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Mutations in the *Corynebacterium glutamicum* Proline Biosynthetic Pathway: a Natural Bypass of the *proA* Step

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Two chromosomal loci containing the *Corynebacterium glutamicum* ATCC 17965 *proB* and *proC* genes were isolated by complementation of *Escherichia coli proB* and *proC* auxotrophic mutants. Together with a *proA* gene described earlier, these new genes describe the major *C. glutamicum* proline biosynthetic pathway. The *proB* and *proA* genes, closely linked in most bacteria, are in *C. glutamicum* separated by a 304-amino-acid open reading frame (*unk*) whose predicted sequence resembles that of the 2-hydroxy acid dehydrogenases. *C. glutamicum* mutants that carry null alleles of *proB*, *proA*, and *proC* were constructed or isolated from mutagenized cultures. Single *proC* mutants are auxotrophic for proline and secrete Δ^1-pyrroline-5-carboxylate, which are the expected phenotypes of bacterial *proC* mutants. However, the phenotypes of *proB* and *proA* mutants are unexpected. A *proB* mutant has a pleiotropic phenotype, being both proline auxotrophic and affected in cell morphology. Null *proA* alleles still grow slowly under proline starvation, which suggests that a *proA*-independent bypass of this metabolic step exists in *C. glutamicum*. Since *proA* mutants are complemented by a plasmid that contains the wild-type *asd* gene of *C. glutamicum*, the *asd* gene may play a role in this bypass.

Exposure of bacteria from the genus *Corynebacterium* (8) to hyperosmotic stress is correlated with biosynthesis and accumulation of some endogenous substrates, also termed compatible solutes. The most conspicuous are proline (15, 21), trehalose (19), glutamine (13), and ectoines (7). Though the causes of this response are not understood, it is accepted that the biosynthesis and accumulation of these substrates is somehow related to the maintenance of the bacterial osmotic equilibrium. In *Corynebacterium glutamicum*, *Escherichia coli*, and *Bacillus subtilis*, proline accumulation is the common feature of the osmotic shock response (14, 15, 36). As part of a systematic approach to study this phenomenon, we report here the cloning and characterization of the *proB* and *proC* genes and the isolation of *proB*, *proA*, and *proC* mutants. The characteristics of these mutants reveal some peculiar aspects of the *C. glutamicum* proline biosynthetic system.

MATERIALS AND METHODS

The bacterial strains and plasmids used are described in Tables 1 and 2. Recombinant DNA methods, plasmid banks, and standard culture techniques for *E. coli* and *C. glutamicum* were as previously described (15, 29). Nutritional complementation of *proC* mutants by synthetic ^{1}-pyrroline-5-carboxylate (P5C), or produced in vivo by the secretor *E. coli* strain χ342 (*proC29*), was performed as described previously (6). Strains G9 (*proA9*) and χ340 (*proA28*) were used for P5C biological detection.

Fully inactivated (null) alleles of *C. glutamicum* ATCC 14752 genes were constructed by (i) single recombination with pBR322 clones containing internal gene fragments and a contiguous *aph3* cartridge or (ii) double recombination with a gene fragment containing an inserted *aph3* cartridge, in some cases linked to a deletion (22, 29) (Fig. 1). The introduction of mutations at genes whose mutant phenotype is compatible with standard *C. glutamicum* ATCC 14752 growth conditions (*proA* and *proC*) proceeded as efficiently as described for *C. glutamicum* ATCC 14752 and B115 (1×10^3 to 2×10^3 integrative transformants per kbp of homology per μg of nonreplicative DNA, with 10 to 30% of double-recombination events [2, 22, 29]). Our transformation conditions gave about 2×10^6 to 3×10^6 transformants per μg of a replicative plasmid. Integrative DNA able to transform the highly DNA restrictive strain *C. glutamicum* ATCC 14752 (35) was prepared from a JM110 (*dam dcm*) transformant or from PCR-synthesized DNA as described previously (2). Conformity (data not shown) to the theoretically predicted mutant structure was examined by conventional Southern blot hybridization analysis of restriction nuclease digests of total DNA, PCR-amplified DNA, or both, using a Plex Luminescent kit (Millipore Corporation, Bedford, Mass.).

Sequences. Overlapping clones of single-stranded DNA cloned in M13mp18 and M13mp19 or double-stranded DNA cloned in Bluescript-II SK(+) were sequenced in an Applied Biosystems model 373A DNA sequencer, using the standard PRISM DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) protocol. Sequence patches were assembled and interpreted by using the DNA Strider version 1.2 (25), AssemblyLIGN 1.0.5/MacVector 4.0 (International Biotechnologies Inc.), and Genetics Computer Group (University of Wisconsin) software packages. The SwissProt database was searched for sequence similarities by the PROFILEMAKE and PROFILESEARCH programs. Sequences that had the best scores were aligned, and a consensus was generated, by the BESTFIT, PILEUP, and PRETTY programs. Phylogenetic trees of ProC and ProB proteins were constructed with the PAUP (version 3.1.1; Smithsonian Institution) and neighbor-joining method programs (31), using the default settings. Blocks of similarity between *unk* and diverse D-2-hydroxy acid dehydrogenases were generated with the PROTOMAT system on the Block E-mail Searcher (18).

