Proline Excretion by *Escherichia coli* K12*

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Proline excretion from proline overproducing strains of *E. coli* K12 has been studied as a model chemical production system. We have isolated proline overproducing mutants of *E. coli* and have shown that uncontrolled synthesis is not sufficient to cause excretion of this amino acid. An episomal mutation causing proline overproduction has been introduced into a series of otherwise isogenic strains that bear well defined, chromosomal lesions affecting the active uptake and catabolism of L-proline. A syntropism test reveals that L-proline is excreted by overproducing strains only if transport and/or catabolism are impaired. Dansyl derivatization and chromatographic analysis of culture supernatants shows that proline is the only amino acid excreted. Batch cultures of an excreting strain in an amino acid production medium yield culture supernatants containing 1 g proline/L, whereas no proline is detectable in supernatants derived from cultures of an overproducing strain with normal transport and catabolic activities. These data reveal that genetic lesions eliminating active uptake can be used to specifically enhance metabolite excretion.

INTRODUCTION

Chemical production by fermentation is a major commercial enterprise. Systematic efforts to improve fermentation productivity have been designed primarily to amplify biosynthesis and reduce catabolism of desired metabolites.[1,2] Increased chemical yield and simplified recovery procedures should also result if microorganisms can be programmed to specifically excrete valued compounds. Metabolite excretion is particularly critical to the operation of immobilized cell reactors, where release of products is desired but non-specific cell permeabilization can be detrimental.[2]

The critical role of membrane permeability in fermentation has been recognized, and measures to enhance glutamic acid excretion are routine to its production.[2] Halsall has correlated lysine excretion by *Escherichia coli* with a lysine uptake defect[3] and other workers have suggested that permeability changes may contribute to enhanced chemical production. No general strategy for the enhanced excretion of particular metabolites has been provided, however.

The movement of molecules across the cell wall may be either passive or mediated. Bacterial membrane permeability has been considered primarily in terms of solute uptake, and such studies have revealed a variety of highly specific porters which facilitate the diffusion or catalyze the active translocation of organic solutes into the cytoplasm.[4] Active uptake processes commonly yield 100- to 1000-fold accumulation of solutes within bacterial cells; passive transmembrane equilibration of solutes becomes virtually imperceptible by comparison. In some cases, energy is supplied to effectively unidirectional uptake processes by coupling to chemical bond cleavage reactions, but frequently organic solute uptake is coupled with the flux of ions down independently generated transmembrane electrochemical gradients. The porters catalyzing ion-linked transport processes are thought to be functionally symmetrical, the net direction of solute flux being imposed by coupling to the anisotropic energy supply.[5]

Three questions arise regarding the relationship between membrane permeability and metabolite production. Does the rate of solute efflux limit metabolite production by fermentation? What is the mechanism of metabolite efflux; is it passive or mediated? Can genetic or biochemical manipulation of membrane permeability be employed to enhance metabolite excretion and, hence, productivity? It is our premise that only specific enhancement of solute efflux is of value, since nonspecific membrane perforation will reduce the efficiency and productive lifetime of the cellular catalysts. The function of solute uptake systems is, of course, relevant to these questions. Do the uptake porters limit net metabolite excretion by catalyzing the reaccumulation of extracellular metabolites, or can they also serve as efflux catalysts?

We are examining the role of membrane permeability in L-proline production by *E. coli* K12. The pathways of proline metabolism are simple[6] and the genetics and biochemistry of proline transport by *E. coli* are relatively well understood.[7,8] We have isolated dominant mutations causing L-proline overproduction. A set of proline overproducing strains has been constructed that bear well-

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defined, chromosomal lesions affecting L-proline transport and/or catabolism. Syntropism testing and chemical analysis have been used to assess proline production by these strains when they are grown aerobically on glucose and NH_3.

