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CONTROLLED LYSIS OF *ESCHERICHIA COLI* DOUBLE-LYSOGEN OF BACTERIOPHAGES λHL1 AND φ434

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Abstract—A novel phage double-lysogen was developed to produce an intracellular protein and disrupt the host cell in the same reactor. Using this double-lysogen, we could simplify the recovering processes without cell harvest and disruption. Construction of the double-lysogen is based on the fact that a lysogen of a phage can be superinfected by another phage with different immunity. The single-lysogen of *Escherichia coli*, P90c/λHL1, was superinfected with bacteriophage φ434 to produce a double-lysogen, in which phage genomes from each phage coexisted in the host chromosome. Two different inducers were used to induce the double-lysogen to produce a protein and to lyse the host cell. The first phage genome, λHL1, the prophage of the original lysogen, containing the temperature sensitive cI_{857}, *lac*Z and defective *Q* genes was induced by increasing temperature to produce β-galactosidase, an intracellular reporter protein. The overproduction of β-galactosidase was carried out without experiencing the cell lysis due to the defective *Q* gene. After the temperature shift, the second prophage from the lysogen MS21/φ434 was induced by mitomycin C or ultra-violet light to lyse the cell. The lysis of the cell releases the intracellular protein to the outer space. The cell lysis was confirmed by the decrease of cell density and the increase of the extracellular activity of β-galactosidase at the same time.

Key words: *Escherichia Coli, Double-Lysogen, Induction, Lysis, β-Galactosidase*

INTRODUCTION

Genetic engineering has found wide applications in the production of valuable biochemicals using bacterial expression systems. A lot of research activities from both the basic and applied sciences have been devoted to the overproduction of recombinant products and economical recovery processes since the beginning of the new biotechnology era [Bailey et al., 1986; Shuler et al., 1992]. Recombinant products such as enzymes are mostly intracellular, and should be released to the extracellular space to be recovered. Product recovery processes are in general time-consuming and labor-intensive tasks, comprising a major portion of the manufacturing cost of the product. Many areas of biotechnology could benefit from cell disruption, a central unit in the isolation of intracellular bioproducts. There are a number of ways to disrupt the cells, including temperature shock, enzymes, and chemicals [Foster, 1992]. Most of the cell disruption methods need pre-filtration or centrifugation steps to isolate the cells from the fermentation broth. For better economics, cell disruption *in-situ* has been sought for long among the bioprocess engineers.

The vector, a λ phage or a plasmid including the product gene is the centerpiece of a recombinant expression system. Manipulation of the promoters which control the expression of the product gene in the vector, consequently becomes important. The bacteriophage λ promoters, p_L and p_R, are controlled by the temperature-sensitive repressor gene (cI_{857}) product [Hendrix et al., 1983]. A temperature-sensitive system can be regulated easily by manipulating the temperature and does not require any inducer or derepressor to be added from outside. The promoters are derepressed and the cloned gene is expressed by deactivating the repressor gene product simply by increasing the temperature. Recently, Lin [1992] developed a mutant lysogen (P90c/λHL1) which has cI_{857} and Q_{am} in its prophage. In that sytem, the phage DNA freed from the host chromosome by the temperature increase remains naked rather than packaged because of the defective *Q* gene. Therefore, the higher production of target product is observed to be related to the availability of the naked DNA for transcription.

The gene product thus produced is contained as an intracellular protein, and the host cell should be disrupted to recover the intracellular protein. A phage double-lysogen was developed in this study to overproduce an intracellular enzyme and be disrupted in the same system. This technique was based on the fact that a lysogen of a phage can be superinfected by another phage with different immunity [Hendrix et al., 1983; Ptashne, 1992]. An *E. coli* lysogen, P90c/λHL1, was superinfected with bacteriophage φ434 to produce a double-lysogen in which the genomes from each phages coexist in the host chromosome. Two different inducers could be used to induce the double-lysogen to produce a protein and to lyse the host cell. The first phage, λHL1, the prophage of the original lysogen, containing the temperature-sensitive cI_{857}, *lac*Z and defective *Q* genes was induced by the temperature increase to produce β-galactosidase, an intracellular reporter protein. Following the temperature shift, the second prophage from the lysogen φ434 was induced by mitomycin C, or ultra-violet light, among other inducers [Hendrix et al., 1983] to lyse the cell. The lysis of the cell released the intracellular protein to the extracellular space. The experimental construction and inductions

