Effective Cloning of Unmarked DNA Fragments in the Bacillus subtilis 168 Genome

Mitsuhiro Itaya†
Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194-8511, Japan

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The Bacillus subtilis 168 genome is being used to clone DNA segments, particularly unmarked DNA segments between the two available cloning systems. To facilitate this cloning, a counter selection method was developed in which loss of a cl repressor gene rendered the host strain resistant to neomycin. This method is promoted to use the B. subtilis genome as a general cloning vehicle.

Key words: positive selection; repressor binding; inducible promoter

The strain Bacillus subtilis 168, an endospore forming Gram-positive bacterium, has novel features such as developing competence to take up DNAs. If the same nucleotide sequence as the taken-up DNA is created in the genome, the DNA goes into a recombinational process and is integrated in the genome. As there is no sequence preference in the process of DNA uptake and recombination, the B. subtilis genome can be used as a cloning vehicle based upon a different principle from those of widely used host-vector cloning systems. The significant aspect to integrate DNAs in the genome by homologous recombination seems particularly advantageous for direct cloning of a segment flanked by the two cloneable DNA regions. However, as the target DNA segments normally lack selectable markers for B. subtilis, a counter-selection method is required for its application to general use. It was examined if a cl repressor protein and its binding promotor sequence from an Escherichia coli bacteriophage lambda was suitable for this method.

The lambda cl857 gene was prepared from pMET424. The fragment including the cl gene of pMET424 was prepared by EcoRI and PstI digestion and it was ligated in the EcoRI and SmaI site of the pBRBHind vector, which had a bacterial S resistance gene (brr) in the pBR322 (unpublished but shown in Fig. 1). In the pBRCI-BS, thus obtained, the cl857 gene joined by the brr gene will be hereafter called the [cl-BS] cassette (Fig. 1).

A neomycin resistance gene (neo), the expression of which is directed by the pr promoter, was constructed as follows. The PstI fragment, approximately 5 kb isolated from pBS25 after being blunt-ended by DNA polymerase I, was ligated in the SmaI site of pBEST15. As the neo gene of pBEST15 had no functional promotor for B. subtilis, the pr promoter-directed neo gene is designated as the [Pr-neo] cassette. The unique EcoRV site coming from pBS25 was converted to a NotI site using an NotI linker, producing pBEST515C (Fig. 1). The NotI fragment prepared from pBEST515C carried the [Pr-neo] cassette, and an erythromycin resistance gene (erm) of pBS25 was inserted in the NotI site of a NotI-linking clone pNEXT4, producing pNEXT4PN1 (not shown). The NotI site is present in the yfc gene, the function of which is unknown but dispensable for cell viability and competence.

Type II restriction enzymes and T4 DNA ligase for manipulation of these recombinant plasmids were obtained from Toyobo (Tokyo, Japan), except NotI (Takara Shuzo, Kyoto, Japan). All the plasmids were cloned in E. coli strain JA221 selected and amplified by ampicillin (100 µg/ml) as described previously.

The [Pr-neo] cassette was integrated in the NotI site of 3.516 kb of the BEST2007 genome by transformation using pNEXT4PN1 and a strain BEST2007 was selected by erythromycin. BEST2007 showed neomycin resistance at the concentration of 3–10 µg/ml. This indicated that neomycin phosphotransferase was expressed from the single copy [Pr-neo] in the genome in sufficient amount to render B. subtilis neomycin resistant. BEST2007 had a pBR322 and a chloramphenicol resistance gene (cat) at the NotI site of 1.378 kb (in the proB gene) as illustrated in Fig. 1. The cat gene in the EcoRI site of the integrated pBR322 of BEST2007 was replaced by the [cl-BS] cassette, as a result of double crossings-over between the pBR322 sequence of pBRCI-BS and that of the BEST2007 genome (Fig. 1). BEST2007 was obtained after selection by blasticidin S, being sensitive to chloramphenicol accordingly. DNA structure of BEST2007 was verified by Southern analysis (using pBRCI-BS as a probe, data not shown).

