Multiple Genes for the Last Step of Proline Biosynthesis in *Bacillus subtilis*

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The complete *Bacillus subtilis* genome contains four genes (*proG, proH, proI,* and *comER*) with the potential to encode Δ^1-pyrroline-5-carboxylate reductase, a proline biosynthetic enzyme. Simultaneous defects in three of these genes (*proG, proH,* and *proI*) were required to confer proline auxotrophy, indicating that the products of these genes are mostly interchangeable with respect to the last step in proline biosynthesis.

The pathway of proline synthesis from glutamate, the most common mechanism of proline biosynthesis, comprises three enzymatic steps (Fig. 1). The corresponding genes of *Escherichia coli*, *proB, proA,* and *proC,* encode γ-glutamyl kinase, γ-glutamyl phosphate reductase, and Δ^1-pyrroline-5-carboxylate (P5C) reductase, respectively (21). The *proBA*-dependent pathway of proline synthesis was shown to function also in *Bacillus subtilis*; mutations within the *proBA* locus cause auxotrophy for proline (8, 29). While *B. subtilis* has a single *proA*-like gene, a second *proB*-like gene, *proJ* of the *proHJ* locus (B. R. Belitsky and A. L. Sonenshein, GenBank accession number AF006720) has been found. In a manner unique to this bacterium, either ProB-like enzyme can provide enough γ-glutamyl kinase activity to support growth in the absence of exogenous proline (unpublished results). Apparently, previously described mutations to auxotrophy in the *proBA* locus either affect *proA* or are *proB* alleles that are polar on *proA* expression. No *proC* mutant of *B. subtilis* has been described, and four genes have the potential to encode ProC-like proteins with P5C reductase activity: *proH* (also called *orf257* and *proC*), *comER* (also called *comED*), *proI* (also called *yqjO*), and *ykeA* (here renamed *proG*) (1, 14, 20). The four genes are located at 172.3°, 225.5°, 211.2°, and 116.1° on the chromosomal map (http://genolist.pasteur.fr/SubtiList [26]) and code for proteins of 271, 273, 278, and 272 amino acids, respectively (the originally reported coding region of *proH* [1, 20] was extended by resequencing the *proH* 3′ end [GenBank accession number AF006720]). ProH and ProI are 42% identical to each other and up to 35% identical to many other P5C reductases from bacteria, archaea, and eukaryotes. ProG and ComER have more limited similarity to other P5C reductases and to each other. The functions of the four *B. subtilis* genes are not known. In this work we sought to identify the gene(s) responsible for the last step of proline biosynthesis.

Construction and properties of a *proG* (*ykeA*) null mutant. To create pBB1081, the 1.56-kb *Pvu*II-*Eco*RI fragment from pCM103 (23) containing most of the *proG* gene and the 5′ end of the *dppA* gene was cloned between the *Pst*I (blunt-ended) and *Eco*RI sites of pJPM1, a derivative of pBS (Stratagene) containing a chloramphenicol resistance marker (27). Methods for plasmid isolation, agarose gel electrophoresis, use of restriction and DNA modification enzymes, DNA ligation, PCR, Southern hybridization with digoxigenin-labeled DNA probes, and electroporation of *E. coli* JM107 or DH5α cells were as described by Sambrook et al. (30). DNA and protein sequences were analyzed using the DNA Strider (22) or BLAST (2) programs. A deletion-insertion mutation within the *proG* gene was created by replacing the 0.10-kb *Pst*I-*Sac*I fragment of pBB1081 with a 1.43-kb *Pst*I-*Sac*I *ble* cassette determining resistance to phleomycin, excised from pJPM136 (6). The orientation of the *ble* gene in the resulting Δ*proG*::*ble* plasmid, pBB1082, coincides with that of the *proG* gene. pBB1082 was introduced into *B. subtilis* SMY, and phleomycin-resistant, chloramphenicol-sensitive transformants, arising from double-crossover homologous recombination events, were selected. Growth of *B. subtilis* cells, transformation by chromosomal or plasmid DNA, and isolation of chromosomal DNA were as described previously (6). The replacement of the chromosomal *proG* gene by the Δ*proG*::*ble* allele in strain BB1951 was confirmed by comparing sizes of the PCR products from the wild-type and mutant *proG* chromosomal loci. Strain BB1951 (*proG*::*ble*) had the growth characteristics of a wild-type strain in the presence and absence of proline.

