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# Synchronized Disruption of *Escherichia coli* Cells by T4 Phage Infection

KAZUHIRO ASAMI, XIN-HUI XING, YASUNORI TANJI,* AND HAJIME UNNO

*Department of Bioengineering, Faculty of Biotechnology, Tokyo Institute of Technology, 4259 Nagatuda-cho, Midori-ku, Yokohama 226, Japan*

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**For development of an autolytic *Escherichia coli* protein expression system, T4 bacteriophage (T4)-mediated *E. coli* disruption was investigated. At least two types of *E. coli* cell lysis, "lysis from without" (LO) and "lysis from within" (LI), are known to be induced by T4. The efficiency of cell disruption was monitored by the release of β-galactosidase from the cells. In the case of multiplicity of infection (m.o.i.) of 100, the infected cells were lysed without proliferation of the progeny phage (LO). When the cells were infected at a m.o.i. of 5, slow cell lysis (LI) was observed. The β-galactosidase activity detected in the supernatant of the culture subjected to LO was the same as that in the lysate produced by chloroform treatment or sonication of the T4-uninfected culture, but about twice that in the supernatant of the culture subjected to LI. At a m.o.i. of 0.01, delayed onset of cell lysis, called lysis inhibition (LIN), was observed. However, the cells in LIN state were simultaneously lysed upon shifting of the temperature from 37°C to 0°C, which was accompanied by an increase in extracellular β-galactosidase activity.**

[**Key words:** phage-mediated cell lysis, *Escherichia coli*, T4 phage, β-galactosidase, lysis inhibition collapse, temperature-induced lysis]

For recovery of proteins produced in engineered cells, cell-free extracts of the cells must be prepared, generally by cell disruption in the first stage of the recovery process. At present, a variety of techniques to disrupt bacterial cells are available (1, 2). However, these techniques require extensive improvement especially for industrial use. Mechanical methods are often accompanied by generation of heat which reduces the activity of the protein to be recovered. In the case of lysozyme use, addition of detergents for efficient disruption of the outer membrane reduces the yield of active target proteins due to denaturation. In addition, lysozyme itself was suggested to exhibit protease activity (3).

As an alternative approach to cell disruption, a programmed cell lysis system might be useful. This technique is based on expression of a cloned T4 gene to produce lytic proteins which function at the most convenient time for target protein recovery. In the present study, various features of T4-mediated *E. coli* cell disruption were examined as the first step to determining the feasibility of the programmed cell lysis system.

At least two types of *E. coli* cell lysis are known to be induced by T4 (4–6): "lysis from within" (LI) and "lysis from without" (LO). LI is normal vegetative lysis and occurs at m.o.i. of less than 20. LO is lysis due to adsorption of a large number of T4 particles on the cell wall and occurs at m.o.i. of more than 20. When *E. coli* cells are infected with phages at m.o.i. of less than 1, the cells show delayed onset of cell lysis, called lysis inhibition (LIN), which is observed in case of superinfection of cells (7, 8). However, little is known about the trigger of LIN. In the present study cells in the LIN state were lysed simultaneously upon lowering of the temperature. This phenomenon was defined as "temperature-induced LIN collapse". Utilization of phage-encoded genes for cell disruption and product release is discussed below in terms of three different types of phage-mediated cell lysis, *i.e.*, LO, LI and LIN collapse.

* Corresponding author.

## MATERIALS AND METHODS

**Bacterial strain, phage, and conditions of cultivation** *E. coli* BE was used as the host strain for the T4 phage. Wild-type T4D phage was used for cell lysis. The medium M9L was used for *E. coli* culture. M9L consists of 6 g of $Na_2HPO_4$, 3 g of $KH_2PO_4$, 0.2 g of $MgSO_4 \cdot H_2O$, 0.5 g of NaCl, 1 g of $NH_4Cl$, 2 g of lactose, and 10 g of Casamino Acids (Difco Laboratories), per liter of water. Two hundred ml of culture was incubated at 37°C with aeration in a 1 *l* culture vial. Cells were harvested periodically and stored at 0°C until the cell disruption. Contamination of the culture by phage was tested for by plating the culture on an agar plate (9).

**Cell disruption** Phage-mediated cell disruption was carried out as follows: diluted T4 phage stock was inoculated into the *E. coli* culture at 0°C for uniform phage adsorption. After the adsorption of the phage onto the *E. coli* cells for 5 min, the culture was incubated at 37°C on a reciprocating shaker.

