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Mutations in the *trpD* Gene of *Corynebacterium glutamicum* Confer 5-Methyltryptophan Resistance by Encoding a Feedback-Resistant Anthranilate Phosphoribosyltransferase

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The *trpD* gene from tryptophan-hyperproducing *Corynebacterium glutamicum* ATCC 21850 was isolated on the basis of its ability to confer resistance to 5-methyltryptophan on wild-type *C. glutamicum* AS019. Comparative sequence analysis of the genes from the wild-type AS019 and ATCC 21850 *trpD* genes revealed two amino acid substitutions at the protein level. Further analysis demonstrated that the *trpD* gene product from ATCC 21850, anthranilate phosphoribosyltransferase, was more resistant to feedback inhibition by either tryptophan or 5-methyltryptophan than its wild-type counterpart. It is proposed that phosphoribosyltransferase insensitivity to tryptophan in ATCC 21850 contributes to an elevated level of tryptophan biosynthesis.

Classically, strains hyperproducing amino acids have been selected as mutants resistant to normally lethal concentrations of amino acid analogs. Such resistance emanates from the elevated expression of certain genes or the deregulated activity of enzymes involved in amino acid biosynthesis (12). Tryptophan-hyperproducing *Corynebacterium glutamicum* ATCC 21850 was isolated from a tyrosine and phenylalanine double auxotroph on the basis of its resistance to analogs of all three aromatic amino acids, including the tryptophan analog 5-methyltryptophan (5MT) (4). Here, we report on the identification of mutations that result in resistance to 5MT in this strain.

The tryptophan genes from *C. glutamicum* ATCC 21850 have previously been cloned on a 14.5-kb *Bam*HI fragment and shown to confer resistance to 5MT and 6-fluorotryptophan on *Escherichia coli* (5). To isolate the locus responsible for resistance to 5MT, subcloning from the 14.5-kb *Bam*HI fragment into plasmid pBGS8 (13) was performed (Fig. 1). The recombinant plasmids generated were tested for their ability to confer resistance to 5MT on both *E. coli* JM109 and wild-type *C. glutamicum* AS019 (16) by the zone of inhibition method previously described (5). Vector pBGS8 used in the transformations replicates in *E. coli* but not in *C. glutamicum*. Maintenance of pBGS8 derivatives in *C. glutamicum* is facilitated only by homologous recombination of pBGS8 into the chromosome, which occurs via a single crossover between the cloned *C. glutamicum* DNA on the plasmid and its complementary region on the chromosome. Electroporation into *C. glutamicum* AS019 was performed according to the method of Dunican and Shivnan (3).

A 5-kb *Bgl*II fragment encompassing the *trpE*, *trpG*, and *trpD* genes (pOG1) (Fig. 1) was able to confer resistance to 5MT in both *E. coli* JM109 and *C. glutamicum* AS019. A 1.7-kb *Pst*I-*Bgl*II fragment (pOG7) (Fig. 1) retained the ability to confer resistance to 5MT on *C. glutamicum* AS019 only and not on *E. coli*. This 1.7-kb fragment contained the entire *trpD* gene, which encodes the second-step tryptophan biosynthetic enzyme, anthranilate phosphoribosyltransferase (PRT), and part of the *trpC* gene. Integration of pOG7 at the *trpD* locus does not result in tryptophan auxotrophy because of the ability of both the *lac* promoter on the plasmid and the constitutive internal *trp* operon promoter (located upstream of the *trpB* and *trpA* genes) to express the downstream tryptophan genes (9). The inability of the *trpD* gene from pOG7 to confer resistance to 5MT on *E. coli* may stem from the different aggregation properties of the *E. coli* and *C. glutamicum* PRT enzymes (10, 14). Whereas the *trpD* gene alone in *E. coli* does not increase resistance to 5MT, when the *trpE*, *trpG*, and *trpD* genes from *C. glutamicum* are introduced together, they appear to act independently of their *E. coli* counterparts and thus confer a dominant phenotype.

To test whether resistance to 5MT was linked simply to the introduction of a second copy of the *trpD* gene into the wild-type host, we introduced the wild-type *trpD* gene from AS019 back into the same strain. For this experiment, a 1.7-kb fragment containing the *trpD* gene was amplified by PCR from AS019 genomic DNA. Introduction of the cloned wild-type gene into AS019 was not accompanied by any increase in resistance to 5MT.

