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Enhancement of 1,4-Dihydroxy-2-Naphthoic Acid Production by *Propionibacterium freudenreichii* ET-3 Fed-Batch Culture[▽]

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The production of 1,4-dihydroxy-2-naphthoic acid (DHNA) was investigated using a fed-batch culture of *Propionibacterium freudenreichii* ET-3. DHNA is a precursor of menaquinone (MK) and is transformed to MK by combination with an isoprenoid unit. We found that ET-3 stopped MK production and increased DHNA production in an anaerobic fed-batch culture by maintaining the lactose concentration at approximately zero. The maximum DHNA concentration observed in the anaerobic fed-batch culture was markedly higher than the maximum DHNA concentration observed in an anaerobic batch culture. Moreover, MK or DHNA production was affected by the lactose feeding rate; this suggests that lactose metabolism participates in the syntheses of these products. On the other hand, accumulation of propionate was found to inhibit DHNA production in the fed-batch culture. Based on the fact that ET-3 increases DHNA production in an aerobic culture by consuming propionate, we carried out a cultivation experiment in which an anaerobic fed-batch culture was switched to an anaerobic batch culture and found that the DHNA production was increased to a greater extent than the DHNA production in an anaerobic fed-batch culture. These results suggest that DHNA production by ET-3 is markedly influenced by carbon source limitation and the oxygen supply.

In a previous study (4) we found that *Propionibacterium freudenreichii* ET-3 produces menaquinone (MK) and 1,4-dihydroxy-2-naphthoic acid (DHNA) in an anaerobic batch culture. MK is known as vitamin K and is an essential cofactor for the posttranslational synthesis of γ-carboxyglutamic acid that is present in specific proteins in the blood and bone in animals (11, 13). Furthermore, MK acts as an electron transfer agent in the respiratory chain in bacteria (9, 10, 16). DHNA is a precursor of MK in bacteria (1); however, there is little information concerning its function in bacteria. DHNA has been reported to particularly stimulate the growth of bifidobacteria (7, 8), thus improving the conditions in the human intestine by increasing the number of bifidobacteria (5, 17). Therefore, addition of an ET-3 culture broth containing DHNA to foods is expected to be used for the development of functional foods improving the intestinal conditions. To be able to utilize ET-3 cultures in the manufacture of a wide variety of foods, it is important to develop an effective method for DHNA production.

As shown in Fig. 1, the biosynthesis of MK comprises two independent syntheses (1); one is DHNA synthesis, and the other is isoprenoid unit synthesis. DHNA is transformed to MK by combination with an isoprenoid unit. Therefore, it is thought that a decrease in isoprenoid synthesis decreases the amount of DHNA utilized for MK synthesis and increases the amount of DHNA released from the cell. The precursors of isoprenoids are glyceraldehyde-3-phosphate (G3P) and pyruvate (Pyr) (14, 15, 19). These precursors are involved in the Embden-Meyerhof-Parnas (EMP) pathway, and the propionibacteria catabolize carbohydrates via this pathway (Fig. 2A) (12, 24). Previously, we carried out a batch culture analysis in which anaerobic conditions were switched to aerobic conditions when the lactose in the medium was depleted (4). This analysis revealed that under aerobic conditions, ET-3 stopped MK production and increased DHNA production. Under aerobic conditions, propionibacteria utilize the reverse methylmalonyl coenzyme pathway that does not produce G3P (Fig. 2B) (24, 26). It is thought that the absence of the isoprenoid precursors stops isoprenoid production and that the termination of isoprenoid production increases DHNA production. This means that DHNA production can be increased by modifying the cultivation method based on the MK biosynthesis pathway.

In this study, we grew an anaerobic fed-batch culture with lactose limitation to change the flux of the EMP pathway and investigated the effect of lactose limitation on DHNA and MK production. We also investigated the effect of the lactose feeding rate on DHNA and MK production and thus determined the optimal lactose feeding rate for enhanced DHNA production. In addition, we carried out a cultivation experiment in which an anaerobic fed-batch culture was switched to an aerobic batch culture at the end of the lactose feeding period to further increase the DHNA production.