Three conventional cell disruption methods were also used: *E. coli* cells (2 ml) not infected with phage were sonicated with an ultrasonicator (Ohtake Works Co. Ltd., Japan) 8 times for 30 s each at 0°C.

Lysozyme treatment was performed by the method of Marston with slight modification (10). A 40 ml aliquot of the *E. coli* culture was centrifuged and the pellet was resuspended in ice-cold lysis buffer containing 50 mM Tris·Cl (pH 8.0), 1 mM EDTA and 100 mM NaCl. Then white egg lysozyme was added to the suspension to a final concentration of 270 μg/ml, followed by stirring of the suspension for 20 min at 4°C.

Chloroform treatment was carried out according to a method described elsewhere (11).

**Cell growth and cell number determination** Cell

growth was monitored in terms of optical density at 600 nm ($OD_{600}$). Bacterial cell number was determined by hemocytometer.

**β-Galactosidase assay** The efficiency of cell disruption was determined in terms of the level of β-galactosidase activity in the supernatant and cell pellet of T4-infected culture centrifuged at 8,000×*g* for 10 min. The pellet was resuspended in saline. The β-galactosidase activity was determined by a method described elsewhere (11). The total β-galactosidase activity in the culture was monitored in terms of the activity in the lysate produced by chloroform disruption of T4-infected culture.

## RESULTS AND DISCUSSION

**Characterization of LO** It has been reported that the gene 5 product (gp5) of T4 particle has lysozyme activity and is responsible for LO (4). At the onset of infection, gp5 penetrates into the cell membrane where it catalyzes peptidoglycan degradation, which facilitates the injection of phage DNA into the cell. At large m.o.i., adsorption of a large number of phage particles on the surface of *E. coli* cells leads to large-scale peptidoglycan degradation and thus cell membrane disruption. For characterization of *E. coli* cell disruption by LO, *E. coli* BE cells were infected with T4 at a m.o.i. of 100. For achieving uniform phage adsorption on the surface of the *E. coli* cells, the phage adsorption was performed at 0°C. Figure 1 shows the profiles of *E. coli* cell lysis in various growth phases. The broken line in the figure indicates the growth of phage-uninfected *E. coli* cells. The culture was sampled in the early exponential growth phase (2 h), middle exponential growth phase (3 and 4 h) and late exponential growth phase (5.3 h), and each sample was infected with T4. When the *E. coli* cells were infected in the early exponential growth phase, a prompt decrease in $OD_{600}$ was observed: the $OD_{600}$ decreased to a minimum within 5 min after infection. A prompt decrease in $OD_{600}$ was also observed when the phage was introduced into the culture in the middle exponential growth phase. On the other hand, when the phage was added to the culture in the late exponential growth phase, cell disruption was retarded: it took about 2 h for complete cell disruption to occur. The slight turbidity of the medium after the completion of the cell disruption was due to light scattering by the cell debris, which was confirmed by microscopic observation.