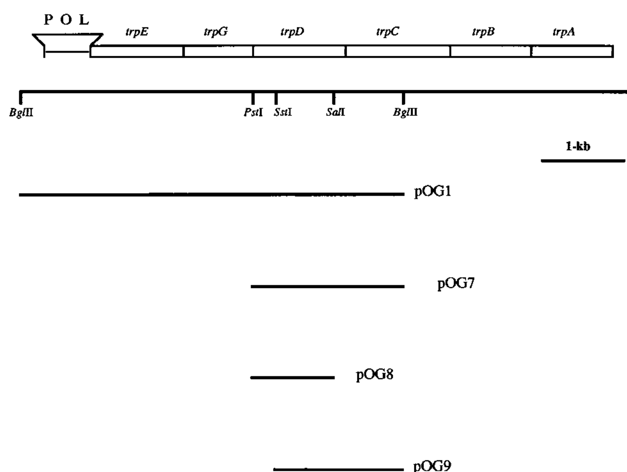

FIG. 1. Locations of subclones encompassing the *trpD* gene from *C. glutamicum* ATCC 21850 relative to the entire tryptophan operon.

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ATG ACT TCT CCA GCA ACA CTG AAA GTT CTC AAC GCC TAC TTG GAT AAC CCC ACT CCA ACC CTG GAG GAG GCA  72
 M   T   S   P   A   T   L   K   V   L   N   A   Y   L   D   N   P   T   P   T   L   E   E   A
ATT GAG GTG TTC ACC CCG CTG ACC GTG GGT GAA TAC GAT GAT GTG CAC ATC GCA GCG CTG CTT GCC ACC ATC  144
 I   E   V   F   T   P   L   T   V   G   E   Y   D   D   V   H   I   A   A   L   L   A   T   I
CGT ACT CGC GGT GAG CAG TTC GCT GAT ATT GCC GGC GCT GCC AAG GCG TTC CTC GCG GCG GCT CGT CCG TTC  216
 R   T   R   G   E   Q   F   A   D   I   A   G   A   A   K   A   F   L   A   A   A   R   P   F
CCG ATT ACT GGT GCA GGT TTG CTA GAT TCC GCT GGT ACT GGT GGC GAC GGT GCC AAC ACC ATC AAC ATC ACC  288
 P   I   T   G   A   G   L   L   D   S   A   G   T   G   G   D   G   A   N   T   I   N   I   I
ACC GGC GCA TCC CTG ATC GCA GCA TCC GGT GGA GTG AAG CTG GTT AAG CAC GGC AAC CGT TCG GTG AGC TCC  360
 T   G   A   S   L   I   A   A   S   G   G   V   K   L   V   K   H   G   N   R   S   V   S   S
AAG TCC GGC TCC GCC GAT GTA CTG GAA GCG CTG AAT ATT CCT TTG GGC CTT GAT GTG GAT CGT GCT GTG AAG  432
 K   S   G   S   A   D   V   L   E   A   L   N   I   P   L   G   L   D   V   D   R   A   V   K
                 T                                                   A
TGG TTC GAA GCG TCC AAC TTC ACC TTC CTG TTC GCA CCT GCG TAC AAC CCT GCG ATT GCG CAT GTG CAG CCG  504
 W   F   E   A   S   N   F   T   F   L   F   A   P   A   Y   N   P   A   I   A   H   V   Q   P
                 F                                                   E
GTT CGC CAG GCG CTG AAA TTC CCC ACC ATC TTC AAC ACG CTT GGA CCA TTG CTG TCC CCG GCG CGC CCG GAG  576
 V   R   Q   A   L   K   F   P   T   I   F   N   T   L   G   P   L   L   S   P   A   R   P   E
CGT CAG ATC ATG GGC GTG GCC AAT GCC AAT CAT GGA CAG CTC ATC GCC GAG GTC TTC CGC GAG TTG GGC CGT  648
 R   Q   I   M   G   V   A   N   A   N   H   G   Q   L   I   A   E   V   F   R   E   L   G   R
ACA CGC GCG CTT GTT GTG CAT GGC GCA GGC ACC GAT GAG ATC GCA GTC CAC GGC ACC ACC TTG GTG TGG GAG  720
 T   R   A   L   V   V   H   G   A   G   T   D   E   I   A   V   H   G   T   T   L   V   W   E
CTT AAA GAA GAC GGC ACC ATC GAG CAT TAC ACC ATC GAG CCT GAG GAC CTT GGC CTT GGC CGC TAC ACC CTT  792
 L   K   E   D   G   T   I   E   H   Y   T   I   E   P   E   D   L   G   L   G   R   T   T   L
GAG GAT CTC GTA GGT GGC CTC GGC ACT GAG AAC GCC GAA GCT ATG CGC GCT ACT TTC GCG GGC ACC GGC CCT  864
 E   D   L   V   G   G   L   G   T   E   N   A   E   A   M   R   A   T   F   A   G   T   G   P
GAT GCA CAC CGT GAT GCG TTG GCT GCG TCC GCA GGT GCG ATG TTC TAC CTC AAC GGC GAT GTC GAC TCC TTG  936
 D   A   H   R   D   A   L   A   A   S   A   G   A   M   F   Y   L   N   G   D   V   D   S   L
AAA GAT GGT GCA CAA AAG GCG CTT TCC TTG CTT GCC GAC GGC ACC ACC CAG GCA TGG TTG GCC AAG CAC GAA 1008
 K   D   G   A   Q   K   A   L   S   L   L   A   D   G   T   T   Q   A   W   L   A   K   H   E
GAG ATC GAT TAC TCA GAA AAG GAG TCT TCC AAT GAC TAG 1047
 E   I   D   Y   S   E   K   E   S   S   N   D   Z
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FIG. 2. Complete nucleotide sequence of the *C. glutamicum* AS019 *trpD* gene. The mutations which were identified in a comparison with the *C. glutamicum* ATCC 21850 *trpD* sequence and which result in amino acid substitutions are indicated above the nucleotides.