Localization of transposon and insertion sites. The transposon used to mutagenize *C. glutamicum* ATCC 14752, Tn*5531* (GenBank accession number U53587; a 4.546-kbp artificial transposon that contains the *aph3* Km^r gene flanked by tandem IS*1207* repeats [7a]), has a single *Eco*RI site linked to the *aph3* gene; hence, the *Eco*RI fragment that contains *aph3*, identified by its ability to confer a Km^r phenotype, must contain one of the transposon-chromosome junctions. The corresponding fragment from each mutant was cloned in pBluescriptII SK(+) and used to sequence the Tn*5531*-chromosome junctions. The sequences of the transposon junctions to the carboxy-terminal *proC* fragments were obtained with an oligonucleotide homologous to a Tn*5531* border. The *proA18* allele 0.9-kbp insertion inactivates the *Pst*I recognition site at bp 362 (GenBank accession number X82929 coordinate). The sequence of the insertion junction to the carboxy-terminal *proA18* fragment was confirmed with a *proA*-specific oligonucleotide.

Levels of proline enzymes. Cell extracts were made in a Retsch MM2000 homogenizer (top speed, 106-μm-diameter glass beads, 10 min at room temperature). Our disruption conditions liberate 6.93 ± 0.05 μg of protein per unit of optical density at 590 nm (OD_{590}). No further increase in the total ProA or ProC activities released per OD_{590} unit was obtained by the use of more extreme cell disruption conditions. Total glutamate kinase was assayed as described for *Brevibacterium flavum* (38). Total (glutamate semialdehyde plus P5C) dehydrogenase, associated with the reverse ProA reaction (this work), and P5C reductase (biosynthetic ProC reaction) were measured as described previously (16). Activities are expressed in micromoles of substrate produced per minute per milligram of protein. Secretion of P5C was measured (6) in the supernatant of cell cultures

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TABLE 1. Bacterial strains used in this study[a]

Strain	Genotype	Source and/or reference
C. glutamicum		
ATCC 17965	Carries cryptic plasmid pCGL500	ATCC
AG1	*proC1*, NG induced from ATCC 17965	A. Guyonvarch
SA12	*proA33* (Fig. 1)	This work
ATCC 14752		ATCC
SA2	ProA and/or ProB deficient, *proC13*::Tn*5531* (Fig. 1)	C. Bonamy and this work
SA3	*proA18 proC16*::Tn*5531* (Fig. 1)	C. Bonamy and this work
SA5	*proC16*::Tn*5531* (Fig. 1)	C. Bonamy and this work
SA6	*proB2* (Fig. 1)	This work
SA8	Δ*proA72* (Fig. 1)	This work
SA10	*proA33* (Fig. 1)	This work
SA13	SA5 (*proC16*) transformed with plasmid pCGL1336 (ProC$^+$) (Table 3)	This work
SA14	Δ*proC1349* (Fig. 1)	C. Bonamy and this work
E. coli		
G9	*proA9 leu thi thr lac rpsL*	VKPM, B3315 (33)
χ340	HfrPOA2 *proA28 metB1 relA1 spoT1*	CGSC, 4513 (33)
χ342	HfrPOA2 *proC29 metB1 relA1 spoT1*	CGSC, 4515 (33)
G14	*proB14 leu thi thr lac rpsL recA56 srl*::Tn*10*	VKPM, B3316, (33)
JM83	*ara* Δ*lac-proAB rpsL* Ø80 d*lacZ*Δ*M15*	37
JM110	*dam dcm supE44 hsdR17 thi leu rpsL lacY galK galT ara tonA thr tsx* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)	37

[a] Abbreviations: ATCC, American Type Culture Collection; VKPM, Collection of Industrial Microorganisms of Soviet Union, Moscow, Russia; CGSC, *E. coli* Genetics Stock Center, Yale University, New Haven, Conn.; NG, nitrosoguanidine. The assignments of proline mutants were recently reconsidered (33).

grown (glucose-minimal medium supplemented with 10 mM proline) to an OD_{590} of 4 to 5, washed twice, and [15] resuspended in 0.5 volume of growth medium devoid of proline and starved by shaking 5 h at 30°C. Secretion activity is expressed in micromoles of total P5C liberated per OD_{590} unit. One OD_{590} unit is the quantity of cells in 1 ml of culture at an OD_{590} of 1.0. Since P5C secretor *proC* mutants frequently yield faster-growing nonsecretor secondary mutations (this work and reference 1a), the P5C secretion phenotype of a sample of colonies isolated from each *proC* cultures was routinely examined.

Nucleotide sequence accession numbers. The sequences reported here can be found in the GenBank database under accession numbers U31224 (*ppx* fragment), U31225 (*proC*), and U31230 (*proB* and *unk*).

RESULTS

Cloning and sequence of the *proC* gene region. We previously (34) showed that complementation of *E. coli proA* alleles by *C. glutamicum proA* is dependent on NaCl addition to the culture medium, a condition referred to hereafter as high osmolarity. Hence, all of our attempts to clone *C. glutamicum* proline genes by complementation of *E. coli* mutants were done both at high and at normal osmolarity. No osmolarity effects were apparent in the transformation of *E. coli* χ342 (*proC29*) with a *C. glutamicum* ATCC 17965 genomic bank. Plasmid extracted from six randomly chosen clones contained identical 6.5-kbp inserts. Mutant *proA*, *proB*, or Δ*proBA E. coli* hosts transformed by these plasmids failed to grow in minimal medium at either high or low NaCl osmolarity. Analysis of *Pst*I deletions of one such plasmid (pPCU1) located the *proC29* complementing activity in a 2.49-kbp insert fragment. This fragment was subcloned (plasmids pPCB1 and pPCB2), and deletion derivatives were submitted to single sequence runs. Subclones whose partial sequence contained homologies to known ProC proteins were completely sequenced, and these sequences were assembled to obtain that of the *C. glutamicum proC* region.

