Construction of a Promoter-Probe Vector for a Bacillus subtilis Host by Using the trpD\(^+\) Gene of Bacillus amyloliquefaciens

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The trp gene cluster of Bacillus amyloliquefaciens was found to be structurally similar to that of the Enterobacteriaceae. The translation termination codon of the putative trpE gene and the initiation codon for the putative trpD gene overlap at the trpE-trpD junction, and a promoter for the putative trpC gene is suggested to exist. A promoter-probe vector of Bacillus subtilis, pFTB281, was constructed with a DNA fragment of B. amyloliquefaciens, complementing the trpC and trpD mutations of B. subtilis, a 42-base-pair DNA fragment of M13mp7, and the larger EcoRI-PvuII fragment of pUB110, which confers an autonomous replication function and the kanamycin-resistance phenotype to the chimeric plasmid. pFTB281 has BamHI, EcoRI, and SalI cloning sites in the 5'-upstream region of the protein-coding region of the putative trpD gene, and the insertion of a certain DNA fragment at any of these sites allowed the plasmid to transform a trpD mutant of B. subtilis to the His\(^+\) phenotype. DNA fragments showing the promoter function for the trpD gene were obtained from B. amyloliquefaciens and Saccharomyces cerevisiae chromosomes and p11 and \(\lambda\) phage DNAs, but rarely from the DNAs of Escherichia coli and pBR322.

Bacillus subtilis has emerged as a major organism for studies in genetics and molecular biology and for industrial application. The development of a molecular cloning system for this organism will further expand its usefulness. For this purpose, we constructed a nonintegrative cloning vector in the recombination-competent B. subtilis host with DNA fragments heterologous with the host chromosome, including a DNA fragment of Bacillus amyloliquefaciens (39). During the course of the construction, we cloned a DNA fragment of B. amyloliquefaciens which complemented the trpC2 mutation of B. subtilis. It was known that the order of the trp genes in B. subtilis is trpE-trpD-trpC-trpF-trpB-trpA (5) and that the gene cluster appears to constitute a transcriptional unit in the direction from trpE to trpA (28). The same gene order and transcriptional organization of the trp genes have been suggested for Bacillus licheniformis (12). Since the above arguments suggest that the individual genes lack their own promoters, we were interested in the possibility of construction of a promoter-probe vector (3, 9, 11, 38, 40) with the DNA fragment from B. amyloliquefaciens.

This communication deals with a DNA fragment of B. amyloliquefaciens that encodes the putative trpD gene. No DNA sequences homologous to the -35 or -10 consensus sequences of B. subtilis promoter were detected in the 5'-upstream region of the trpD gene, whereas the ATG codon of the trpD gene overlapped the TGA termination codon of the preceding open reading frame. The cloned trpD gene of B. amyloliquefaciens was able to complement the trpD mutation of B. subtilis on insertion of a certain DNA fragment in its 5'-upstream region, and various DNA fragments exhibiting promoter function were obtained from several DNA sources.

MATERIALS AND METHODS

Organisms and plasmids. The major microorganisms used in this study are listed in Table 1. In addition, B. subtilis strains 1A62 (trpA5), 1A63 (trpB4), 1A64 (trpD2), 1A65 (trpE26), 1S12 (trpF7 spo0A12), 1A83 (hisH2 aroB2), 1A117 (aroH1 pig-73), and 1A134 (aroF5), obtained from the Bacillus Genetic Stock Center, were used for complementation tests of cloned DNA fragments. Plasmid pUB110 prepared from B. subtilis M113 was used as a vector. The replicative form of phage M13mp7 DNA prepared from Escherichia coli JM103 (23) was used as a source of DNA carrying the BamHI and SalI sites. M13mp7 phage was purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. DNAs of plasmid pE194 prepared from B. subtilis 1E18 and pBR322 prepared from E. coli JA221 and phage p11 prepared from B. subtilis 168T1 and \(\lambda\) c1857 S7 (purchased from Takara Shuzo Brewing Co., Kyoto, Japan) were used for trial cloning of promoter sequences.