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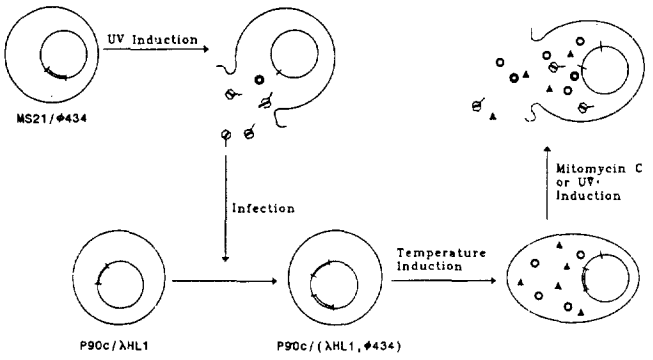

Fig. 1. Schematics of construction and inductions of double-lysogen [P90c/(λHL1, φ434)].
□; prophage φ434, ■; prophage λHL1, ▲; β-galactosidase.

of the double-lysogen will be presented with the discussions for the possible industrial applications.

MATERIALS AND METHODS

1. Bacterial Strains and Phages

An *E. coli* bacterial strain, P90c (ara Δ(lac-pro) thi) [Simons et al., 1987], a lysogen of a bacteriophage λ derivative vector [Lin, 1992; Sambrook et al., 1989; Schechtman et al., 1980] λHL1, containing *lacZ*, cI_{857}, and Q_{am}, was used as a host strain. An *E. coli* lysogen (MS21/φ434), kindly provided by Dr. Jeffrey W. Roberts (Cornell University) was used as a source strain to obtain the phage φ434 for the infection of the single-lysogen P90c/λHL1, and the confirmation of the double-lysogen. Wild type λ phage was also used to confirm the double-lysogen. *E. coli*, JM109, served as a host strain for the preparation of wild type phage stock solution.

2. Construction of Double-lysogen of λHL1 and φ434

The schematics of the construction procedures of a double-lysogen are presented in Fig. 1. The preparation of plating bacteria, plating phages and the infection of bacterium mostly followed the descriptions of Sambrook et al. [1989]. Phage φ434 was obtained from MS21/φ434 by UV induction. The UV (254 nm) irradiation was carried out for 10 ml culture (OD_{600} 0.4) in M9a media which was evenly spreaded on a Petri dish (I.D. 10 cm). MS21/φ434 was irradiated with a dosage of 300 μJ/cm^2 (UV Stratalinker 1800, STRATAGENE). MS21/φ434 began to lyse and released φ434 at 1.5 hour after the irradiation. Plating bacterium, the single-lysogen (P90c/λHL1) was superinfected by φ434 on agar plate. The resulting double-lysogen [P90c/(λHL1, φ434)], picked from the turbid plaque was identified by various methods, such as induction by the UV irradiation or mitomycin C, infection by φ434 or wild type λ, and β-galactosidase activity. The identification methods, based upon the immunity and β-galactosidase activity are described in Table 1.

3. Inductions of Double-lysogen for Protein Production and Cell Lysis

An induction of the double-lysogen by the temperature shift to express the product gene, was followed by another induction to lyse the host cell. The temperature of 30℃ for the cell growth in LB media was shifted to 35℃ or higher to induce the prophage λHL1. After the temperature shift, the double-lysogen was induced again to lyse by either the mitomycin C addition or the UV irradiation. Mitomycin C (2 mg/5 ml) was mainly used to induce the double-lysogen in a culture of 5 ml in 100 ml flask. The UV (254 nm) irradiations were carried out to the culture of LB media in 250 ml flask. The intensity of the UV light (Pen-Ray, UVP) was measured by the UV radiometer (Blak-Ray, UVP). The culture volume was 25 ml. The cell density was measured spectrophotometrically (Spectronic 601, Milton Roy) at 600 nm. The β-galactosidase activity was measured using o-nitrophenyl-β-D-galactoside, a color-developing substrate, following the procedures suggested by Miller [1972]. The extracellular activity of β-galactosidase was measured after the cells and cell debris were removed by centrifugation at 12,000 rpm for 5 minutes in the microcentrifuge (MICROSPIN 12S, Sorvall).

