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Plasmid Stability in Long-Term *h*G-CSF Production Using L-Arabinose Promoter System of *Escherichia coli*

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Abstract To examine the feasibility of the long-term production of the human granulocyte colony stimulating factor (*h*G-CSF) using the L-arabinose promoter system of *Escherichia coli*, flask relay culture and cyclic fed-batch culture were performed. In the flask relay culture, it was found that the plasmid was maintained stably up to about 170 generations in an uninduced condition, whereby the cells could also maintain the capability of expressing *h*G-CSF upon induction. However, in an induced condition, the structural damage of the plasmid occurred after about 100 generations, and thereafter the *h*G-CSF expression decreased gradually. In both cases, it was observed that the plasmid and the *h*G-CSF expression were maintained stably up to at least 100 generations. In contrast, in the cyclic fed-batch culture, segregational plasmid instability was observed within about 4 generations after induction, even though the cell growth and *h*G-CSF production reached their maximum values, 78.0 g/l of dry cell weight and 7.0 g/l of *h*G-CSF, respectively. It would appear that, when compared to the flask relay culture, the high-cell density and high-level expression of *h*G-CSF in the cyclic fed-batch culture led to the segregational plasmid instability; in other words, a severe metabolic burden existed on the cells due to the high-level expression of *h*G-CSF. Accordingly, based on these long-term cultures, the segregational and structural plasmid instability was observed and a strategy to overcome such problems could be designed.

Key words: L-Arabinose promoter system, human granulocyte colony stimulating factor (*h*G-CSF), plasmid stability

The L-arabinose promoter system is a positively regulated system, by which the L-arabinose *ara*BAD operon is positively regulated by the AraC protein [18]. Induction in the L-arabinose promoter system results from the positioning of an activation domain of the regulatory protein, AraC, in the -35 region of the promoter [15]. The positioning occurs when a subunit of AraC dissociates from a distal binding site, thereby opening a DNA loop, and rebinds to the -35 region [10]. When the domain is placed at this position, the transcription activation occurs, presumably as a result of the direct AraC-RNA polymerase interactions [6].

With the L-arabinose promoter system, many recombinant proteins have been expressed on a lab-scale [2, 5, 14, 16, 22]. The 20 liter-scale fermentation of the recombinant cholera toxin B subunit using the L-arabinose promoter system [20] and the kinetic study of the L-arabinose promoter on a lab-scale have also been reported [19]. Recently, the production characteristic of interferon-α using an L-arabinose promoter system was examined in a high-cell-density culture [9]. However, there have been few reports on the use of the L-arabinose promoter system regarding long-term cultivation, such as continuous culture and repeated fed-batch culture, and its application to high-cell-density cultures.

In general, in the long-term culture of recombinant microorganisms, plasmid stability was known to be a critical factor affecting the productivity of the whole process and thus must be maintained above a certain level. Two types of plasmid instabilities, structural and segregational instabilities, have been studied [3, 12, 13, 24]. It has also been observed that modifications of the plasmid in the host cell can cause the loss of high-level production of recombinant proteins [4].

Flask relay cultures and cyclic fed-batch cultures have been introduced for the investigation of plasmid stability in a shake flask and fermentor, respectively. In particular, a cyclic fed-batch culture is an alternative to a continuous culture and fed-batch culture. The cyclic fed-batch culture is similar to the fed-batch culture except that a portion of the culture broth is periodically removed from the vessel, therefore, long-term production can be accomplished by a repeated 'draw and fill' process [7].

In this report, to examine the feasibility of the long-term production of *h*G-CSF using the L-arabinose promoter system of *E. coli*, a flask relay culture and cyclic fed-batch culture were performed, and the plasmid stability, especially

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in terms of the structural and the segregational stabilities, and *h*G-CSF production were analyzed.

