STABILITY OF A RECOMBINANT PLASMID CONTAINING α-AMYLASE GENE IN CHEMOSTAT

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Continuous cultivation of *Escherichia coli* harboring a recombinant plasmid containing α-amylase gene was carried out, and the stability of the plasmid was examined under different cultivation conditions. *E. coli* maintained the plasmid stably when grown in L-broth medium. Production of α-amylase, however, decreased as the dilution rate increased. When the cells were grown in a synthetic medium (Davis medium) supplemented with glucose as a carbon source, the plasmid was unstable. Plasmid-free organisms, organisms harboring a plasmid having a deletion in α-amylase gene and organisms harboring the plasmid whose copy numbers decreased appeared along with organisms harboring an intact plasmid. When starch was used as a carbon source, the plasmid was reasonably stable.

Introduction

In producing gene products using recombinant DNA technology, stable maintenance of plasmids is one of the most important problems. From stock culture to tank-scale cultivation, organisms must be cultivated through considerable generation numbers. As a consequence, industrial applications of microorganisms harboring unstable recombinant plasmids might be difficult even if they produced gene products at a high level in the first generation.

Jones *et al.*[7] indicated that plasmid (pBR322)-free segregants were observed after 30 generations under both glucose and phosphate limitations in antibiotic-free chemostat. In contrast, Noack *et al.*[12] showed that plasmid pBR322 was stably maintained in antibiotic-free chemostat under both glucose and ammonium chloride limitations, whereas plasmid pBR325, a derivative of plasmid pBR322, was unstable under the same conditions. Dwivedi *et al.*[4] and Skogman *et al.*[14] also reported that the recombinant plasmid became more unstable than the vector plasmid itself. We had carried out the fed-batch culture of *E. coli* harboring a recombinant plasmid and encountered similar problems.[11]

In the present study, the effects of medium components and selective pressure on the stability of a recombinant plasmid containing α-amylase gene were examined in chemostat. The organism appeared to maintain the plasmid stably under some culture conditions.

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1. Experimental

1.1 Microorganism

The organism used in this study was *Escherichia coli* HB101/pHI301.[16] Genotype of *E. coli* HB101 is F^- *leu*$^-$ *pro*$^-$ *lac*$^-$ *gal*$^-$ *thi*$^-$ *recA*$^-$ r^-m^- *st*^{r}.[3] The recombinant plasmid consisted of α-amylase gene derived from *Bacillus stearothermophilus* DY-5 chromosomal DNA and pBR322, and its phenotypic expression was ampicillin-resistant and tetracycline-sensitive.

1.2 Media

L-broth (1 kg bacto-tryptone (Difco), 0.5 kg yeast extract, 5 kg NaCl, 1.23 kg $MgSO_4 \cdot 7H_2O$ and 500 g ampicillin per m^3, pH 7.0) and Davis medium (7 kg K_2HPO_4, 2 kg KH_2PO_4, 0.05 kg $MgSO_4 \cdot 7H_2O$, 1 kg $(NH_4)_2SO_4$, 0.1 kg proline, 0.05 kg leucine and 1 g thiamine·HCl per m^3) supplemented with 0.4 kg glucose or soluble starch and, when necessary, 250 g ampicillin per m^3 were used for continuous culture.

Microorganisms during continuous cultures were plated on L-agar (L-broth containing 25 kg agar/m^3). In the replica method, L-agar was supplemented with 1 kg soluble starch per m^3 (LS-agar) or 1 kg soluble starch and 100 g ampicillin per m^3 (LSA-agar).

1.3 Cultivation

The seed culture was prepared as follows. A loop of the microorganism stocked on L-agar supplemented with 100 g ampicillin per m^3 was inoculated into 100 ml of L-broth or Davis medium in an Erlenmeyer flask. The seed culture medium was the same as that in continuous cultivation. After overnight incubation at 37°C, this culture was transferred into a jar-fermentor (working volume: 350 ml). After several

hours of batch culture, continuous cultivation was started under the limitation of carbon source. Culture broth (about 5 ml) was sampled at appropriate intervals.

