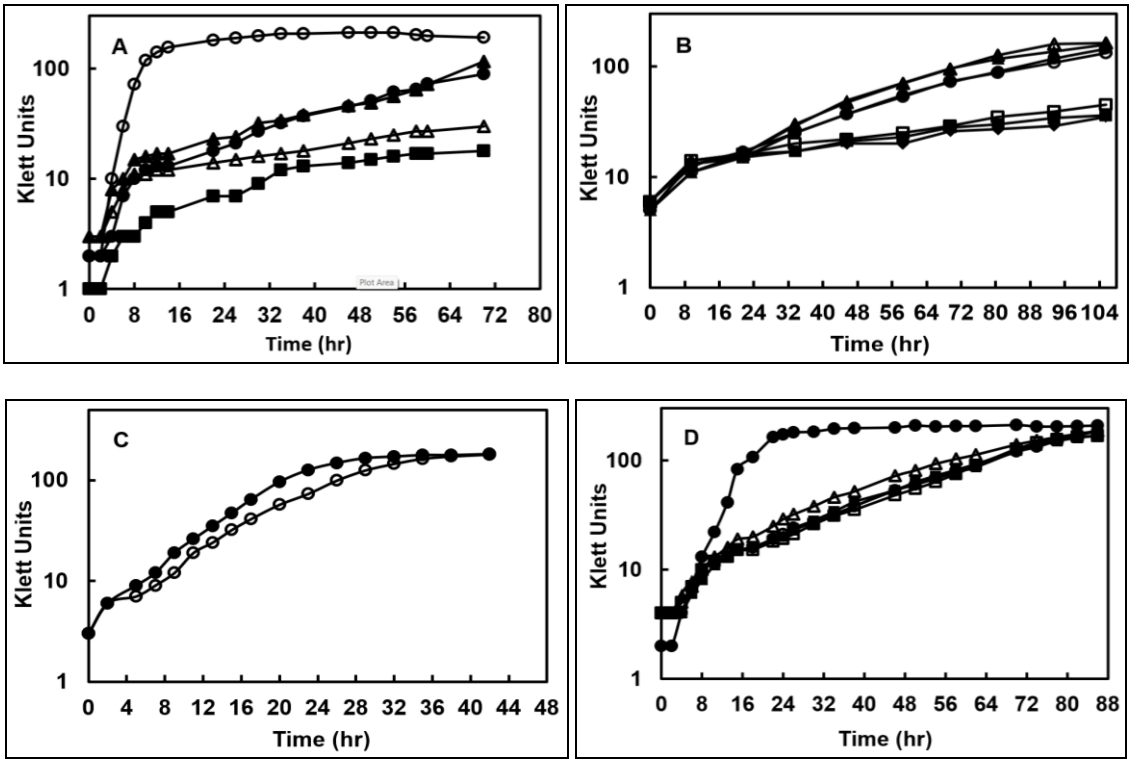
These results were consistent with the deletion of the genes for the three known L-proline transporters and suggested some other mechanism was involved in proline accumulation. To determine if any other amino acids could compete with L-proline and reduce the proline-dependent growth, cultures were treated with 5 mmol l^{-1} concentrations of various pure L-amino acids. Some amino acids such as glutamate, glutamine, aspartate, and asparagine were not tested because they are known to be good nitrogen sources. The amino acids fell four general groups (Fig. 3 panel A). Group 1, as illustrated by L-alanine and including L-serine and glycine, stimulated growth and the bacteria grew better

than with L-proline alone. Group 2, as illustrated by L-methionine and including L-leucine, had no effect. Group 3, as illustrated by L-isoleucine and including L-threonine and L-histidine to a lesser extent, were partially inhibitory and the bacteria grew slower than with L-proline alone. Group 4 as illustrated by L-valine was strongly inhibitory. The inhibitory effect of this amino acid is well documented for *E. coli* and reflects s a specific inhibition of branched chain amino acid synthesis [45].