Media and cultivation conditions. For cultivation of Bacillus strains, nutrient media, brain heart infusion and Luria broth were prepared as described (39) with or without supplementation with kanamycin (5 \(\mu\)g/ml; Meiji Seika, Tokyo, Japan). Spizizen minimal medium (33) supplemented with Casamino Acids (2 mg/ml, for the vitamin assay; Difco Laboratories, Detroit, Mich.) was used for testing the tryptophan auxotrophic trait (T\(\)r) of B. subtilis. DM3 (7) and mR2 (29) media were used for the regeneration of B. subtilis protoplasts. In the case of DM3 medium, kanamycin was added at a concentration of 150 \(\mu\)g/ml (recommended by F. Kawamura and H. Saito, personal communication). All cultivations were performed at 37°C with or without shaking.

Preparation and analysis of DNA. Methods for preparation of bacterial and yeast DNAs, digestion with restriction enzymes, and analysis by agarose gel electrophoresis of DNA fragments were as described previously (39). Southern hybridization (32) of DNAs was carried out with \(^{32}\)P-end-labeled DNA (21) under the same moderately stringent conditions (65°C, 3 \(\times\) SSC [1 \(\times\) SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) as previously described (39). Small DNA
molecules were analyzed by polyacrylamide slab gel (7.5%; 15 by 18 by 0.3 cm) electrophoresis at 2 V/cm overnight in 89 mM Tris-borate buffer (pH 8.3) containing 2.5 mM EDTA. For estimation of the molecular size of DNA, λ c1857 S7 DNA digested with HindIII (34) and PBR322 DNA digested with Sau3A1 (31) were used as standards. The replicative form of M13mp7 phage DNA was prepared by the method of Messing et al. (24). Phage p11 was induced from a lysogenic strain of *B. subtilis* 168T1 with mitomycin C (Sigma Chemical Co., St. Louis, Mo.), and phage DNA was purified by the method of Kawamura et al. (19).

DNA sequencing was performed by the method of Maxam and Gilbert (21). The gels were autoradiographed with Kodak X-Omat RP film (Eastman Kodak Co., Rochester, N.Y.) at -80°C with intensifying screens (Eastman Kodak).

**Transformation.** Transformation of *B. subtilis* was carried out with protoplasts, and kanamycin resistance (Km') transformants were selected on DM3 medium after expression of the antibiotic resistance gene as described by Chang and Cohen (7).

**RESULTS**

Cloning and characterization of a DNA fragment of *B. amyloliquefaciens* complementing the *B. subtilis trpC* and *trpD* mutations. Chromosomal DNA of *B. amyloliquefaciens* V (ca. 3 μg/100 μl of reaction mixture) and pUB110 DNA (ca. 1 μg) were each digested simultaneously with EcoRI and XbaI, mixed, and ligated with T4 ligase. When cells of *B. subtilis* M1114 (*trpC2 r- m-*) were transformed with the ligation mixtures, many Km' transformants appeared on the DM3 medium. Seven Trp' colonies were isolated after replicating ca. 50,000 Km' transformants onto Spizizen minimal medium containing Casamino Acids, and plasmid DNAs were extracted from them by the rapid alkaline extraction procedure (4) and electrophoresed on an agarose gel. The extracted plasmids fell into four groups by size, which were separated by cesium chloride-ethidium bromide density gradient centrifugation. Three plasmids, pFTB232, pFTB233, and pFTB239, (Fig. 1) were isolated as representatives of three of the groups and were subjected to further study. For the remaining group, a single plasmid could not be isolated, as it was readily degraded to smaller molecular size. Since all of the Km' transformants of *B. subtilis* M1114 (*trpC2*) with pFTB232, pFTB233, and pFTB239 showed the Trp' phenotype, the plasmids bear a gene complementing the *trpC* mutation.