Table 1. Identification of possible lysogens

Method	Strains			
	Without phage	MS21/φ434	P90c/λHL1	P90c/ (λHL1, φ434)
Induction by				
UV	−	+	−	+
Mitomycin-C	−	+	−	+
Infection with				
wild type λ	+	+	−	−
φ434	+	−	+	−
Production of				
β-galactosidase	−	−	+	+

RESULTS AND DISCUSSION

1. Identification of Double-Lysogen

A double-lysogen was selected among the single colonies picked from the streakings of the turbid plaque, using the protocols shown in Table 1. A double-lysogen has immunity against the infection of both λ and φ434. The colonies which were not infected (lysed) by either wild type λ or φ434 were irradiated with the UV light. All the colonies, lysed upon the UV irradiation, showed lysis by the addition of mitomycin C and high β-galactosidase activities. These colonies were finally identified as the double-lysogen carrying both phages of λHL1 and φ434. Wild type λ was used to confirm the double-lysogen instead of λHL1. Note that λHL1 can not be obtained as a separate phage progeny due to its defective Q_{am} gene which is responsible for the expressions of the head and tail of a phage.

2. Inductions for β-Galactosidase Production and Cell Lysis

The double-lysogen was induced by the temperature increase and the mitomycin C addition to produce an enzyme and lyse the cell, respectively. The cell growth of the double-lysogen and the single-lysogen are compared in Fig. 2. While the double-lysogen grew at 30℃, a 5 ml portion of the culture was taken at proper cell densities (OD 0.4, 1.9, and 4.1), and put to 100 ml flask in shaking incubator at 35℃, followed by the addition of mitomycin C. During the exponential growth phase at 30℃, the double- and the single-lysogens didn't exhibit much difference in their growth patterns. The double-lysogen showed lysis at about 1.5 hour after the addition, while the single-lysogen was not affected much. Up to the middle exponential growth phase (OD 2-3), the double-lysogen lysed and the culture broth looked clear at about 4 hours after the induction. The addition of mitomycin C to the double-

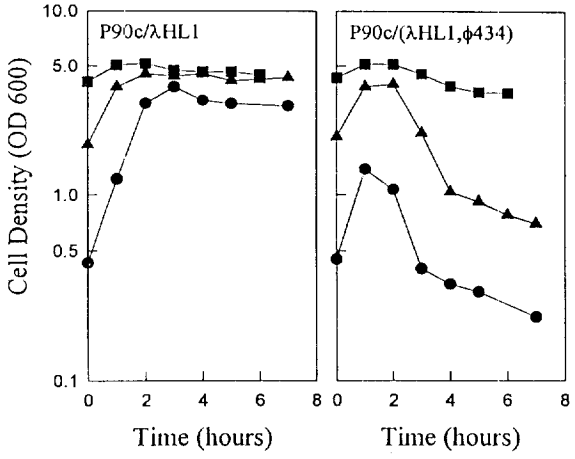

Fig. 2. Comparison of growth curves between P90c/λHL1 and P90c/(λHL1, φ434) after the mitomycin C addition at 35°C.

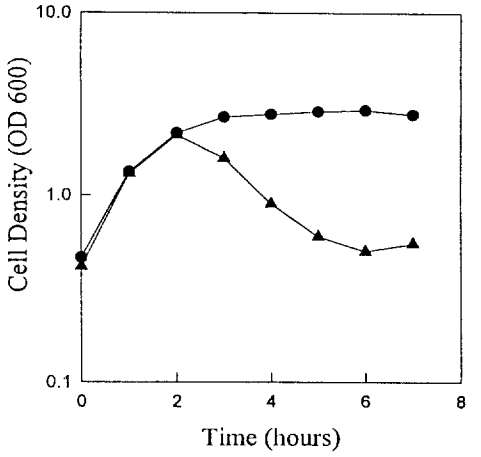

Fig. 4. Growth curves of P90c/λHL1 (●) and MS21/φ434 (▲) after the addition of mitomycin C at 35°C.