MATERIALS AND METHODS

Chemicals

Chemical sources were as previously described[8] with the following exceptions. The L-[U-^{14}C] proline and [^{3}H]-dansyl chloride were obtained from Amersham (Oakville, Ontario). Unlabelled dansyl chloride, obtained from the Pierce Chemical Co. (Rockford, IL) was recrystallized from benzene and petroleum ether. Analytical grade triethylamine was obtained from British Drug Houses (Toronto, Ontario). Acetone, pyridine, toluene, 95% ethanol, formic acid, benzene, acetic acid, *n*-butylacetate, and methanol were all of reagent grade from Fisher Scientific Co. (Pittsburgh, PA). Aquasol was from New England Nuclear Corp. (Boston, MA).

Microbiological Media

Microbiological media were as previously described,[8] with the exception of proline production medium which is described below. Solid media contained D-glucose (2 mg/mL), as carbon source and NH_4Cl (95m*M*) as nitrogen source, unless otherwise stated. Proline production medium, based on that of Nakamori et al.,[9] contained (per L): 50 g D-glucose, 25 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.8 g $MgSO_4 \cdot 7H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $Mn_2SO_4 \cdot 4H_2O$, 1 mg thiamine HCl, 0.5 g Difco yeast extract, 0.2 g L-tryptophan, and was adjusted to pH 7.0 with KOH. The complete medium was sterilized by filtration and 100 mL was introduced into each culture flask prepared as follows: 5 g of $CaCO_3$ were weighed into each 500 mL culture flask. Each flask was heated to 400°C for 4 h, then capped and autoclaved for 1 h. This procedure was essential to eliminate contamination arising from the $CaCO_3$.

Bacterial Strains

The bacterial strains prepared and used during this study were all derivatives of *E. coli* K12.[10] The chromosomal variants are listed in Table I. Bacteriophage λ*cI857* containing Tn*10* was a gift from Dr. Claire Berg (University of Connecticut) and bacteriophage f2 was from Dr. K. F. Gregory (University of Guelph). Genetic manipulations were performed as described by Miller[11] or by Davis and co-workers.[12] The derivations and characteristics of many of the strains employed in this study are described fully elsewhere.[7,13] Briefly, WG145 is a uracil-requiring Tn*10* insertion mutant obtained by mutagenizing strain CSH4 with bacteriophage λ*cI857* containing Tn*10* and screening the resulting mutant pool for auxotrophs.[12] Both WG146 and WG95 were selected as tetracycline-resistant transductants and identified as proline utilizers (*put*$^+$) by screening on TTC indicator medium.[14] WG170 is a spontaneous, 3,4-dehydroproline resistant mutant of RM2. WG138 is a uracil prototrophic transductant of WG95 selected on minimal medium lacking uracil. WG148 was selected as a kanamycin resistant transductant. It is more sensitive to AC and DHP than its parent, WG170, and is unable to utilize L-proline (see the Results section). WG140 is a kanamycin resistant transductant whose proline auxotrophy was confirmed by streaking on defined media. WG176 is a tetracycline resistant transductant whose uracil auxotrophy was confirmed by streaking on defined media. Both WG177 and WG96 are transconjugants of WG176 and WG2300, respectively, from E5014. They were isolated by spot-mating[11] on minimal medium containing lactose as carbon source and tetracycline as counterselective agent. The same conjugation procedure was used for all other episome transfers unless otherwise stated.

Table I. *Escherichia coli* K12 strains.[a]