By digestion with EcoRI and XbaI, pFTB232 generated 1.2-, 2.9-, and 4.1-kilobase (kb) fragments; pFTB233 generated 0.5-, 2.9-, and 4.1-kb fragments; and pFTB239 generated 0.5-, 2.9-, 3.0-, and 4.1-kb fragments (data not shown). Since double digestion of pUB110 DNA with EcoRI and XbaI gives rise to 0.5- and 4.1-kb fragments, the 1.2- and 2.9-kb fragments of pFTB232, the 2.9-kb fragment of pFTB233, and the 2.9- and 3.0-kb fragments of pFTB239 might have originated from chromosomal DNA of *B. amyloliquefaciens* V. The 2.9-kb EcoRI fragment seems to bear the gene complementing the *trpC* mutation, because all three of the Trp' plasmids share this fragment (Fig. 1). To confirm that the 2.9-kb EcoRI fragment is a fragment of chromosomal DNA of *B. amyloliquefaciens* V, the chromosomal DNA was digested with EcoRI and electrophoresed on agarose gel in parallel with similar digests of *B. subtilis* M1114 and pFTB233. Fragments were transferred onto a nitrocellulose filter by Southern blotting and hybridized with the 32P-end-labeled 2.9-kb EcoRI fragment of pFTB233 as probe under moderately stringent conditions. One fragment of *B. amyloli-

**TABLE 1. Microorganisms used**

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<thead>
<tr>
<th>Microorganism and strain</th>
<th>Genotype</th>
<th>Source (reference)</th>
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<tr>
<td><em>Bacillus subtilis</em></td>
<td></td>
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<tr>
<td>M1113</td>
<td>arg-15 trpC2 r- m-</td>
<td>K. Sakaguchi</td>
</tr>
<tr>
<td>M1114</td>
<td>leu7C trpC2 r- m-</td>
<td>K. Sakaguchi</td>
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<td>RM125</td>
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<td>K. Sakaguchi (Uozumi et al. [36])</td>
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<tr>
<td>168T1</td>
<td>trpC2 thyA thyB [p11]</td>
<td>H. Saito (Farmer and Rothman [10])</td>
</tr>
<tr>
<td>JU2</td>
<td>leu-8 trpD2 r- m-</td>
<td>Constructed by congresional transformation of RM125 with the DNA of <em>B. subtilis</em> 1A64 (trpD2) obtained from Bacillus Genetic Stock Center</td>
</tr>
<tr>
<td>1E18</td>
<td>[pE194]</td>
<td>Bacillus Genetic Stock Center</td>
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<tr>
<td><em>Bacillus amyloliquefaciens</em></td>
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</tr>
<tr>
<td>V</td>
<td>Wild type</td>
<td>Daiva Kasei Co., Ltd., Osaka, Japan</td>
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<tr>
<td>F</td>
<td>Wild type</td>
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<td><em>Escherichia coli</em></td>
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<td>JM103</td>
<td>Δ(lac-pro) thi rpsL supE endA sbcB15 hsdR4 F' traD36 proAB ΔlacF' ZM15 Δlac leuB6 trpC930 rpsL hsdR- hsdM'</td>
<td>Bethesda Research Laboratories, Inc., Gaithersburg, Md. (23)</td>
</tr>
<tr>
<td>MC1065</td>
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<td>Our stock culture (Casadaban et al. [6])</td>
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<tr>
<td>JA221</td>
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<td><em>Saccharomyces cerevisiae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-28-24C</td>
<td>Wild type</td>
<td>Our stock culture</td>
</tr>
</tbody>
</table>

* The genetic symbols for *B. subtilis* and *E. coli* follow those of Henner and Hoch (13) and Bachmann (1), respectively, or those of the original authors listed in the table.
to complement other mutations, various trp, aro, and hisH2 mutants of B. subtilis were transformed with the above three plasmids. Km' transformants were selected, and the colonies were replicated onto appropriate test media for the auxotrophic traits. pFTB232 and pFTB233 were found to complement the trpD mutations, whereas pFTB239 could not (Fig. 1). None of the plasmids complemented the trpA, trpB, and trpE mutations (no data were obtained for a trpF mutation because no Km' transformants were obtained with the trpF mutant as the host) or the aroB, aroH, aroF, and hisH mutations, which flank the trp cluster on the B. subtilis chromosome (13).

To confirm the location of the trpC and trpD genes on pFTB233, various deletion plasmids were constructed by digesting pFTB233 with restriction endonucleases, and their complementation activities were tested. The results indicated that the protein-coding region of trpD is located in the 1.55-kb EcoRI-HindIII fragment within the 2.9-kb fragment and the trpC gene lies in the 1.0-kb BglII-PvuII fragment (Fig. 1). Since the molecular sizes of the trpD+ and trpC+ gene products of B. subtilis were estimated to be, respectively, 57,000 and 30,500 by gel filtration (15), a DNA fragment of ca. 1.55 kb is necessary for trpD and one of 0.83 kb is necessary for trpC. The DNA sizes observed in the deletion analysis agree with these estimates.