Construction and properties of a *proH* null mutant. The 0.27-kb *Eco*RI-*Pst*I 3′-end fragment of the *proH* gene was subcloned in several steps from pLS23-17 (7) between the *Eco*RI and *Pst*I sites of pBB544, a derivative of pBluescript SK(−) (Stratagene) containing a neomycin resistance marker (5). The resulting plasmid, pBB575, was integrated via a single-crossover recombination event into the chromosome of *B. subtilis* strain SMY at the *proH* locus. To clone DNA adjacent to the site of integration of pBB575, the chromosomal DNA of the resulting strain was digested with *Hin*dIII, self-ligated, and introduced by electroporation into *E. coli* cells. The isolated plasmid, pBB576, had a 1.32-kb insert of chromosomal DNA carrying most of *proH*. A deletion-insertion mutation within the *proH* gene was created by replacing the 0.55-kb *Bcl*I-*Eco*RI

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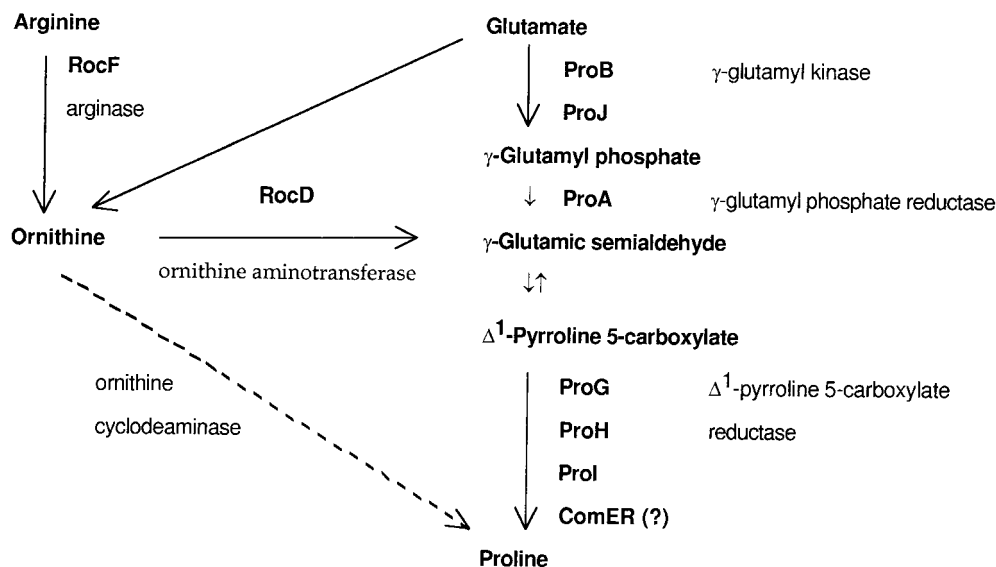
FIG. 1. Pathways of proline biosynthesis in *B. subtilis*. The major pathway of proline synthesis from glutamate is shown as a descending series of reactions. Some proline is also apparently synthesized from glutamate via ornithine (an intermediate in arginine synthesis) by the action of the RocD product (unpublished results). Since no ortholog of *E. coli* ArgE protein is present in *B. subtilis,* conversion of *N*-acetylglutamic γ-semialdehyde (an intermediate in arginine synthesis) to γ-glutamic semialdehyde (21) apparently does not occur. Details of the anabolic pathway from glutamate to ornithine and the catabolic reaction from γ-glutamic semialdehyde to glutamate have been omitted. The catabolism of citrulline includes its conversion to ornithine (unpublished data) but has not been characterized further. Ornithine cyclodeaminase (dashed line) is not present in *B. subtilis*. The *proG* and *proI* genes have been previously known as *ykeA* and *yqjO,* respectively.

fragment of pBB576 with a 1.9-kb *Bam*HI-*Eco*RI *tet* cassette, excised from pBEST307 (17). The orientation of the *tet* gene in the resulting plasmid, pBB734, coincides with that of the *proH* gene. Strain BB286 (*proH*::*tet*) was constructed as described above for strain BB734, using pBB734 and selecting for tetracycline-resistant, neomycin-sensitive transformants. Strain BB286 (*proH*::*tet*) had the growth characteristics of a wild-type strain in the presence and absence of proline.