The inhibition of CDC11-1 by L-isoleucine was of particular interest in light of previous studies on the possible cryptic proline transporter ProY that was first identified in *Salmonella enterica* (formerly *S. typhimurium*) [28]. ProY is a hydrophobic transmembrane H^+ symporter whose expression allows $PutP^-$ mutants of this bacterium to use L-proline as the sole nitrogen source. Expression of ProY could be activated by mutations in an unlinked gene called *proZ* which mapped to a site at about 76 min on the *Salmonella* chromosome near the genes for the major branched-chain amino acid transporter LIV-1 [46]. The *proY* gene itself mapped to a site at about 8 min on the *Salmonella* chromosome adjacent to a gene called *brnQ* that encodes part of the LIV-II branched-chain amino acid transporter [28]. Overexpression of *proY* on a multicopy plasmid led to enhanced growth in medium with L-proline as the nitrogen source in a $PutP^-$ background. Polar mutations in *proY* did not affect utilization of branched chain amino acids and polar mutations in *brnQ* did not affect proline utilization, suggesting that the relationahip between L-proline transport and branched chain amino acid transport might be just coincidental. *E. coli* has a gene homologous to *proY*, which is also designated *yajM* (ECK0396) that again is located adjacent to the *brnQ* gene [47]. A bioinformatic analysis indicated that the *E. coli* ProY amino acid sequence (P0AAE2 in the UniProt KB data base) has 100% sequence identity to that of *S. enterica* but there is no evidence the protein is expressed.

To study this issue in more detail, the triple mutant strain CDC11-1 was grown in Medium A without ammonium sulfate containing 0.5% glycerol, 10 mmol l^{-1} L-proline, and varying concentrations of L-leucine or L-isoleucine (Fig. 3 panel B). The growth curves with with 1, 2, or 5 mmol l^{-1} L-leucine were similar and showed no inhibition. The growth curves with 1, 2, or 5 mmol l^{-1} L-isoleucine were also similar but showed a similar amount of inhibition by this amino acid. By contrast, the parental strain BW25113 showed faster growth in Medium A without ammonium sulfate containing 0.5% glycerol and 10 mmol l^{-1} L-proline and there was little inhibition by 5 mmol l^{-1} L-isoleucine (Fig. 3 panel C).

To clarify the role of the *proY* and *brnQ* genes in this pattern of growth, the deletion mutation *proY::kan* from strain JW5055 and the deletion mutation *brnQ::kan* from strain JW0391 were introduced into *E. coli* strain CDC11-1 by transduction. Loss of the wild type gene in the original strain and in the derivatives of the triple mutant was confirmed by PCR analysis. Strains GCC104 and GCC108 (CDC11-1 *proY::kan*) showed the same pattern of growth in Medium A without ammonium sulfate containing 0.5% glycerol and 10 mmol l^{-1} L-proline as strain CDC11-1 (Fig. 3 panel D). The same was true for strains GCC201 and GCC 210 (CDC11-1 *brnQ::kan*). When 5 mmole l^{-1} L-isoleucine was added to cultures of these mutants in this medium, there was again an inhibition of growth as shown for GCC104 and GCC210 (Fig. 3 panel E). Thus, there is no support for the hypothesis that the uptake of L-proline by the triple mutant requires or is affected by the *proY* or *brnQ* genes. The primary high affinity leucine isoleucine valine transport systems in *E. coli* (LIV-1 and LS) are encoded by six genes, two of which specify periplasmic binding proteins [48-49]. The LS-BP encoded by *livK* is highly specific for L-leucine while the LIV-BP encoded by *livJ* is less specific and can facilitate the uptake of L-isoleucine and L-valine as well as several L-leucine peptides and analogues [50-51]. Because the growth of the triple mutant strain CDC11-1 on L-proline was not inhibited by L-leucine, we did not test additional mutants with deletions in the LIV-I system.



