Original

Molecular cloning of sucrose-hydrolyzing enzyme
genes from *Streptococcus mutans* in
bacteriophage lambda L 47

バクテリオファージラムダ L 47を用いた *Streptococcus mutans*
スクロース分解酵素の遺伝子クローニング

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Received July 18, 1989; accepted August 31, 1989

概要: *gtfB*, *C* 遺伝子を欠損している *Streptococcus mutans* NHS 1 株の染色体 DNA を *Sau* 3 AI で部分切断した後, *Bam*HI で切断したバクテリオファージラムダ L47ベクターにつないだ。*in vitro* パッケージング後, ラムダファージを指示菌の大腸菌 C600P 2 に感染させたファージクローンバンクを作製した。スクロースを唯一の炭素源とする M9 培地上では大腸菌が生育できない事を利用してクローンバンクからスクロース分解酵素遺伝子を組み込んだラムダファージを検出した。即ち, スクロース分解酵素遺伝子を組み込んだラムダファージのプラーク周囲ではスクロースが分解し, グルコースとフルクトースが生じる。このため大腸菌の生育が良くなりプラークの周囲に暈輪が観察されるようになる。この方法で13クローンが選択された。このうち5クローンには強いインペルターゼ活性が認められた。更にそのうちの1つのクローンにはグルコシル基の転移活性が認められたが, グルカン合成活性はなかった。

Key words: Recombinant DNA technique, Bacteriophage lambda, Sugar metabolism, *Streptococcus mutans*

索引用語: 組み換え DNA 技術, バクテリオファージ ラムダ, 糖代謝, ストレプトコッカスミュータンス

Introduction

Streptococcus mutans has been recognized as the principal causative bacterium of human dental caries¹⁾. This organism possesses a variety of pathways for metabolizing dietary sucrose. These pathways are believed to be an important factor in the ability of *S. mutans* to cause dental caries. The conversion of dietary sucrose to glucan is the most important cariogenic property of *S. mutans*. This organism also uses dietary sucrose as a source of carbon and energy. The enzymes involved in metabolism of sucrose,

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both intra- and extracellularly, are greatly complicated. Recombinant DNA techniques are suitable for the analysis of such complex situations because the genes responsible for individual functions can be isolated. Such genes can be cloned into a novel genetic circumstance. Recently, the three glucosyltransferase genes (*gtf*) of *S. mutans* were cloned^{2,4)}. These genes are important for dental caries. In order to get more progressive information about dental caries, we need to find all sucrose-hydrolyzing enzyme (invertase) genes in *S. mutans*. In this paper we described the application of a bacteriophage vector system for the molecular cloning of several sucrose-hydrolyzing enzyme genes from *S. mutans*.

Materials and Methods

1. Bacterial strains

Escherichia coli C600 and C600 (P2) were kindly provided by Howard K. Kuramitsu, Northwestern University, Chicago, Illinois. *S. mutans* strain NHS1, which lacks most of the *gtfB* and C genes, was described previously³⁾. Lambda L47 DNA was obtained from Amersham Japan Corp., Tokyo. *E. coli* strains were grown in L broth (10 g of tryptone [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract [Difco], 5 g of NaCl per liter). Phage stocks were prepared on LB agar plates in the top agar overlays⁵⁾.

2. DNA manipulations

Chromosomal DNA from *S. mutans* NHS1 was purified essentially as previously described⁶⁾, extracted with phenol, phenol/chloroform and chloroform, precipitated with ethanol (2x), and dissolved in TE buffer (10 mM Tris hydrochloride, 1.0 mM EDTA [pH 8.0]). These cycles were repeated ten times.

3. Construction of the *S. mutans* clone bank

Chromosomal DNA from *S. mutans* NHS1 was partially digested with restriction endonuclease enzyme *Sau3A*I. DNA fragments of over 6 kb were isolated from an agarose gel. These fragments were mixed with lambda L47 DNA which was completely digested with *Bam*HI in a 1 : 1 ratio (total DNA concentration was 300 µg/ml). DNA fragments were ligated at 12°C for 16 h in the presence of T4 DNA ligase. After ligation, the mixture was phenol extracted, phenol/chloroform extracted, chloroform extracted and ethanol precipitated (2x). The ligation mixture was dissolved in 11 µl TE buffer. *In vitro* packaging of the ligation mixture (5 µl) was done with a packaging kit following the directions of the supplier (Amersham Japan). *E. coli* C600 (P2) cells were infected with the packaging mixture for 20 min at 37°C in 10 mM MgSO₄. Infected *E. coli* were mixed with 3.0 ml of LB top agar and plated on LB agar plates at 37°C for 8 to 16 h. The resultant plaques were harvested by scrubbing each plate with 3.0 ml of lambda dilution buffer (10 mM Tris, 10 mM MgSO₄ [pH 7.5]). The clone bank was stored at 4°C and was used to infect C600 cells for subsequent screening.