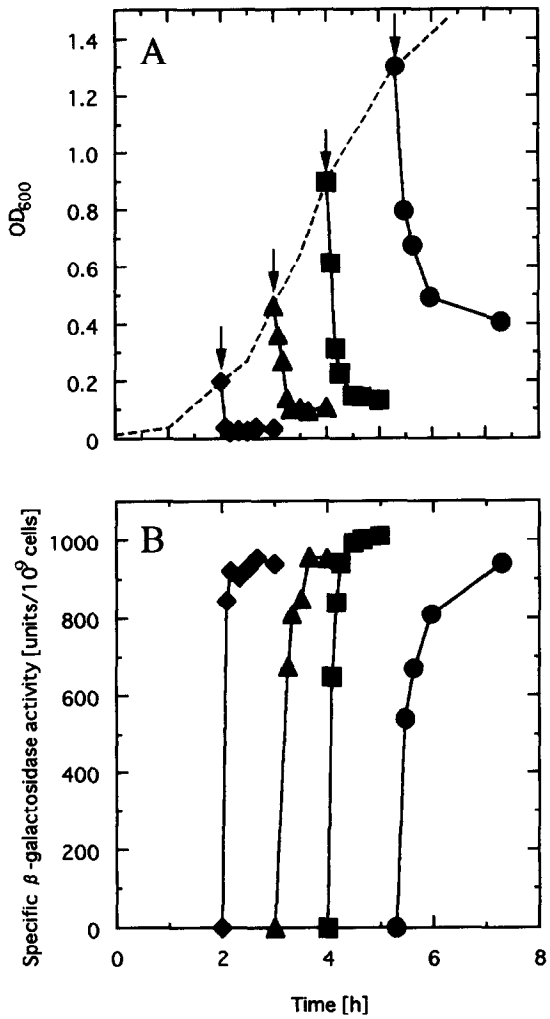


FIG. 1. Profiles of *E. coli* cell lysis by T4 phage-mediated LO at a m.o.i. of 100 (A), and changes in specific β-galactosidase activity (B). The broken line indicates the growth of phage-uninfected *E. coli* cells. A 2 ml aliquot of an *E. coli* culture, grown in a 1 *l* culture vial with aeration, was transferred into a test tube (φ18 mm × 180 mm) at each of the time points indicated by the arrows. Then the aliquot was infected with phage and incubated at 37°C on a reciprocating shaker (2 cm stroke, 180 cycles/min).

According to the decrease in $OD_{600}$, the extracellular β-galactosidase activity increased rapidly (Fig. 1B). Table 1 shows the maximum β-galactosidase activity detected in the supernatant of lysate produced by LO and β-galactosidase activity in the lysate produced by chloroform disruption of T4-uninfected culture at various incubation times. The relative activity of β-galactosidase in the supernatant of lysate produced by LO was almost the same at each incubation time, which suggests that production of β-galactosidase in the cells was not affected by LO.

Figure 2 shows the β-galactosidase activity in the supernatant and cell pellet of the culture infected with the phage in the late exponential growth phase, *i.e.*, at 5.3 h of incubation as indicated in Fig. 1. The β-galactosidase activity in the supernatant and cell pellet was also compared with the total β-galactosidase activity in the lysate produced by chloroform disruption of T4-infected culture. No β-galactosidase activity was detected in the supernatant of culture prior to the infection. The β-galactosidase activity observed in the cell pellet of the culture at time zero was likely due to the penetration of *o*-nitrophenyl galactoside (ONPG) into the cells, where β-galactosidase hydrolyzes ONPG to *o*-nitrophenol, a yellow chromophore. After the phage infection the β-galactosidase activity in the supernatant and cell pellet increased rapidly. The sum of the β-galactosidase activity in the supernatant and cell pellet reached 80% and 98% of the total β-galactosidase activity in the lysate

TABLE 1. β-Galactosidase activity at various incubation times

| Incubation time (h) | β-Galactosidase activity (units/ml) | | Relative activity (A/B) |
|---|---|---|---|
| | (A) Disrupted by LO | (B) Disrupted by chloroform | |
| 2.0 | $(0.19\pm0.00)\times10^3$ | $(0.19\pm0.01)\times10^3$ | 1.00 |
| 3.0 | $(1.00\pm0.02)\times10^3$ | $(1.05\pm0.02)\times10^3$ | 0.95 |
| 4.0 | $(2.41\pm0.01)\times10^3$ | $(2.57\pm0.02)\times10^3$ | 0.94 |
| 5.3 | $(6.43\pm0.06)\times10^3$ | $(6.81\pm0.48)\times10^3$ | 0.94 |

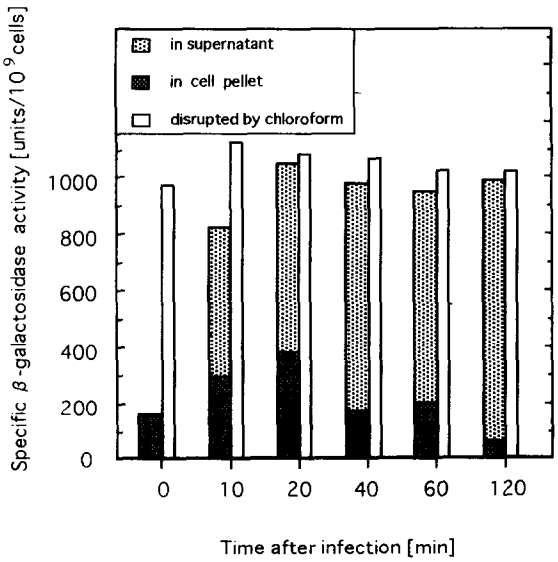


FIG. 2. Changes in specific β-galactosidase activity in the supernatant and cell pellet resuspended in saline for the culture subjected to LO. The left bar in each pair indicates the β-galactosidase activity in the supernatant and cell pellet of the T4-infected culture and the right bar indicates that in lysate produced by chloroform treatment of T4-infected culture.