1.4 Analytical method

Concentration of the microorganism was determined by measuring the optical density at 570 nm of the culture broth with a Shimadzu Spectronic 20 photometer and converting its value into dry cell weight. Maximum growth rate of the organism was determined by the method of Pirt *et al.*[13] The culture broth obtained by sampling was sonicated using a cell disrupter (Ohtake Works Co., 5202). Cell debris was removed by centrifugation, and 1 ml of the supernatant diluted appropriately with 50 mM acetate buffer (pH 6.0) was mixed with 2 ml of 0.5% soluble starch in the same buffer. After incubation at 40°C for 10 min, α-amylase activity (dextrinizing power) was assayed as described by Fuwa.[5] Hydrolysis of 0.1 mg soluble starch per minute was defined as 1 unit of enzyme activity.

The phenotype of the organism cultivated in Davis medium was examined as follows. The culture broth obtained by sampling was diluted appropriately with 0.9% NaCl solution. An aliquot was plated on L-agar, and 100 colonies from each plate were tested for the resistance to ampicillin and the production of α-amylase by transferring to LS-agar plates and LSA-agar plates using toothpicks. After overnight incubation at 37°C, the plates were stained with 1.7 mM I_2-KI solution. The number of colonies producing α-amylase which were surrounded by clear zone was counted.

For isolation of plasmid DNA, the rapid alkaline extraction method was applied.[2] Restriction endonucleases were purchased from Wako Pure Chemical Industries, and were used as recommended by the supplier.

Agarose gel electrophoresis was performed using vertical slab gel in a buffer consisting of 40 mM Tris(hydroxymethyl)aminomethane, 20 mM sodium acetate and 2 mM EDTA, pH 8.0. λDNA cleaved with *Hin*dIII was used as a molecular weight reference. After electrophoresis, the gel was stained in EtBr solution (0.5 μg/ml), and the DNA bands were visualized and photographed under ultraviolet light.

2. Results

Figure 1 shows the result of continuous cultivation in L-broth medium. After the start of continuous cultivation, α-amylase production increased with the growth of the organism. Within initial two days, both cell concentration and α-amylase activity in culture reached steady state and this state continued for 10 days. The specific activity in the steady state was 394 U/mg dry cells. 98% of α-amylase activity was

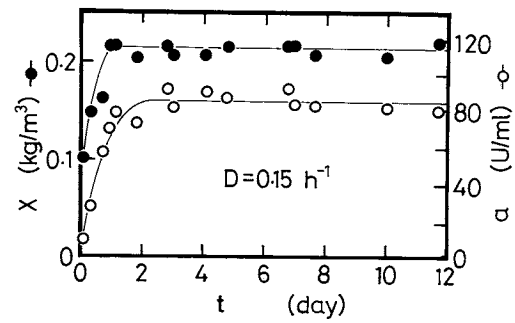

Fig. 1. Continuous cultivation in L-broth medium.

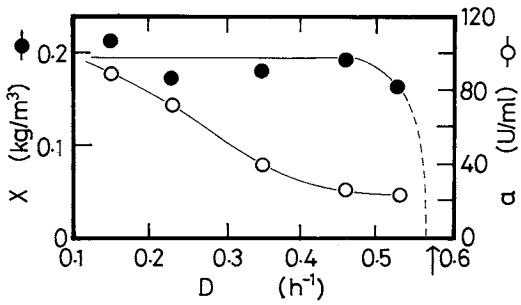

Fig. 2. Effect of dilution rate on α-amylase activity and cell concentration. Arrow shows the maximum specific growth rate.