Construction and properties of a *proI* (*yqjO*) null mutant. The 1.85-kb *'yqjP proI yqjN'* chromosomal region was amplified by PCR using custom synthesized oligonucleotides as primers. To create pJS18, the internal 1.62-kb *Cla*I-*Nsi*I fragment of the PCR product, including the entire *proI* gene and the flanking regions of the *yqjP* and *yqjN* genes, was cloned in pBluescript SK(−) (Stratagene), cleaved with *Cla*I and *Pst*I. For construction of pJS20 (Δ*proI*::*spc*), the 0.25-kb *Bcl*I-*Stu*I fragment of pJS18 that is internal to *proI* was replaced with the 1.3-kb *Bam*HI-*Xba*I (filled-in) fragment, excised from plasmid pRMK65 (18), which contains the *spc* gene. The orientation of the *spc* gene in this construction is opposite to that of the *proI* gene. Strain JSB9 (Δ*proI*::*spc*) was isolated by transformation of strain JH642 (*trpC2 pheA1*) (obtained from J. Hoch) with *Bam*HI-linearized DNA of pJS20. Spectinomycin-resistant transformants were selected, and the correct double-crossover integration event was verified by Southern hybridization using the *Sal*I-*Not*I fragment of pJS18 as a probe. Strain JSB9 (*proI*::*spc*) had the growth characteristics of a wild-type strain in the presence and absence of proline.

Construction and properties of multiple mutants. *comER* mutants were constructed previously and shown to be prototrophic (14, 16). Strains containing all possible combinations of two or three mutations in the *proG, proH, proI*, and *comER* genes and the corresponding quadruple mutant were constructed by transformation of strain SMY with chromosomal DNAs from appropriate mutants. The Pro phenotype of some of the double and triple mutants is shown in Table 1. The growth rate in glucose-ammonia medium of any of the double mutants was identical to the growth rate of a wild-type strain. No contribution of the *comER* gene to the cells' ability to grow without proline was detected for any of the mutants. The *proG proH proI* triple mutant required proline for growth, demonstrating that the P5C reductase enzymes are in fact required for proline synthesis in *B. subtilis* as in other organisms but that

TABLE 1. Growth of mutant strains in minimal medium[a]

Strain	Genotype	Nitrogen source	
		Ammonium	Arginine ± ammonium
SMY	Wild type	+	+
BB1954	*proG proH*	+	+
BB1960	*proG proI*	+/−	+/−
BB1969	*proH proI*	+	+
BB1973	*proG proH proI*	−	−
BB1980	*proB proG proH*	−	+
BB1979	*proB proG proI*	−	+/−
BB1978	*proB proH proI*	−	+
BB1983	*proB proG proH proI*	−	−

[a] Strains were grown at 37°C in liquid TSS minimal medium (12) with 0.5% glucose as a carbon source and 0.2% nitrogen source. The + sign indicates the generation time of 55 to 65 min in ammonium or arginine medium. The − sign indicates no cell growth. The +/− sign indicates a long lag period before initiation of growth. The *proB*::*cat* mutation, which is apparently polar on the expression of the downstream *proA* gene, was described previously (29). Strains carrying this mutation are not able to use the glutamate pathway of proline synthesis and therefore cannot grow in ammonium medium.

this function can be taken over by any one of three proteins, ProG, ProH, or ProI. In some experiments the *proG proI* mutant exhibited a long lag period before initiating growth in minimal medium without proline. This effect probably reflects the need for *proH* to be induced in order to provide enough enzyme to support proline synthesis. We cannot exclude the possibility that spontaneous mutations which elevate expression of *proH* accumulate in the culture of the *proG proI* mutant.