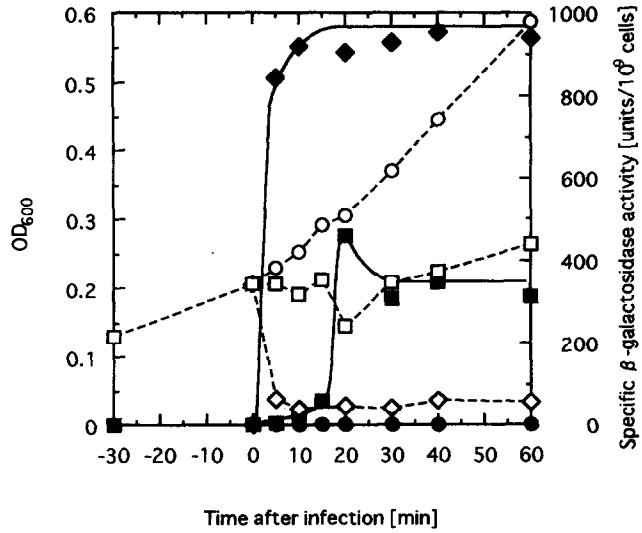


FIG. 3. Profiles of *E. coli* cell lysis by T4 phage-mediated LI (m.o.i. of 5) and LO (m.o.i. of 100). *E. coli* cells were grown to $2 \times 10^8$ cells/ml and infected with phage at time zero. $OD_{600}$ (□, ◇, ○) and specific β-galactosidase activity in supernatant (■, ◆, ●) were determined in the culture subjected to LI (□, ■) and LO (◇, ◆) and the culture without phage infection (○, ●).

produced by chloroform disruption of T4-infected culture at 10 min and 20 min, respectively. The total β-galactosidase activity in T4-infected culture remained almost constant throughout the incubation period. The β-galactosidase activity in the cell pellet of T4-infected culture reached a maximum at 20 min, then decreased. On the other hand, the β-galactosidase activity in the supernatant of lysate produced by LO increased rapidly in the first 10 min of incubation, and then increased gradually, which indicates sudden disruption of the cells followed by gradual release of β-galactosidase from the cells into the medium.

**Characterization of LI** Figure 3 shows the profiles of *E. coli* cell lysis by T4 phage-mediated LI and LO. The cells were infected with T4 phage at a m.o.i. of 100 or 5, for LO and LI, respectively. When the cells were infected at the m.o.i. of 5, 99.4% of the cells were considered to be infected by at least one phage particle (9). In this case, the $OD_{600}$ remained almost constant for 1 h after phage infection. Typically, when one *E. coli* cell is infected by a small number (less than 20) of phage particles, the cell does not undergo to immediate disruption (9). Since microscopic observation revealed that the amount of viable cells was small in the culture at 30 min after phage infection, the $OD_{600}$ at that time was considered to be due to the presence of large fragments of cell debris, which remained there for an extended period of incubation.

On the other hand, a rapid increase in the β-galactosidase activity in the supernatant of the culture subjected to LI was observed at 20 min after infection. The occurrence of LI is suggested to depend on the activity of both the phage-encoded lysozyme (gene E product, gpE) and the inner membrane protein (gene t product, gpt) (6). Gpt is considered to form a tunnel-like structure in the cell membrane, through which gpE accesses the periplasmic space and the layer of peptidoglycan as its substrate at around 20 min after infection (12). Therefore, the increase in β-galactosidase activity in the supernatant was due to cell disruption by LI.

The maximum β-galactosidase activity in the supernatant of the culture subjected to LI was about half of that detected in the supernatant of the culture subjected to LO. This is because that phage infection results in termination of host DNA replication and enhancement of proteolytic activity in the host cell (12), and thus degradation of the protein in the cell during the period of phage proliferation.