MATERIALS AND METHODS

Strain

E. coli pWAGF, which was derived from the *ara*$^-$ host strain *E. coli* MC1061, was used to produce the recombinant human granulocyte colony stimulating factor (*h*G-CSF) [21]. Figure 1 shows the plasmid pGW2.0, in which the structural gene of *h*G-CSF was cloned under the control of the L-arabinose promoter.

Flask Relay Culture Experiments

In the group I experiment, a vial from the cell bank was inoculated into a 500-ml shaker flask containing 100 ml of a fortified Luria Broth (FLB) medium (10 g/l Bactotrypton, 20 g/l Bacto yeast extract, 5 g/l NaCl, 70 μg/ml ampicillin), and incubated for 12 h at 30°C. At the end of the culture, 100 μl of the culture was transferred to a fresh FLB medium and also incubated for 12 h. This procedure was repeated 24 times. By these procedures, the main flask relay culture was maintained without induction of the L-arabinose promoter. Induction of the L-arabinose promoter was carried out in another sub-culture flask. At the end of each culture cycle, 100 μl of the culture broth was transferred to another fresh FLB medium containing 1% L-arabinose, and sub-cultured for 12 h.

In the group II experiment, a vial from the cell bank was inoculated into a 500-ml shaker flask containing 100 ml of a FLB medium and incubated for 12 h. At the end of the cultivation, 100 μl of the culture was transferred to a fresh FLB medium containing 1% L-arabinose. After 12 h of culture, 100 μl of the culture broth was reinoculated into another FLB medium containing 1% L-arabinose. This procedure was also repeated 24 times. By these procedures, the main flask relay culture was maintained in induced condition.

The growth was monitored by measuring the OD at 600 nm. The generation number was calculated for each culture period by measuring the OD at 600 nm before and after growth.

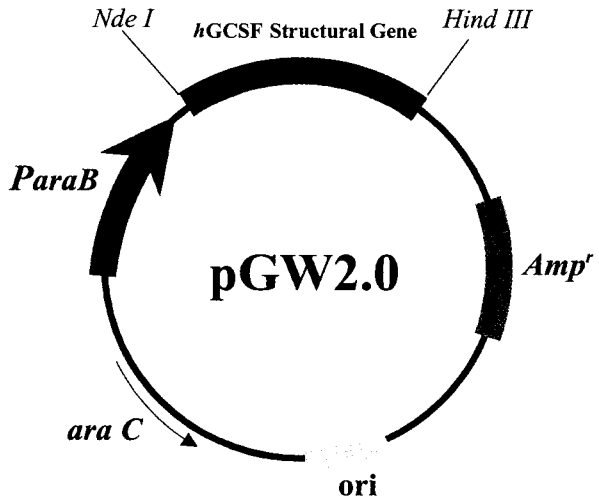

Fig. 1. Structure of the pGW2.0 plasmid.

Plasmid Isolation and Transformation

The cell pellet harvested from 3 ml of the culture broth was used for the extraction of the plasmid DNA. The plasmid DNA was isolated using the alkaline lysis method and suspended with a TE buffer, pH 7.4 [10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0)] [17].

Restriction mapping by double digestion with *Nde*I and *Hin*dIII was performed to identify the integrity of the structural gene. The plasmid DNA that was treated with the restriction enzymes was electrophoresed on a 0.8% agarose gel. The isolated plasmid DNA was then retransformed into the fresh *E. coli* MC1061 host cells using the calcium chloride/rubidium chloride procedure [11].