Role of *proC*-like genes in proline generation through the arginase pathway. In addition to the anabolic pathway of proline synthesis from glutamate, two catabolic pathways can lead to proline from ornithine; neither of these pathways requires the first two steps of proline synthesis from glutamate (Fig. 1). *B. subtilis* does not contain any gene that could code for ornithine cyclodeaminase (cyclase) (20, 28, 31) but has a well-characterized arginase pathway for arginine degradation (11). In that pathway, ornithine aminotransferase, the product of the *rocD* gene, generates γ-glutamic semialdehyde, a substrate of P5C reductase, from ornithine (Fig. 1) (4, 13). *B. subtilis* cells are able to utilize extracellular ornithine or the related amino acids arginine and citrulline as sources of proline (8), and they failed to do so in a *rocD* mutant, indicating that the arginase pathway is essential for proline generation under these conditions (Fig. 1). Formation of proline from ornithine, arginine or citrulline was also dependent on the presence of *proG*, *proH*, or *proI*, the same genes that can support proline synthesis from glutamate. The *proG proI* double mutant, whose only P5C reductase is encoded by *proH*, again had a small growth defect under these conditions (Table 1). To ensure that no proline was derived from glutamate, which can be formed from ornithine and related amino acids, we introduced a *proB::cat* mutation into our strains and confirmed the requirement for either *proG*, *proH*, or *proI* (Table 1). Thus, *proG*, *proH*, or *proI* is essential for proline formation, both through the glutamate pathway and through the arginase pathway.

Possible roles of multiple P5C reductases. Participation of at least three P5C reductase isoenzymes in the last step of proline synthesis is unique to *B. subtilis* among characterized organisms and may reflect specialized functions or regulation or both. Multiple genes with potential to code for P5C reductase isoenzymes have been detected in the genomes of the gram-positive bacteria *Bacillus halodurans* (32), *Bacillus anthracis* (http://www.tigr.org), *Enterococcus faecalis* (http://www.tigr.org), and *Clostridium difficile* (http://www.sanger.ac.uk) and the gram-negative bacterium *Pseudomonas putida* (http://www.tigr.org), but their functions have not been verified.

Though we could not detect a unique role for ProG, ProH, or ProI in proline synthesis from either glutamate or arginine, it is possible that such a role exists under some physiological conditions. Transcription of the *proBA* and *proI* genes is increased during proline limitation (unpublished data) and seems to be regulated by a termination-antitermination control mechanism, the T-box system (15); both *proBA* and *proI* contain 18-bp T-box-like sequences with the predicted proline specifier codons CCU and CCC (9). Coordinate induction of *proI* and *proBA* by proline limitation suggests that ProI is the major P5C reductase under such conditions. The *proHJ* locus, encoding enzymes for the first and the last steps of proline synthesis, is induced by high concentrations of salt (unpublished data), in keeping with the role of proline as the major endogenously produced osmoprotectant in *B. subtilis* (19, 25, 33). Finally, multiple P5C reductase isoenzymes may be involved in removal of excess P5C, which was reported to be toxic in *Aspergillus nidulans* (3) and in human cells (24) and has also been shown to be toxic for *B. subtilis* cells (unpublished data).

The role of the *comER* gene remains unknown (16). *comER* itself and its unusual overlapping, divergent orientation with respect to that of the *comEA-EB-EC* operon (14) are conserved in *B. halodurans*, *B. anthracis*, and *Bacillus stearothermophilus*, i.e., all *Bacillus* species for which sequencing information is available. *comER* expression decreases in stationary phase of growth in competence medium (14); no effect of *comER* mutations on cell competence was observed in earlier work (14, 16). The *comER* gene is at least partially under sporulation control, and its putative σ^E-dependent promoter has been identified (10). We could not detect any effect of *comER* mutations on sporulation efficiency in nutrient broth medium or in minimal medium either without proline or in the presence of a limiting amount of proline when the *proG proH proI* mutant was used.

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