4. Screening of clone bank

The recombinant phage clone bank was plated with *E. coli* C600 in soft agar made with LB on top of M9 minimal salts agar medium (6 g of Na₂HPO₄, 3 g of KH₂PO₄, 0.5 g of NaCl, 1 g of NH₄Cl and 15 g of agar per liter) containing 0.4% sucrose as the only carbon source and supplements (1 µg/ml of thiamine hydrochloride, 8 µg/ml of L-leucine and 8 µg/ml of L-threonine) required by *E. coli* C600. Plates were incubated 2 or 3 days at 37°C. Recombinant plaques suspected of containing sucrose-hydrolyzing enzyme genes were picked and checked for their invertase activity.

5. Enzyme assays

Invertase activity was determined as previously described⁷⁾ in a standard incubation mixture containing 100 mM potassium phosphate buffer (pH 6.0), 2% sucrose, 0.2% saline-NaN₃, enzyme, and water in a total volume of 0.1 ml. The reaction mixtures were incubated for 30 min at 37°C and analyzed for reduc-

Fig. 1 Restriction enzyme digestion profiles. (a) Molecular size marker, lambda DNA *Hind*III fragments (23, 9.4, 6.6, 4.4, 2.3 and 2.0kb), (b) Lambda phage L47 DNA *Bam*HI digestion, (c) *S. mutans* NHS1 chromosomal DNA *Sau*3AI partial digestion.

Fig. 2 Ligation profile of the *S. mutans* clone bank. (a) Lambda DNA *Hind*III fragments, (b) After ligation of *S. mutans* NHS1 chromosomal DNA and lambda phage DNA utilizing T4 ligase.

ing sugar formation by the Somogyi-Nelson procedure. The difference of the amounts of free glucose and fructose in the reaction mixture was determined by the F-kit (Boehringer Mannheim, FRG), a biochemical assay of fructose/glucose⁸⁾.

Results

1. Construction of clone bank

Fig. 1 shows the digestion profiles of the *S. mutans* chromosomal DNA and lambda phage L47 DNA. Fig. 2 shows the ligation profile of the *S. mutans* lambda phage clone bank. The DNA concentration and the purity of DNA fragments were important factors during the ligation and *in vitro* packaging procedures.

2. Detection of sucrose-hydrolyzing enzyme recombinants

The phage detection method depends on the fact that *E. coli* K12 is unable to transport sucrose into the cell. But *E. coli* fermented monosaccharides (glucose or fructose). In this experiment monosaccharides were produced by cleavage of sucrose which existed in the bottom agar. Monosaccharides in the top agar layer allowed a faint lawn of *E. coli* to grow, sufficient to support phage multiplication and formation of plaques. The production of monosaccharides in plaques where sucrose-hydrolyzing enzyme genes were expressed supported much more growth and plaques were surrounded by haloes of *E. coli*.

Thirteen of a total of 2000 plaques from the lambda phage clone bank showed such haloes.

3. Enzyme activity of the recombinants

The invertase activity of 13 phage clone lysates prepared from cross-feeding recombinants was examined by Somogyi-Nelson procedures. Strong invertase activity was detected from 5 clones. Weak activities were detected from 4 clones. In spite of the existence of haloes, we could not detect any invertase activity in the remaining 4 clones. The amount of free glucose and fructose in the reaction mixture was

determined by the F-kit (Table 1). Fructose/glucose ratio showed that clones No. 1 to No. 4 had no detectable glucosyl residue transfer activity. Clone No. 7 showed glucosyltransferase-like activity. But no glucan formation activity was detected at this time (data not shown).

Discussion

The data presented in this communication demonstrate that the sequence encoding the sucrose-hydrolyzing enzyme genes of *S. mutans* has been cloned in *E. coli* on a Lambda L47 replacement vector.

We expected to have glucan forming activity from phage lysates. But we could not find such plaques with this cloning method. Clone No. 7 probably had transfer activity of glucosyl residue. However, we could not get any glucan forming activity from No. 7 clone. We intend to characterize clone No. 7 in future.

A screening procedure similar to that described here was earlier used by Franklin⁹⁾ to detect lambda *trp* recombinants, by Gilpin *et al.*¹⁰⁾ in studies of *gtf* genes and by Russell *et al.*¹¹⁾ in studies of *scr* and *gtfA* genes. *E. coli* K12 is normally unable to transport sucrose into the cell so that cloning systems based upon plasmid vectors require the devising of different methods for uptake of the sugar¹²⁾. The advantage of the lambda system is that sucrose-hydrolyzing enzymes from *S. mutans* carried by recombinant phage are produced and released to the outside of the *E. coli* cells. This is why we can detect their activity within the resultant plaques.

The characterization of these sucrose-hydrolyzing enzymes and analysis of the complex pathways of sugar metabolism will be greatly helped by the development of recombinant DNA techniques for cloning and expression of *Streptococcus mutans* genes in *E. coli*.

Acknowledgement

This study was supported in part by a Grant (1989) 01870090, in aid for Basic Scientific Research, from the Education Ministry, Japan.

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Table 1 Detection of sucrose-hydrolyzing enzyme recombinants

clone number	invertase activity	fructose/glucose
1	+	1.33
2	+	0.82
3	+	1.10
4	+	0.88
5	+/-	N.D. ^a
6	-	N.D.
7	+	2.35
8	+/-	N.D.
9	+/-	N.D.
10	-	N.D.
11	-	N.D.
12	+/-	N.D.
13	-	N.D.

^a N.D., Not determined

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