Plasmids pFTB232 and pFTB239 contain, respectively, a 1.2-kb XbaI-EcoRI fragment or a 3.0-kb EcoRI fragment, as indicated by the thick lines on Fig. 1. These fragments might originate from the B. amyloliquefaciens chromosome but be unrelated to the trp genes, being accidentally inserted at the site during the cloning. When the 3.0-kb EcoRI fragment of pFTB239 was eliminated by EcoRI digestion and self-ligation, the resultant plasmid could transform B. subtilis JU2 (trpD2) to the Trp+ phenotype. This result suggests that the 3.0-kb fragment inhibits the expression of the trpD' gene on pFTB239.

Plasmid pFTB255 (Fig. 1) was constructed by digestion of pFTB233 with EcoRI and self-ligation, transformation of B. subtilis MI114 (trpC2), and selection of TrpC+ transformants. It was confirmed that the 2.9-kb EcoRI fragment of pFTB255 was connected with pUB110 DNA in the reverse direction to that in pFTB233 by restriction analysis with BamHI and SalI (data not shown). Since pFTB255 has the same 2.9-kb fragment as pFTB233 and could complement the trpC mutation but not the trpD mutation, the 5'-upstream region of trpD in the 2.9-kb fragment must lack the promoter necessary for its expression in pFTB255, whereas the trpC gene must have an effective promoter region. The expression of trpD observed with pFTB233 is probably due to a promoter function of a DNA sequence in the PvuII-BamHI-XbaI-EcoRI region of pUB110 (Fig. 1) operating in the direction from the PvuII to EcoRI sites. This is also supported by the description by Williams et al. (38), who reported that plasmid pPL601, constructed by cloning an EcoRI fragment bearing the intact structural gene for chloramphenicol acetyltransferase of Bacillus subtilis at the same EcoRI
Fig. 2. Restriction map of pFTB281. The thin line indicates the DNA fragment originated from pUB110, the double line is the B. amyloliquefaciens DNA, and the 42-bp sequence illustrated outside the circle is a DNA fragment originated from replicative form DNA of phage M13mp7. The inner arcs with arrowheads at both ends indicate possible coding regions for the trpC+, trpD+, and kanamycin resistance (km') genes.

Site of pUB110, was unable to confer chloramphenicol resistance to the B. subtilis host, whereas the same DNA fragment of B. pumilus conferred high-level resistance to the host cells when it was connected with pUB110 at the same EcoRI site but in the reverse orientation of that in pPL601. The structure and expression characteristics of plasmids pCPP-3 and pCPP-4 that are described by Band et al. (3) are consistent with the above arguments.

Construction of a promoter-probe vector. Since the common 2.9-kb EcoRI fragment of pFTB232, pFTB233, and pFTB239 contains the complete coding sequence for the protein that complements the trpD mutation, but probably not its promoter, we attempted to construct a promoter-probe vector with this fragment. We constructed a plasmid, pFTB275, in which the 1.5-kb PvuI fragment bearing the possible promoter sequence was deleted from pFTB255 (Fig. 1). pFTB275 should have intact trpC+ and trpD+ genes but, like pFTB255, it expresses only trpC+. It also has a single EcoRI site, probably in the 5′-upstream region of the trpD+ gene. Cloning a DNA fragment of the B. amyloliquefaciens F chromosome into the EcoRI site of pFTB275 allowed the chimeric plasmids to transform B. subtilis JU2 (trpD2) to the TrpD+ phenotype (data not shown). This suggested that insertion of a certain DNA fragment at the EcoRI site of pFTB275 might allow expression of the trpD+ gene.

To give a variety of cloning sites to pFTB275, the 42-base pair (bp) EcoRI fragment of the replicative form DNA of phage M13mp7 (23), which contains two sites each for BamHI and SalI, was inserted into the EcoRI site of pFTB275. Thus, we obtained pFTB281 (Fig. 2). Although the 42-bp EcoRI fragment contains a single PstI site, this site is not useful for the cloning as the trpC-trpD fragment also contains four PstI sites (Fig. 1).