Strain	Genotype	Source or derivation
CBK130	F$^-$ *thyA36 proC*::Tn*5*	Claire Berg (University of Connecticut)
CSH4	F$^-$ *trp lacZ rpsL thi*	Cold Spring Harbor Laboratory
CSH26	F$^-$ *ara Δ(lac pro) thi*	Cold Spring Harbor Laboratory
E5014	(F′$_{128}$ *lac*$^+$ *proA*$^+$ *B*$^+$) *Δ(lac pro) supE spc thi*	Cold Spring Harbor Laboratory (as CSH23)
JT31	CSH4 *putA1*::Tn*5*	this laboratory (refs. 7 and 8)
JT34	CSH4 *putP3*::Tn*5*	this laboratory (refs. 7 and 8)
RM2	CSH4 *Δ(putPA)101*	Rolf Menzel (University of Utah) (refs. 7 and 8)
WG95	CSH4 *pyr-76*::Tn*10* *proP219*	P1 transductant of WG170 from WG146 (ref. 14)
WG96	(F′$_{128}$ *lac*$^+$ *proA*$^+$ *B*$^+$) WG2300	transconjugant of WG2300 from E5014
WG138	CSH4 *proP219*	P1 transductant of WG95 from CSH4 (ref. 14)
WG140	CSH4 *proC*::Tn*5*	P1 transductant of CSH4 from CBK130 (ref. 14)
WG146	CSH4 *pyr-76*::Tn*10*	see Materials and Methods Section (ref. 14)
WG148	CSH4 *putA1*::Tn*5* *proP219*	P1 transductant of WG170 from JT31 (ref. 14)
WG170	CSH4 *Δ(putPA)101* *proP219*	see the Materials and Methods section (ref. 14)
WG176	CSH26 *pyr-76*::Tn*10*	P1 transductant of CSH26 from WG146
WG177	(F′$_{128}$ *lac*$^+$ *proA*$^+$ *B*$^+$) WG176	transconjugant of WG176 from E5014
WG2300	CSH4 *Δ(putPA)101* *recA56 srl300*::Tn*10*	this laboratory (ref. 8)

[a] The genetic nomenclature is that of Bachmann and Low (ref. 10). The deletion in strain RM2 was previously named incorrectly as *Δ(putPA)100*, and *proP* is defined in the text.

Phenotypic Tests

Mutant phenotypes with respect to proline utilization (TTC indicator medium), resistance to the proline ana-

logues AC and DHP (radial streak tests) and proline dehydrogenase (*o*-aminobenzaldehyde plate test) were determined as described before.[8] Acquisition and retention of F'_{128} and its variants were detected as maintenance of the Lac^+ Pro^+ phenotype and by cross-streaking with the male-specific bacteriophage, f2.[11]

Syntropism was used to measure proline excretion. A loopful of a fresh LB overnight culture of each putative proline excreting strain was spotted at the periphery of a minimal agar plate using a 3-mm (diameter) soldered loop. A fresh LB overnight culture of strain WG140 (*procC*::Tn*5*) was streaked across the plate, reaching within 1mm of each peripheral test spot. After 36 h incubation at 37°C, the zone of stimulation of the growth of strain WG140 was measured as an indication of proline excretion.

Cultivation of Bacteria for Proline Production

One-hundred milliliters of sterile proline production medium in a 500-mL erlenmeyer culture flask was inoculated with 0.5 mL of a fresh LB[11] overnight culture of the strain to be tested. The flask was incubated at 37°C with rotary agitation at 200 rpm. At designated intervals samples were withdrawn aseptically and treated as follows. To determine culture density a 0.1-mL culture sample was diluted 26-fold with 0.1*N* HCl, mixed vigorously, and the optical density of the resulting suspension determined at 600 nm with a Bausch and Lomb Spectronic 70.[9] A second 0.1-mL aliquot was subjected to serial dilution with sterile water as required and aliquots were spread on Lactose McConkey indicator medium. Red and white colonies appearing after 18–24 h incubation at 37°C were counted to determine the segregation frequency for F'_{128} and its variants. A 0.4- or 0.5-mL sample was clarified by centrifugation for 4 min at 10^4g in a Beckman Microfuge B and the supernatant stored frozen. At the end of the growth period, a sample was removed aseptically from each flask and exhaustive phenotypic tests were performed to detect contamination or genetic alteration of the cultured bacteria. No contaminants or variants were found. Finally, the remaining $CaCO_3$ and bacteria were removed by centrifugation from the remaining culture and the pH of the culture supernatant was determined with a Fisher pH electrode and meter.