Cloning and sequence of the *C. glutamicum proB* gene and the *proA-proB* interval. A *C. glutamicum* ATCC 17965 gene bank (29) was introduced by transformation into the *E. coli* mutant G14 (*proB14*), and the plasmids of 19 proline prototrophic transformants were analyzed. Four types of inserts, which shared a common chromosomal region, were found. All of these plasmids complemented strain JM83 (Δ*proBA*) but not strain χ342 (*proC29*), which indicates that they carry both the *proB* and *proA* genes.

In the absence of the *proA* gene, plasmid pCGL347 (*C. glutamicum* wild-type *proB*) complements *E. coli proB* mutants at NaCl concentrations higher than 0.45 M in the culture medium. The complementation of *E. coli proA* mutants by the *C. glutamicum proA* gene is optimal at an NaCl concentration of 0.2 M (34). No osmolarity effect was observed in the complementation of *E. coli proB*, *proA*, or Δ*proBA* mutants by plasmid pCGL1341, which carries the *C. glutamicum proB* and *proA* genes. These observations suggest that the dependence of complementation on high osmolarity is only observed in conditions in which heterologous $ProB_{C.\ glutamicum}$-$ProA_{E.\ coli}$ or $ProB_{E.\ coli}$-$ProA_{C.\ glutamicum}$ complexes must be formed. Clone pRBU1 (ProB$^+$ ProA$^+$; 5.3 kbp) was sequenced after subcloning on M13mp18 and M13mp19 vectors. The sequences of the *proB* and *proA* regions bracket a 1-kbp DNA stretch, which encodes a hypothetical product of 305 amino acids (*unk*) of unknown function located upstream from the *C. glutamicum proA* gene (Fig. 1).

Identification of *C. glutamicum proC* alleles in a collection of proline auxotrophic mutants. We characterized four proline auxotrophic mutants (Table 3). The mutants tested were first classified by their ability to accumulate the proline precursor P5C. In *E. coli*, mutants blocked in the *proC* gene cannot grow in the absence of an external proline source, although they accumulate P5C (6). This intermediate is excreted to the culture medium and can be detected by cross-feeding of *proB* or *proA* mutants or by direct chemical assay (Table 3). The *C. glutamicum* proline auxotrophic mutants were also characterized by complementation with the ProC$^+$ clone pCGL1354 and by their reversion frequency to prototrophy. The proline auxotrophy of strains AG1, SA2, SA3, SA5, and SA12 is not complemented by plasmids that carry either the ProB$^+$ or the ProA$^+$ region, as is expected from an extreme *proC* mutant.

These criteria defined two distinct groups. Strains from the first group, including AG1 and SA5, excrete P5C but are not supplemented by P5C (Table 3). Strain SA5 is complemented by the wild-type *proC*$^+$ clone pCGL1354, as expected from a

TABLE 2. Plasmids and vectors used in this study

Plasmid or vector	Description
Plasmids	
pPCU1	Smallest primary pUN121 clone (6.5-kbp insert) that complement a *proC* mutant of *E. coli* (this work)
pPCB1	2.91-kbp *Pst*I-*Nsi*I fragment of pPCU1 containing 2.49 kbp of *C. glutamicum* DNA and 417 bp from the pUN121 vector cloned into pBluescript II KS(−); complements *E. coli proC* mutants (this work)
pPCB2	As pPCB1, but inserted in the opposite orientation (this work)
pSAC13	*Eco*RI chromosome fragment (2.4 kbp) of SA2 cloned, together with the *aph3* gene of Tn*5531*, in pBluescript II SK (+) *Eco*RI site (this work)
pRBU1	Primary pUN121 clone (5.3-kbp insert) that complement *proB* mutants of *E. coli* (this work)
pCGL308	Low-copy-number plasmid Tet^s-Cm^r derivative of pACYC184 (30); contains a unique *Sac*I site; constructed from a *Hae*II digest of pACYC184 (28)
pCGL324	Low-copy-number Am^s plasmid containing the *lac* and multiple cloning site of pBluescript II SK(+) and the *cat* gene (Cm^r) and replication origin of pACYC184 (28); constructed from a *Hae*II digest of both plasmids (2)
pCGL347	Smallest *Nsi*I fragment (3.3 kbp) of pCGL1341 that complements *E. coli proB* mutants, as cloned in plasmid pCGL324. The fragment extends from the unique *Nsi*I site in pUN121 to the *Nsi*I site of *unk* (coordinate 2362 in GenBank accession no. U31230; this work).
pCGL467	4.44-kb *C. glutamicum* partial *Sau*3A digestion fragment ligated to a*Bcl*I pUN121 digest; carries *lysC* and *asd* genes of *C. glutamicum* (34)
pCGL472	*Sac*II *aph3* cartridge from pCGL243 (29) inserted in the *Sac*II site of pCGL452 (34) within the *proA* gene
pCGL1314	*Sac*I fragment of pCGL243 (29) containing an *aph3* cartridge, cloned into pBluescript SK II (+) *Sac*I site (this work)
pCGL1327	*Sac*I fragment of pCGL472 containing part of the *proA* gene and the *aph3* insert cloned in the *Sac*I site of pCGL308 (this work)
pCGL1338	Shuttle vector containing the pCGL243 (29) pBL1 replicon, the *cat* gene of pACYC184 (28), and the *Xba*I-linearized *C. glutamicum* wild-type *proA* plasmid pCGL452 (34)
pCGL1341	Primary pUN121 clone (4.7-kbp insert) that complements *proB* and *proA* mutants of *E. coli* (this work)
pCGL1345	*Sac*I-*Xho*I *aph3* cartridge ligated to a *Sac*I-*Xho*I digest of pCGL1341, generating an insertion-deletion within *proA* (this work)
pCGL1346	Shuttle vector containing the pCGL243 (29) pBL1 replicon ligated to pCGL467 *Asp* 718-*Xho*I fragment (this work)
pCGL1347	*Sma*I fragment of pCGL1314 containing an *aph3* cartridge inserted in a *Dra*I digest of pUN121. The *bla* (Amp^r) gene is deleted in this construct, and the *aph3* cartridge is inserted in the orientation opposite to that of *tet* (this work)
pCGL1352	pCGL1347 derivative in which an internal *Pst*I fragment of *proB* of *C. glutamicum* is cloned in the *Nsi*I site of pUN121 (this work)
pCGL1354	Shuttle vector containing the pCGL243 pBL1 replicon (29), *Nhe*I-linearized pPCU1, and the *Nhe*I-*Hae*II *cat* gene fragment of pACYC184 (28)
pCGL1356	*Xba*I deletion of pCGL1346 that remove *lysC*α promoter (this work)
pCGL1357	Shuttle vector containing the pCGL243 (29) pBL1 replicon and *Nhe*I-linearized pCGL347 (this work)
Vectors	
M13mp18	Sequencing vector (26, 37)
M13mp19	Sequencing vector (26, 37)
pBluescript II KS(−)	Cloning and sequencing vector (Stratagene; accession no. X52329)
pBluescript II SK(+)	Cloning and sequencing vector (Stratagene; accession no. X52325)