MATERIALS AND METHODS

Microorganism and media. ET-3 was used throughout this study. This strain was isolated from Swiss cheese and was stocked at the Food Functionality Research Institute of Meiji Dairies, Odawara, Japan. The preculture medium contained 10% (wt/wt) whey powder (Meiji Dairies, Tokyo, Japan) and 0.1% (wt/wt) beer yeast extract (Asahi Food and Health, Tokyo, Japan). The fermentation medium contained 3.0% (wt/wt) skim milk powder (Meiji Dairies, Tokyo, Japan), 3.4% milk protein concentrate (Murray Goulburn, Melbourne, Australia), and 0.5% (wt/wt) beer yeast extract (Asahi Food and Health). The whey powder was dissolved in a volume of deionized water that was 50 to 70% of the volume that was used to dissolve the medium and was digested using 0.07%

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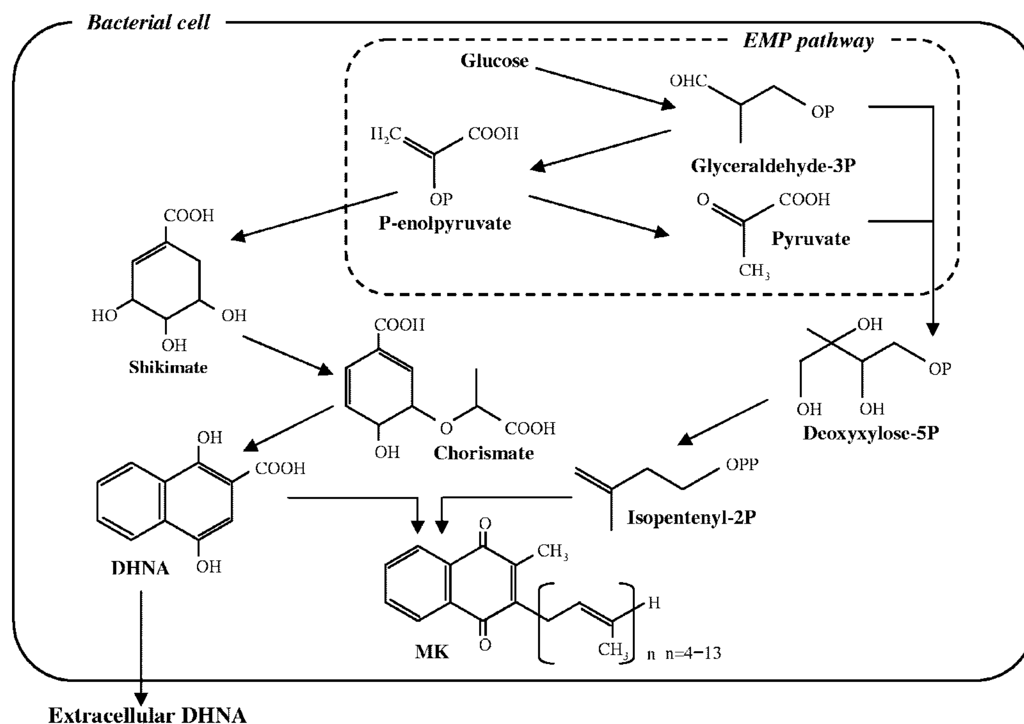

FIG. 1. Pathway for biosynthesis of MK in bacteria. MK is synthesized by combining DHNA and an isoprenoid unit (1). The pathways for biosynthesis of DHNA and isoprene were described by Bentley et al. (1) and Rohmer et al. (15, 16, 19), respectively.

(wt/wt) protease (Amano A; Amano Pharmaceutical, Tokyo, Japan) at 47°C for 3 h. The skim milk powder and milk protein concentrate were also dissolved after mixing using the procedure described above and were digested using 0.25% protease (Amano A) at 47°C for 6 h. During enzyme digestion, the pH was controlled in the range from 6.6 to 7.0 using K_2CO_3. The media were autoclaved at 121°C for 15 min.

Culture method. One milliliter of a frozen ET-3 culture stored at −80°C was inoculated into 100 ml of the preculture medium. After static incubation for 48 h at 37°C, 20 ml of the culture was inoculated into a 3-liter jar fermentor (BMS 03PI; Able, Tokyo, Japan) containing 2.0 liters of the fermentation medium. The temperature of the culture was controlled at 33°C, and the pH of the medium was adjusted to 6.5 using K_2CO_3 during the culture period. An anaerobic culture was grown using filter-sterilized nitrogen gas. The agitation speed and the flow rate of nitrogen in the anaerobic culture were controlled at 100 rpm and 0.4 liter min^{-1}, respectively. All the fed-batch cultures in this study were grown under anaerobic conditions and were started 72 h after the anaerobic batch culture was started by feeding a lactose solution at a constant rate. The concentration of the lactose solution fed was 1.5 M, and the lactose feeding rate was maintained in the range from 0.62 to 2.54 ml h^{-1}. The lactose in the anaerobic batch culture was almost entirely depleted after 72 h of culture. Samples of the cultures were taken at 24-h intervals. The volume of the samples withdrawn from the fermentor was almost the same as the volume of the lactose solution added for each 24-h period, which maintained the working volume of the fed-batch cultures at approximately 2.0 liters. For the aerobic batch culture, oxygen gas was supplied at a rate of 0.4 liter min^{-1}, and the agitation speed was controlled at 100 rpm. These aerobic conditions provided the minimum oxygen transfer rate (0.38 mg $liter^{-1}$ h^{-1}) necessary to induce ET-3 to change its metabolism (5).