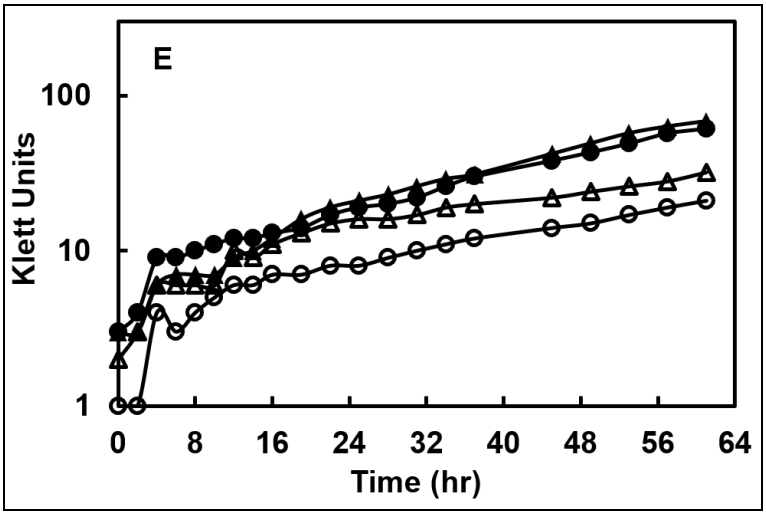


Fig 3: Effects of amino acids on the growth of *E. coli* strain CDC11-1 in Medium A without ammonium sulfate at 37 °C. Panel A shows the growth of cultures in medium containing 0.5% (v/v) glycerol, 10 mmol l-^{1} L-proline, and no additional amino acid (●), 5 mmol l^{-1} L-alanine (○), 5 mmol l^{-1} L-methionine, (▲),5 mmol l^{-1} L-isoleucine (△), or 5 mmol l^{-1} L-valine (■). Panel B shows the growth of cultures containing no additional amino acid (●), 1 (○), 2 (▲), or 5 (△) mmol l^{-1} L-leucine, or 1(○), 2 (▲), or 5 (△) mmol l^{-1} L-isoleucine. Panel C shows the growth of the parental strain BW25113 in the same medium with no additional amino acid (●) or 5 mmol l^{-1} L-isoleucine. Panel D shows the growth of the parental strain BW25113 (●), strain CDC11-1 (○), strain GCC 104 (CDC11-1 *proY::kan* ▲), strain GCC 108 (CDC11-1 *proY::kan* △), strain GCC 201 (CDC11-1 *brnQ::kan* ■) or strain GCC 210 (CDC11-1 *brnQ::kan* □) in minimal medium without ammonium sulfate containing 0.5% glycerol, 10 mmol l-^{1} L-proline, and 5 mmole l^{-1} L-isoleucine. Panel E shows the growth of strain GCC 104 (CDC11-1 *proY::kan*) in minimal medium without ammonium sulfate containing 0.5% glycerol, 10 mmol l-^{1} L-proline, and 0 (●) or 5 (○) mmole l^{-1} L-isoleucine and strain GCC 210 (CDC11-1 *brnQ::kan*) in minimal medium without ammonium sulfate containing 0.5% glycerol, 10 mmol l-^{1} L-proline, and 0 (▲) or 5 (△) mmole l^{-1} L-isoleucine.

3.5 Effects of other potential inhibitors on the growth of *E. coli* strain CDC11-1

The effects of several other compounds whose transport systems have been implicated in the uptake of L-proline in other organisms were tested as well. The GabP transporter in *Bacillus subtilis* which normally mediates the uptake of γ-aminobutyric acid (4-amino butyrate, GABA) was found to facilitate the uptake of L-proline in a mutant strain lacking the primary proline transporters PutP and OpuE [52]. *E. coli* has a *gabP* gene that encodes a similar protein (P25527 in the UniProt KB data base) which has 46.3% sequence similarity to the one from *B. subtilis* [53]. This protein has a relatively broad substrate specificity and can take up or be inhibited by a number of GABA analogues including cyclic forms [54]. To assess the effects of GABA on the growth of *E. coli* strain CDC11-1, the bacteria were first grown in minimal Medium without ammonium sulfate containing with 0.5% (v/v) glycerol, 10 mmol l^{-1} L-proline and 0, 10, or 20 mmol l^{-1} GABA (Figure 4 panel A). The presence of GABA in the cultures resulted in a small increase in the growth rate and the final yield of bacteria. Strain CDC11-1 was then grown in the same medium with 5 mmol l^{-1} azetidine-2-carboxylate and 0, 10, 20, 30, and 50 mmol l^{-1} GABA. The presence of the A2C again caused a marked inhibition of growth to strain CDC11-1 (Figure 4 panel B). The presence of GABA in the medium did not block the inhibitory effect of A2C, suggesting this proline analogue was not taken up by a GABA-dependent transporter. To confirm these results, the *gapP::kan* mutation from the Keio collection strain JW2678 was introduced into strain CDC11-1 by transduction. Several kanamycin-resistant mutants were selected and their growth compared to the parental strains and strain CDC11-1 As expected, both strain BW25113 and strain JW2678 grew much better in medium with L-proline as the sole nitrogen source than strain CDC11-1 (Figure 4 panel C). The new mutants with a deletion in *gapP* showed the same pattern of growth as CDC11-1. Thus the GabP transporter does not appear to be involved in the uptake of proline in the triple mutant strain of *E. coli*.