Cyclic Fed-Batch Culture

In the cyclic DO-stat fed-batch culture, the cell growth was divided into three phases: the inoculum, batch, and post-induction cyclic fed-batch. The inocula were initiated by adding 100 μl of the thawed stock cell bank to a flask containing 100 ml of a FLB medium. The flask was incubated at 30°C in a shaker at 150 rpm for 14 h. The flask content was then added to a 2.5 l Bioflow III bioreactor (New Brunswick Scientific, Edison, U.S.A.) containing 1.0 l of the sterilized batch medium, as outlined in Table 1. The temperature was maintained at 37°C and the pH was controlled at 7.0 by automatic addition of 15% (v/v) NH_4OH and 30% (v/v) H_3PO_4, respectively. The dissolved oxygen level was maintained above 20% saturation by the automatic adjustment of the agitation speed and oxygen partial pressure of inlet gas. The aeration rate was maintained at 1.0 vvm and the oxygen partial pressure of the inlet gas was controlled by mixing air and pure oxygen in the inlet gas stream using the computer-controlled Two Gas Mixer (New Brunswick Scientific, Edison, U.S.A.). The cells were allowed to grow in the batch mode until all the glycerol was exhausted (dry cell weight >25 g/l). At this

Table 1. Medium compositions for DO-stat fed-batch culture.

Component	Batch	Feed
Glycerol	40 g/l	390 g/l
Yeast Extract	40 g/l	292 g/l
Na_2HPO_4	10.22 g/l	
KH_2PO_4	2.31 g/l	
$MgSO_4 \cdot 7H_2O$		5.76 g/l
Ampicillin	70 μg/ml	70 μg/ml

point, 50 ml of 20% (w/v) L-arabinose solution was added to a final concentration of 1% (w/v) to induce the *h*G-CSF expression; thereafter, the DO-stat fed-batch was performed with the feed media as shown in Table 1. When the DO level rose above the set point (50% air-saturation), a feed pump was turned on and this pump then stopped when the DO level fell below the set point. This procedure was repeated by checking the DO level every 30 seconds using the computer aided Advanced Fermentation Software (AFS, New Brunswick Scientific, Edison, U.S.A.). One liter of feed media was consumed during the DO-stat fed-batch period. At the end of the fed-batch period, 1.0 l of culture broth was drained. At this point, 50 ml of 20% (w/v) L-arabinose solution and 50 ml of buffer solution (10% (w/v) sodium phosphate, dibasic and 2.3% (w/v) potassium phosphate, monobasic) were also added to maintain the L-arabinose and buffer concentration, and then the supply of the feed media was restarted over 24 h. These procedures were repeated four times.

Computer Hardware and Software

An IBM486DX2-based computer system was used for on-line data collection, calculation, and control. The computer was linked with the fermentor through an RS422 interface. The data, including the dissolved oxygen level, pH, temperature, agitation speed, and nutrient pump speed were collected, analyzed, and controlled by Advanced Fermentation Software (AFS, New Brunswick Scientific, Edison, U.S.A.).

Analytical Methods

The cell density was determined by a dry cell weight (DCW) measurement. Ten milliliter of the culture broth was centrifuged at 9,000 rpm, and the cell pellet was washed with phosphate buffered saline (PBS) and then recentrifuged. This procedure was repeated twice. Finally, the cell pellet was resuspended in distilled water and dried using an electronic moisture analyzer (Sartorius MA 40, Sartorius, Germany).

The glycerol and acetic acid concentrations were measured by gas chromatography (HP5890 series II, Hewlett Packard, U.S.A.). OV-17 (19001A-B12, Hewlett Packard, U.S.A.) and DEGS (19001A-L12, Hewlett Packard, U.S.A.) columns were used for the glycerol and acetic acid measurements, respectively.

The segregational plasmid stability was measured by tooth picking 200 colonies from the LB agar to an LB agar plate containing 70 μg/ml ampicillin sulfate.