Quantification of DHNA, MK, organic acids, and biomass. DHNA, MK, and organic acid contents were measured using a high-performance liquid chromatography system. The details of the measurement procedures used for these products have been described previously (4). Biomass was expressed in terms of dry cell weight.

RESULTS

Effect of lactose limitation on DHNA production. To investigate the effect of lactose limitation on DHNA production, ET-3 was cultured by feeding a lactose solution at a constant rate under anaerobic conditions (Fig. 3). The initial lactose concentration in this culture was 0.08 M, and 0.20 mol of lactose was fed during the fed-batch culture experiment. An anaerobic batch culture was also grown as a control (Fig. 3). To the anaerobic batch culture, 0.20 mol of lactose was added before the culture was started. As shown in Fig. 3, no lactose was detected throughout the lactose feeding period. MK production stopped during the lactose feeding period, although it increased with cell growth in the anaerobic batch culture. In contrast, DHNA production increased in the anaerobic fed-batch culture, and the maximum DHNA concentration in the anaerobic fed-batch culture was 1.7 times that in the anaerobic batch culture. There was no substantial difference in propionate or acetate production between these two cultures.

Effect of lactose feeding rate on DHNA production. In a preliminary experiment, an anaerobic fed-batch culture in which a lactose solution was fed at a rate of 1.90 ml h^{-1} was grown for 140 h to add 0.40 mol of lactose (data not shown). In this culture, DHNA production stopped after 0.24 mol of lactose had been added, and the maximum DHNA concentration was 0.30 mM. In our previous study (4), propionate accumulation inhibited DHNA production, and the propionate concentration at the time that DHNA production stopped was 0.35 M; this concentration was higher than the concentration at which DHNA production is inhibited. Therefore, 0.24 mol of lactose was used as the total amount of lactose that was added during the anaerobic fed-batch culture in the subsequent anaerobic fed-batch cultures.

To investigate the optimal lactose feeding rate for DHNA production, anaerobic fed-batch cultures were grown with var-

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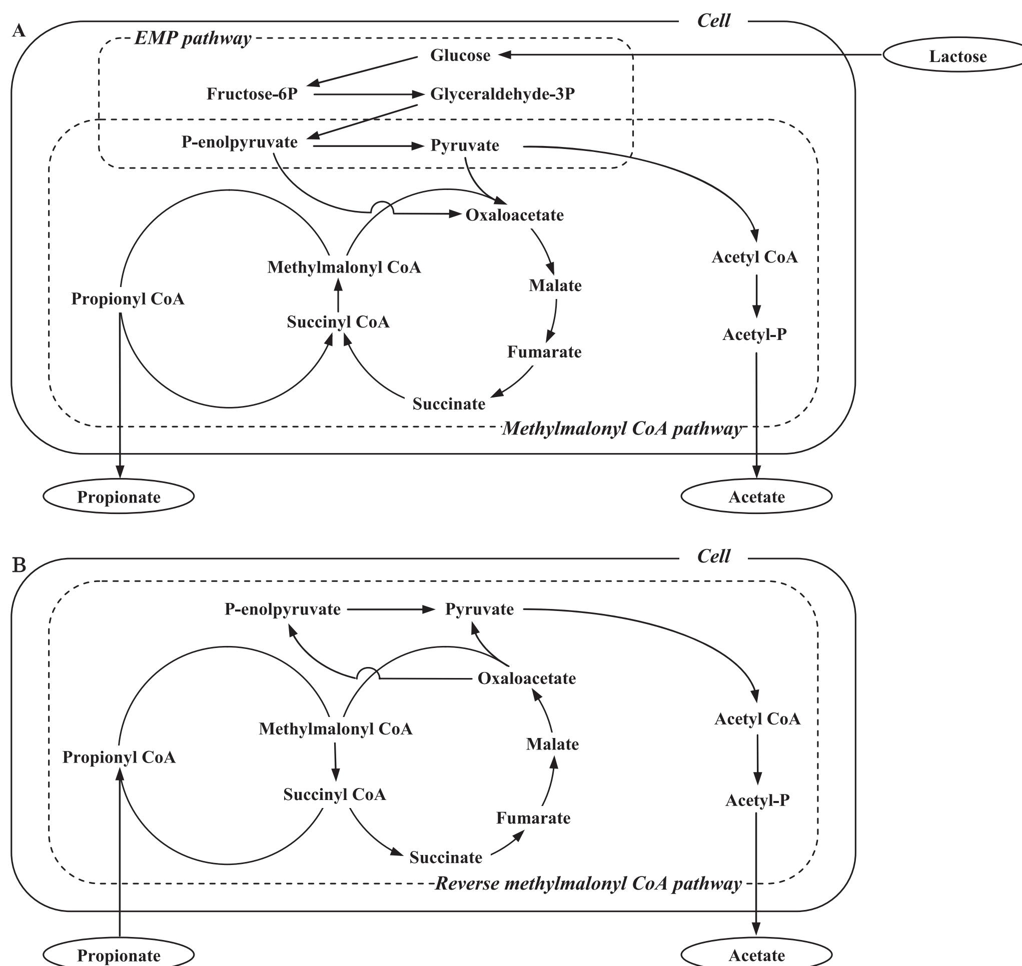

