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CAROTENOID-BIOSYNTHESIS GENES AS A GENETIC MARKER FOR THE PURPOSE OF GENE CLONING

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A cloning vector pSL775 (7.0 kb) was constructed using the carotenoid-biosynthesis genes of *Erwinia herbicola* Eho13 (ATCC 53489). This vector contained a ColE1 origin, an ampicillin resistant gene, and a total of 11 single cloning sites: *Asp*718, *Ava*I, *Bam*HI, *Ban*II, *Eco*RV, *Hin*dIII, *Kpn*I, *Mlu*I, *Nco*I, *Not*I, and *Sma*I. Transforming the vector into an *Escherichia coli* strain could result in pink clones. On the other hand, insertion of a DNA fragment into one of these cloning sites resulted in nonpigmented clones. The color differential between the two types of colonies could be detected visually on agar medium after culturing the cells at 37°C for 18 hours.

Cloning vectors such as the plasmids of the pUC series (1) normally contain an ampicillin resistant gene and a fragment encoding the amino-terminus of the β-galactosidase (*lacZ'*). These plasmids, when transformed into a proper host, give a blue color on media containing a color indicator, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and the *lac* inducer, isopropropyl-β-D-thiogalactoside (IPTG). When a DNA fragment is inserted into one of the cloning sites in the *lacZ'* region, the resulting colonies become white. This type of plasmids has an advantage over the vectors which use two antibiotic resistant markers for selection because a time-consuming replica-plating step can be avoided. In this article, I report the use of carotenoid-biosynthesis genes to replace the *lacZ'* for the purpose of selection. This selection marker has an advantage over the *lacZ'* because the selection can be carried out in any *E. coli* strains on a common culture medium such as LB-ampicillin agar.

MATERIALS AND METHODS

Bacterial strains

E. coli strains HB101 (2), RR1 (2), DH5α (BRL), MG1063 (3), P678-54 (4), C600 (5), JM107 (1), MC1061 (6), CSH50 (7), and LE392 (8) were used as hosts for cloning experiments. Plasmid pSL525 contained the entire *crt* gene cluster of *E. herbicola* Eho13 (ATCC 53489) (9).

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Media and culture condition

Cells were cultured in LB broth or in LB agar (7). Ampicillin at 100 μg/ml was used in the media for the purpose of selection. Cells were cultured at 37°C.

DNA manipulation

Plasmid purification was carried out by the clear-lysate method of Perry *et al.* (9). Plasmid screening was done according to the method of Kado and Liu (10). Restriction digest, DNA cloning, Klenow reaction, T4 DNA polymerase reaction, and BAL31 reaction were carried out according to the methods described by Sambrook *et al.* (11).

RESULTS AND DISCUSSION

The carotenoid biosynthesis genes used for the construction of the vector pSL775 was originally isolated from a cosmid library of *Erwinia herbicola* Eho13. This yellow cosmid, pSL469 (48 kb) was able to synthesize yellow pigment on LB-ampicillin agar. The size of pSL469 was reduced to 9.5 kb by two successive rounds of *Sau*3AI digests and followed by *Pvu*II-partial digest. The resulting plasmid, pSL525 (Fig. 1) was the smallest subclone which still gave a yellow phenotype in *E. coli* (12). Sequencing analysis revealed that the DNA insert in pSL525 was 6,965-bp long (Genbank accession number M90698). According to the sequence homology with the *crt* genes of *Erwinia uredovora* (13), this gene cluster consisted of five genes — *crtEXYIB*. With the exception of *crtX*, these *crt* genes were responsible for the synthesis of yellow-colored β-carotene from farnesyl pyrophosphate (13, 14). Gene *crtX*, on the other hand, was responsible for the conversion of zeaxanthin to zeaxanthin-β-diglucoside (15, 16). Since the gene cluster in pSL525 did not contain a gene which was able to convert β-carotene to zeaxanthin, the yellow pigment synthesized in a clone containing pSL525 should be β-carotene. Plasmid pSL525 was further partially digested by Bal31 at the *Sal*I site. This deletion process has removed a 1,477-bp sequence, starting from n.t. 2,183 to n.t. 3659 of the *crt* gene cluster (GenBank accession number M90698), including the first 537-bp of the 5' sequence of *crt*Y. Since *crtY* was responsible for the conversion of lycopene to β-carotene, removal of the 5' sequence of this gene has abolished this function and resulted in the accumulation of lycopene in the cells. Therefore, cells containing this plasmid (pSL647) were pink due to the presence of lycopene. Plasmid pSL647 was partially digested by Bal31 at the *Eco*RI sites to generate plasmids pSL772 and pSL774 (Fig. 1). Plasmid pSL772 (7.3 kb) had a 822-bp deletion at the *Eco*RI site upstream from *crtE* (Fig. 1). This deletion eliminated the promoter sequence of the *crt* operon (unpublished result) and the sequence encoding the first 17 amino acids of the *crtE* gene product. Because *crtE* was required for the synthesis of a precursor of lycopene (14), elimination of this gene would have resulted in nonpigmented colonies. Therefore, deletion in pSL772 probably generated a translational fusion with a vector-encoded protein. Plasmid pSL774 (7.8 kb) had a 0.3-kb deletion at the *Eco*RI site downstream from *crtB* (Fig. 1). Plasmids pSL772 and pSL774 were digested by *Nde*I and by