The *h*G-CSF concentration was assayed by the gel electrophoresis procedure with a 4-20% Tris-Glycine precast gel (Novex, U.S.A.). In the fed-batch culture, 10 μl samples of 1/10 diluted culture broth were loaded on the gel along with 2.8– 11.6 μg of the purified *h*G-CSF standards. After gel electrophoresis, the Coomassie-stained gel was scanned with a laser scanner (DeskScan II v2.1, Hewlett Packard, U.S.A.) and then the *h*G-CSF concentration was quantified by comparing the integrated peak absorbance of the samples with those of the standards. Image analysis software (Biomed Instruments, Zenith programs, Universal Software, U.S.A.) was used for the gel analysis. In the flask relay culture experiments, a western blot was used to analyze the expression level of *h*G-CSF. The samples fractionated on the SDS-PAGE gel were transferred to nitrocellulose membranes. The membranes were then soaked overnight in a glass tray containing blocking buffer (PBS with 5% skim milk and 0.2% Tween 20) and the primary antibody (goat anti-G-CSF antibody, 50 μg/10 ml in PBS containing 2% skim milk) was applied. After washing the membranes three times with PBST buffer (PBS with 2% Tween 20), the secondary antibody (anti-goat IgG) coupled to horseradish peroxidase was challenged. After shaking for about 2 h, the membranes were reacted with 100 ml of the reaction mixture (3,3-diaminobenzidine, 60 mg/100 ml of 10 mM Tris-Cl, pH 7.5 and 100 μl of H_2O_2) and the reaction was stopped with deionized water.

Results and Discussion

Long-Term Flask Relay Culture

To examine feasibility of the long-term production of *h*G-CSF by the L-arabinose promoter system of *E. coli*, the plasmid stability and *h*G-CSF production were investigated using flask relay cultures. These cultures were divided into two groups. In the group I experiment, flask relay cultures were performed for over 170 generations without L-arabinose. In addition, at the end of each flask relay culture, a portion of the main flask relay culture broth was also transferred into 1% L-arabinose-containing FLB media and subcultured for 12 h to check the expression level of *h*G-CSF and the plasmid stability. That is, each flask relay culture was eventually divided into two different cultures: the uninduced main culture and an induced subculture. In the group II experiment, flask relay cultures for 170 generations were performed in 1% L-arabinose-containing FLB media. All the flask relay cultures were carried out in an induced condition.

As shown in Fig. 2A, in both experimental groups, there was no segregational plasmid loss or significant change in the OD at 600 nm at the end of each relay culture. In the expression level of *h*G-CSF, however, a significant difference was observed between the two experimental groups. In the group II experiment, the expression level of *h*G-CSF gradually decreased after about 100 generations and eventually no *h*G-CSF was expressed after about 150 generations, whereas no decrease in the expression level of *h*G-CSF was observed throughout all the generations in the group I experiment (Figs. 2B and 2C).

Since no segregational plasmid loss was observed in either an induced or uninduced conditions (Fig. 2A), it

would appear that the impaired expression observed under an induced condition was due to structural instability of the expression system rather than the segregational plasmid instability. Two explanations for structural instability have been suggested in previous reports: One of them was structural damage to the plasmid itself [3, 24], and the other was the modification of the host cell machinery during the protein synthesis [4]. To investigate which was responsible for the apparently reduced function of the L-arabinose promoter system, the plasmids from the last cultures of the two experimental groups were isolated and then re-transformed into fresh host cells. Next, the cells were inoculated into FLB media and subsequently induced for 10 h. If the damage to the host cell machinery during protein synthesis was the major cause of the decreased expression level in an induced condition, the newly transformed cells with an intact protein synthesis machinery would then be able to express *h*G-CSF properly. However, if the damage to the plasmid itself was the reason, the expression would either decrease or would not be detected. As shown in Fig. 3A, the analysis of the plasmids re-isolated from the newly transformed cells confirmed that the plasmids were properly transformed into the new host cells and there was no deletion of the *h*G-CSF structural gene from the plasmids in both the induced and the uninduced 170-generation cells. Yet, when the clones were induced with 1% L-arabinose, no *h*G-CSF expression was detected in the newly transformed cells with the plasmid