FIG. 2. Metabolic pathways in propionibacteria. (A) Metabolic pathway under anaerobic conditions, as reported by Piveteau (12) and Wood (24). (B) Metabolic pathway under aerobic conditions, as reported by Wood (24) and Ye et al. (26). CoA, coenzyme A.

ious feeding rates (Fig. 4). The feeding rates in these anaerobic fed-batch cultures were maintained at 2.54, 1.90, 1.31, 0.90, and 0.62 ml h^{-1}. As shown in Fig. 4, the lactose concentration during all the fed-batch cultures remained constant at approximately zero. The MK concentration and dry cell weight decreased as the lactose feeding rate decreased. However, the DHNA concentration increased as the lactose feeding rate decreased. The maximum DHNA concentration in these anaerobic fed-batch cultures was observed in the cultures with lactose feeding rates of 0.90 and 0.62 ml $liter^{-1}$ and was 0.40 mM. Therefore, a lactose feeding rate of 0.90 ml $liter^{-1}$ was employed for the subsequent anaerobic fed-batch cultures by considering the time needed to reach the maximum DHNA concentration. The rates of production of propionate and acetate decreased in proportion to the lactose feeding rate; however, the final concentrations of these organic acids were practically the same in all the cultures (Fig. 4). The maximum DHNA and MK concentrations in each fed-batch culture are shown in Table 1. Table 1 also shows the sum of the maximum DHNA and MK concentrations in each fed-batch culture. The sums are almost the same, ranging from 0.41 to 0.44 mM, regardless of the lactose feeding rate.

Table 2 shows the specific lactose consumption, DHNA production, MK production, and cell growth rates in the anaerobic fed-batch cultures. The specific lactose consumption rate decreased in proportion to the lactose feeding rate. The specific MK production and cell growth rates markedly decreased as the lactose feeding rate decreased. In contrast to these results, the specific DHNA production rate remained constant at approximately 0.18 μmol g^{-1} h^{-1} when the lactose feeding rate was maintained at more than 0.90 ml h^{-1}.

DHNA production after a switch from an anaerobic fed-batch culture to an aerobic culture. As described above, DHNA production was inhibited by propionate that accumulated in the anaerobic fed-batch culture. However, in our previous study (4) we showed that ET-3 consumed propionate and produced DHNA continuously even after propionate accumulated by switching from anaerobic conditions to aerobic conditions at the time of lactose depletion. As shown in Fig. 2B, under aerobic conditions, propionibacteria consumed propionate and produced acetate via the reverse methylmalonyl coenzyme A pathway. To decrease the inhibitory effect of propionate on DHNA production, an anaerobic fed-batch culture was switched to an aerobic batch culture at the end of the lactose feeding period (Fig. 5). The propionate concentration started to decrease immediately after the switch to aerobic