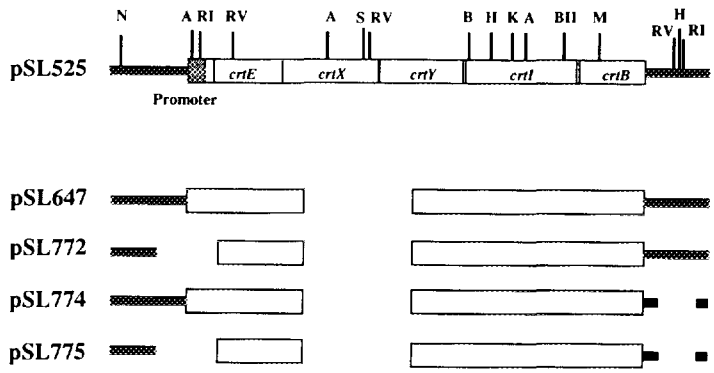

Fig. 1. Physical map of the plasmids derived from pSL525. A: *Aval*; B: *Bam*HI; BII: *Ban*II; H: *Hin*dIII; K: *Kpn*I; N: *Nde*I; M: *Mlu*I; RI: *Eco*RI; RV: *Eco*RV; S: *Sal*I.

*Bam*HI. The restriction fragments generated from these two plasmids were reconstituted to form plasmid pSL775 (Fig. 1, 2). Plasmid pSL775 has a total of 11 unique restriction sites within the *crt* genes. Insertion of a DNA fragment into any one of these sites could inactivate the function of *crtE*, *crtI*, or *crtB* (Fig. 2) and resulted in nonpigmented colonies. The selection of nonpigmented colonies could be done on LB-ampicillin agar or M9-ampicillin agar supplemented with casamino acids. Pigmented and nonpigmented colonies could be distinguished on LB-ampicillin agar within 18 to 24 hours. Fig. 3 shows the DNA sequence adjacent to the cloning sites. This sequence information is useful for the synthesis of primers for the purpose of DNA sequencing.

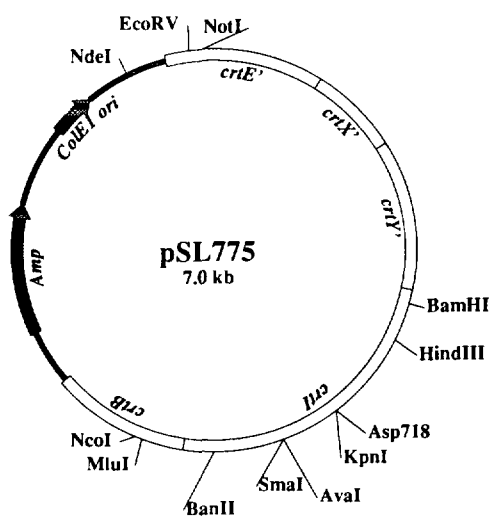

Fig. 2. Restriction map of pSL775.

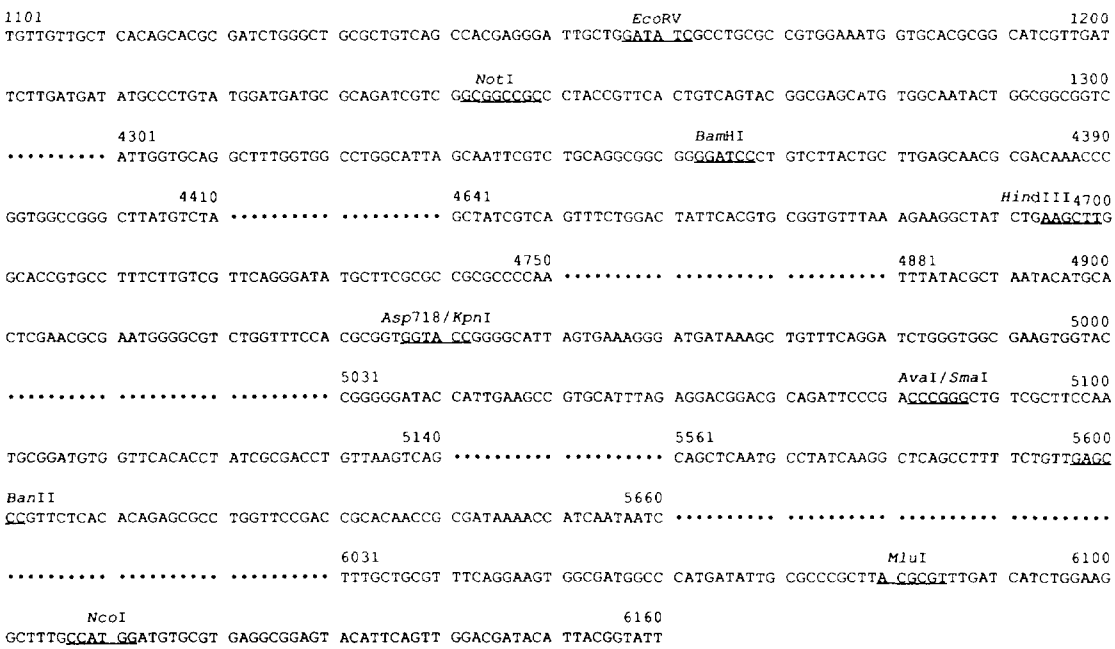

Fig. 3. The *crt* sequences adjacent to the cloning sites in pSL775. The sequence information can be used for designing primers for the purpose of sequencing of DNA fragment cloned in pSL775. The numbers used indicate the nucleotide positions of the *crt* gene cluster (GenBank accession number M90698).

Plasmid pSL775 was transformed into at least 10 different commonly used *E. coli* strains. This plasmid was stable in most of the strains except in LE392. When *E. coli* LE293(pSL775) was plated on LB-ampicillin agar, at least 30% of the colonies were nonpigmented. Agarose gel electrophoresis revealed that the loss of pigmentation was due to random deletions of the plasmid, suggesting that this strain is not suitable for the cloning with pSL775 or even with other types of vectors.

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