Table 1. Effect of carbon source on α-amylase activity*

Carbon source	Specific α-amylase activity [U/mg dry cells]
Glucose	56
Glycerol	33
Starch	57
Sorbitol	52
Fructose	54
Succinate	87
Maltose	43

* *E. coli* HB101/pHI301 was cultivated at 37°C for about 16 h in a test tube containing the synthetic (Vogel and Bonner) medium[17] supplemented with 0.1 kg proline, 0.05 kg leucine, 1 g thiamine·HCl, 5 kg carbon source and 100 g ampicillin per m^3.

observed within the cells.

Effect of dilution rate on enzyme production is shown in **Fig. 2**. As the dilution rate increased, α-amylase production decreased almost linearly. In L-broth medium, the maximum specific growth rate of the organism was 0.58 h^{-1}, and the relationship between cell concentration and dilution rate was similar to textbook examples.[1]

Table 1 shows the effect of carbon source on α-amylase activity in a synthetic medium. α-Amylase was not repressed by glucose.

Figure 3 shows the result of the chemostat in Davis medium supplemented with starch and ampicillin (DSA medium) at a dilution rate of 0.28 h^{-1}. Both

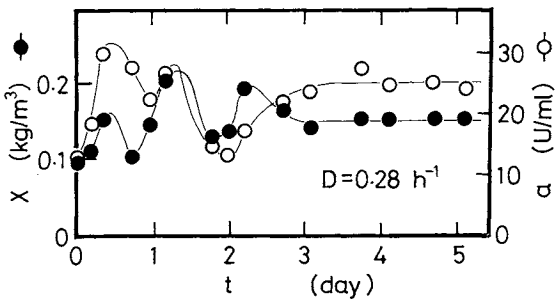

Fig. 3. Continuous cultivation in Davis medium supplemented with starch and ampicillin.

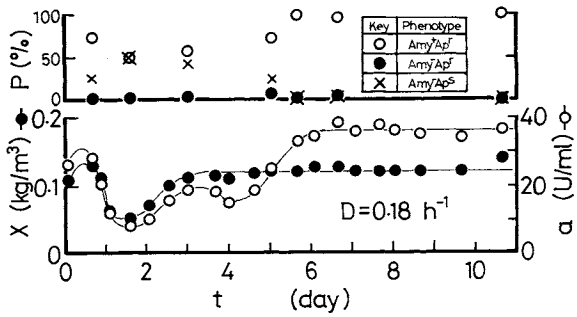

Fig. 4. Continuous cultivation in Davis medium supplemented with starch.

cell concentration and α-amylase activity oscillated during the initial 3 days, and then reached steady state. In the outlet culture, neither starch nor glucose was detected in the steady state. The specific activity in the steady state was 165 U/mg dry cells, which was 55% of that in L-broth medium based on the same dilution rate.

Cultivation result of the chemostat in Davis medium supplemented with starch (DS medium) is shown in **Fig. 4**. After an oscillation, both cell concentration and α-amylase activity reached quasi-steady state at 3 days. However, α-amylase activity began to increase, reached steady state at 6 days and was remained constant for 5 days. The specific activity in the steady state was 250 U/mg dry cells, which was 60% of that in L-broth medium based on the same dilution rate. According to the phenotype analysis, the population of Amy^+Ap^r organism decreased and that of Amy^-Ap^s organism increased with cultivation time. They were each about 50% from 2 to 3 days, and then the population of Amy^+Ap^r organism increased to almost 100%. During the cultivation, a population of Amy^-Ap^r organism was also observed although its percentage was less than 6%.