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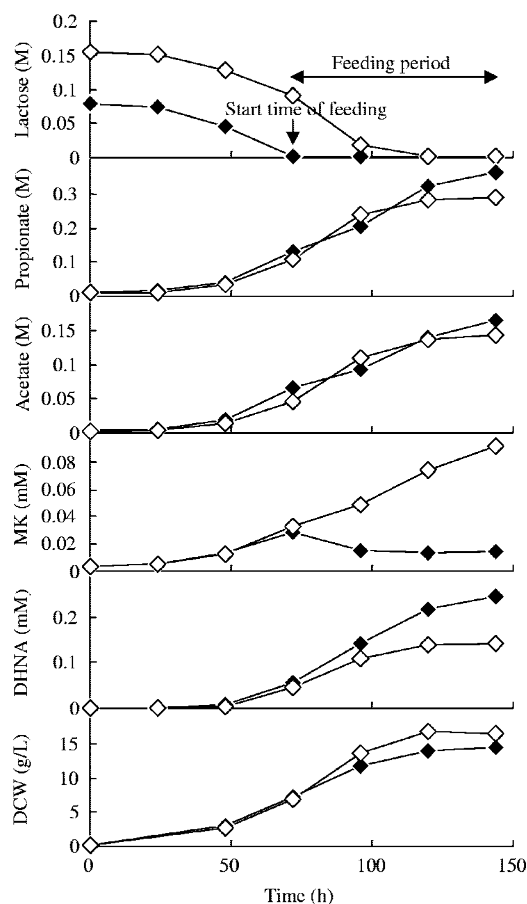

FIG. 3. Profiles of anaerobic batch and anaerobic fed-batch cultures of ET-3. The anaerobic fed-batch culture was started 72 h after the anaerobic batch culture was started. During the fed-batch culture, 0.20 mol of lactose was fed, and the lactose feeding rate was maintained at 1.90 ml h^{-1}. The concentration of the lactose solution fed was 1.5 M. In the anaerobic batch culture, 0.20 mol of lactose was added before the culture was started. The vertical and horizontal arrows indicate the start time and the period of lactose feeding, respectively. ◇, anaerobic batch culture (control experiment); ◆, anaerobic fed-batch culture. DCW, dry cell weight.

conditions, and the acetate concentration increased continuously even after the end of lactose feeding. DHNA was produced continuously after the end of lactose feeding, although the level of MK produced was low throughout the cultivation. However, an increase in the dissolved oxygen (DO) concentration was observed from 96 to 120 h in the aerobic batch culture (data not shown). The DHNA concentration decreased sharply during this time because the structure of DHNA is sensitive to oxidation. The maximum DHNA concentration in this culture was 0.51 mM.

DHNA production by repeated alternation between anaerobic fed-batch and aerobic cultures. Propionibacteria are sensitive to oxygen (2, 21, 25). In our previous study (3, 4) we also showed that in aerobic cultures grown for a long time there was an increase in the DO concentration, resulting in a marked decrease in the DHNA concentration; this was despite the fact that the concentration of propionate as a carbon source at the time was sufficiently high. To protect ET-3 from exposure to oxygen for a long period, a cultivation method involving repeated alternation between anaerobic and aerobic batch cultures was developed in our previous study (3). This cultivation method increased the DHNA concentration continuously by maintaining the DO concentration at approximately zero. In this study, we applied this method to a cultivation in which an anaerobic fed-batch culture was switched to an aerobic batch culture (Fig. 6). As shown in Fig. 6, the aerobic batch culture was switched to the anaerobic fed-batch culture before an increase in the DO concentration occurred, and the anaerobic fed-batch and aerobic batch cultures were repeated twice alternately. Throughout the experiment, the DO concentration remained constant at approximately zero, and the propionate concentration was lower than the concentration at which DHNA production is inhibited. However, DHNA production stopped immediately after the start of the second fed-batch culture, and the maximum DHNA concentration in this cultivation was 0.52 mM; this concentration was almost the same as that observed in the cultivation in which an anaerobic fed-batch culture was switched to an aerobic batch culture, as described above.

At the time that DHNA production stopped in this cultivation, the acetate concentration was 0.45 M. Acetate also inhibits DHNA production, although the inhibitory effect of acetate is much weaker than that of propionate (4). To investigate the effect of acetate accumulation on DHNA production, 0.84 mol of acetate was added 48 h after the start of an anaerobic batch culture with a 2.0-liter working volume (data not shown). In this culture, DHNA production stopped immediately after the addition of acetate, whereas DHNA was produced continuously for 120 h in the anaerobic batch culture to which acetate was not added (Fig. 3).

