Note

Construction of a Novel Tetracycline Resistance Gene Cassette Useful as a Marker on the Bacillus subtilis Chromosome

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Many chromosomal markers are needed for the genome analysis and constructions of increasingly complicated mutants. Because of the advantage of selection for drug resistance, I constructed a tetracycline resistance gene, tet, which renders B. subtilis tetracycline resistant (Tc'), adding this selectable marker to the genes cat, bsr, and neo.

The plasmid pH300PK (Ap', Tc') was constructed by Ishiwa and Shibahara as a vector to shuttle between Escherichia coli and B. subtilis. The tet gene in pH300PK is from pAMZ1, which was isolated from Streptococcus faecalis. A 1.7-kb HaeIII fragment carrying the intact tet gene, including its promoter, was isolated from pH300PK and inserted at the Smal site of pBEST201 (Fig. 1), yielding pBEST301. The effectiveness of the tet gene cassette derived from pBEST301 was tested by its insertion in the leuB gene (RSF2124-B-leuB) or the pro gene (pNEXT41) as described previously. The plasmids prepared from E. coli carrying leuB::tet (pBMAP05T) or pro::tet (pNEXT41T) were linearized and used for transformation of OA101, a prototrophic B. subtilis strain. Tc' transformants could be directly selected for on LB plates containing tetracycline (5 μg/ml). Their gene structure, leuB::tet or pro::tet, was confirmed by Southern analysis, indicating a single copy of the tet gene per genome.

The minimum inhibitory concentration (MIC) of tetracycline was estimated to be about 2 μg/ml for several B. subtilis 168 derivative strains in this laboratory, so the effective range of tetracycline concentrations was narrow (3–6 μg/ml). Tc' transformants grew slowly and colonies were of different sizes. Therefore, to make a more effective tet gene, the 1.7-kb segment was placed under the control of a promoter for the bsr gene.

Use of a new construct generated from pBEST35 (Fig. 1) in which the promoter region for the bsr gene was fused with the tet gene gave better results, pBEST309 (Fig. 1) was constructed as a tet gene carrier using the several steps shown in Fig. 1. Results of the following two experiments showed that there was no recombination between the endogenous cryptic tetracycline resistance gene (tetBS908) of B. subtilis and the tet gene of the transforming DNA. When the tet cassette gene was inserted at met, pro, or leuB on the B. subtilis 168 chromosome, all of the Tc' transformants tested (up to 200 for each) showed the expected auxotrophic phenotypes. The results indicated that tetracycline resistance of B. subtilis was equivalent to a gene disruption by the single tet gene cassette. When chromosomal DNA isolated from various leuB::tet transformants was digested by the restriction enzymes SfiI or NotI and fractionated by pulsed-field gel electrophoresis, I observed no amplification of the endogenous tet gene, tetBS908, whose amplification was reported by Amano et al. When the 1.9-kb tet gene cassette generated from pBEST309 was inserted at the NotI site of several NotI-linking clones and the resulting plasmids were used to transform several different B. subtilis 168 strains, effectiveness was as follows. 1. Tc' B. subtilis transformants could be directly selected on Antibiotic Medium III (Difeo) or LB plates containing tetracycline at 15 μg/ml, and 2. during the standard transformation, induction by tetracycline was not necessary.

Therefore, the novel tet gene cassette (1.9 kb long) generated from pBEST309 is useful for the study of B. subtilis. Particularly, the tet cassette in pBEST307 carries a sequence of 18 nucleotides (TAGGAT-AACAGGTAAT) recognized and cleaved by omega endonuclease (I-SceI), so it makes possible introduction of a unique I-SceI site on the B. subtilis 168 chromosome (unpublished data).

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References
Fig. 1. Construction of pBEST307 and 309.

pHY300PLK was purchased from Takara Shuzo (Kyoto, Japan). bla, beta-lactamase gene; tet, tetracycline resistance gene; hsr, blasticidin S resistance gene. pBEST201 was constructed by an insertion of the hsr gene generated from pBEST-4023 with SmaI digestion at the DraI site of pGEM4 (Promega Biotec, U.S.A.), so that the bla gene was replaced by the hsr gene. pBR322B was derived by the insertion of the hsr gene generated from pBEST4023 with EcoRI digestion at the EcoRI site of pBR322. pBEST301 had an as yet unidentified fragment, 2.6 kb long, enclosed here by broken lines. pBEST309 was described previously.7 pBEST307 and pBEST313 carries respectively a SfiI linker (5'GGGCCCTGCAGGGCCC3') and an 1-SceI linker (5'GATCCGGTAGGAAAAAGGGTAATAT3'); Boehringer Mannheim, U.S.A.) at the BamHI site of pBEST402.8 pBEST305 was derived by the insertion of a NotI linker (TGCCGCCGGCA, New England Biolabs., U.S.A.) at the Smal site of pBEST304. The promoter parts of the hsr gene and tet gene are indicated as ▶ and ◄, respectively.

Bars connecting plasmids represent steps of enzymatic digestion. T4 indicates a ligation step with T4 DNA ligase. Before the ligation of pBEST308 and pBEST307, both fragments were made blunt-ended with a Takara Blunting Kit (Takara Shuzo), restoring the original restriction sites, EcoRI and XhoI, of pBEST304. Bold-faced letters: R, EcoRI; S, SphI; P, PstI; L, Sali; X, XhoI; B, BamHI; C, SdiI; M, Smal; N, NotI; F, SfiI; K, KpnI; G, BgIII; Y, EcoRV; and I, I-SceI.

Transformed E. coli (JA221) cells were selected in LB medium containing either ampicillin (100 μg/ml), blasticidin S (150 μg/ml), or tetracycline (20 μg/ml).