The nucleotide sequence of the ATCC 21850 and AS019 *trpD* genes was determined by the method of Sanger et al. (11) with a Pharmacia T7 Sequencing kit. DNA and deduced protein sequences were analyzed with the DNA Strider program. Sequence alignments were performed with the CLUSTAL program (6). Four base pair differences were identified, two of which translate into amino acid substitutions at positions 149 (serine to phenylalanine) and 162 (alanine to glutamic acid). A comparison of the TrpD sequences from AS019 and the very closely related organism *Brevibacterium lactofermentum* revealed only two differences, neither of which was at position 149 or 162, thus suggesting that mutations had not been inadvertently introduced during PCR of the wild-type gene. Protein sequences from both of these bacteria are very highly conserved, and a recent report proposed that *B. lactofermentum* be reclassified as *C. glutamicum* (8). The obtained sequence (1,047 bp) of the AS019 *trpD* gene is given in Fig. 2, with the amino acid substitutions in the ATCC 21850 sequence highlighted.

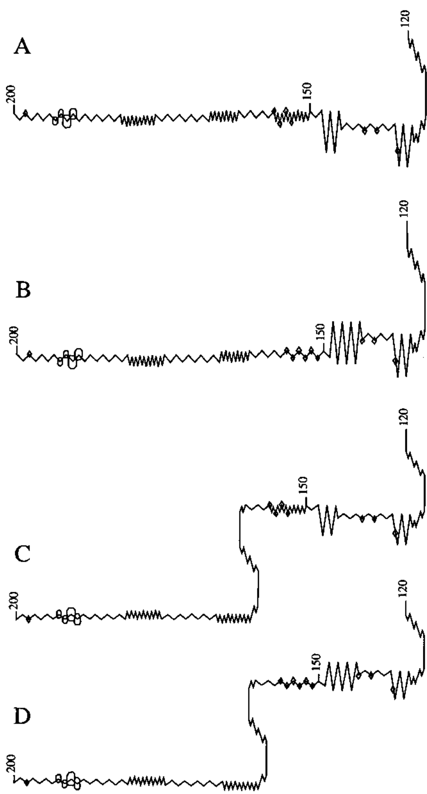

FIG. 3. Predicted secondary structures of the *C. glutamicum trpD* gene products between residues 120 and 200. (A) AS019 (wild type); (B) serine-to-phenylalanine substitution at position 149; (C) alanine-to-glutamic acid substitution at position 162; (D) ATCC 21850 (substitutions at positions 149 and 162). The diamonds and larger circles superimposed on the plots represent hydrophilic and hydrophobic amino acids, respectively.

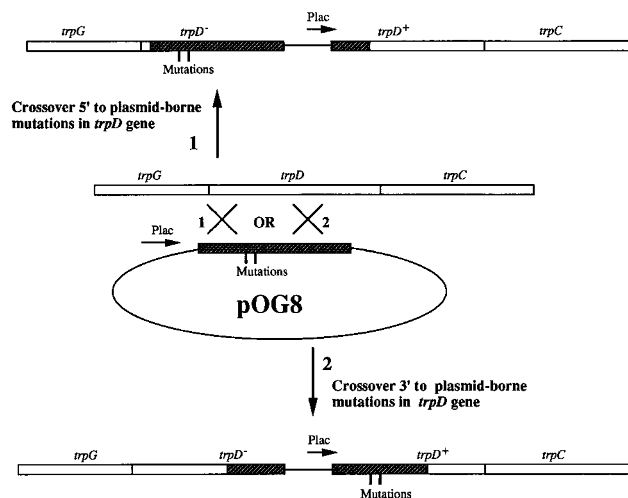

FIG. 4. Diagrammatic representation of two possible physical structures at the *trpD* locus following the integration of pOG8. If the crossover event occurs 5′ to the plasmid-borne mutations responsible for resistance to 5MT (crossover 1), then the mutations eventuate in the incomplete copy of the *trpD* gene on the chromosome and the strain remains sensitive to 5MT. However, if the crossover occurs 3′ to the mutations (crossover 2), then the mutations eventuate in the complete copy of the *trpD* gene and resistance to 5MT is conferred on the strain.