To confirm the utility of pFTB281 as a promoter-probe vector, we cloned Sau3AI fragments of pE194 DNA at the BamHI sites of pFTB281 by mixing BamHI-digested pFTB281 DNA and Sau3AI-digested pE194 DNA, and the ligation mixture was used for transformation of B. subtilis JU2 (trpD2). Many Km' transformants appeared, of which ca. 2% showed the TrpD+ phenotype. Plasmid DNAs were prepared from five independent Km' TrpD+ transformants by cesium chloride-ethidium bromide centrifugation and analyzed by electrophoresis after digestion with Sau3AI (Fig. 3). All the TrpD+ derivatives of pFTB281 contained various Sau3AI fragment(s) originated from pE194. According to Horinouchi and Weisblum (16), pE194 has five (A through E) open reading frames capable of encoding proteins consisting of more than 100 amino acids, of which frames C,

Fig. 3. Polyacrylamide gel electrophoresis of Sau3AI fragments of chimeric plasmids constructed by cloning various Sau3AI fragments of pE194 on pFTB281. Chimeric plasmids pFTB303, pFTB305, pFTB306, pFTB308, and pFTB313, which conferred the TrpD+ phenotype on the B. subtilis trpD mutant host, were digested with Sau3AI and run on the polyacrylamide gel in parallel with the DNAs of pFTB281 and pE194 similarly digested. Molecular sizes of the Sau3AI-digested fragments of pE194 are 1,587 bp (lane A), 929 bp (lane B), 541 bp (lane C), 409 bp (lane D), and 262 bp (lane E) (16).
D, and E do not start with ATG and are not flanked by possible ribosome-binding sites in their 5′-upstream regions (Fig. 4). Frame A starts from Sau3AI fragment E; frame B, which is identical with the ermC gene (Emr': macrolide, lincosamide, and streptogramin type B resistance determinant), starts in Sau3AI fragment D; and the C, D, or E frames start from different points in Sau3AI fragment A (16). It was obvious that four of the five derivative plasmids of pFTB281 have Sau3AI fragment E inserted upstream of trpD, and the remaining plasmid had the Sau3AI fragment carrying frame B. Restriction maps of the TrpD+ derivative plasmids of pFTB281 were constructed (Fig. 4) with the data from double digestion with several restriction enzymes (data not shown) along with the data shown in Fig. 3. No TrpD+ plasmids in which expression of trpD+ was promoted by the C, D, or E frame on Sau3AI fragment A were obtained. These observations are in agreement with the findings of Horinouchi and Weisblum (16) and strongly suggest that pFTB281 is useful for cloning a promoter sequence.

**Nucleotide sequence of the 5′ end of the trpD+ gene.**

The nucleotide sequence containing the 5′ end of the putative trpD+ gene from the EcoRI site (position 1) to the leucine codon at the 164-bp position in the 0.6-kb EcoRI-KhoI fragment of pFTB281 (Fig. 2) was determined (Fig. 5). One open reading frame starts with an ATG codon at positions 33 to 35, and this probably encodes the trpD+ protein. As expected, there were no sequences homologous to the −35 and −10 consensus sequences (TTGACA and TATAAT) of B. subtilis promoters (25) in the 5′-upstream region of the putative trpD gene. The ATG codon of the putative trpD+ gene overlapped with the TGA termination codon of the preceding open reading frame (most probably the trpE gene).

The nucleotide sequence 5′-ACAAAGGTCCGT-3′, which is complementary to the 3′ end of 16S rRNA (3′-OH-UUCUUUCCUCUCACUG...5′; C. Woese [unpublished result] cited in reference 22), was found 17 bp upstream from the ATG codon. The corresponding distances in other B. subtilis genes range from 7 to 14 bp (22, 25). In addition, the predicted free energy of base pairing (ΔG) of the corresponding mRNA from this sequence with 16S rRNA was calculated as −9.6 Kcal (ca. −40.2 kJ) according to the rules of Tinoco et al. (35). This value is apparently lower than that necessary for efficient translation with B. subtilis ribosomes, for which values ranging from −14 to −23 Kcal (ca. −58.6 to −96.2 kJ) have been calculated for other B. subtilis genes (22, 25). These facts suggest that the binding affinity of the mRNA to the 16S rRNA might be low.

