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GROWTH AND PLASMID STABILITY OF RECOMBINANT *E. coli* CELLS PRODUCING HEPATITIS B SURFACE ANTIGEN

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Abstract—The self-made recombinant plasmid vector, pKSB292, harboring the amino terminal-deleted surface antigen gene of hepatitis B virus (HBsAg) under the control of *lpp*+*lac* double promoters with the signal peptide region of the lipoprotein gene was tested for its effect on host cell growth and for the stability behaviour under various environmental conditions. The selection pressure imposed by the antibiotic ampicillin resulted in the more hindered cell growth as compared with the metabolic load caused by the recombinant plasmid in the host cell induced by the IPTG in the antibiotic-free medium. However the simultaneous application of both the antibiotic selection pressure and the metabolic load from the IPTG induction exhibited the lowest cell growth rate. The host cells harboring the recombinant plasmid, pKSB292, accounted for the majority of cell population under antibiotic selection pressure showing the apparently higher stability. The better growth rate of the recombinant cells and the higher HBsAg production rate could be achieved in the nonselective complex medium.

INTRODUCTION

Various plasmid vectors have been developed for the production of hepatitis B vaccine in *E. coli* by Charnay et al. [1] and Edman et al. [2], in *S. cerevisiae* by Miyanohara et al. [3] and Hitzeman et al. [4], and in animal cell lines by Gouch and Murray [5] and Dubois et al. [6]. In contrast to yeast and mammalian cells the direct production in *E. coli* of HBsAg in substantial quantity has been rather difficult because the produced antigen gave harmful effects on hosts: growth inhibition and rapid proteolytic degradation in cytoplasm [2,7].

Recently our laboratory succeeded in the direct production of a substantial quantity of HBsAg in the cytoplasmic, periplasmic, and/or extracellular spaces of *E. coli* [8]. In this study the HBsAg gene lacking a short sequence in the amino terminal region was hooked to the control region of a plasmid vector comprised of *lpp*+*lac* double promoters and the DNA segment for lipoprotein signal peptide. Consequently the growth inhibition of *E. coli* host cell upon HBsAg expression could be avoided and much of the produced antigen was led to the periplasmic space with the help of *E. coli* lipoprotein signal peptide facilitating the product isolation.

Other than the growth inhibitory effect of the whole HBsAg protein on the host cell, little information is available on the stability of the recombinant plasmid itself carrying the HBsAg gene. The current research was conducted based upon our interest on the plasmid maintenance in the case of HBsAg production from the recombinant cells constructed for ourselves. The effects of the kinds of *E. coli* host strains used, the selection pressure, and the use of expression inducer on the growth rates of the recombinant cells, the HBsAg production level, and the plasmid stability are reported.

MATERIALS AND METHODS

E. coli Host Strains and Plasmid Vector

E. coli strains C600[9], HB101[10], JA221[11], JE5505[12], and RB791[13] were used as host strains for transformation with the recombinant plasmid DNA (Table 1). The plasmid vector, pKSB292[8] (Fig. 1), which has the *lpp* and *lac* double promoters, the DNA segment for lipoprotein signal sequence, the structural gene for HBsAg/*adyw* lacking the segment for amino terminal end, the *lac* I regulatory gene, and the ampicillin marker gene, was used for HBsAg production and stability test.

Media and Culture Condition

Nonselective medium was LB and selective medium had 50μg/m*l* of ampicillin added to the nonselective one. 1 mM of IPTG (isopropyl β-D-thiogalactopy-

Table 1. *E. coli* host strains used in plasmid stability test.