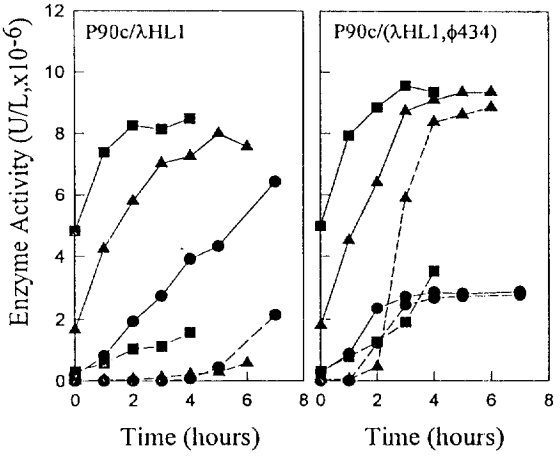

Fig. 3. Comparison of β-galactosidase activity between P90c/λHL1 and P90c/(λHL1, φ434) after the mitomycin C addition at 35 °C.

—; total enzyme activity after the mitomycin C addition, ---; extracellular enzyme activity after the mitomycin C addition, ●; addition at OD 0.4 (I), ▲; addition at OD 2.1 (II), ■; addition at OD 4.3 (III).

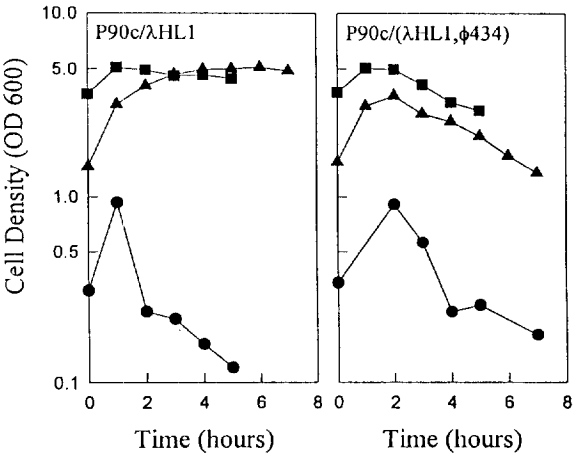

Fig. 5. Comparison of growth curves between P90c/λHL1 and P90c/(λHL1, φ434) after the mitomycin C addition at 38°C.

lysogen at high cell density didn't cause much cell lysis in comparison to those with lower cell densities. This verifies that young cells in their early exponential phase are sensitive to the inducers.

Another way to measure the extent of cell lysis is to compare the total and the extracellular concentrations of β-galactosidase which is normally produced intracellularly. The concentrations of β-galactosidase produced by the lysogens with the temperature shift to 35°C are compared in Fig. 3. With the double-lysogen, the extracellular concentration of β-galactosidase, shown as dashed lines, began to increase abruptly at about 1.5 hour after the mitomycin C addition. The extracellular concentration approached 90% of the total concentration, shown as solid lines, at about 4 hours after the addition. The approach was slow and incomplete when the mitomycin C was added at high cell density of OD 4.3. The total β-galactosidase production was much less with the mitomycin C addition at low cell density of OD 0.4 than those at higher densities due to the cell lysis before the exponential growth phase. The increase of the extracellular concentration of β-galactosidase were observed to start at the same time with the decrease of cell density shown in Fig. 2. The degree of cell lysis, defined as the ratio of the extracellular concentration to the total concentration of β-galactosidase was observed to be roughly proportional to the ratio of the cell densities after and before lysis shown in Fig. 2. These confirmed that the intracellular β-galactosidase was released to the extracellular space by the cell lysis induced by the mitomycin C addition. Exact proportionality between the extent of protein release and the cell density was not expected due to the effect of cell debris after lysis to the spectral absorbancy for the cell density measurement. During the stationary growth phase of the single-lysogen, the extracellular concentrations of β-galactosidase increased to certain values. This can be interpreted as a result of the autolysis which naturally occurs during the stationary phase, or the incomplete blockage of transcription through the genome of λHL1 prophage by the defective *Q* gene. For reference, the growth curves of P90c/λHL1 and MS21/Φ434 after the addition of mitomycin C are presented in Fig. 4. The fact that P90c/λHL1 did not lyse with the mitomycin C assured that the lysis of double-lysogen is driven by the expression of φ434, the second prophage.