Determination of L-Proline in Culture Supernatants

The concentration of L-proline in culture supernatants was determined by the dansylation double-labelling procedure described by Brown and Perham.[15] All manipulations were performed with chromic acid-washed glassware or new, disposable plastic labware to avoid chemical contamination. Dansyl chloride stock solutions were prepared in toluene and stored, dessicated, at −17°C. Prior to use, they were evaporated to dryness under a stream of N_2 and redissolved in acetone, all at 4°C.

In order to eliminate interference by ammonium salts present at high concentration in some samples, culture supernatants were treated as follows. Culture supernatant (25 μL) was mixed with 25 μL of triethylamine in a 0.5-mL microfuge tube. The mixture was evaporated to dryness at 30°C *in vacuo* and redissolved in 25 μL of glass-distilled H_2O.

Dansylation was performed by mixing 5 μL of a triethylamine-treated sample (above) with 5 μL each of L-[U-^{14}C] proline (0.019m*M*, 280 mCimmol^{-1}), triethylamine (0.1*M* in H_2O), and [^{3}H]dansyl chloride (2m*M*, 61 mCimmol^{-1}), in that order, in a 0.5-mL microfuge tube. The reaction mixture was agitated vigorously, collected at the bottom of the tube by centrifugation in the Beckman Microfuge B for 2 min, and incubated at 45°C for 15 min in a Fisher Isotemp Dry Bath (model 145). It was then evaporated to dryness at room temperature, *in vacuo*, and redissolved in 5 μL of pyridine.

The Pyridine solution (1 μL) was spotted at the corner of a 7.5 × 7.5-cm micropolyamide sheet (Schleicher and Schuell, F1700) and the chromatogram was developed with three solvent systems in two dimensions as described by Woods and Wang.[16] The dansyl derivatives were visualized by UV irradiation and identified by their position and color in comparison with standard dansylated compounds. The dansyl proline spot was cut out of the chromatogram and placed in a scintillation minivial (New England Nuclear, 7 mL capacity) with 125 μL of 95% ethanol. After 45 min at room temperature, 3 mL of Aquasol were added and the radioactivity in the sample determined with a Beckman LS7000 liquid scintillation spectrometer. Proline concentrations were determined by comparison of $^3H/^{14}C$ ratios for supernatant samples with those for standard L-proline solutions prepared in proline production medium and treated exactly as described above. The assay readily detected L-proline at concentrations as low as 20μ*M*.

RESULTS

Isolation of Proline Overproducing Mutants

Proline biosynthesis from L-glutamate is initiated by glutamate kinase, the *proB* gene product.[17] Indirect evidence suggests that the enzymes of proline biosynthesis are constitutive and the pathway is controlled by feedback inhibition of glutamate kinase.[6] Proline overproducing mutants of *E. coli* are thought to lack feedback control due to a mutation at *proB*.[18]

Mutants of *E. coli* resistant to the toxic L-proline analogue, L-azetidine-2-carboxylate (AC) occur at high frequency. They include proline transport mutants,[7,13,19] proline overproducing mutants altered at *proB* or *argD*,[18] and others, as yet undefined. Proline overproducers are usually identified as strains that excrete proline, stimulating the growth of a proline auxotroph (syntropism).[18]

We have been unable to identify proline excretors among AC resistant mutants arising spontaneously from *E. coli* strain CSH4 (S. Korycan and J. M. Wood, unpub-

lished data). We therefore followed the procedure of Csonka[20] to isolate AC-resistant mutants whose genetic lesions were localized near *proAB*. Mutants of strain WG177 [(F'_{128} *lac*$^+$ *proA*$^+$*B*$^+$) Δ (*lac pro*)] resistant to AC (100 μg/mL) arose at a frequency of 3×10^{-5} on minimal medium containing uracil. A grid of those mutants on the same medium was replicated onto the same medium lacking uracil and spread with ca. 10^8 cells of strain CSH26 [F^- Δ(*lac pro*)]. Putative proline overproducing transconjugants were identified as AC-resistant colonies. They were purified and screened for AC and 3,4-dehydroproline (DHP) resistance as well as proline excretion. Proline excreting strains with increased proline analogue resistance were identified but their phenotypes were complex. They had also lost the ability to utilize proline as detected by TTC indicator medium. Presumably they contained spontaneous mutations in the *put* region of the recipient chromosome as well as episomal mutations.