Strains	Genotype
C600	F⁻, thi-1, thr-1, leuB6, lacY1, ton A21, Sup E44, λ^-
HB101	F⁻, pro, leu, thi, lacY, str^r, $r_k^- m_k^-$, endA⁻, recA⁻
JA221	hsdM⁺, hsdR⁻, leuB6, lacY, trpE5
JE5505	F⁻, lpp-2, pps, his, proA, argE, thi, gal, lac, xyl, mtl, tsx
RB791	W3110 lacI^QL8

ranoside) was used as the inducer of *lac* operon. For the preparation of an inoculum for batch fermentation, a single colony of transformed *E. coli* was transferred into 5 m*l* of the selective medium. After overnight incubation, 1 m*l* each of culture broth was inoculated into each of the four 250 m*l* flasks, each containing 50 m*l* of LB, 50 m*l* of LB+ampicillin, 50 m*l* of LB+IPTG, and 50 m*l* of LB+IPTG+ampicillin, respectively. Cells were cultured at 37°C in a shaking incubator.

HBsAg Assay

Cells were harvested and the crude extract of cells was prepared [8]. The extract sample was analyzed by the use of HBsAg RIA kit AUSRIA II (Abott Laboratory).

Cell Growth Rate and Stability Analysis

Using five different strains, maximum specific growth rates (μ_{max}) were compared with each other in each of the above-mentioned media according to the Pirt's method [14]. For the stability analysis of plasmid, cells were successively cultured in new media using 2% inoculum in order to sustain the exponential growth phase and the identical generation condition. Culture broth was then sampled, diluted, and spread on selective and nonselective agar plates simultaneously. The stability figure was computed using the ratio of the colony number on the selective plate to that on the nonselective plate.

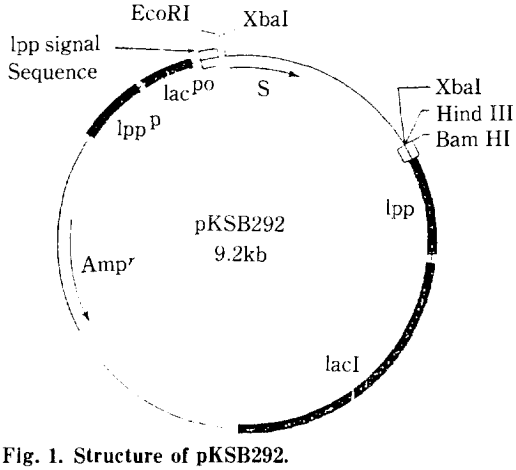

Fig. 1. Structure of pKSB292.
lpp: lipoprotein, S: HBsAg gene
lac: lactose

RESULTS AND DISCUSSION

Effect of Plasmid pKSB292 on the Growth of *E. coli*

The maximum specific growth rates (μ_{max}) were compared among the five different kinds of host cells with or without plasmid in the nonselective medium (Table 2). The cells without plasmid grew faster than those carrying plasmid. This is in accordance with the general theory of the added reproduction load and additional metabolic stress [15,16]. The strain JA221 harboring the plasmid pKSB292 exhibited the lowest μ_{max} value.

Effect of Media on the Growth of *E. coli* Harboring pKSB292

The effect of different media on the growth of recombinant cells carrying pKSB292 is shown in Table 3. The maximum specific growth rates in LB+ampicillin+IPTG medium were 70-80% of those in LB+IPTG medium regardless of the specific host strains used. The fact that the μ_{max} values in LB+ampicillin medium are lower than those in LB+IPTG medium indicates that the physiological stress caused by the antibiotic selection pressure is more detrimental to the cell growth than the metabolic load for additional protein production upon IPTG induction at the current level of foreign protein manufacture. The strains JE5505 and HB101 showed the smallest reduction in μ_{max} value when IPTG or ampicillin was added. Final cell densities of each strain in different media corresponded to the trends in the μ_{max} values (Table 4). It is

Table 2. Specific growth rate of *E. coli* strains with and without plasmid in LB medium.

Host Strains	μ_{max} (hr^{-1})		Growth Ratio
plasmid	without plasmid	with	
C600	0.84	0.83	0.99
HB101	1.02	0.82	0.80
JA221	1.25	0.88	0.70
JE5505	1.10	0.84	0.76
RB791	0.78	0.73	0.94

Table 3. Effect of medium composition on the growth rate of recombinant *E. coli* cells.