DISCUSSION

In this study, we found that growing a fed-batch culture with lactose limitation is one of the optimal methods for increasing DHNA production. As shown in Table 1, the maximum DHNA concentration increased when the lactose feeding rate decreased, and the maximum MK concentration decreased. The sums of the maximum DHNA and MK concentrations were almost the same regardless of the lactose feeding rate (Table 1). In our previous study (4) we showed that MK accumulates in ET-3 cells and that DHNA that is not utilized for MK synthesis is released from the cells. Based on these results, an anaerobic fed-batch culture is thought to increase the amount of DHNA released from the cells by decreasing MK production.

Here, we discuss the changes in DHNA and MK production by an anaerobic fed-batch culture on the basis of the MK biosynthesis pathways (Fig. 1) and the metabolic pathways of propionibacteria (Fig. 2A and B). As shown in Fig. 1, DHNA is transformed to MK by combination with an isoprenoid unit (Fig. 1). Rohmer et al. (14, 15, 19) reported that isoprenoids are synthesized from G3P and Pyr. Their discovery revealed that various bacteria utilize this pathway for isoprenoid synthesis (6, 22, 23). Although to our knowledge there have been no studies on the biosynthesis of isoprenoids in propionibacteria, we believe that propionibacteria synthesize isoprenoids by the pathway reported by Rohmer et al. Thus, it is possible

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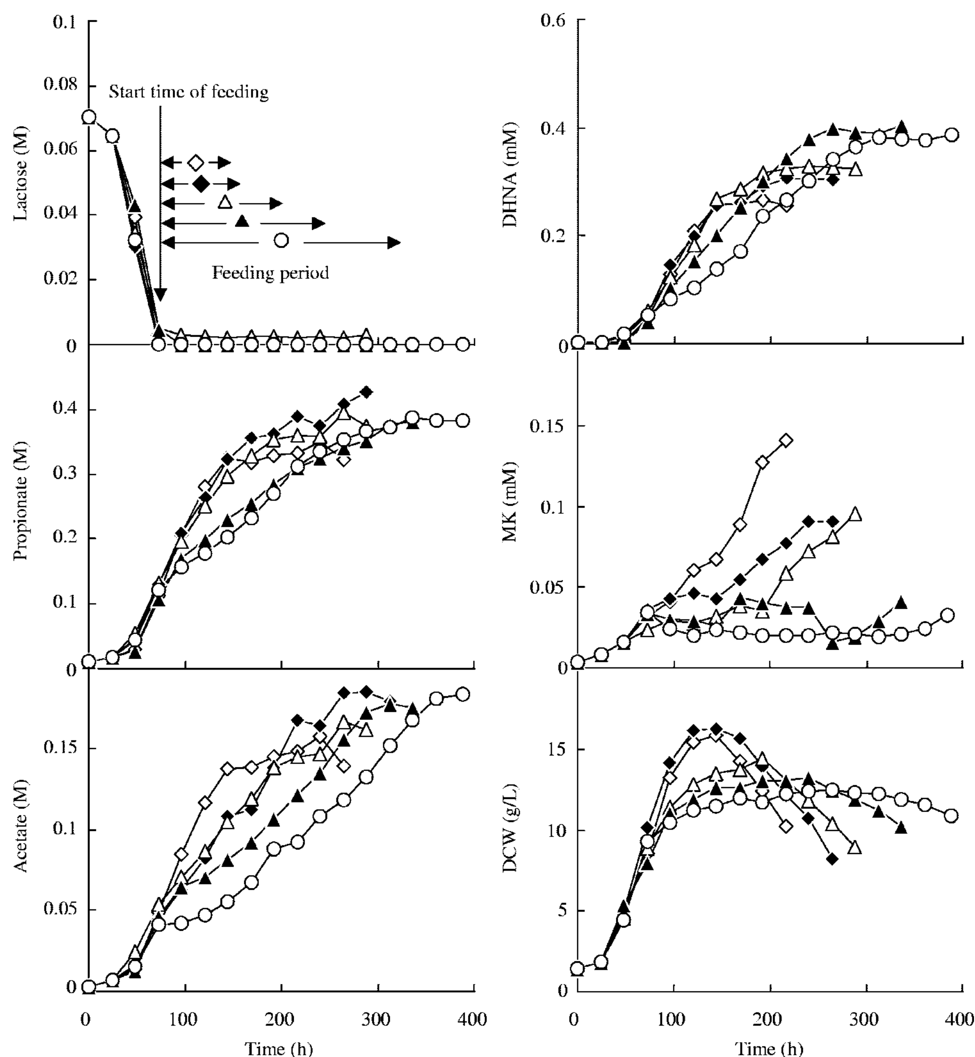