To resolve effects of chromosomal mutations from those attributable to variants of F'_{128}, episomes were transferred from three analogue resistant transconjugants of CSH26 that showed proline excretion back into strain WG176. Each episome was also transferred to strain WG2300 (*recA56*) to ensure stable maintenance of any episomal mutations. Control strains were constructed containing unmodified F'_{128} (strains WG177 and WG96). In each case, Lac$^+$ transconjugants were selected, and their phenotypes are shown in Table II.

Table II. Phenotypes of strains harboring variants of F'_{128}.

Strain[a]	Relevant genotype: Chromosome	Relevant genotype: Episome	Analogue resistance[b]: AC	Analogue resistance[b]: DHP	Proline dehydrogenase[c]
WG176	Δ(*lacpro*)	Nil	d	d	d
WG2300	*recA56*	Nil	5	3	I
WG177	WG176	*proA*$^+$*B*$^+$	4	3	I
WG96	WG2300	*proA*$^+$*B*	5	3	I
WG178	WG176	*pro-202*	1	1	C
WG97	WG2300	*pro-202*	5	3	C
WG179	WG176	*pro-203*	1	1	C
WG98	WG2300	*pro-203*	5	1	C
WG180	WG176	*pro-204*	1	1	C
WG99	WG2300	*pro-204*	5	1	C

[a] Strain constructions are described in the text. Each strain is Lac$^+$, insensitive to arginine and can utilize L-proline.

[b] Proline analogue resistance was measured by the radial streak test (ref. 8). Relative analogue sensitivities are designated numerically, where strains designated 1 are least sensitive, 5 are most sensitive to growth inhibition by AC or DHP. The designation 1 represents no growth inhibition by up to 1 mg of AC or DHP.

[c] Proline dehydrogenase was detected qualitatively by the *o*-aminobenzaldehyde plate test described before (ref. 8): (I) bacteria yield a positive response when cultured on glycerol minimal medium plus glycyl-L-proline; (C) bacteria yield a positive response when cultured on glycerol minimal medium with or without glycyl-L-proline (0.2%).

[d] Proline analogue resistance cannot be determined since this strain is a proline auxotroph. It does express proline dehydrogenase.

The episomes thought to bear *proB* mutations did confer enhanced proline analogue resistance on their host strains. They were designated as bearing alleles *pro-202*, *pro-203*, and *pro-204*. While each conferred increased resistance to both AC and DHP on strain WG176, they conferred only DHP-resistance on strain WG2300. The level of proline analogue resistance conferred by allele *pro-202* was consistently less than that conferred by *pro-203* or *pro-204*. Each strain retained the ability to catabolize proline as shown by TTC indicator medium (*putP*$^+$*A*$^+$) and was insensitive to arginine (*argD*$^+$).[18] Surprisingly, none of these strains excreted proline as detected by syntropism with strain WG140.

Proline dehydrogenase is induced by L-proline.[21] It is normally undetectable in bacteria grown in the absence of proline or glycyl-L-proline as inducer.[21] Proline overproducing mutants would be expected to maintain elevated cytoplasmic L-proline pools and might therefore show apparently constitutive synthesis of proline dehydrogenase. We examined the production of proline dehydrogenase by our putative proline overproducing mutants (Table II). Those strains bearing allele *pro-202*, *pro-203*, or *pro-204* do synthesize proline dehydrogenase in the absence of inducer whereas the control strains require induction. We therefore believe that the mutant alleles do cause proline overproduction but that the amino acid is not excreted from strains with the genetic constitution of WG176 and WG2300 (see below). The failure of these alleles to confer either AC resistance or proline excretion on strain WG2300 (CSH4 *recA56 sr1300*::Tn*10*) explains our failure to detect proline excretion among spontaneous, AC-resistant mutants of strain CSH4.

