Two Single-Base-Pair Substitutions Causing Desensitization to Tryptophan Feedback Inhibition of Anthranilate Synthase and Enhanced Expression of Tryptophan Genes of *Brevibacterium lactofermentum*

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*Brevibacterium lactofermentum* is an industrially important gram-positive bacterium used for the production of various amino acids. We have focused our efforts on the tryptophan biosynthesis of this microorganism, because tryptophan is an important ingredient in medicines and animal feed as an essential amino acid.

Tryptophan biosynthesis in *Brevibacterium* spp. has been found to be regulated by two mechanisms, feedback inhibition by tryptophan of anthranilate synthase (AS) (6), anthranilate phosphoribosyltransferase (10), and tryptophan synthase (9) and repression by tryptophan of the synthesis of AS and the other tryptophan enzymes (8), as is the case for *Escherichia coli* and *Bacillus subtilis*. Furthermore, analysis of the nucleotide sequence of the *B. lactofermentum* trp operon revealed the presence of operator-attenuator-like sequences (3, 5). Thus, we have assumed that repression of the synthesis of tryptophan enzymes in *B. lactofermentum* is carried out by both the repressor-operator system and the attenuation system, as in *E. coli* (13). To breed improved tryptophan-producing strains, it is necessary to remove these regulatory mechanisms. For this purpose, we have isolated a 5-fluorotryptophan-resistant (4,000 μg/ml) spontaneous mutant from wild-type *B. lactofermentum* AJ12036. The spontaneous mutant, termed 1041, was revealed to have an altered AS, which was fully active even in the presence of 10 mM tryptophan, while the activity of the wild-type AS from AJ12036 under the same conditions was less than 1% of that in the absence of tryptophan. Furthermore, the specific activities of AS and anthranilate phosphoribosyltransferase in 1041 were 29- and 23-fold higher than those in parental strain AJ12036, respectively. Determination of the mutation site in 1041 should be useful in clarifying the molecular mechanisms of the regulation of tryptophan biosynthesis in *B. lactofermentum*.

Recombinant plasmids ptrPE97 and ptrPE36 carry the trp operon of 1041 and the 5'-proximal region of the trp operon of AJ12036, respectively (2) (Fig. 1). The DNA fragment of ptrPE97 containing the trpE, trpG, trpD, and trpC genes but no major trp promoter was inserted downstream of the lac promoter of pUC18 (4). The resulting plasmid, ptrPE101, conferred 5-fluorotryptophan resistance to *E. coli* JM109 (11). To detect the mutation site in 1041, we substituted the 5' half of the mutant trpE gene for the 5' half of the wild-type gene; a hybrid trpE gene was constructed by replacing the *XmnI-HindIII* fragment of ptrPE101 with the corresponding *XmnI-HindIII* fragment of the wild-type trpE gene cloned in ptrPE36 (Fig. 1). The resulting plasmid, ptrPE102, did not confer 5-fluorotryptophan resistance to JM109, suggesting that the mutation site exists in the *XmnI-HindIII* fragment within the trpE gene cloned in ptrPE97. Comparison of the nucleotide sequences of the *XmnI-HindIII* fragments of ptrPE97 and ptrPE36 (3) revealed only one adenine-to-cytosine single-base-pair substitution at codon Ser-38. The Ser codon (AGC) was changed to an Arg codon (CGC) (Fig. 2). This mutation was also confirmed by restriction enzyme digestion. One (AGCGCT) of the *HaeII* sites (PuGCAGPy) existing in the wild-type trpE gene was disrupted by this substitution. No other alteration was found in the trpE and trpG gene sequences. Therefore, we concluded that the substitution of Arg for Ser caused the desensitization to feedback inhibition by tryptophan of the AS in 1041. Furthermore, we found that the particular amino acid sequence from Leu-35 to Ser-38, Leu-Leu-Glu-Ser, in the *B. lactofermentum* trpE gene was conserved in the corresponding regions of *E. coli* (13) and *B. subtilis* (1). These data suggest that Ser-38 is essential and furthermore that the region Leu-Leu-Glu-Ser is essential for the allosteric regulation of AS. So far, we have very little information about the allosteric inhibition mechanism of this AS. However, we can suggest three possibilities: (i) Ser-38 may be the binding site for tryptophan; (ii) the difference in charge between Ser and Arg may influence the binding of tryptophan to AS; and (iii) the substitution of amino acid residues may cause a conformational change in AS.