Sarcosine (*N*-methylglycine) is a nonproteogenic amino acid that can inhibit L-proline uptake in the single cell eukaryotes *Histoplasma capsulatum* and *Candida albicans* [55-56]. It also inhibits proline uptake by the mammalian proline transporters PAT2 and PAT4 [57-58]. While many bacteria form an enzyme called sarcosine oxidase that can catalyze the degradation of this compound to form glycine, formaldehyde, and hydrogen peroxide [59], cloning and expression of the gene for a similar protein in *E. coli* indicated it had very low activity in cell extracts [60]. Thus sarcosine alone cannot be used as a nitrogen source. To determine if sarcosine affects the growth of *E. coli* strain CDC11-1 in minimal Medium A without ammonium sulfate containing with 0.5% (v/v) glycerol and 10 mmol l^{-1} L-proline, cultures were treated with sarcosine concentrations up to 10 mmol l^{-1}. There was no inhibition of growth at any of the tested concentrations (Figure 4 panel D). Several other chemicals that have structural similarities to L-proline were also tested as inhibitors of the growth of strain CDC11-1. These included choline, nicotinic acid, orotic acid, and uracil, but none of these compounds had any effect.

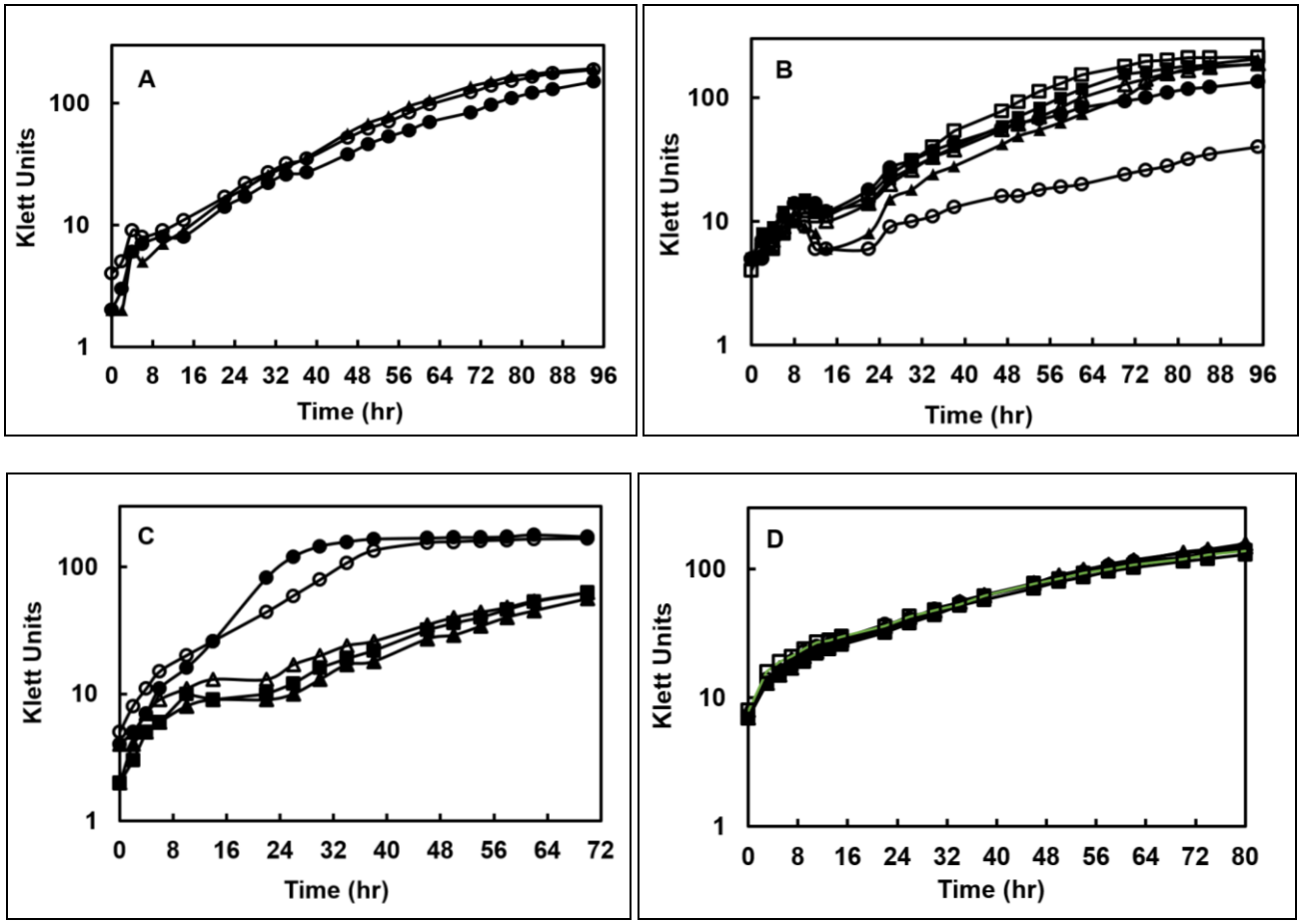


Fig 4: Effects of potential inhibitors on the growth of *E. coli* strain CDC11-1 in Medium A without ammonium sulfate supplemented with 0.5% (v/v) glycerol and 10 mmol l^{-1} L-proline at 37 °C Panel A shows the growth curves of cultures supplemented with 0 (●), 10 (○), or 20 (▲) mmole l^{-1} γ-aminobutyric acid (GABA). Panel B shows the growth curves of cultures supplemented with no azetidine-2-carboxylate (A2C) (●), with 5 mmol l^{-1} A2C (○). or with 5 mmol l^{-1} A2C along with 10 (▲), 20 (△), 30 (■), or 50 (□) mmol l^{-1} GABA. Panel C shows the growth curves of cultures of strain BW25113 (●), strain JW2678 (*gabP::kan*) (○), strain CDC11-1 (▲), strain GCC301 (CDC11-1 *gabP::kan*) (△), and strain GCC307 (CDC11-1 *gabP::kan*) (■). Panel D shows the growth curves of strain CDC11-1 supplemented with 0 (●), 1 (○), 2 (▲), 3 (△), 5, (■), or 10 (□) mmol l^{-1} sarcosine.

4. Conclusions