proC single mutant. In strain SA5, inactivation of the *proC* gene by a transposon insertion (*proC16* [Fig. 1]) produces all of the SA5 phenotypes. This finding was confirmed by genetic construction of a *proC* mutant (SA14 [Fig. 1]) containing a deletion which removes the amino-terminal end of the *proC* gene. The proline-independent revertants of SA5 do not secrete P5C (like the wild-type ATCC 14752 strain), became Km^s (50 tested), and excised the transposon (2 tested).

Strains from the second group of proline auxotrophic mutants, including SA2 and SA3, do not secrete P5C and are not supplemented by P5C (Table 3). The minimal hypothesis explaining the behavior of these mutants is that SA2 and SA3 contain at least two mutations, one at the *proC* and the other elsewhere.

Strain SA3 proved to contain (Fig. 1) an uncharacterized 0.9-kbp insertion within the *proA* coding region, at 74 bp from the amino-terminal end (34). This allele (*proA18*) does not fully inactivate the *proA* gene, since SA3 is complemented to prototrophy by pCGL1354. This finding is consistent with the elevated SA3 revertion frequency to a prototrophic phenotype (Table 3), similar to that of the single ProC mutant SA5, whose *proC16* allele is identical to the SA3 allele *proC17* (Fig. 1).

Strain SA2 is not complemented by pCGL1354. The nature of the P5C biosynthetic block of SA2 is not yet known and is probably associated to the existence of a second mutation at an unknown locus. No genetic rearrangements were found in the neighborhood of strain SA2 regions linked to the *proB* and *proA* genes after a systematic examination by PCR amplification and Southern blot hybridization.

A *proB* mutant of *C. glutamicum* ATCC 14752. A *proB* mutant was obtained by integration of a 1.6-kbp nonreplicative synthetic DNA circle containing the 688-bp *Pst*I fragment inside the *C. glutamicum proB* gene and a *aph3* cartridge for the selection of host recombinants (2). A single Km^r transformant per μg of DNA (strain SA6, *proB2*), which had a proline auxotrophic phenotype and the predicted gene structure (Fig. 1), was obtained. Transformation of SA6 with plasmid pCGL1357, containing the wild-type *proB* gene, restored a fully prototrophic phenotype. In minimal medium, the SA6 mutant reverted to prototrophy at high frequency (1×10^{-3} to 3×10^{-3}). These revertants probably arise by recombination at the 688-bp duplication bracketing the *aph3* insertion, since they are Km^s (20 tested) and recover the normal *proB* structure (2 tested). Less than 1% prototrophic revertants are found in