Host Strains	μm (hr^{-1})				b/a	c/a	d/a
	LB(a)	LB+IPIG(b)	LB+Amp(c)	LB+Amp+IPTG(d)			
C600	0.83	0.80	0.73	0.63	0.96	0.88	0.76
HB101	0.82	0.79	0.77	0.71	0.96	0.94	0.87
JA221	0.88	0.78	0.72	0.60	0.89	0.82	0.68
JE5505	0.84	0.81	0.79	0.73	0.96	0.94	0.87
RB791	0.73	0.72	0.62	0.61	0.99	0.84	0.84

also noteworthy that the recombinant cells harboring the plasmid with HBsAg gene lacking the amino terminal end shows a cell growth rate comparable to that of plasmid-free host cells especially considering the fact that the recombinant plasmid with the whole HBsAg gene interferes with *E. coli* cell growth leading to enentual cell death probably via the interaction between HBsAg protein and cell membrane [3].

Effect of Selection Pressure on the Production of HBsAg

Table 5 summarizes the data on the activity levels of HBsAg when the recombinant cells are induced by IPTG in the selective or nonselective medium. The total activities were higher in LB+IPTG medium than in LB+ampicillin+IPTG medium, but the specific activities were in reverse order. This change in the trend for the specific activities may be at least partly due to the increase in segregant population in the nonselective medium (Figs. 2 and 3). Among the five strains tested JE5505 showed the highest HBsAg activity level and the highest "b/a" value (Table 5), indicating that the unfavorable influence by the antibiotic selection pressure was the least for this particular strain. The probable cause for this phenomenon may be that the strain JE5505 has a mutation in the chromosomal *lpp* gene and, therefore, the competition for secretion into the periplasmic space is far more favorable for the vector-produced *lpp* signal peptide than for the chromosome produced lipoprotein.

Table 4. Effect of medium composition on the final cell density of recombinant *E. coli* cells.

Host Strains	Final Cell Density (g/l)*			
	LB	LB+IPTG	LB+Amp	LB+Amp+IPTG
C600	2.76	2.68	2.47	2.40
HB101	3.69	3.62	3.24	2.80
JA221	2.49	2.40	2.12	2.02
JE5505	2.76	2.68	2.39	2.34
RB791	2.91	2.72	2.46	2.33

* Cell density is expressed by dry cell wt (g)/unit vol. of culture (l) at 10 hr from inoculum.

Stability of pKSB292 with Generation Number

The stability of plasmid pKSB292 in each recombinant cell was examined through 30 generations. The relative proportion of plasmid-harboring recombinant cells in the selective medium although higher than that in the absence of the antibiotic selection pressure still decreased constantly with generation number. This signifies that even the presence of the antibiotic selection pressure at the current concentration level is

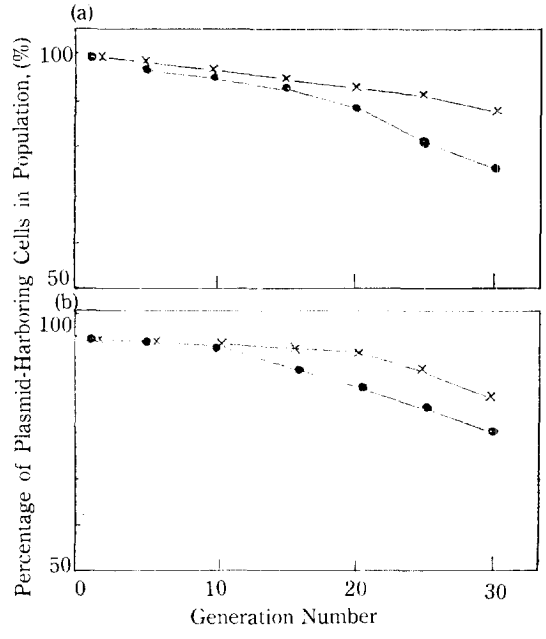

Fig. 2. Plasmid stability of recombinant *E. coli* cells harboring pKSB292 (A).
(a) C600, (b) HB101
●: Stability at LB+IPTG Culture
×: Stability at LB+Amp+IPTG Culture

Table 5. Effect of ampicillin selection on production of HBsAg from pKSB292.