FIG. 4. Effects of lactose feeding rate on the production of organic acids, MK, and DHNA and cell growth. During the fed-batch cultures, the lactose feeding rates were maintained at 2.54, 1.90, 1.31, 0.90, and 0.62 ml h^{-1}, and it took 63, 86, 122, 178, and 260 h to feed 0.24 mol of lactose, respectively. The concentration of the lactose solution fed was 1.5 M. The vertical and horizontal arrows indicate the start time and the periods of lactose feeding, respectively. The symbols between the horizontal arrows correspond to the symbols in the plots, as follows: ◇, 2.54 ml h^{-1}; ◆, 1.90 ml h^{-1}; △, 1.31 ml h^{-1}; ▲, 0.90 ml h^{-1}; ○, 0.62 ml h^{-1}. DCW, dry cell weight.

that lactose limitation decreases the amount of G3P and Pyr available for isoprenoid synthesis because these metabolites are involved in the EMP pathway (Fig. 2A). It is also possible that the decrease in isoprenoid production decreased MK production. On the other hand, phosphoenolpyruvate (PEP), which is a precursor of DHNA (Fig. 1), is also involved in the EMP pathway. This means that lactose limitation may decrease DHNA production. However, there are significant differences between the amounts of these precursors required for isoprenoid unit synthesis and for DHNA synthesis. The MK produced by propionibacteria is (II, III)-tetrahydromenaquinone-9 (18, 20), and we also confirmed that it was produced by ET-3 (4). Therefore, the synthesis of isoprenoid units for 1 mol of MK by ET-3 requires 9 mol each of G3P and Pyr. In contrast, the synthesis of 1 mol of DHNA requires only 1 mol of PEP. The decrease in the flux of the EMP pathway is thought to strongly decrease MK synthesis more than it decreases DHNA synthesis. This conclusion is supported by the finding that the specific MK production rate markedly decreased as the lactose feeding rate decreased and by the finding that the specific DHNA production rate was almost constant regardless of the lactose feeding rate (Table 2). However, to

TABLE 1. Maximum DHNA and MK concentrations and sums of these concentrations in fed-batch cultures with various lactose feeding rates

Lactose feeding rate (ml h^{-1})	Maximum DHNA concn (mM)	Maximum MK concn (mM)	Sum of maximum DHNA and MK concn (mM)
2.54	0.27	0.14	0.41
1.90	0.31	0.10	0.41
1.31	0.33	0.08	0.41
0.90	0.40	0.04	0.44
0.62	0.40	0.03	0.43

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TABLE 2. Specific lactose consumption, DHNA production, MK production, and cell growth rates in batch and fed-batch cultures[a]

Culture conditions	Lactose feeding rate (ml h^{-1})	Specific rates			
		Lactose consumption (mmol g^{-1} h^{-1})	DHNA production (μmol g^{-1} h^{-1})	MK production (μmol g^{-1} h^{-1})	Cell growth (h^{-1})
Batch		0.299	0.270	0.182	0.036
Fed-batch	2.54	0.132	0.183	0.048	0.012
	1.90	0.093	0.185	0.014	0.010
	1.31	0.076	0.184	0.010	0.006
	0.90	0.056	0.170	0.000	0.002
	0.62	0.041	0.126	0.000	0.001

[a] The specific rates in the batch and fed-batch cultures were calculated by using the data for the exponential growth phase and the entire period of lactose feeding, respectively.

clarify this, it is necessary to investigate the activities and amounts of the enzymes involved in the reaction synthesizing isoprenoids from G3P and Pyr and in the reaction synthesizing DHNA from PEP.

Note that approximately 2 mol of propionate and 1 mol of acetate were produced from 1 mol of lactose in all the cultures shown in Fig. 3 and 4. These results suggest that the anaerobic fed-batch culture with lactose limitation did not drastically change the lactose metabolism. Therefore, the anaerobic fed-batch culture with lactose limitation apparently decreased the