BEST2007 did not form colonies at 30–37°C on Penassay plates (Difco Laboratory, Michigan, USA) containing neomycin at concentrations ranging 3–10 µg/ml. BEST2007 became neomycin resistant when incubated at 45°C. This observation indicated that the Cl repressor expressed from a single copy [cl-BS] in the genome binds to the pr promoter sequence of the [Pr-neo] to repress the neo gene expression. The heat labile Cl product was unable to bind the pr promoter at the elevated temperature. Thus, strains that lose the cl gene out of the BEST2007 genome should be selected by neomycin.

Linearized pBRcm by PvuII digestion was added to...
Fig. 1. A Method to Clone a cat Gene in the B. subtilis Genome by Selection with Neomycin.

Plasmids and materials used to construct the [Pr-neo] cassette or [cl-BS] cassette are described in the text. B. subtilis strains BEST2007, BEST2087, and BEST2097 are shown with their genome structures. The pBR322 sequence of pBRCI-BS, pBRCm, and their equivalent in the B. subtilis genome are divided into two halves by EcoRI and PstI. The closed box region includes the tetracycline resistance determinant gene, and the open box region includes the β-lactamase gene.

The pBR322 part of the BEST2087 genome interacts with that of the incoming plasmid pBRCI-BS by double crossovers over (X X) as shown in the left rectangle so as to give neomycin-sensitive BEST2097 (downward, NmS). In BEST2087 neomycin phosphotransferase is expressed by the pr promoter activity in the [Pr-neo] cassette (indicated by wavy lines). In contrast, the constitutively expressed CI repressor protein in BEST2097 specifically binds to the pr promoter and shuts off the promoter activity. When the cl gene in the [cl-BS] cassette of the BEST2097 genome is replaced by the cat gene of pBRCm as shown in the right rectangle, the pr promoter is activated again so that neomycin resistant BEST2097 can be selected by neomycin (NmR, upward).

[NmR], [BSR] and [EmR] are phenotypes of chloramphenicol resistant, blasticidin S resistant, and erythromycin resistant bacteria. Enzymes are, E: EcoRI, P: PstI, V: EcoRV, S: SmaI, Ss: Sse8387I, Pv: PvuII, and N: NcoI. cat indicates the chloramphenicol acetyl transferase gene.10

competent BEST2097 cells prepared as previously described, and transformation solution was incubated for 2.5 hours at 37°C before selection. This long incubation for outgrowth was adopted to dilute the pre-existing CI protein by cell division. Half of the transformation solution was selected on Penassay plates containing chloramphenicol (5 μg/ml) and the rest was selected by neomycin (5 μg/ml). In a typical case, 375 chloramphenicol resistant transformants and 340 neomycin resistant ones were obtained per μg of plasmid DNA. No chloramphenicol-resistant colonies and twenty-two spontaneous neomycin-resistant colonies were obtained in the control experiment where no DNA was added. All the 375 clones selected by chloramphenicol were blasticidin S-sensitive and neomycin-resistant. Among the clones selected by neomycin, 299 showed blasticidin S sensitivity and chloramphenicol resistance as expected. The remaining 41 clones, roughly similar to 22 false-positives, appeared when no DNA was added, were chloramphenicol-sensitive and were not analyzed further. Genome structures of six representatives of 299 clones were identical to that of BEST2087 verified by Southern hybridization (using pBRCm as a probe, data not shown). This demonstrated that the cat gene, regarded as unmarked DNA flanked by pBR322 sequences, can be efficiently cloned in the B. subtilis genome by neomycin. An alternative system using the lacI repressor and spacer promoter was similarly constructed. However, the system did not provide clear selection conditions, probably due to insufficient expression from the spacer promoter.

It was concluded that B. subtilis cells that integrated the unmarked DNA sequence into the genome can be positively selected without using vectors. The positive
selection will facilitate not only direct cloning of long un-marked DNA but also an assembly process of giant DNA segments in the *B. subtilis* genome. Large DNAs, once integrated in the *B. subtilis* genome, can be manipulated in similar manner to cognate genomic regions. This type of DNA handling is specific to *B. subtilis* genome technology allowing any DNA rearrangement on the cloned inserts. Applications of the method to other DNA sources are underway.

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References