Effects of Proline Transport and Catabolic Lesions on Proline Production and Excretion

E. coli and *S. typhimurium* can utilize L-proline as sole source of carbon or nitrogen for growth. Mutations at *putP*, which eliminate proline transport via proline porter I (PP-I) and mutations at *putA*, which eliminate proline dehydrogenase, prevent proline utilization.[7,12] The *put* genes are adjacent at 22 min on the chromosome map and are coordinately regulated.[7,22,23] Strains lacking the *putA* gene product show constitutively high transport activity, probably because that protein acts as a negative genetic effector controlling its own expression and that of *putP*.[24]

Proline auxotrophic strains defective at *putP* can grow if provided with media low in proline (25μ*M*), whereas those defective at an additional locus, *proP*, require high proline for growth (2.5m*M*).[13,25] Mutations at *proP* inactivate a second proline transport system, PP-II, which is induced by amino acid starvation.[13,26] The *proP* locus of *S. typhimurium* is located at 92 min on the chromosome map[25]; the location of the *E. coli proP* mutations has not been determined. The *S. typhimurium proP* mutations are, however, complemented by *E. coli* F'_{117}(94-98 min).[25]

Episome F'_{128} and its variant bearing allele *pro-203* were transferred to a set of strains that are isogenic but for lesions in *putP*, *putA*, or *proP*. The episomes were transferred by spot mating with strains WG177 and WG179 as donors and selecting lactose utilizing transconjugants on medium lacking uracil. All transconjugants shared the expected phenotype: $Lac^+ Trp^- Str^r$ and bacteriophage f2 sensitive. Those that were $putA^+$ showed apparently constitutive expression of proline dehydrogenase when the episome bearing allele *pro-203* was introduced.

Proline overproduction would be expected to increase the cytoplasmic L-proline concentration, increasing resistance to the proline analogues AC and DHP. The proline analogue resistance phenotypes of the transconjugants and their parents are shown in Table III. The effects of mutations at *putP*, *putA*, and *proP* on AC and DHP resistance in the absence of F'_{128} have been discussed elsewhere.[7,13] Introduction of unaltered F'_{128}, which makes each strain diploid for *proAB* (wild type), had no effect on AC or DHP resistance. The episome bearing *pro-203* eliminated the remaining DHP sensitivity in all strains, but it reduced AC sensitivity only in some genetic backgrounds. These data imply that AC and DHP exert their toxic effects by acting at different intracellular sites.

The excretion of proline by the same set of strains has been assessed by testing for syntropism with strain WG140 (Table IV). Again, bacteria with wild type transport and catabolic functions do not excrete proline, regardless of their biosynthetic activity. Those unable to degrade proline ($putA^-$) or to accumulate it via proline porter I ($putP^-$) do excrete proline when its synthesis is amplified. Transport lesions (*putP* or *proP*) further enhance excretion by $putA^-$ bacteria, although the effects of the *putP* defect are the more pronounced. During these experiments all cultures were routinely tested to confirm their phenotypes with respect to antibiotic resistance, nutritional requirements, proline utilization capacity and sensitivity or resistance to bacteriophage f2. In no case was evidence obtained for segregation of F'_{128} or its derivatives. However, we cannot exclude the possibility of recombination between chromosome and episome.

Table III. Proline analogue resistance.[a]

Parent strain	Chromosomal variation	Proline analogue	Episomal genotype: nil	$proA^+B^+$	*pro-203*
CSH4	$putP^+putA^+$	AC	4	4	4
	$proP^+$	DHP	3	3	1
JT34	*putP3*::Tn*5*	AC	2	2	1
		DHP	1	1	1
WG138	*proP219*	AC	4	4	4
		DHP	3	3	1
JT31	*putA1*::Tn*5*	AC	5	5	3
		DHP	5	5	1
RM2	*Δ(putPA)101*	AC	2	2	1
		DHP	3	3	1
WG148	*putA1*::Tn*5*	AC	3	3	1
	proP219	DHP	4	4	1
WG170	*Δ(putPA)101*	AC	2	2	2
	proP219	DHP	2	2	1

[a] See footnote (b) to Table II; strain constructions are described in the text.