**Comparison of β-galactosidase activity with conventional methods** Table 2 shows a comparison of the β-galactosidase activity in the lysate produced by phage infection and that in the lysate of T4-uninfected culture disrupted by the conventional methods. The β-galactosidase activity in the supernatant of the lysate produced by LI was lower than those in the lysate produced by chloroform disruption and sonication of T4-uninfected culture. On the other hand, the β-galactosidase activity in the supernatant of the lysate produced by LO was

TABLE 2. β-Galactosidase activity in the lysate or the supernatant of the lysate produced by various disruption methods

| Method of disruption | Specific β-galactosidase activity (units/$10^9$ cells) |
|---|---|
| Early exponential growth phase | |
| Lysis from without | 930±20 |
| Lysis from within | 460±9 |
| Sonication | 964±24 |
| Lysozyme | 772±34 |
| Chloroform | 925±34[a] |
| Late exponential growth phase | |
| Lysis from without | 918±9 |
| Lysis from within[b] | — |
| Sonication | 996±25 |
| Lysozyme | 802±27 |
| Chloroform | 973±68[a] |

[a] The activity of the lysate not in the supernatant.
[b] Could not be performed.

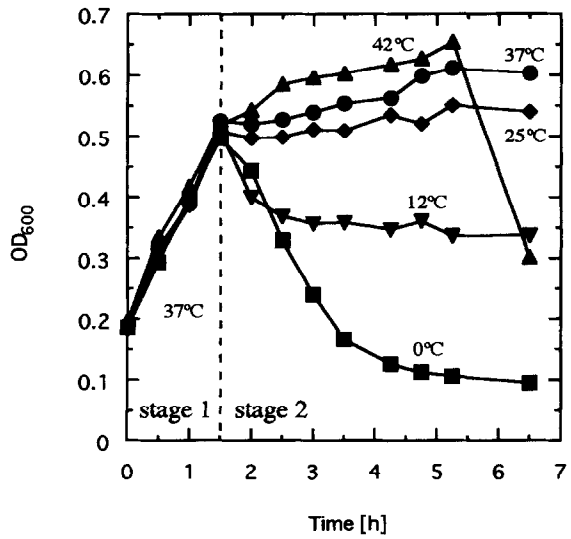


FIG. 4. Lysis profiles of *E. coli cells* in LIN state by a temperature shift at 1.5 h after infection. *E. coli* cells were grown to $2 \times 10^8$ cells/ml with aeration in a 1 *l* culture vial and a 20 ml aliquot of the culture was transferred into a test tube ($\phi$30 mm × 200 mm). The cells in this aliquot were infected with phage at a m.o.i. of 0.01 at time zero and then incubated for 1.5 h at 37°C on a reciprocating shaker (2 cm stroke, 180 cycles/min) (stage 1) and then incubated statically at 42°C (▲), 37°C (●), 25°C (◆), 12°C (▼) or 0°C (■) (stage 2).

almost the same as those in the lysate produced by chloroform disruption and sonication, and slightly higher than those in the lysate produced by lysozyme treatment. These findings imply that prompt disruption of the cells is important for efficient product recovery from the host cells.

**Characterization of inducible LIN collapse** Figure 4 shows lysis profiles of *E. coli* cells in LIN state by a temperature shift at 1.5 h after infection. The LIN was realized by phage infection at a m.o.i. of 0.01, followed by incubation at 37°C for 1.5 h in a shaking water bath (Fig. 4, stage 1). During this stage, the phage proliferated in the host cells and was released into the medium at around 25 min after infection. Since the progeny phage release was not synchronized, superinfection of the cells followed by LIN was realized. During the second stage, aliquots were incubated at various temperatures, 0°C, 12°C, 25°C, 37°C and 42°C, without shaking. Gradual increases in $OD_{600}$ at 25°C, 37°C and 42°C were observed. The LIN collapse occurred at 7 h of incubation when the LIN-state cells were incubated at 42°C. LIN collapse also observed at 37°C after 20 h of incubation (data not shown). Incubation at 25°C did not lead to LIN collapse during the present experimental period. On the other hand, the temperature shift from 37°C to 12°C or 0°C, especially 0°C, led to immediate cell disruption, indicated by a rapid decrease in $OD_{600}$.