These experiments indicated that a triple mutant of *E. coli* lacking the PutP, ProP, and ProU transport systems can still grow with L-proline as the sole nitrogen source. The new mutant was constructed using strains from the Keio collection because the complete genomic sequence of the parent strain BW25113 is available and because the deletion mutations are almost complete and entirely stable. Growth of the new triple mutant was reduced in the presence of the inhibitory proline analogue L-azetidine-2-carboxylate (A2C). Growth with L-proline could be partially inhibited by L-isoleucine but not by L-leucine, other amino acids, γ-aminobutyric acid, or sarcosine. Deletion of the genes *proY*, *brnQ*, and *gabP* had no effect. The mechanism of proline uptake in the new triple mutant is still unclear. By using a deletion mutation of *proW*, the possibilities that L-proline bound directly to the membrane component of the ProU system or that another periplasmic binding protein facilitated its delivery to this system were eliminated. It is most likely there is another membrane transporter that has a broad substrate specificity and that can take up L-proline as a secondary effect. It is unlikely that this transporter is the branched chain amino acid transporter LIV-I or LIV-II since growth on L-proline was not inhibited by L-leucine. It is also unlikely that this transporter facilitates the uptake of glycine betaine or another solute which is accumulated during osmotic stress. The cryptic transporter ProY does not appear to play a role in *E. coli* since deletion of the *proY* gene had no effect. Identification of the transporter that is functional in the triple mutant would require a fuller screening of the Keio collection or a metabolomic analysis. These studies were beyond the scope of this project, but I will make my mutant available to anyone who might be able to use it.

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6. Contributor Role

All of the experiments were designed and performed by Charles E. Deutch as was preparation of the manuscript.

7. Conflicts of Interest

The author declares there are no conflicts of interest.

8. Financial Support
Not available

9. References

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