Table IV. Proline excretion.[a]

Parent strain	Relevant chromosomal genotype	Episomal genotype: nil	$proA^+B^+$	*pro-203*
CSH4	$putP^+$ $putA^+$ $proP^+$	−	−	−
JT34	*putP3*::Tn*5*	−	−	+
WG138	*proP219*	−	−	−
JT31	*putA1*::Tn*5*	−	−	+
RM2	*Δ(putPA)101*	+	+	++
WG148	*putA1*::Tn*5 proP219*	−	−	++
WG170	*Δ(putPA)101 proP219*	+	+	++

[a] Strain constructions are described in the text. Excretion is estimated as the zone of stimulation of the growth of strain WG140 (see the Materials and Methods section).

Proline Production in Liquid Culture

The specificity of proline excretion and quantity of proline produced were examined employing strain WG105 [(*F'128 pro-203*)RM2]. Batch cultures were prepared in amino acid production medium (see the Materials and Methods section) which employs D-glucose as carbon source, $(NH_4)_2SO_4$ as nitrogen source, and $CaCO_3$ as pH stat. The medium contained 5% (w/v) D-glucose and 2.5% (w/v)$(NH_4)_2SO_4$. A twofold increase in glucose and $(NH_4)_2SO_4$ concentrations yielded a prolonged lag phase and no greater net growth, whereas lower nutrient concentrations [2% glucose and 1% $(NH_4)_2SO_4$] yielded a lower final culture density. The WG105 was grown to stationary phase and the culture supernatant composition was monitored by dansylation (see the Materials and Methods section). Dansylamide, Dansyltryptophan, dansylproline, and the hydrolysis product, dansoic acid, were the only quantitatively significant fluorescent derivatives detected by two-dimensional thin-layer chromatography of the dansylated supernatants. The dansyl proline spot was the only one that increased in intensity with culture age.

The cultures of strain WG105 took approximately 70 h to reach stationary phase at an optical density of 9. Proline production began after a lag of ca. 7 h and reached a maximum concentration of 8m*M* as the cells entered stationary phase. The fraction of Lac^- segregants in the culture never exceeded 6% and did not increase with culture age. Final culture pHs were always between 6.5 and 7.5, implying that growth ceased as a result of nutrient limitation, not acidification of the culture medium.

Cultures of each of the strains harboring *pro-203* that are listed in Table IV showed analogous growth characteristics to WG105. In each case qualitative analysis of the final culture supernatant by dansylation revealed no

major dansyl derivatives other than dansoic acid, dansylamide, dansyltryptophan, and (in some cases) dansylproline. Dual-labelling analysis of a 96-h culture supernatant from strain WG102 [(F'_{128} *pro-203*) CSH4] revealed no detectable proline.

DISCUSSION

We have examined the effects of genetic lesions preventing proline transport and catabolism on the production of extracellular proline by *E. coli*. Maximum proline excretion, as detected by syntropism, is attained in strains defective for proline catabolism (*putA*$^-$) and for proline porter I (*putP*$^-$) or II (*proP*$^-$) (Table IV). Indeed, no proline is detectable in culture supernatants from bacteria whose biosynthetic pathway is amplified but whose transport and catabolic functions are intact (see text and Table IV). Thus proline efflux does limit proline production, and specific genetic manipulations have been shown to enhance both proline excretion and production.