Figure 5 shows the effects of incubation conditions on LIN collapse and the release of β-galactosidase into the medium. The cells were infected with phage at a m.o.i. of 0.01 at time zero. LIN was realized at around 30 min after infection, based on the assumption that at that time released progeny phage began to infect new host cells. After 1.5 h of incubation (stage 1), the culture was incubated at 0°C (in an ice bath) with or without shaking. In both cases, with and without shaking, $OD_{600}$ and the cell number started to decrease following the temperature downshift (Fig. 5A and B, stage 2).

Figure 5C shows changes in β-galactosidase activity in the supernatant of LIN-state culture and the total β-galactosidase activity in LIN-state culture disrupted by chloroform treatment. The total β-galactosidase activity increased for 1.5 h after infection, and then reached a plateau, indicating cessation of protein synthesis in the cells at that time. After the temperature shift at 1.5 h, the β-galactosidase activity in the supernatant of LIN-state cultures increased corresponding to the decrease in cell number (Fig. 5B, C). The release of β-galactosidase into the medium in the shaking culture was more extensive than that in the static culture, possibly due to the higher shear stress on the LIN-state cells in the shaking culture than in the static one (Fig. 5C). In all three cases, the final β-galactosidase activity in the supernatant reached the total β-galactosidase activity in LIN-state culture disrupted by chloroform treatment. The total β-galactosidase activity in the LIN-state cultures, in stage 2, was constant, indicating that the β-galactosidase produced was not inactivated in the cells.

Figures 5D–F show the distributions of β-galactosidase activity in the supernatant and cell pellet of LIN-state cultures. Under these three sets of conditions, the β-galactosidase activity in the cell pellet at 0 h was approximately 50 units/ml. At 0.5 h of incubation, expected to be the time of LIN-state onset, the β-galactosidase activity in the cell pellet was about 4 times larger than that at 0 h. The increase in the β-galactosidase activity was likely due to the enhanced penetration of ONPG into the LIN-state cells and the increase in cell number. The sum of the β-galactosidase activity in the supernatant and that in the cell pellet reached approximately 950 units/ml at 1.5 h after infection, and remained constant until 5 h of incubation in all three cases. However, rapid release of β-galactosidase into the medium was detected when the LIN-state cells were incubated at 0°C (Fig. 5E, F). The release of β-galactosidase at 0°C was enhanced by shaking (Fig. 5F).

As shown in Fig. 5B, the cell number determined by hemocytometer did not change from 0.5 to 3 h after infection, indicating lack of *E. coli* proliferation. Even though the *E. coli* proliferation ceased at LIN onset, the $OD_{600}$ increased from 0.5 to 3 h after infection (Fig. 5A), implying enlargement of individual cells during this period. Enhancement of cell disruption and release of β-galactosidase into the medium were detected when the LIN-state culture was subjected to a low temperature, which has not been reported previously. The mechanism of this enhanced cell disruption is not fully understood. Since phage-encoded gpE is considered to be expressed in LIN-state cells (4), this enhanced cell disruption was attributed to the degradation of the peptidoglycan layer by gpE, which was released from the cytoplasm into the periplasmic space due to a combined effect of the cell fragility in the LIN state and the damage induced by the sudden drop in temperature. Further investigations such as morphological observations and cell permeability tests are needed for elucidation of the mechanisms underlying the temperature-induced LIN collapse.

**Conclusion** Intracellular β-galactosidase was released into the medium when the cells underwent LO or LI. The maximum β-galactosidase activity in the supernatant of the lysate produced by LO was the same as those in the lysate produced by conventional cell disruption methods. In the case of temperature-induced LIN

