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Iron Feeding Optimization and Plasmid Stability in Production of Recombinant Bacterial Magnetic Particles by *Magnetospirillum magneticum* AMB-1 in Fed-Batch Culture

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The production of bacterial magnetic particles (BMPs) by recombinant *Magnetospirillum magneticum* AMB-1 harboring the plasmid pKML was enhanced in pH-regulated fed-batch culture. The addition of fresh nutrients was feedback-controlled as a function of the pH of the culture. The yield of BMPs was optimized by adjusting the rate of ferric iron addition. Feeding ferric quinate at 15.4 μg/min resulted in a BMP yield of 7.5 mg/l, which is the highest yield so far reported. Expression of a plasmid-encoded fusion protein and segregation of the plasmid during bacterial growth were both stable during fed-batch culture. More than 75 % of the cells retained the plasmid for 130 h under antibiotic-free conditions. In addition, the fusion protein permitting the display of a specific protein on the BMP surface was also stably expressed.

[**Key words:** *Magnetospirillum magneticum* AMB-1, bacterial magnetic particles (BMPs), fed-batch culture, iron source, BMP production, plasmid stability]

Magnetic bacteria have been found in freshwater, marine sediment, and soil (1, 2). They usually contain 10–30 magnetic particles (Fe_3O_4) aligned in a chain. Bacterial magnetic particles (BMPs) are covered with a thin lipid membrane and have average diameters of 50–100 nm (3). Antibodies immobilized on BMPs have been used for highly sensitive immunoassays (4, 5). Genetic analysis using a transposon to elucidate the BMP synthesis has lead to the isolation of the *magA* gene from *Magnetospirillum magneticum* AMB-1 (6). This gene encodes a membrane protein, which works as an iron-transport protein on the BMP membrane. Furthermore, through gene fusion, novel technology for the display of functional proteins on BMPs has been developed using the MagA protein as an anchor (7). BMPs on which proteinA is displayed have been successfully applied to the immunoassay development (8, 9).

In previous work, we have developed efficient techniques for the mass cultivation of magnetic bacteria and the production of BMPs by employing fed-batch culture using nitrate/succinate feeding (10). We have also found that the feeding of iron as ferric quinate in fed-batch culture can enhance growth and total magnetite production; by this means a maximum BMP yield of 6 mg/l was achieved using recombinant AMB-1 (11). However, optimization of the iron feeding process and plasmid stability under the fed-batch condition were not investigated.

With a view to the industrial application of BMPs, in the work reported here we investigated bulk production of BMP complexes (luciferase-displayed BMPs) in fed-batch culture with iron feeding and plasmid stability without the use of an antibiotic.

MATERIALS AND METHODS

Bacterial strain and plasmid Recombinant *M. magneticum* AMB-1 harboring the plasmid pKML derived from pRK415 (Tc^r, *lacZ*, *mob*$^+$) by cloning the luciferase gene (*luc*) downstream of the 3′ end of *magA* was used throughout this study (7). Plasmid pRK415, a broad host-range vector for gram-negative bacteria, is replicated in *Magnetospirillum magneticum* AMB-1 (12).

Fed-batch cultivation Fed-batch culture of *M. magneticum* AMB-1 transformed with the expression plasmid pKML was carried out in a 10-*l* jar fermentor (BMS-10L; ABLE; Tokyo) under microaerobic conditions. AMB-1 cells were precultured to the exponential phase (10^8 cells/ml) in a 50-ml flask, and 40 ml of the preculture was then transferred as the inoculum to a jar fermentor containing 8-*l* magnetic spirillum growth medium (MSGM) (13) with tetracycline (5 μg/ml). The starting cell density after inoculation was 5×10^5 cells/ml. The culture was maintained at 25°C and continuously agitated at 100 rpm with a paddle. Consumption of the electron donor (succinic acid) in the medium was followed by measuring the pH of the culture. The substrate solution was prepared by dissolving 60 g succinic acid, 40 g sodium nitrate, and 20 ml 60% (w/v) nitric acid to a final volume of 1000 ml. This solution was fed in a pH-controlled manner into the fermentor via a peristaltic pump, which was set to activate whenever the pH exceeded 6.75. This system met the demands of succinate, but not of those nitrate. For the latter, a solution consisting of 0.96 g sodium nitrate dissolved in 20 ml distilled water was injected into the fermentor whenever the concentration of nitrate dropped below 0.2 mM. A series of fed-batch cultures was established, with ferric iron feeding rates of 5.1, 10.3, 15.4, 20.5, 25.7, 35.9, and 46.2 μg/min. The cultures were maintained for 48 h after the cell concentration reached 1×10^7 cells/ml. The total iron added respectively corresponded to 2-, 3-, 4-, 5-, 6-, 8-, 10-times that in the original MSGM. The rate of addition was optimized in terms of the yield of BMPs. Microaerobic conditions were achieved by sparging the medium with argon gas for 30 min prior to use.