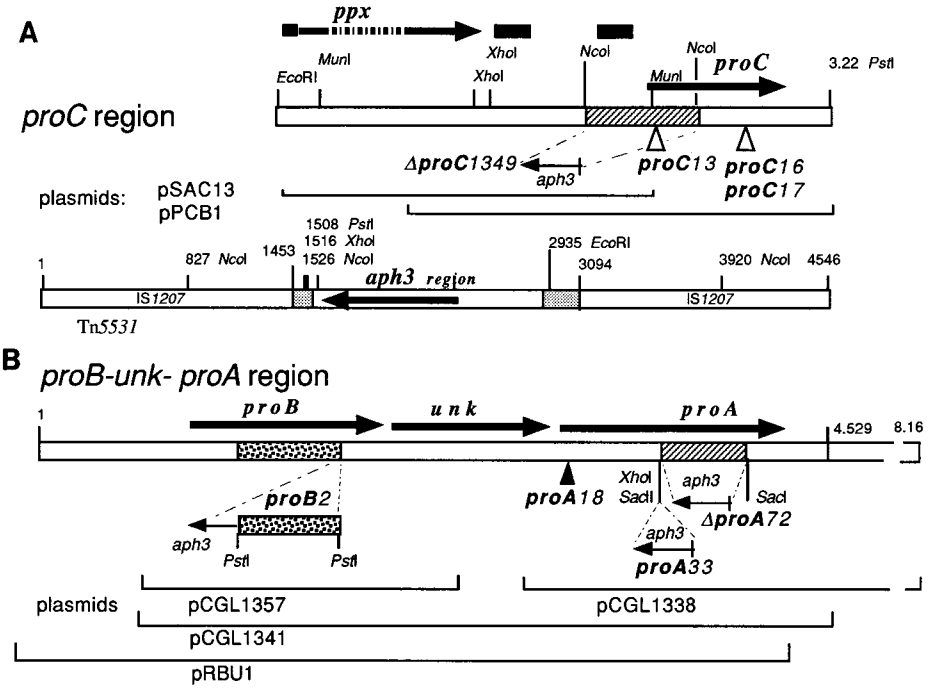

FIG. 1. The *proC* and *proB-unk-proA* regions of *C. glutamicum* (A) The *proC* gene and upstream regions of *C. glutamicum* ATCC 17965. Black arrows and black boxes, regions of strong homology between the proteins encoded by *C. glutamicum* and *M. leprae* (GenBank accession number U00018); thin arrow (not to scale), *Streptococcus faecalis aph3* (Km^r) gene. Homologies to the *ppx* and *proC* genes have been registered under GenBank accession numbers U31224 and U31225, respectively. Unreliable sequence data within the presumed *ppx* gene are shown by a discontinuous line. White wedges show the positions (GenBank accession number U31225 coordinates) of the Tn*5531* inserts (GenBank accession number U53587), 984 bp (*proC13*) and 1,514 bp (*proC16* and *proC17*). Tn*5531* orientation in the *proC* mutants, and genetic landmarks, are shown below. (B) *proB-unk-proA* region, reconstructed from the overlapping GenBank accession numbers U31230 (*proB-unk*, coordinates 1 to 3005) and X82929 (*proA*, coordinates 259 to 1783). Black arrows, *proB*, *unk*, and *proA* genes; dotted boxes, duplicated internal gene fragment in the insertion mutant *proB2*; black wedge, position of a 0.9-kbp uncharacterized insertion in allele *proA18*; hatched boxes, deleted DNA; brackets, plasmid inserts length. The allele *proA33* is an *aph3* insertion at a *Sac*II site.

proB2 cultures after 30 generations of growth on nonselective conditions (brain heart infusion broth).

The *proB2* strain SA6 makes small colonies on brain heart infusion medium. Phase-contrast microscopic observation (not shown) of SA6 cultures reveals that about 20% of the cells have constrictions that give them a string-of-pearls appearance. Such structures are not observed in the wild-type strain or in the proline prototrophic revertants of the SA6 strain.

TABLE 3. Proline auxotrophic mutants of *C. glutamicum*

Strain	Known genotype	P5C utilization[a]	*proA28* cross-feeding[b]	P5C secretion	Revertion frequency to proline independence	Complementation to prototrophy[c]			Enzyme sp act (U)	
						pCGL1357 (ProB$^+$)	pCGL1338 (ProA$^+$)	pCGL1354 (ProC$^+$)	ProA[d]	ProC[e]
ATCC 14752	Wild type	NA[f]	−	2.5	1.0	+[g]	+[g]	+[g]	<0.01	4.22 ± 0.13
SA6	*proB2*	+	−	2.3	$(1–3) \times 10^{-3}$	+	−	−	<0.01	4.80 ± 0.5
SA10	*proA33*	+	−	3.2	ND[h]	−[g]	+	−[g]	<0.01	4.35 ± 1.35
SA5	*proC16*	−	+	12.4	10^{-4}	−	−	+	0.42 ± 0.03	<0.05
SA13	*proC16*; high *proA* dosage	ND	±	8.5	ND	NA	NA	NA	2.48	ND
SA2	*proC13* *proB*?-*proA*?	−	−	1.2[i]	10^{-8}	ND	−	−	0.37 ± 0.02	<0.05
SA3	*proA18* *proC17*	−	−	4.0	10^{-4}	ND	−	+	0.31 ± 0.03	<0.05

[a] Tested with synthetic P5C or with a P5C donor strain (*E. coli* χ342 [*proC29*]). Symbols: +, grows in the absence of proline if supplemented with P5C; − does not grow in the absence of proline even if supplemented with P5C.
[b] Ability to work as a P5C donor in a biological test. P5C tester strain is *E. coli* χ340 (*proA28*).
[c] Done with plasmids described in Table 2. In the case of bradytrophic strains, the recovery of a wild-type growth rate in liquid medium was also tested.
[d] The estimated detection threshold of our system is 0.02 U.
[e] The estimated detection threshold of our system is 0.05 U.
[f] NA, not applicable.
[g] The phenotype tested on the nontransformed parent was unchanged in the transformed strain.
[h] ND, not done.
[i] The estimated detection threshold of our system is 0.2 P5C production units.

Transformation of SA6 with plasmid pCGL1357 ($ProB^+$) also restores a normal morphology. This finding suggests that the SA6 morphology does not result from a polar effect of the *proB2* insertion on the downstream genes.