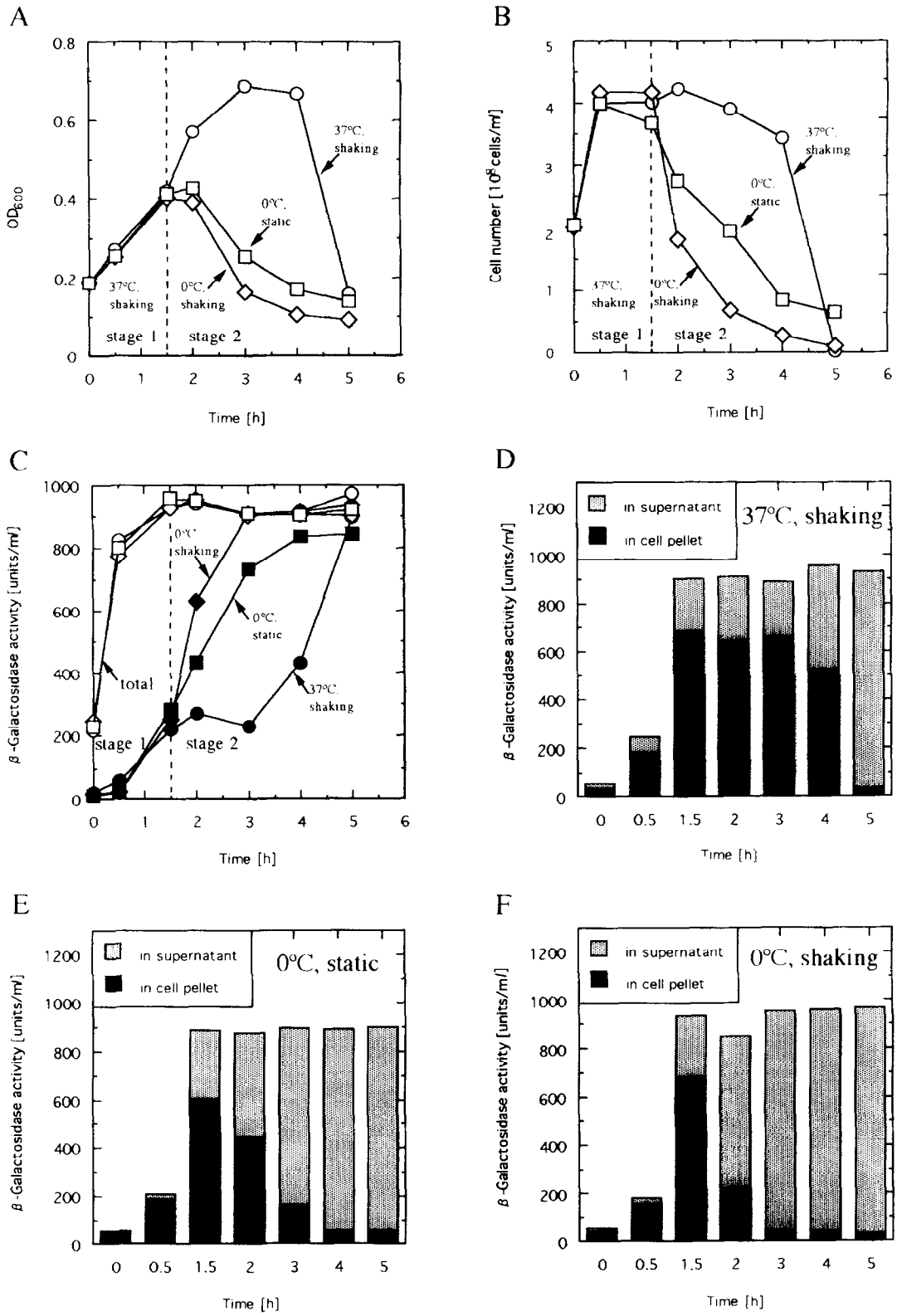


FIG. 5. Effects of incubation conditions on LIN collapse and the release of β-galactosidase into the medium. The cells were infected with phage at time zero and then incubated for 1.5 h as noted in the caption of Fig. 4 (stage 1). Then the phage-infected cultures were incubated in an ice bath statically with shaking (stage 2). As a control, a culture was incubated continuously at 37°C on reciprocating shaker. $OD_{600}$ (A), cell number determined by hemocytometer (B), total β-galactosidase activity produced by chloroform disruption of LIN-state culture (◇, shaking at 0°C; □, without shaking at 0°C; ○, shaking at 37°C) and β-galactosidase activity in the supernatant of LIN-state culture (◆, shaking at 0°C; ■, without shaking at 0°C; ●, shaking at 37°C) (C) were determined. The distributions of β-galactosidase activity in the supernatant and cell pellet for the culture conditions of shaking at 37°C (D), without shaking at 0°C (E) and shaking at 0°C (F) were also determined.

collapse, the final β-galactosidase activity reached the total β-galactosidase activity in the lysate produced by chloroform disruption of the T4-infected culture. These results suggest that expression of the phage-encoded lytic genes, such as gene 5 responsible for LO and gene E responsible for LIN collapse, might be applied to cell disruption for efficient protein recovery.

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