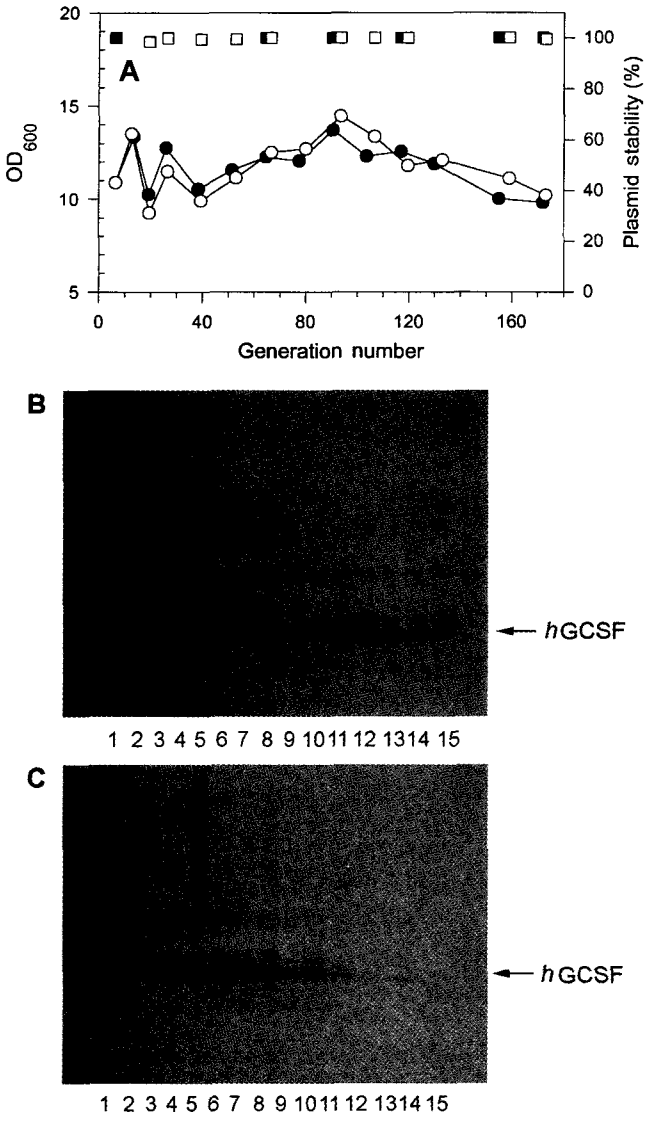

Fig. 2. Comparison of flask relay culture results between two experimental groups.

(A) Cell growth profiles between group I (●) and group II (○) and segregational plasmid stability profiles between group I (■) and group II (□) at each generation number. (B) Western blot profile for expression of *h*G-CSF at generation numbers from 6.45 to 171.99 (from lane 2 to 15) in the group I experiment. Lane 1: uninduced negative control; lane 2: 6.45; lane 3: 13.39; lane 4: 19.22; lane 5: 25.84; lane 6: 38.33; lane 7: 51.34; lane 8: 64.48; lane 9: 77.30; lane 10: 90.44; lane 11: 103.42; lane 12: 116.89; lane 13: 129.82; lane14: 154.91; lane 15: 171.99 (C) Western blot profile for expression of *h*G-CSF at generation numbers from 6.45 to 173.12 (from lane 2 to 15) in the group II experiment. Lane 1: uninduced negative control; lane 2: 6.45; lane 3: 12.55; lane 4: 19.50; lane 5: 26.46; lane 6: 39.32; lane 7: 52.95; lane 8: 66.56; lane 9: 79.90; lane 10: 93.56; lane 11: 106.62; lane 12: 119.55; lane 13: 132.91; lane14: 158.99; lane 15: 173.12.