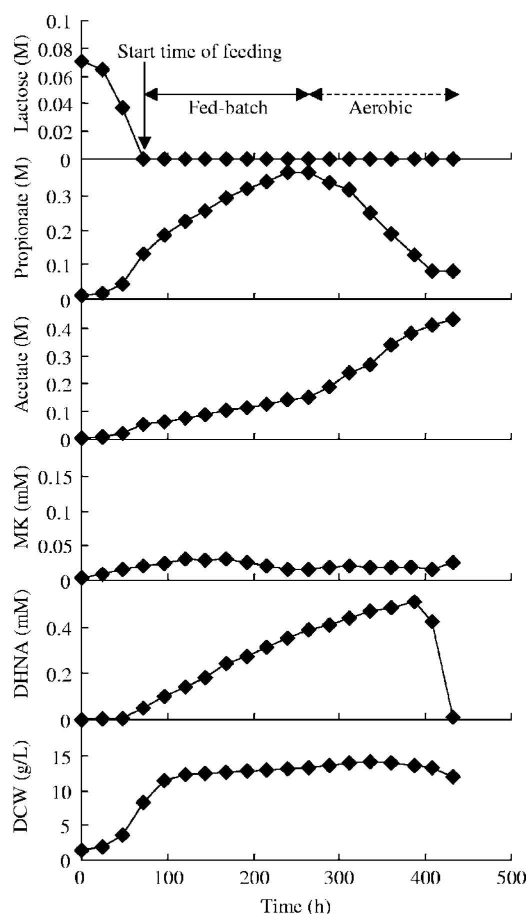

FIG. 5. Profiles of cultivation in which an anaerobic fed-batch culture was switched to an aerobic culture. During the fed-batch culture, the lactose feeding rate was maintained at 0.90 ml h^{-1}, and 0.24 mol of lactose was fed. The concentration of the lactose solution fed was 1.5 M. The vertical arrow indicates the start time of lactose feeding. The solid and dotted horizontal arrows indicate the periods of lactose feeding and aerobic culture, respectively. DCW, dry cell weight.

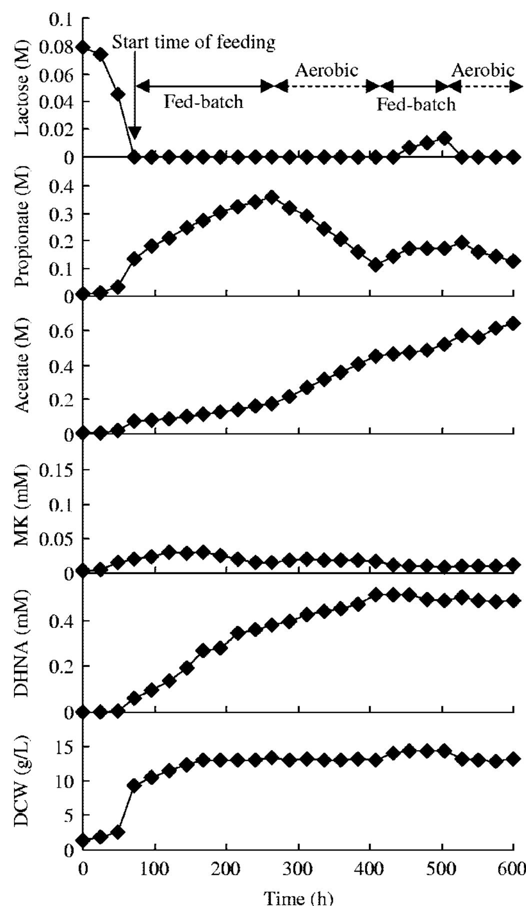

FIG. 6. Profiles of cultivation in which an anaerobic fed-batch culture was repeatedly alternated with an aerobic culture. During the fed-batch culture, the lactose feeding rate was maintained at 0.90 ml h^{-1}. During the first and second feeding periods, it took 178 and 90 h to feed 0.24 and 0.12 mol of lactose, respectively. The concentration of the lactose solution fed was 1.5 M. The vertical arrow indicates the start time of lactose feeding. The solid and dotted horizontal arrows indicate the periods of lactose feeding and aerobic culture, respectively. DCW, dry cell weight.

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flux of the EMP pathway without changing the lactose metabolism.

The cultivation shown in Fig. 6 maintained the DO concentration at approximately zero and the propionate concentration at a concentration lower than the concentration at which DHNA production is inhibited. However, DHNA production was inhibited early in the second fed-batch culture. Acetate accumulation might have been responsible for the termination of DHNA production in this cultivation because acetate addition at 48 h during incubation of the anaerobic batch culture prevented ET-3 from producing DHNA.

In conclusion, DHNA production was enhanced by a cultivation method that combined anaerobic fed-batch and aerobic batch cultures. The mechanism resulting in the increase in DHNA production is thought to be related to the metabolic change involved in synthesis of the precursors of MK. Our results should be useful for understanding the biosynthesis of DHNA and MK and for developing an effective method for DHNA production.

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