Extraction of bacterial magnetic particles Cells were harvested at the stationary phase (1 *l*) by centrifuga-

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tion (8000 rpm, 15 min, 4°C). Pelleted cells were subsequently washed three times with phosphate-buffered saline (PBS, pH 7.4) by three cycles of resuspension and centrifugation (8000 rpm, 15 min, 4°C), resuspended in 20 ml of 1 N NaOH, and boiled for 20 min to lyse the cells. BMPs were collected magnetically using a neodymium-boron magnet, washed with PBS at least three times by repeated dispersion using a sonicator (VS-30, 35W; Velvo-Clear, Tokyo), and then stored at 4°C in PBS buffer.

For determination of enzyme activities, luciferase-BMPs were isolated by a different procedure. Cells were suspended in 20 ml PBS and disrupted by three passes through a French Press (Ohtake Works, Tokyo) at 1500 kg/cm^2 on ice. Luciferase-BMPs were then separated from the cell debris using a neodymium-boron magnet, washed at least three times with PBS buffer, and stored at 4°C.

Analytical procedures The cell concentration was measured by haemocytometry. The iron concentration was determined using an atomic absorption spectrophotometer (AA6600G; Shimadzu, Kyoto). The nitrate and succinate concentrations in the culture were determined every 6 h by HPLC on a Shimadzu Shim-Pack IC-A2 ion-exchange column. The optical density (A_{660}) of the luciferase-BMP suspension was measured and converted to dry weight. Luciferase activity on the BMPs was determined as follows: 20 μl of a recombinant BMP suspension (0.06 mg/ml) was mixed with 100 μl PicaGen luminescence substrate solution (Wako Pure Chemical Industries, Osaka) and the luminescence intensity was measured using a luminometer (BLR-301; Aloka, Tokyo) at 37°C.

Evaluation of plasmid stability To evaluate plasmid modification in the cell, plasmid isolated by a standard method (14) from 10^{10} cells sampled at 0, 56, 80, and 104 h in fed-batch culture was digested with a restriction enzyme, *Sma* I, and electrophoresed on agarose. Analysis was performed by Southern hybridization, using as probes a 0.8 kb fragment of *tet* and *magA* (1.3 kb) labeled with a DIG chemiluminescence kit (Boehringer Mannheim, Germany). Plasmid stability was also determined by evaluating the ratio of cells harboring pKML to the total number of cells. Cell samples collected at various intervals during fed-batch cultivation (in a tetracycline-free medium) of *M. magneticum* AMB-1 transconjugants were plated out in triplicate on MSGM with or without tetracycline. After microaerobic incubation for three weeks, the ratio of cells harboring the plasmid was calculated as the number of colonies on plates with tetracycline divided by the number of colonies on plates without tetracycline.

RESULTS AND DISCUSSION

Fed-batch cultivation with iron feeding Since AMB-1 cells take up a tremendous amount of iron to meet the requirements for BMP synthesis, optimization of the iron source feeding process is one of the key points for successful large-scale production of BMPs. We therefore investigated the relationship between the BMP yield and the feeding rate of ferric quinate solution during fed-batch cultivation. Alterations in the iron concentration in the culture and the cell growth curve obtained at each iron feeding rate are respectively shown in Figs. 1A and 1B. Feeding rates above 20.5 μg/min resulted in rapid accumulation of iron in the culture medium, indicating the presence of excess iron that could not be utilized for growth or BMP synthesis. Growth inhibition begin to appear at 25.7 μg/min, and notable inhibition was observed at iron feeding rates of 35.9 and 46.2 μg/min. The relationship between the feeding rate and the BMP yield is shown in Table 1. Feeding of ferric ion at 15.4 μg/min resulted in a BMP yield of 7.5 mg/*l*, with a final cell density of 0.34 g cell dry weight/*l*. This is the highest yield reported in the literature to date with MSGM.