Figure 5 shows the chemostat result in Davis medium supplemented with glucose and ampicillin (DGA medium). At first, α-amylase activity increased with the growth of the organism, but decreased sharply and became 2 U/ml at 2 days. On the other

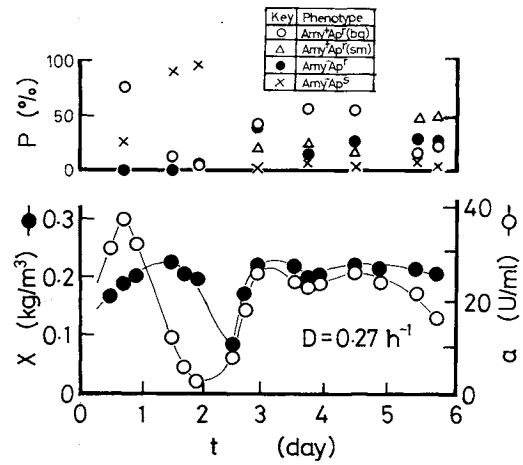

Fig. 5. Continuous cultivation in Davis medium supplemented with glucose and ampicillin.

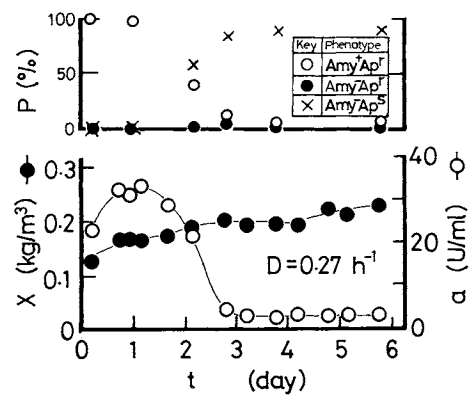

Fig. 6. Continuous cultivation in Davis medium supplemented with glucose.

hand, cell concentration began to decrease after 1.5 days, became minimum at 2.5 days and then increased. α-Amylase activity also started to increase after 2 days, reached an almost constant value, and then began to decrease after 5 days.

Expression of phenotype changed rather drastically in this chemostat. At first, the population of Amy^+Ap^r organism decreased and that of Amy^-Ap^s organism increased with cultivation time. At 2 days, the former and the latter became 2% and 95%, respectively. However, the latter decreased drastically to 1% at 3 days. At the same time, the population of Amy^-Ap^r organism increased drastically to 38%. In the phenotype of Amy^+Ap^r, the organism having a clear zone of small size (sm) when determined α-amylase activity on plates appeared at 20% and the organism having a clear zone of big or normal size (bg) increased to 41% at 3 days. After 5 days, "sm" cells became predominant, as shown in Fig. 5. Change of α-amylase activity well reflected the phenotypic variations.

Figure 6 shows the chemostat result in Davis medium supplemented with glucose (DG medium).

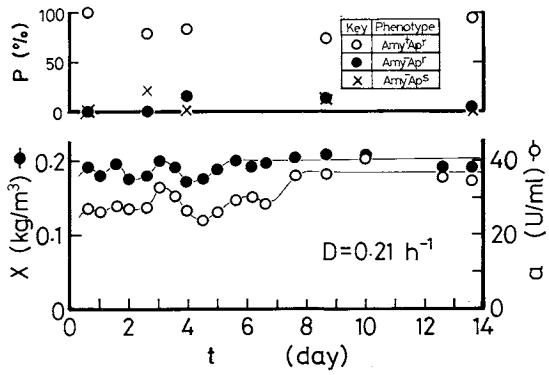

Fig. 7. Continuous cultivation of HB101/pHI301-A in Davis medium supplemented with glucose.

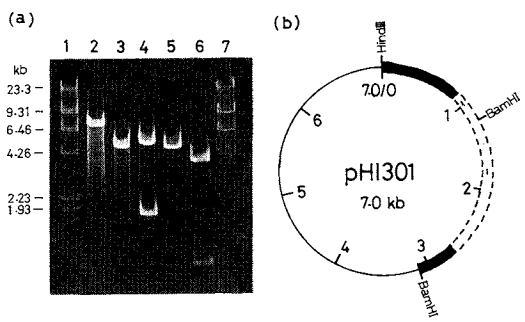

Fig. 8. (a) Agarose gel electrophoretic analysis of pHI301 and pHI301-L. Lane 1, 7, λDNA (*Hin*dIII); lane 2, pHI301 (*Hin*dIII); lane 3, pHI301-L (*Hin*dIII); lane 4, pHI301 (*Bam*HI); lane 5, pHI301-L (*Bam*HI); lane 6, pHI301-L (*Hin*dIII & *Bam*HI).
(b) Physical map of plasmid pHI301. The thick line represents the *B. stearothermophilus* DY-5 DNA and the dotted line shows deleted portion.