Host	Total Activity*			Specific Activity**	
	LB+IPTG[a]	LB+IPTG+Amp[b]	a/b	LB+IPTG	LB+IPTG+Amp
C600	3610	3215	0.89	331	338
HB101	3825	3150	0.82	261	281
JA221	3440	2500	0.73	327	336
JE5505	4235	3970	0.93	395	424
RB791	4020	3455	0.86	369	371

* Total activity was measured in cpm in 1 m*l* of *E. coli* extract from 10 hour culture.

** Speific activity was measured in 10^{-2} × cpm per 1g of dry cell weight.

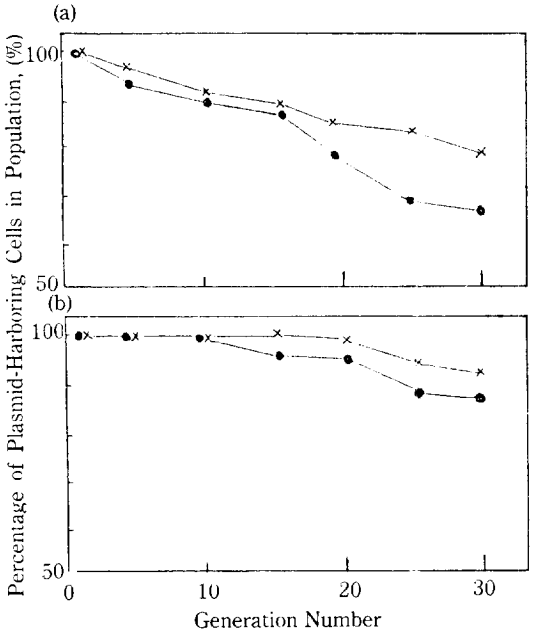

Fig. 3. Plasmid stability of recombinant *E. coli* cells harboring pKSB292 (B).

(a) JA221, (b) JE5505

●: Stability at LB+IPTG Culture

×: Stability at LB+Amp+IPTG Culture

not absolutely effective in preventing the existence of plasmid-free cells (Figures 2 and 3) The reason for this may be the partial resistance of the host itself or the decrease in the efficacy of the antibiotic in the course of fermentation. In the presence of the antibiotic the broadly used host strains HB101 and C600 exhibited good stability of over 90% through 30 generations. JA221 was least stable, the plasmid-harboring cells occupying less than 90% of total population even at the generation number around 15. JE5505 showed the best plasmid maintenance among the strains tested. RB791 was similar to C600 in plasmid stability (data not shown). Although JA221 had the least stable plasmid maintenance among the five host strains tested, the overall stability of pKSB292 in *E. coli* cells was relatively better than that of other *E. coli* plasmids reported elsewhere [17, 18, 19]. Several probable causes for the plasmid instability including the metabolic or physiological stresses have been proposed [20, 21].

CONCLUSIONS

The reduction in the growth rate of *E. coli* cells caused by the antibiotic selection pressure was greater than that caused by IPTG addition for gene expression, which suggests that the physiological stress from the use of antibiotic in the growth medium has more inhibitory effect on cell growth than the extra metabolic load for the manufacture of the heterologous protein at the level of productivity experienced in the present study.

Among the five strains used in the present study, JE5505 was found to be the most suitable host for the HBsAg production with pKSB292. The nonselective medium despite the low stability of pKSB292 was adequate for a large scale HBsAg production because of the higher growth rate and cell density achievable, the low cost of medium, and the absence of the procedure for the removal of drug residues in the product recovery step following fermentation.

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