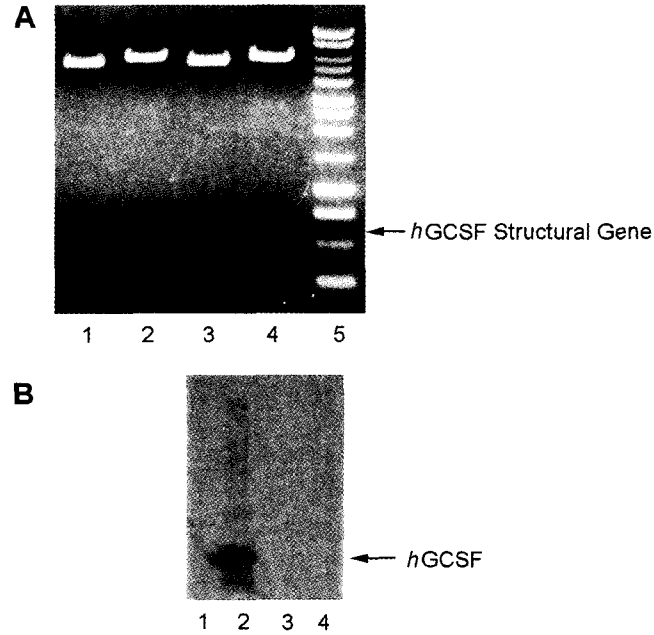

Fig. 3. Re-transformation of plasmids into fresh host cells.

(A) Gel electrophoresis of plasmids as single and double digested forms. Lane 5 is the size markers. The plasmids isolated from the group I experiment were double digested with *NdeI*/*Hin*dIII (lane 1) and single digested with *Nde*I (lane 2). The plasmids isolated from group II were also double digested with *NdeI*/*Hin*dIII (lane 3) and single digested with *Nde*I (lane 4). (B) Western blot analysis of *h*G-CSF expression in new transformants. Western blot results before (lane 1) and after (lane 2) induction for the new transformants from group I. Western blot results before (lane 3) and after (lane 4) induction for the new transformants from group II.

isolated from 170-generation cells which had grown under an induced condition, whereas a significant expression of *h*G-CSF was detected in the cells transformed with the plasmid isolated from 170-generation cells which had grown under an uninduced condition (Fig. 3B).

From the above results, it was demonstrated that the reason for the decrease in the expression level of *h*G-CSF under an induced condition was not damage to the host cell machinery during protein synthesis, but rather damage in the plasmid itself, probably in the essential components for the expression of protein such as the promoter system. Furthermore, it was found that the plasmid and *h*G-CSF production in the L-arabinose promoter system were maintained stably in both induced and uninduced conditions up to 100 generations.

One-Stage Cyclic Fed-Batch Culture

Since it was demonstrated in a long-term flask relay culture that no operational problem associated with plasmid stability and *h*G-CSF production were found within 100 generations of culture, regardless of induced or uninduced conditions, a scheme for the long-term production of *h*G-CSF using a high-cell-density culture was designed. In particular, a one-stage cyclic fed-batch culture was employed in which a DO-stat fed-batch culture was used as a basic strategy.

In the cyclic fed-batch culture, the medium was fed by the DO-stat control mode, and the culture broth was repeatedly withdrawn (Fig. 4B). As shown in Fig. 4A, the L-arabinose induction was carried out at the end of the batch culture period of the first cycle and the L-arabinose concentration was maintained by adding a feed medium (Table 1) which contained 1% L-arabinose. At the end of the second cycle, the cell growth and production of *h*G-CSF reached their maximum values, 78.0 g/l of DCW and 7.0 g/l of *h*G-CSF, respectively, and then gradually decreased in the following cycles (Fig. 4A). Neither residual glycerol nor acetic acid was detected throughout all the cycles, indicating that the carbon source was properly added through the DO-stat feeding and all the culture cycles were thus performed under favorable conditions. In the previous flask relay cultures, it was found that under an induced condition, the plasmid was maintained stably during at least 100 generations. However, in the cyclic fed-batch culture, the plasmid stability started to decrease just after the arabinose induction of the first cycle and, by the third cycle, decreased to below 5%, which unfortunately occurred within about 4 generations after induction. It was observed that, after the third cycle, segregational plasmid instability was the major cause for the decrease in both the plasmid stability and the production of *h*G-CSF. Accordingly, it would appear that the cell growth and production of *h*G-CSF in a flask relay culture were maintained at relatively low levels compared to those in the cyclic fed-batch culture and, as a result, there was probably no severe metabolic