Mutants that excrete proline have previously been isolated by selection with the toxic proline analogues AC and DHP.[9,18,19] In our strains which retain intact proline transport and catabolic functions, proline overproduction yields resistance to DHP but it produces neither AC resistance nor proline excretion (Tables II, III, and IV). Both AC and DHP can also be used to select mutants defective for proline transport and catabolism.[7,13,22,25,27] Since *putA* (proline dehydrogenase) and *putP* (related to PP-I) are adjacent on the *E. coli* chromosome, deletion mutants lacking both genes are readily isolated.[7,22] It is therefore probable that previously isolated proline excreting organisms were multiple mutants bearing transport and/or catabolic lesions as well as altered biosynthesis. Indeed, Nakamori and co-workers have suggested that their dehydroproline-resistant, proline-producing *Brevibacteria* may be permeability mutants.[9] Proline overproducers that retain *putA* show apparently constitutive expression of proline dehydrogenase (Table II). This phenomenon could therefore be exploited to screen bacterial populations for organisms that overproduce but do not excrete proline.

Proline production by our proline overproducing strains is also enhanced if PP-II is defective (*proP219*, Table IV). The syntropism test is not sufficiently precise to determine whether the effects of the *putP* and *proP* lesions are additive in proline overproducing, *putA*$^-$ strains. PP-II is induced during amino-acid-limited growth and its activity is expected to be low during growth on glucose and NH_3 if amino acids are supplied in excess.[13,26] The energetics of uptake via PP-II have not been examined, but our data suggest that, under the conditions of this study, it serves to catalyze the active reaccumulation of extracellular proline.

The mechanism of proline excretion by bacteria lacking both PP-I and PP-II is unclear. A minimum permeability coefficient for proline efflux of 8.6×10^{-10} L/s/mg cell protein or 1.3×10^{-8} L/s/mg membrane protein accounts for the production of $8 \times 10^{-3}M$ medium L-proline if we assume that level is achieved by passive efflux of proline from a culture at an optical density of 9 (600 nm) in 50 h (see Fig. 1). An optical density of 1 is assumed to correspond to 0.5 mg cell protein/mL, membrane protein is assumed to constitute 5% of total cellular protein, and the bacteria are assumed to maintain a constant cytoplasmic L-proline concentration of 20mM. (The estimated permeability varies from 0.6×10^{-8} to 4×10^{-8} L/s/mg membrane protein if the assumed cytoplasmic proline concentration varies from 40mM to 10mM.) Our own measurements of L-proline uptake reveal an uptake component in *E. coli* and *S. typhimurium* whose rate is a linear function of L-proline concentration between $10^{-6}M$ and $10^{-3}M$ that has a permeability coefficient on the order of 10^{-8} L/s/mg cell protein.[13,26] Stevens and co-workers have observed a Na^+-independent L-proline uptake component by rabbit jejunal brush border membrane vesicles with a permeability coefficient of 1.6×10^{-8} L/s/mg membrane protein.[28] These two values represent widely divergent estimates of passive membrane permeability for proline, but either would be adequate to account for the observed proline excretion according to our very approximate estimate of the required permeability. Further experiments are underway to characterize the efflux mechanism and to determine whether efflux rate continues to limit proline productivity in the absence of active uptake processes.

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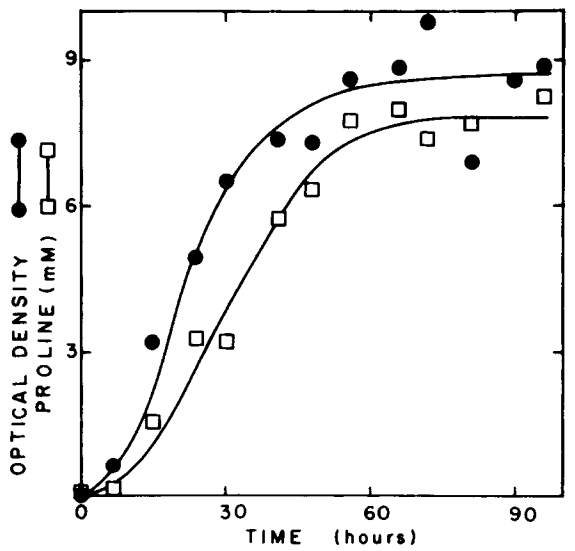

Figure 1. L-proline production by *E. coli* strain WG105. Culture optical density and culture supernatant L-proline concentration were determined as described in the Materials and Methods section.

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