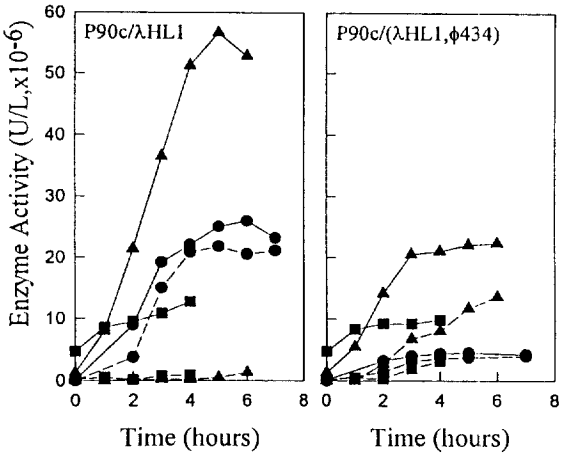

Fig. 6. Comparison of β-galactosidase activity between P90c/λHL1 and P90c/(λHL1, φ434) after the mitomycin C addition at 38 °C.

—; total enzyme activity after the mitomycin C addition, ---; extracellular enzyme activity after the mitomycin C addition, ●; addition at OD 0.3 (I), ▲; addition at OD 1.4 (II), ■; addition at OD 3.6 (III).

The results of cell lysis of the double-lysogen by the temperature shift to 38℃ are shown in Fig. 5. The trends of cell lysis were similar to those with the temperature shift to 35℃. The lysis induced by the mitomycin C at the middle of exponential growth phase (cell density of OD 1.5) was not as quick as that with the temperature shift to 35℃. This incomplete cell lysis was shown more clearly in Fig. 6. The extracellular concentration of β-galactosidase was only 38% of the total concentration at 4 hours after the mitomycin addition. Comparison between the total β-galactosidase production by the double- and the single-lysogens at 38℃ showed that the induction for the β-galactosidase production by the temperature shift is most effective with the culture of cell density of about OD 1.5. The low degree of cell lysis for the double-lysogen at 38℃, when the total enzyme concentration was high, may be ascribed to the fact that the amino acids resources supplied from nutritional media were depleted by the overexpression of the enzyme. Note that the cell lysis is accompanied by the liberation of many phages with complete head and tail which are mostly proteins, composed of amino acids. The exact causes remain unknown and are subject to further studies. The unexpected lysis of P90c/λHL1 at 38℃ at the early exponential phase could be caused by the leaking of Q_{am} mutation in the single-lysogen. Some genetic modifications in regulation by mutation is inherently apt to function incompletely. Mitomycin C was observed to enhance the leakage. The degrees of cell lysis with different timings of the mitomycin C addition are shown in Table 2. In most cases, P90c/(λHL1, φ434) showed more lysis than P90 c/λHL1, especially at 35℃.

The optimization of the induction processes should be subject to the engineering studies to obtain the most efficient protein production and cell lysis. The temperature and the timings of induction are considered to be the most important parameters in the optimization studies. A study for the successful lysis at high cell density remains as a future work.