Analysis of the predicted *trpD* gene products from both strains revealed that the alanine-to-glutamic acid substitution at position 162 of the ATCC 21850 enzyme altered the secondary structure in that region, as could be predicted by the method of Chou and Fasman (2) (Fig. 3). Interestingly, the serine-to-phenylalanine substitution at position 149 does not affect the predicted secondary structure, thus pointing to a more significant role for the substitution at position 162 in the 5MT resistance phenotype. It may also be significant that the alanine residue at position 162 of the AS019 TrpD sequence is fully conserved in *Pseudomonas aeruginosa* and conservatively substituted in *E. coli* and *Bacillus subtilis*. There is no conservation of the AS019 serine residue at position 149 in any of these organisms.

Confirmation that the mutations in the *trpD* gene alone were responsible for resistance to 5MT was obtained when the 5′ 556 bp of the *trpC* gene were removed from the 1.7-kb *Pst*I-*Bgl*II clone in pOG7. When the resulting construct, pOG8 (Fig. 1), was introduced into AS019, it conferred resistance to 5MT on 60% of the integrants. The failure of 40% of the integrants to display resistance to 5MT may be explained by consideration of where the crossover between the ATCC 21850 *trpD* gene on pOG8 and its chromosomal AS019 homolog occurs (Fig. 4). Removal of the 356 bp at the 5′ end of the *trpD* gene also implicated the ATCC 21850 *trpD* gene in resistance to 5MT.

TABLE 1. Anthranilate PRT activities in crude extracts from strains AS019 and AS019::pOG7 in the presence and absence of tryptophan and 5MT[a]

Strain and condition	Anthranilate PRT activity (mU/mg)	Relative anthranilate PRT activity[b]
AS019	49	1
AS019 with Trp (0.83 mM)	11	0.22
AS019 with 5MT (0.32 mM)	12.5	0.25
AS019::pOG7	40	1
AS019::pOG7 with Trp (0.83 mM)	32	0.8
AS019::pOG7 with 5MT (0.32 mM)	24	0.6

[a] The assays were performed in triplicate over three experiments. Individual enzyme activities did not deviate from the mean by more than 15%.

[b] Anthranilate PRT activities without tryptophan and 5MT were defined as 1 for purposes of calculating relative activity.

Introduction of the generated construct, pOG9 (Fig. 1), into AS019 did not confer any increase in resistance to 5MT.

To investigate the activity of the *trpD* gene products from AS019 and ATCC 21850, PRT assays were performed on crude cell extracts of AS019 and AS019::pOG7 (AS019 carrying the integrated *trpD* gene from ATCC 21850) in the presence and absence of 5MT and tryptophan. The protein concentration was determined according to the method of Bradford (1), and PRT assays were performed according to the method of Sugimoto and Shiio (15). In a manner similar to that of its counterpart in *B. flavum* (15), the PRT enzyme from AS019 was sensitive to inhibition by tryptophan and 5MT. However, the ATCC 21850 PRT enzyme was 3.6-fold more active in the presence of 0.83 mM tryptophan and 2.4-fold more active in the presence of 0.32 mM 5MT than its wild-type counterpart (Table 1). In contrast, separate experiments revealed that anthranilate synthase activity from AS019 and AS019::pOG7 remained sensitive to feedback inhibition by tryptophan (0.25 mM) and 5MT (0.25 mM) (data not shown). This result suggested that as in *B. flavum* (15) and opposite to the situation with *E. coli* (10), the anthranilate synthase and PRT enzymes of *C. glutamicum* are functionally independent.

A recent report has described the requirement for a deregulated PRT enzyme in the molecular breeding of a tryptophan-hyperproducing *C. glutamicum* strain (7). Our observation that mutations in the *trpD* gene of ATCC 21850 by themselves result in increased resistance to 5MT corroborates this evidence, further emphasizing the significance of PRT in the regulation of tryptophan biosynthesis and its potential to become a metabolic bottleneck. The presence of a tryptophan-resistant form of PRT appears to contribute to a shift in the equilibrium of the *C. glutamicum* ATCC 21850 tryptophan pathway towards overproduction.

Nucleotide sequence accession number. The GenBank accession number for the *C. glutamicum* ATCC 21850 *trpD* sequence is U11545.

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