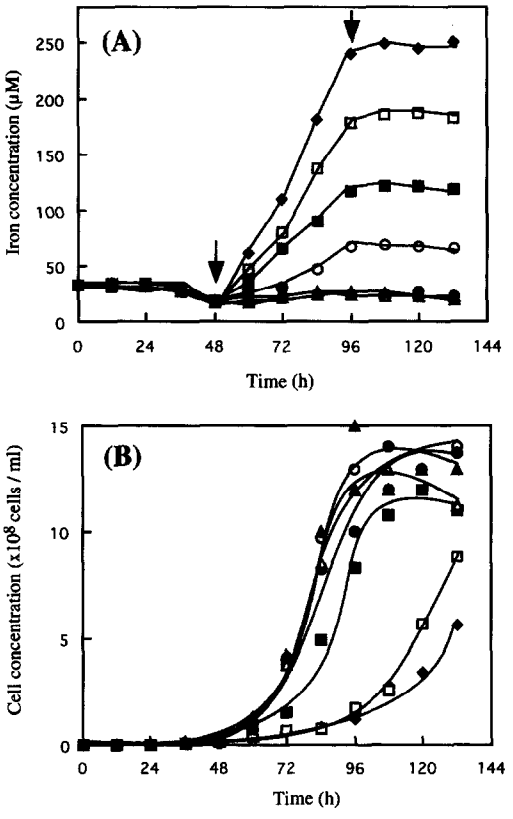

FIG. 1. Alteration of iron concentrations (A) and recombinant AMB-1 growth curves (B) in fed-batch cultures under various ferric iron feeding rates. Ferric iron was fed at 5.1 (▲), 10.3 (△), 15.4 (●), 20.5 (○), 25.7 (■), 35.9 (□), and 46.2 (◆) μg/min. Arrows in (A) indicate the starting and finishing of iron feeding.

In our previous work, batch culture of AMB-1 wild-type with an initial iron concentration of 33 μM yielded 2.6 mg BMPs/*l* with a final cell density of 0.09 g cell dry weight/*l* (15), from which it was calculated that 95% of the total available ferric iron was converted into BMPs.

TABLE 1. BMP production under various rates of ferric iron feeding

Iron feeding rate (μg/min)	Final cell density (g cell dry weight/*l*)	BMP production (mg/*l*)	BMP productivity (mg/g cell dry weight)
5.1	0.33	4.2	12.7
10.3	0.35	5.5	15.7
15.4	0.34	7.5	22.1
20.5	0.34	5.7	16.8
25.7	0.31	3.4	11.0
35.9	0.27	3.3	12.2
46.2	0.21	2.2	10.5

High-density fed-batch cultivation with continuous iron feeding at 11.7 μg/min using recombinant AMB-1 harboring pKML increased the absolute BMP production to 6 mg/l with a final cell density of 0.35 g cell dry weight/l (11). This corresponds to a BMP productivity of 17.1 mg/g cell dry weight, which is far lower than that achieved by batch culture (28.9 mg/g cell dry weight). In the present work, not only was the absolute BMP production enhanced, but the BMP productivity was improved to 22.1 mg/g cell dry weight (Table 1).

Schüler and Baeuerlein (16) reported that growth and magnetite formation in *Magnetospirillum gryphiswaldense* strain MSR-1 was dependent on the extracellular iron concentration with growth inhibition being observed at an initial iron concentration of 200 μM in batch culture. In our fed-batch system, feeding rates exceeding 25.7 μg/min led to growth inhibition at lower iron concentrations in the medium. The growth of strain AMB-1 may be inhibited more at lower iron concentrations than that of MSR-1, but it is not clear why growth at lower iron concentration in the medium was inhibited when the iron feeding rate exceeded 25.7 μg/min. Further investigation of the iron uptake ability of the cells and intracellular accumulation of iron will clarify this.

Stability of plasmid in *M. magneticum* AMB-1 The stability of pKML in AMB-1 cells in fed-batch culture with continuous iron feeding at a rate of 15.4 μg/min was investigated by Southern hybridization analysis. Two probes were used: (i) a 0.8-kb *Sma*I-digest fragment of pKML containing the central region of *tet*, and (i) *magA* (1.3 kb). Figure 2 shows the results using both probes. Plasmid DNA isolated from each of the fed-batch culture samples yielded the same hybridization pattern as the control (pKML), indicating that pKML was stably maintained in the cells without any modification during fed-batch culture. Figure 3 shows the growth curve of the AMB-1 transconjugant harboring pKML in fed-batch culture without tetracycline and the stability of the plasmid during growth. Plasmid instability and depression of cell growth have been noted in production using recombinant cells. For industrial application, plasmid stability in the absence of selection pressure is required. In this study, the growth curve of the transconjugant was similar to that of the wild-type (Fig. 3). The percentage of plasmid-bearing cells was maintained at a stable level during the first 72 h. Although the ratio of cells harboring pKML decreased thereafter, it remained above 75% after 130 h cultivation. These findings suggest that this cultivation system is advantageous in terms of its stability and hence for the mass production of BMPs.