The reading direction and the phase of coding frame of the ermC gene of pE194 on pFTB305, for example, fit with those of the putative trpE gene which precedes the putative trpD gene (details not shown), but the triplet frames are not in phase with that of the trpD gene because the termination and initiation codons overlap with the .TGATG.. sequence. This fact strongly suggests that the mRNA specified by the trpD gene on pFTB305 is transcribed by the ermC promoter, whereas the reintiation of translation of the trpD mRNA is required at the putative Shine-Dalgarno sequence but should not result in a fused protein of the ermC and trpD genes.

**FIG. 5.** Nucleotide sequences of the 5′-upstream region of the putative trpD+ gene of B. amyloliquefaciens on pFTB281. Nucleotide sequences from the EcoRI site of B. amyloliquefaciens DNA (position 1) to the leucine codon at position 164 were determined, whereas that of the M13mp7 DNA was adapted from Messing et al. (23).
Thus, it is possible to expect that pFTB281 can select for DNA fragments that contain a promoter but which need not contain a ribosome-binding site or a start codon.

**Cloning of DNA fragments showing promoter function.** To isolate DNA fragments with promoter activity, DNAs from various origins were digested with Sau3A1 or Sall, and the fragments were inserted into the BamHI or Sall site of pFTB281. The ligation mixtures were used for transformation of *B. subtilis* JU2 (trpD2). Km' transformants and TrpD' transformants were selected, independently, on DM3 medium containing kanamycin or mR2 medium containing Casamino Acids. Although the frequency of Km' transformants was almost equal for all the samples (1.8 × 10^5 to 4.5 × 10^3 transformants per μg of DNA), the frequency of TrpD' transformants varied from 3 to 370 per μg of DNA, depending on the DNA sample (Table 2). The DNAs of *Bacillus* phage pl1 and the *B. amyloliquificiens* F chromosome yielded high frequencies of the TrpD' transformants. When *Saccharomyces cerevisiae* chromosomal DNA was used as a donor DNA, a surprisingly high yield of TrpD' transformants was obtained. TrpD' transformants were also obtained with phage λ DNA in relatively high frequency, but rarely with DNAs of *E. coli* chromosome and pBR322.

**DISCUSSION**

A DNA fragment from *B. amyloliquificiens* V complementing the trpC and trpD mutations of *B. subtilis* was cloned and characterized. This fragment was used to construct the promoter-probe vector pFTB281 (Fig. 2) by connecting a 42-bp DNA fragment of the multiple cloning sites of M13mp7 and the larger EcoRI-PvuII fragment of pUB110 plasmid that supplies an autonomously replicating function and the kanamycin resistance phenotype. Since all the DNAs used in the construction of pFTB281 lack sequence homologies with the *B. subtilis* chromosome, no integration of the plasmid into the host chromosome occurred, and no modification of the plasmid molecules was detected in the recombination-competent *B. subtilis* host. Plasmid pFTB281 has BamHI, EcoRI, and Sall cleavage sites in the 5'-upstream region of the initiation codon of the putative trpD' gene, which lacks its own promoter but has a putative Shine-Dalgarno sequence. Insertion of a DNA fragment with promoter activity into the EcoRI, BamHI, or Sall site allowed the expression of the trpD' gene, and the TrpD' transformants were easily selected on mR2 medium containing Casamino Acids. Whether the cloned sequence is functional as a promoter in the native host is not known. Although unlikely, it is possible that an alternative mechanism might operate by which the trpD gene in pFTB281 is expressed by a promoter within the pUB110 DNA, but transcription is prevented from reaching trpD by a termination site located at or near the EcoRI site. Addition of a certain DNA fragment in the cloning site could inactivate the terminator. This will be clarified by transcription mapping of the trpD mRNA.