It seemed unlikely that the mutation in the trpE gene also caused the elevated activities of AS and anthranilate phosphoribosyltransferase observed in 1041. It seemed unlikely that the mutation in the trpE gene also caused the elevated activities of AS and anthranilate phosphoribosyltransferase observed in 1041. Therefore, we have focused our efforts on the tryptophan biosynthesis of this microorganism, because tryptophan is an important ingredient in medicines and animal feed as an essential amino acid.

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phoribosyltransferase in 1041. We expected that there might be another mutation in the 5' control region, comprising the presumed promoter, operator, and attenuator regions (5) of the trp operon of 1041. Therefore, the nucleotide sequence of the 5' control region of the trp operon cloned in ptrpE97 was determined. Only one base pair change was found within the putative attenuator sequence (Fig. 2). This substitution (guanine to adenine) was also confirmed by restriction enzyme digestion. The sequence of 1041 had a newly created Stul site (AGGCCT), the result of the substitution of adenine for guanine. Owing to this substitution, the free energy of the stem structure of a predicted p-independent terminator formed within the putative attenuator in the presence of excess tryptophan was decreased from -18.0 to

FIG. 1. Detection of the mutation site in 5-fluorotryptophan-resistant strain 1041. The organization of the B. lactofermentum trp operon is indicated at the top. The open bars indicate the 1041-derived trp operon carried in plasmid ptrpE97. The filled bars indicate the DNA fragment from wild-type strain AJ12036 in ptrpE36. ptrpE101, a subclone of ptrpE97, contains the trpE, trpG, trpD, and trpC genes from ptrpE97. ptrpE102, a hybrid plasmid constructed from ptrpE101 and ptrpE36, is composed of the 4.8-kilobase-pair (kb) HindIII fragment of ptrpE97, which contains the trpG, trpD, and trpC genes, and the 640-base-pair XmnI-HindIII fragment of ptrpE36. The trp genes in both ptrpE101 and ptrpE102 were located downstream from the lac promoter of pUC18. Abbreviations: B, BamHl; H, HindIII; P, PsiI; X, XmnI; 5F, 5-fluorotryptophan.

FIG. 2. Nucleotide sequence of the 5'-proximal region of the trp operon of B. lactofermentum AJ12036. The 934-base-pair nucleotide sequence from the Alul site through the HindIII site is shown with several restriction enzyme cleavage sites in it. Asterisks, underlinings, and divergent arrows indicate the presumed ribosome-binding sites, promoter, and attenuator, respectively. The changes found in the nucleotide and amino acid sequences of the trpE gene of 1041 are indicated by a large box. Another base substitution found in the attenuator region of 1041 is indicated by a small box.
functions in the predicted attenuator of the terminator matches into the (7, 12). The present data strongly suggest that the putative attenuator predicted from the analysis of the nucleotide sequence of the B. lactofermentum AJ12036 trp operon (5) functions in vivo.

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FIG. 3. Predicted secondary structures formed in the putative attenuator of B. lactofermentum AJ12036 and mutant 1041. (a) Attenuator structure of the wild-type trp operon in the presence of excess tryptophan. (b) Defective attenuator structure of the mutant 1041 trp operon under the same conditions as in panel a. Arrows indicate the mutation site.

−12.4 kcal (ca. −75.3 to −51.9 kJ)/mol. This mutation may disrupt the terminator conformation (Fig. 3). In the case of the E. coli trp operon, the introduction of base pair mismatches into the attenuator destabilizes the terminator structure and caused high levels of readthrough transcription (7, 12). The present data strongly suggest that the putative attenuator predicted from the analysis of the nucleotide sequence of the B. lactofermentum AJ12036 trp operon (5) functions in vivo.