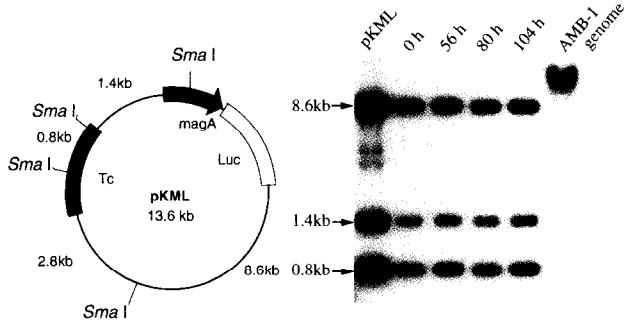

FIG. 2. Southern hybridization of plasmid isolated at various intervals during fed-batch cultivation. In each case, the plasmid was digested by *Sma*I. A. 0.8-kb fragment of *tet* and *magA* (1.3 kb) were used as probes.

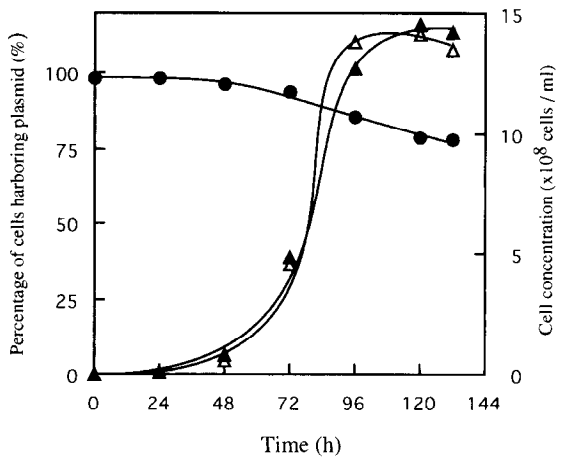

FIG. 3. Growth curves and plasmid stability during fed-batch cultivation without an antibiotic. Symbols: ▲, growth curve of recombinant cells; △, growth curve of wild-type cells; ●, percentage of recombinant cells harboring pKML.

Activity of luciferase expressed on BMPs Since the activity of the *magA* promoter appears to be partly regulated by the iron concentration in the medium (7), the effect of the iron concentration on the expression of BMP-associated luciferase was also investigated. AMB-1 cells were batch cultured with various initial iron concentrations. Considering the inhibition of cell growth noted earlier, the iron concentrations in the medium were set at values up to 60 μM, which gave optimal BMP production in our fed-batch cultivation. The luminescence yield on the BMPs was substantially unchanged in cultivations under the various initial iron concentrations, indicating that the iron concentration has no significant effect on the expression of luciferase (Fig. 4). BMP-associated luciferase activity in fed-batch culture was also compared with that in batch culture. BMPs isolated from pKML–recombinant AMB-1 cultivated in batch culture exhibited an average luminescence yield of about 0.36±0.02 kcounts/min/μg BMPs. A similar yield of 0.41±0.09 kcounts/min/μg BMPs was obtained in fed-batch culture. These data suggest that the *magA–luc* fusion gene was stably expressed on pKML during fed-batch culture.

In this study, we have demonstrated that BMP production by *M. magneticum* AMB-1 can be improved by optimizing the iron feeding rate in a fed-batch culture system. The recombinant plasmid is stably maintained in

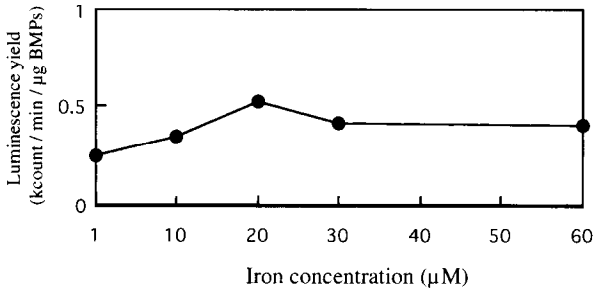

FIG. 4. Luminescence yield on recombinant BMPs from AMB-1 cells batch cultured under various iron concentrations. Cells were cultivated for 5 d.

AMB-1 cells during this process, even under non-selective conditions, and a fusion protein permitting the display of a specific protein on the BMP surface is also stably expressed. These results indicate that it will be possible to achieve efficient and economical production of functional BMPs.

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