Cell concentration increased gradually. α-Amylase activity increased at first and then decreased to 3 U/ml at 3 days. It was interesting that the enzyme activity remained constant afterward although the activity level was low. Phenotype analysis indicated that the population of Amy^-Ap^s organism increased and eventually became about 90%. The population of Amy^-Ap^r organism was about 2–3% throughout the cultivation and "sm" was not observed. Population of "bg" decreased gradually but was maintained at about 7% after 3 days. This organism (HB101/pHI301-A) was isolated and cultivated again in the same condition since the plasmid seemed to be stabilized.

Figure 7 shows the result in DG medium. Contrary to the result shown in Fig. 6, both cell concentration and α-amylase activity were maintained at constant values although some small fluctuations were observed. The specific activity in the steady state was 185 U/mg dry cells, which was 50% of that in L-broth medium based on the same dilution rate. Throughout the chemostat, the predominant population was Amy^+Ap^r. This phenotypic strain was isolated at 13 days (HB101/pHI301-B). After cultivating this strain

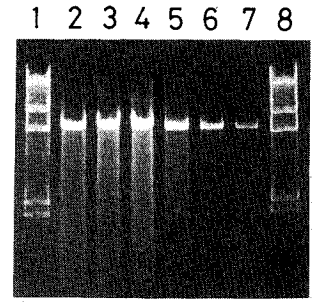

Fig. 9. Direct visualization of plasmid content. Lane 1, 8, λDNA (*Hin*dIII); lane 2, "sm" (×1); lane 3, "bg" (×1); lane 4, "bg" (×1/2); lane 5, "bg" (×1/4); lane 6, "bg" (×1/10); lane 7, "bg" (×1/20). In "sm" (×1) and "bg" (×1), the amount of cell mass was adjusted to be the same.

in a test tube as well as the original strain and HB101/pHI301-A, the plasmids were isolated and examined by agarose gel electrophoresis. As far as we examined, sizes of pHI301, pHI301-A and pHI301-B were equal with one another.

In these chemostat cultures, a population of Amy^-Ap^r organism (HB101/pHI301-L) was observed, especially in DGA medium. This strain was isolated and its plasmid (pHI301-L) was examined by agarose gel electrophoresis as shown in **Fig. 8**. The plasmid pHI301 was digested with *Hin*dIII (lane 2) and *Bam*HI (lane 4), and the plasmid pHI301-L was also digested with *Hin*dIII (lane 3) and *Bam*HI (lane 5), and double-digested with *Hin*dIII and *Bam*HI (lane 6). As shown in lanes 2 and 3 in Fig. 8, pHI301-L was smaller than pHI301 by about 2 kb. From the results shown in lanes 4, 5 and 6, it was found that about 2 kb around a *Bam*HI site at 1.2 kb in the map of pHI301 was deleted.

Concerning "sm", which was only observed in the chemostat with DGA medium, copy number of its plasmid and specific α-amylase activity were examined as follows. Colonies of "sm" and "bg" on the LSA-agar plate were picked up and cultivated in L-broth medium. The aliquots were used for the extraction of plasmids and the measurement of α-amylase activity. Copy numbers of plasmids obtained were compared by direct visualization in agarose gel electrophoresis as shown in **Fig. 9**. The former was about one-fourth of the latter, whereas the specific α-amylase activity of "sm" was one-seventh of that of "bg" (data not shown).