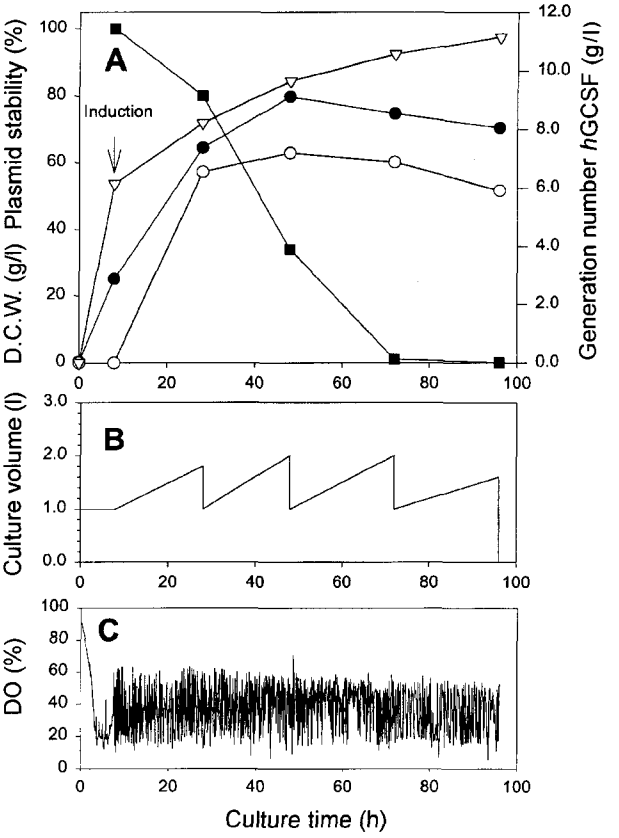

Fig. 4. Time courses of one-stage cyclic fed-batch culture. (A) Time courses of dry cell weight (●), generation number (▽), *h*G-CSF expression level (○), and plasmid stability (■). (B) Time course of culture volume. (C) Time course of dissolved oxygen (DO) level.

burden on the cells. In a one-stage cyclic fed-batch culture, however, *h*G-CSF was overexpressed in a more favorable environment than in a flask relay culture. Consequently, the plasmid-bearing cells had a more severe metabolic burden compared to the plasmid-free cells, resulting in the outgrowth of the plasmid-free cells. Therefore, within about 4 generations, the major cell population in the cyclic fed-batch culture became plasmid-free. These phenomena were in good agreement with many other studies on various promoter systems [1, 8, 23].

CONCLUSION

In the L-arabinose promoter system of *E. coli*, it was found that, in an uninduced condition, the plasmid and *h*G-CSF production were maintained stably up to 170 generations in a flask relay culture. In contrast, in an induced condition, plasmid instability and a decrease in *h*G-CSF production occurred. When the *h*G-CSF expression level was relatively low (3–4% of total cell protein), as in the flask relay culture, structural damage rather than the segregational loss was the major cause of the impaired function of the L-

arabinose promoter system. In contrast, when the *h*G-CSF expression level was high enough (more than 20% of total cell protein), as in the cyclic fed-batch culture, segregational plasmid loss was the major cause.

For long-term *h*G-CSF production, a two-stage fed-batch culture, in which one fermentor is used for cell growth and the other for *h*G-CSF production, that separates the cell growth and *h*G-CSF production, must be designed for an optimal operation strategy, even though the cell growth and *h*G-CSF production in a one-stage cyclic fed-batch culture reached 78 g/l of DCW and 7.0 g/l of *h*G-CSF, respectively.

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