Glutamate kinase activity was undetectable in *C. glutamicum* cell extracts, even under conditions of increased *proB* dosage (transformation with plasmid pCGL1357). Two glutamate kinase activities, related to glutamine synthetase and ProB, were found in an other actinomycete, *B. flavum* (35). However, the total cellular activity of both *B. flavum* enzymes is very low and would not have been detected by our present assay. The large difference between the ProB and the ProC specific activities, both required in proline biosynthesis, suggests that the standard glutamate kinase assay does not represent the biological activity of the *C. glutamicum proB* gene product (see Discussion).

Single mutations at the *proA* gene of *C. glutamicum* ATCC 14752. We have earlier described a *C. glutamicum proA* gene (34 [GenBank accession number X82929]). Two kinds of null alleles of this *proA* gene were introduced in *C. glutamicum* ATCC 14752. Strain SA10 (*proA33* [Fig. 1]) had a generation time of 3.0 h^{-1} on minimal medium, instead of the generation time of 1.5 h^{-1} observed for the wild-type strain. The bradytrophic strain SA10 recovered a normal generation time after addition of proline (up to 10 mM) to the minimal medium or after transformation with the $ProA^+$ clone pCGL1338.

There remained the possibility of a residual enzymatic ProA activity of the *proA* gene fragment downstream from the *proA33* insertion. To rule out this possibility, most of this region was removed and substituted by an *aph3* cartridge (Fig. 1). A strain (SA8) that contained such a substitution (Δ*proA72*) gave results similar to those for strain SA10 for all nutritional phenotypes tested. This finding indicates that the proline bradytrophy of SA10 is not due to the leakiness of the *proA33* allele. The *proA33* allele was also crossed to the *C. glutamicum* ATCC 17965 strain, the original source of the cloned proline genes discussed in this work. The *proA33* derivative of ATCC 17965 was also bradytrophic, which suggests that a bypass of the *proA* step is a characteristic common to many corynebacteria.

Activity of the *proA* gene product. The reverse *proA* reaction is due to the 4-glutamyl-semialdehyde dehydrogenase activity. Since this substrate is in tautomeric equilibrium with P5C, the evaluation of ProA activity interferes with P5C reductase (ProC), which consumes the NADPH formed by the reverse ProA reaction. Thus, ProA activity can be evaluated only in ProC-deficient strains. Other possible sources of interference are the ProA-independent dehydrogenases that use as substrate P5C or its tautomeric 4-glutamyl-semialdehyde form. In *E. coli*, the ProA-independent dehydrogenases are phosphate independent and ProA is phosphate dependent. Our efforts to make a similar distinction in *C. glutamicum* cell extracts were unsuccessful. However, we found that a significant fraction of the combined P5C plus 4-glutamyl-semialdehyde dehydrogenase activity (ProA-like activity) is determined by the *proA* product. This activity is 5.9 times greater in extracts of an SA5 derivative that carries plasmid pCGL1338 ($ProA^+$); similar amplification factors were observed for other gene products cloned in plasmids with the pCGL1338 replication origin (29).

Quantitative measurement of P5C secretion. Since *C. glutamicum* is specialized in converting glucose to glutamate, P5C secretion levels should be determined mainly by the combined activity of the *C. glutamicum* proline biosynthetic enzymes (Fig. 1). This prediction is verified in our mutants. The measurement of P5C secretion activity in *proC*-deficient mutants offers a more reliable correlation with the nutritional phenotypes of our mutants than does the measurement of the ProA-like activity. Thus, the highest P5C production corresponds to strain SA5 ($ProB^+$ $ProA^+$ $ProC^-$), which is a good P5C cross-feeder, and the lowest corresponds to strain SA2, extremely deficient in cross-feeding (not complemented by $ProC^+$). An intermediate P5C secretion level was observed in SA3. Though a poor cross-feeder, SA3 still produced P5C, since it was complemented to prototrophy by $ProC^+$ clones.

As expected, the P5C secretion levels in $ProC^+$ strains are lower. The wild-type ATCC 14752 strain secretes as much P5C as the *proB2* mutant, which is blocked in P5C production. This finding suggests that in the wild-type strain, most of the P5C produced is immediately converted into proline. Paradoxically, the *proA33* mutant secretes significantly more P5C than both the *proB2* and the wild-type *proA* strains. This increase may be due to the absence of reverse *proA* activity in *proA33*, which reduces the potential loss of P5C synthesized through the *proA* bypass. P5C secretion by the *proC16* mutant SA5 is reduced by transformation with the $ProA^+$ clone pCGL1338. Besides that of the $proA^+$ gene, the other sequences on this plasmid are unrelated to known proline metabolism genes (32). Thus, an increased dosage of the $proA^+$ gene is the simplest explanation of this observation.

Suppression of *proA* mutant by the *lysC* β-*asd* operon. The bradytrophic strain SA10 (*proA33*) recovered a normal generation time after transformation with plasmid pCGL1356. This plasmid carries the *C. glutamicum lysC* β-*asd* operon and its promoter but lacks part of the *lysC* α gene and the promoter of the overlapping *lysC*α-*lysC*β operon, which encodes both subunits of the *C. glutamicum* aspartokinase. The product of the *lysC*β gene region, common to both operons, is the target of the aspartokinase feedback inhibition by threonine and lysine, two end products of this pathway. The addition of different combinations of the end products of this pathway (threonine, lysine, and methionine) to the culture medium did not alter the growth rate of the *proA33* mutant in the absence of proline. This result suggests that the *proA* bypass is not subject to the same regulation as threonine, lysine, and methionine biosynthesis. However, the participation of Asd in this bypass cannot be excluded, since Asd activity is not inhibited by threonine and lysine (10) (Fig. 2). Distinction between both alternatives awaits the construction of double *asd proA* mutants.

