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 $\Delta^1$-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 $\gamma$-glutamyl kinase, $\gamma$-glutamyl phosphate reductase, and $\Delta^1$-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, proD 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enzyme can provide enough $\gamma$-glutamyl kinase activity to support growth in the absence of exogenous proline (unpublished results).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 ygjG), and ykceA (here renamed proG) (1, 14, 20). The four genes are located at 172.3°, 221.5°, 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 (ykceA) null mutant.

To create pBB1081, the 1.56-kb PvuII-EcoRI fragment from pCM103 (23) containing most of the proG gene and the 5’ end of the dpyA gene was cloned between the PsI (blunt-ended) and EcoRI sites of pJP1, 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 DH5$\alpha$ 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 PsI-SacI fragment of pBB1081 with a 1.43-kb PsI-SacI ble cassette determining resistance to phleomycin, excised from pJPM136 (6). The orientation of the ble gene in the resulting $\Delta$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 was as described previously (6). The replacement of the chromosomal proG gene by the $\Delta$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 EcoRI-PstI 3’-end fragment of the proH gene was subcloned in several steps from pLS23-17 (7) between the EcoRI and PstI sites of pBS44, 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 HindIII, self-ligated, and introduced by electroporation into E. coli cells. The isolated plasmid, pBB576, had a 1.52-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 BclI-EcoRI
of two or three mutations in the trophic (14, 16). Strains containing all possible combinations of mutants were constructed previously and shown to be proline dependent in the presence and absence of proline. (spc::comER) had the growth characteristics of a wild-type strain in the presence and absence of proline. Integration of the PCR product, including the entire spc gene and the flanking regions of the yqiP and yqiN genes, was cloned in pBluescript SK(−) (Stratagene), cleaved with Clal and PstI. For construction of pJS20 (ΔproI::spc), the 0.25-kb BclI-StuI fragment of pJS18 that is internal to proI was replaced with the 1.9-kb BamHI-EcoRI 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 (yqiN) null mutant.** The 1.85-kb ‘yqiP proI yqiN’ chromosomal region was amplified by PCR using custom synthesized oligonucleotides as primers. To create pJS18, the internal 1.62-kb Clal-NsiI fragment of the PCR product, including the entire proI gene and the flanking regions of the yqiP and yqiN genes, was cloned in pBluescript SK(−) (Stratagene), cleaved with Clal and PstI. For construction of pJS20 (ΔproI::spc), the 0.25-kb BclI-StuI fragment of pJS18 that is internal to proI was replaced with the 1.3-kb BamHI-XbaI (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 (ΔproI::spc) was isolated by transformation of strain JH642 (trpC2 pheA1) (obtained from J. Hoch) with BamHI-linearized DNA of pJS20. Spectinomycin-resistant transformants were selected, and the correct double-crossover integration event was verified by Southern hybridization using the SalI-NoI 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

**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-acetylglutamate to γ-glutamylsemialdehyde (an intermediate in arginine synthesis) to γ-glutamylsemialdehyde (21) apparently does not occur. Details of the anabolic pathway from glutamate to ornithine and the catabolic reaction from γ-glutamylsemialdehyde 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 yqiG and yqiO, respectively.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Growth source</th>
<th>Nitrogen source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMY</td>
<td>Wild type</td>
<td>+</td>
<td>Arginine ± ammonia</td>
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<tr>
<td>BB1954</td>
<td>proG proH</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>+/−</td>
</tr>
<tr>
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<td>proH proI</td>
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<td>+</td>
</tr>
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<td>−</td>
</tr>
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<td>+</td>
</tr>
<tr>
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<td>+/−</td>
</tr>
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<td>+</td>
</tr>
<tr>
<td>BB1983</td>
<td>proB proG proH proI</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*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 proBcat 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 cycloleucinase (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-glutamyl 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 pro 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 pro contain 18-bp T-box-like sequences with the predicted proline specifier codons CCU and CCC (9). Coordinate induction of pro 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-ER-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 with proline or in the presence of a limiting amount of proline when the proG proH proI mutant was used.

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**REFERENCES**