When a DNA fragment with promoter function is cloned at any one of the cloning sites of pFTB281, it is able to confer the TrpD' phenotype to the trpD mutant host. This cloning phenotype differs from that of chloramphenicol resistance in pL603 (38), pGR71 (11), and pCPP-3 and pCPP-4 (3). It also differs from the color assay methods based on the activity of β-galactosidase from *E. coli* (9) or of catechol 2,3-dioxygenase from *Pseudomonas putida* (40). These selection methods have advantages for the rough estimation of the promoter activity on a selection plate. However, selection by chloramphenicol resistance, in general, needs primary selection with a low dose of the antibiotic, and the color assay methods have the disadvantage of limiting the number of cells that can be assayed on a single plate. In contrast, it is possible to test directly 10^6 or more cells with pFTB281 as the vector on a single plate consisting of simple Trp indicator medium. The disadvantage of this procedure is that we have not yet developed a simple method for detection of the anthranilate phosphoribosyltransferase activity (37) encoded by the trpD gene (13), although it is possible to estimate the activity to some extent by measuring the growth rate of each transformant clone.

Nichols et al. (27) determined the nucleotide sequences of the trp(GD)-coding regions of *E. coli*, *Shigella dysenteriae*, *Salmonella typhimurium*, and *Serratia marcescens*. The trpD genes in these *Enterobacteriaceae* species are homologous, and the translation initiation codons for trpD overlap the translation termination codon of trpE at the trpE-trpD junction. The nucleotide sequences of the putative trpE and trpD genes of *B. subtilis* recently determined by Band et al. (2) showed a 29-bp overlap of the regions involving the translation initiation codon of trpD and the termination codon of trpE and an 8-bp overlap at the trpD and trpC junction, leaving no intercistronic regions between the genes. Significant homology (ca. 75%) of nucleotide sequence was observed between the 164-bp fragment of *B. amyloliquificiens* (Fig. 5) and a 158-bp portion (from positions 1119 to 1276 on the nucleotide sequence) of the putative trpE-coding region of *B. subtilis* (2), but not the overlapping region of trpE and trpD. These observations indicate that the structural overlapping of the translation

<table>
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<tr>
<th>DNA sourcea</th>
<th>Cloning site</th>
<th>Transformation frequencyb</th>
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<tr>
<td><em>B. amyloliquificiens</em> F</td>
<td>BamHI</td>
<td>Km' (x10^3)</td>
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<tr>
<td><em>B. amyloliquificiens</em> F</td>
<td>Sall</td>
<td>3.0</td>
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</tr>
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<td>pBR322</td>
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<td>Without DNA</td>
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<td>1.8</td>
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</table>

a DNA samples of *B. amyloliquificiens* F and *E. coli* MC1065 were purified by the method of Marmur (20). DNA of pl1 phage was purified by the method of Kawamura et al. (19), and *S. cerevisiae* P-28-24C was purified by the method of Hereford et al. (14). Purified λ c1857 S7 DNA was purchased from Takara Shuzo Brewing Co. pBR322 plasmid DNA was prepared by cesium chloride-ethidium bromide density gradient centrifugation.

b Number of transformants per microgram of DNA under the conditions described in the text.

pFTB281 DNA was digested with BamHI and religated without addition of other DNA.
termination and initiation codons or the regions involving these codons for the trpE and trpD genes are conserved in a wide variety of bacteria. However, this should be confirmed by protein sequencing data, which remains for future study. The above four species of enteric bacteria have nucleotide sequences complementary to the 3' end of E. coli 16S rRNA in the trpE gene, located 5 to 9 bp upstream from the trpG/GID initiation codon; and a similar sequence was found 17 bp upstream in *B. amyloliquefaciens*. In addition, the existence of a low-level constitutive promoter within the trpD gene of *E. coli*, called trp-p2, was predicted by Morse and Yanofsky (26) and Jackson and Yanofsky (18), and this was recently confirmed by Horowitz and Platt (17). The basal level of expression of trpC, trpB, and trpA is believed to be due to this second promoter in *E. coli*. In the present study, we detected a similar promoter activity for trpC in the cloned DNA fragment encoding the trpC and trpD proteins. These arguments further support the structural similarities of the trp gene cluster between *B. amyloliquefaciens* and *Enterobacteriaceae*.

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LITERATURE CITED