Integration at the *asd* and *unk* loci. We attempted to inactivate *asd* and *unk* by integrating, through a single recombination event, a nonreplicative plasmid which contains an internal gene fragment. Despite the proven efficiency of this method in *C. glutamicum* (reviewed in reference 22), it was inadequate in this case. We observed (i) a frequency of integration 10^2 to 10^3 lower than expected (2, 22, 29), (ii) absence of the expected phenotype among the rare recombinants obtained, and (iii) abnormal recombinant structures. Independently obtained recombinants (four for *asd* and two for *unk*) were analyzed and found to contain tandem copies of the target gene, one of which had the insert (data not shown). The obtention of similar target gene duplications requires at least two different rare events in *C. glutamicum* (22) and *E. coli* (17). This finding suggests that its appearance is associated with the selection of mutants that can survive only with a diploid state of the target gene. In the case of *asd*, *meso*-diaminopimelate, a necessary supplement for Asd-deficient strains, is not taken up by *C. glutamicum* (10). Polarity may be also relevant to this problem. In the case of *proB*, a 1.6-kbp insertion permitted the isolation of the expected *proB* mutant, though the insertion of 6.0 kbp at the same *proB* site produced a *proB* duplication of the type described (one event tested).

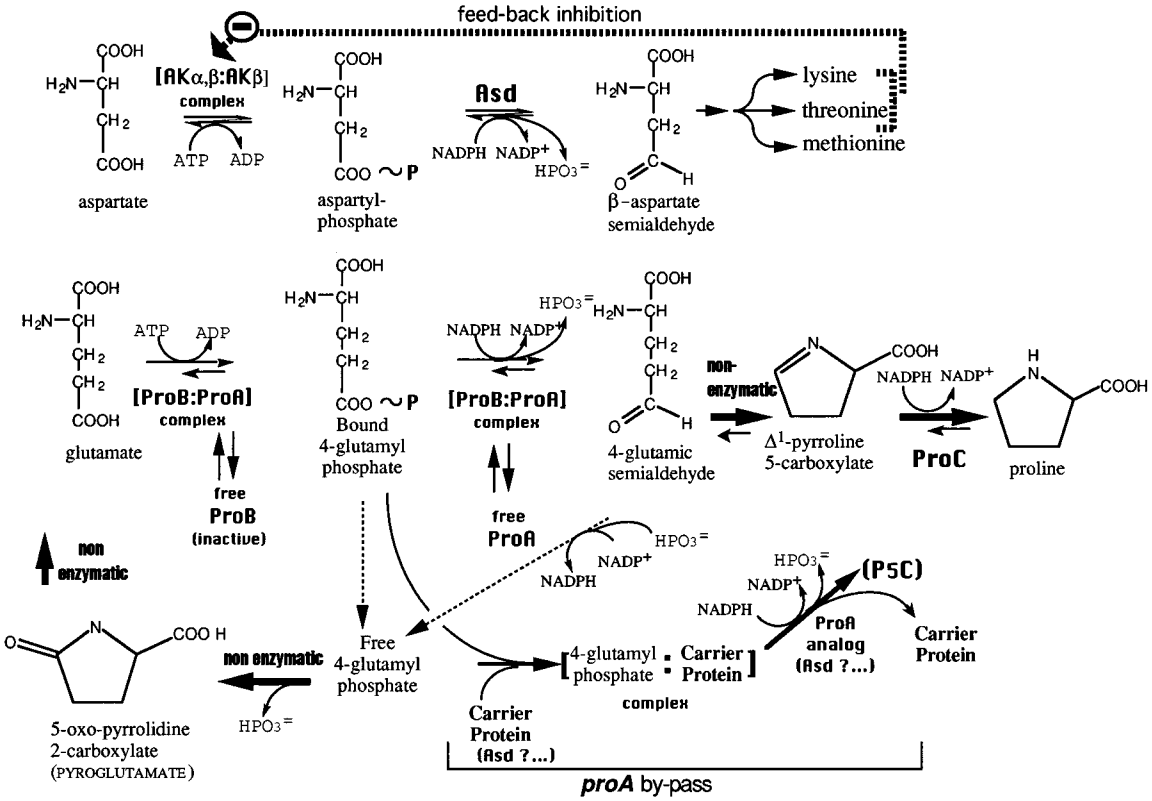

FIG. 2. Possible proline biosynthetic pathways in *C. glutamicum*. Upper row, first three reactions of the lysine pathway; middle row, consensus proline biosynthesis pathway (1); bottom row, fate of free 4-glutamyl-phosphate (left) and presumed *C. glutamicum proA* bypass (right). Note the similarity of the Asd substrates with 4-glutamyl-phosphate and 4-glutamyl-semialdehyde (34).

DISCUSSION

Three *C. glutamicum* genes that complement *E. coli* proline-deficient *proB*, *proA*, and *proC* mutants also determine critical steps of the major proline biosynthetic pathway (references 1 and 23 and this work). The analogy between the proline biosynthetic pathway of *C. glutamicum* and that of other bacteria is supported by sequence comparisons (not shown), indicating a consistency between the phylogenetic position of the predicted *C. glutamicum proB*, *proA*, and *proC* products and the taxonomic position of this organism based on other results. For example, the actinomycetes *C. glutamicum* and *Mycobacterium leprae* have closely related ProC genes. The *C. glutamicum-M. leprae* homology extends to the regions upstream to *proC*. Conversely, *C. glutamicum* ProC shows an increasingly distant relationship with the ProC proteins of gram-positive enterobacteria, higher plants, and *Homo sapiens*. However, many characteristics of *proB*, *proA*, and *proC* mutants indicate that the *C. glutamicum* proline biosynthetic pathway is different from that of other organisms.