3. Cell Lysis by UV Irradiation

There are several ways to induce the dormant prophage to lyse the host cell. UV irradation is known to be an effective inducer [Hendrix et al., 1983]. In Fig. 7, the cell growths of the double- and single-lysogens are compared at various UV dosages. The cells were grown in LB media in the shaking flask at 30℃, and the temperature was shifted to 38℃ at the cell density of about OD 0.5. The double-lysogen began to lyse at 1.5 hour after the UV irradiation. The degree of cell lysis increased with the UV dosage in the experimental ranges up to 96 mJ/cm^2 (at the intensity of 800 μJ/cm^{2}sec for 120 sec). The slow growth of the single-lysogen was resulted from the germicidal effect of UV light. It is interesting to note the response time of 1.5 hour for the cell lysis of the double-lysogen is common to the UV irradiation and the mitomycin C addition. The cell lysis curves by the UV irradiation was not as sharp as that by mitomycin C. It is considered due to the lack of homogeneity in UV dosages among the cells, caused by the blockage of UV light by the cells near the surface. However, the phage induction by UV irradiation has advantage over the method of chemical addition, such as mitomycin C, in the sense that it does not leave any toxic chemicals in the system. As some compounds, especially the ring structured ones, absorb the UV light, the culture in LB media needed more UV dosage than that in the minimal media (M9a).

Table 2. Degree of cell lysis at 4 hours after the addition of mitomycin C with different timings of induction *

Mitomycin C addition timing during growth	Strains			
	P90c/λHL1		P90c/(λHL1, φ434)	
	35℃	38℃	35℃	38℃
I[a)]	0.02	0.94	0.94	0.83
II	0.03	0.01	0.92	0.38
III	0.19	0.07	0.41	0.34

*Degree of cell lysis represents the ratio of the extracellular enzyme activity to the total enzyme activity.

[a)]Data from Figs. 3, and 6.

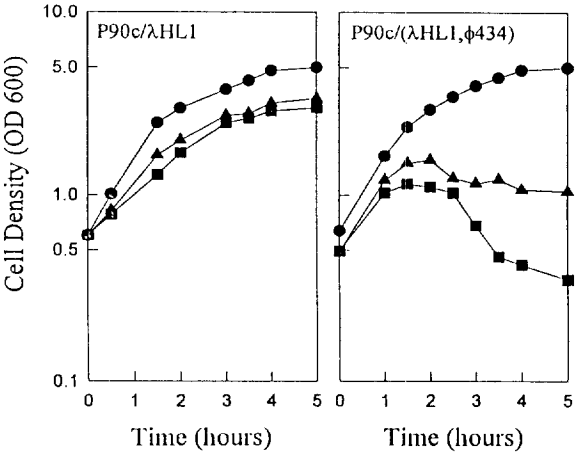

Fig. 7. Comparison of growth curves between P90c/λHL1 and P90c/(λHL1, φ434) after the UV irradiations at the intensity of 800 μJ/cm^{2}sec at time 0 for 0 sec (●), 60 sec (▲) and 120 sec (■).

4. Considerations for the Industrial Applications

Intracellular enzyme should be secreted to the extracellular space to function as a catalyst unless the substrate itself comes into the cell from outside. The reaction rates of many biological

systems are limited by the mass transfer resistance of the substrate and the product, permeating in and out, respectively, through the cell wall. Therefore, an efficient way of disrupting cell, such as one described in this study, is expected to enhance the reaction rate. A bioreactor system for the environmental detoxification would fit to this situation. Recently, the expanded bed adsorption technique [Chase, 1994], where the target product is separated directly from the crude fermentation broth in the fluidized bed of a proper adsorbent, has attracted attention among the bioseparation engineers. In this process, the intracellular product should be released to the extracellular space in advance. Cell lysis *in-situ* described in this work will satisfy the conditions needed in the process of expanded bed adsorption.

CONCLUSIONS

This work has demonstrated the successful construction and verification of the *E. coli* double-lysogen. A double-lysogen (P90c/(λHL1, φ434)) was constructed by infecting the single-lysogen (P 90c/λHL1) with φ434 from another single-lysogen (MS21/φ434), and was identified by the several tests. The double-lysogen at early exponential growth phase was satisfactorily induced by the temperature shift from 32℃ to 35℃ or 38℃. Upon the mitomycin C addition, the lysogen was successfully disrupted at early exponential growth phase, resulting in the extracellular β-galactosidase activities more than 90% and 80% of the total activities at 35℃ and 38℃, respectively. The double-lysogen at early exponential growth phase was also induced and disrupted by the UV irradiation with the dosage of 96 mJ/cm^2. Improved operational conditions to disrupt the cells of higher cell density are needed for this technique to be useful for the industrial applications.

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