3. Discussion

The plasmid was stably maintained in *E. coli* grown in a complex (L-broth) medium as shown in Fig. 1, while the plasmid was unstable in synthetic (DG and DGA) media as shown in Figs. 5 and 6. Skogman *et al.*[14] showed that the stability of plasmids is better in a complex (LB) medium than in a synthetic (Vogel and Bonner) medium. To examine the effect of the

main components of L-broth medium on the stability of the plasmid, continuous cultivations were carried out in DG medium supplemented with 0.2 kg bactotryptone or 0.1 kg yeast extract per m^3. The plasmid was unstable in both cases, although increases of the specific activity of α-amylase were observed. The reason why the plasmid became stable in the complex medium remains to be investigated.

In the present study, the production of α-amylase decreased with the dilution rate (Fig. 2). Toda[15] indicated that several types of relation exist between enzyme specific activity and dilution rate. According to Toda, a decreasing curve may be considered a result of catabolite repression of the enzyme. Although the catabolite repression of α-amylase was examined, α-amylase was not repressed (Table 1). The decrease of α-amylase production with increasing dilution rate may be due to the decrease of plasmid copy number.

As shown in Figs. 3 and 4, the plasmid was almost stably maintained in DS and DSA media although oscillations were observed. Usually, the specific growth rate of a microorganism harboring plasmid is lower than that of plasmid-deleted strain, and plasmid-deleted strain becomes predominant in continuous culture.[6] However, in the present cases, the plasmid-deleted strain cannot grow because *E. coli* cannot utilize starch, and α-amylase activity was only observed within the transformant. This may support the stability of the plasmid in DS and DSA media.

In large-scale cultivation, antibiotics as a selective pressure should not be used if post-fermentation processes are considered. To maintain plasmid stably without using antibiotics, Miwa *et al.*[10] devised a method using an antibiotic-dependent mutant. Kakutani *et al.*[8] also developed a system which use a host lysogenic for phage Φ80 whose cI repressor is temperature-sensitive and plasmid on which a cI repressor gene was cloned. Results in Figs. 3 to 6 show that the stability of the plasmid under selective pressure by starch is better than that by ampicillin. Appearance of the population of both Amy^-Ap^s and Amy^-Ap^r organisms could be prevented by using starch as a carbon source and the plasmid was maintained stably. Using a carbon source (e.g. starch) which host cells cannot utilize is also recommended to maintain the plasmid stably as shown in the present paper.

In the four continuous cultivations shown in Figs. 3 to 6, various phenotypes of the organisms appeared during cultivation. The production of α-amylase and the cell concentration were dependent on the phenotypes. The production of α-amylase was decreased by three different causes: the disappearance of plasmid, the decrease in plasmid copy number and the deletion of α-amylase gene. As shown in Fig. 8, about 2 kb around α-amylase gene on pHI301 was deleted. Dwivedi *et al.*[4] also showed that the organisms which have the plasmid deleted only tryptophan operon on recombinant plasmid emerged in large numbers. To date, the reason is unknown in both cases.

We could isolate the plasmid-stabilized organisms, HB101/pHI301-A and HB101/pHI301-B. As shown in Fig. 7, even after cultivation under non-selective pressure (in DG medium) for 13 days, the percentage of population of Amy^+Ap^r organism was about 90%. It is considered that stabilization may be caused by the mutation of plasmid DNA or chromosomal DNA. We are working to confirm the possibility. Skogman *et al.*[14] used the partition locus of plasmid pSC101[9] to increase the stability of the cloning vectors. Their data, however, show that the partition locus of pSC101 may not be effective enough to obtain full stability and that plasmid-free organisms appear and eventually become dominant. To the contrary, the organism obtained in the present study grew dominantly in spite of the appearance of plasmid-free organisms. This host or plasmid will be useful in large-scale cultivation.

Nomenclature

a	=	α-amylase activity	[U/ml]
D	=	dilution rate	[h^{-1}]
t	=	cultivation time	[day]
X	=	cell concentration	[kg/m^3]
P	=	phenotype of colonies	[%]

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