The null *proB2* allele is pleiotropic, affecting colony morphology and cell shape. These phenotypes are probably unrelated to proline biosynthesis, since they are not observed in other proline auxotrophic mutants. The participation of the ProB biosynthetic product 4-glutamyl-phosphate as a general phosphoryl donor has been proposed for *Bacillus subtilis* (27). A similar role in *C. glutamicum* could explain indirect effects of a *proB* mutation on *C. glutamicum* functions apparently unrelated to proline biosynthesis (septum formation, cell turgor, etc.).

An unusual feature (24) of *C. glutamicum* is that the *proB* and *proA* genes are separated (33). In this place is found a new gene (*unk*) of unknown activity. An analysis (18) of the functional domains of the predicted *unk* product (not shown) suggests a close relationship to a family of dehydrogenases that interconvert D-2-hydroxy acids and the corresponding 2-keto acids (4). The separation of *proB* and *proA* genes has been taken as indicative of their independent expression (24). However, we cannot exclude the possibility of a coordinated expression of the *proB-unk-proA* cluster, perhaps related to an unspecified *unk* role in proline metabolism. Potential substrates for an enzyme with the expected Unk specificity occur in bacterial 4-hydroxyproline catabolism (1).

C. glutamicum naturally bypasses the ProA step (Fig. 2). The ProB and ProC steps are required for successful bypass, since *proB* and *proC* mutants are auxotrophic. This is a unique feature of *C. glutamicum*, since all known alternative proline biosynthetic pathways (1, 12, 23) are independent of both the ProB and the ProA steps or require an increased gene dosage of specific genes. The efficiency of this bypass can be improved by complementation with the plasmid-borne *C. glutamicum lysC*β-*asd* region. In *E. coli*, multicopy suppression of *proA* mutants by *asd* also requires active *proB* and *proC* genes (34). Thus, the conversion of 4-glutamyl-phosphate to 4-glutamyl-semialdehyde by the *proA* bypass could be a consequence, devoid of biological meaning, of the similarity between the Asd and ProA reactions (34). However, such a limited view leaves unresolved two paradoxes. (i) The production of the short-lived 4-glutamyl-phosphate by ProB must be tightly coupled to its efficient utilization by other enzymes (23, 34). The *C. glutamicum proA* bypass should account for this coupling. (ii) The

purified ProB protein is enzymatically inactive but recovers its activity by addition of ProA (20). This finding raises the question of the identity of the protein(s) other than ProA that activates ProB during the function of the *proA* bypass. Both paradoxes could be answered by the single postulate of a carrier protein which, during a transient specific interaction with ProB, both binds 4-glutamyl-phosphate and activates its synthesis.

The biosynthetic ProB activity was undetectable in *C. glutamicum* extracts (Table 3). Low amounts of an activity amplified by an increased *proA* gene dosage, and thus associated with the reverse ProA reaction, were found in cell extracts. The assumption of an unusually tight coupling between substrate utilization and synthesis for both reactions (Fig. 2) may explain both our failure in measuring the ProB substrate 4-glutamyl-phosphate and in showing a phosphate dependence for the reverse ProA reaction. Our attempts to measure the coupled conversion of glutamate into P5C in cell extracts were also unsuccessful. Similar results were found under a variety of extraction conditions (not shown) that permitted the detection of relatively high levels of biosynthetic ProC activity in *C. glutamicum* cell extracts. This finding suggests that either the ProB and ProA activities are unstable or both enzymes determine the rate-limiting steps of proline biosynthesis.

The effects of increased *proA* gene dosage in the *proC16* mutant are (i) an increase in reverse ProA activity and (ii) a decrease of P5C secretion. If P5C production is limited solely by the net ProA biosynthetic activity, an increased dosage of ProA would determine a parallel increase of P5C secretion. Conversely, if P5C production is limited solely by the net ProB biosynthetic activity, an increased dosage of ProA would not increase P5C secretion. Instead, the actual effect of an increased *proA* dosage is reducing P5C secretion, which is consistent with a net increase in reverse ProA activity. These considerations suggests that the ProA/ProB ratio has a critical role in determining P5C levels.

Though strains with *proB-proA* deletions are the most frequent type of *E. coli* proline auxotrophs, single *proA*, *proB*, and *proC* mutants are known (33). Four *C. glutamicum* mutagen-induced and transposon-induced auxotrophs contained at least a *proC* mutation. The rare occurrence of induced *proA*, *proB*, and Δ(*proBA*) mutants is not surprising, since bradytrophic *proA* mutants are difficult to detect, and *proB* mutants are pleiotropic, which may result in an enhanced sensitivity to mutagens. Deletions involving both *proB* and *proA* would also delete *unk* (Fig. 1), which may result in a growth disadvantage. Two auxotrophs (strains SA2 and SA3) contained, in addition to the *proC* mutation, other mutations that reduced or abolished P5C synthesis. Though deficient in P5C production, strains SA2 and SA3 retain significant levels of ProA-like activity. P5C accumulation, the primary phenotype of *proC* mutants, is toxic in *Aspergillus nidulans* (3). A similar toxicity of P5C in *C. glutamicum* would explain why P5C-deficient mutants frequently occur in *proC* cultures. Further characterization of this phenomenon is in progress.

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