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Enhanced Production of Benzoylformate Reductase in *Enterococcus faecalis* under Oxidative Stress Established by Natural Electron Carriers

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Abstract Enhancement of the production of benzoylformate reductase (BFR) was attempted under oxidative stress established by natural electron carriers. α-lipoic acid (LA), flavin adenine dinucleotide (FAD), and ubiquinone (UQ) did not inhibit growth of *E. faecalis* when their concentrations were as high as 10 μM, while H_2O_2 and methyl viologen (MV^{2+}) inhibited the bacterial growth. BFR activity in the bacterial extract had increased rapidly after 1 h of cultivation after the addition of 4 μM of natural electron carriers, and the activity was maintained during further cultivation. BFR activity of the cells treated with the natural electron carriers was 40% higher than that of the control. In the presence of 4 μM H_2O_2 and MV^{2+}, BFR activity increased, reaching the highest activity at about 5 h cultivation, and then decreased with further cultivation. It seems that natural electron carriers not only stimulate the induction of BFR, but also stabilize the enzyme. BFR was hardly affected by LA, FAD, and UQ, while H_2O_2 and MV^{2+} inactivated the crude enzyme. The decrease of BFR activity in the presence of H_2O_2 and MV^{2+} might be ascribed to inactivation of the enzyme by the oxidants.

Key words: Oxidative stress, oxidant, redox-cycling agent, electron carrier, benzoylformate reductase, *Enterococcus faecalis*

Bacteria are able to sense and adjust to a great number of different environmental stresses [1, 11, 12, 13, 16, 21] including reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. These reactive oxygen species are generated by the incomplete reduction of oxygen during respiration or by exposure to light, metals, radiation, and redox-cycling agents such as paraquat (methyl viologen) and menadione [17]. The ROS constitute a stress because they lead to damage of almost all cellular components by causing mutagenesis, enzyme inactivation, and membrane damage [5]. However, many microorganisms possess enzymatic and nonenzymatic antioxidative mechanisms and minimize generation of ROS to levels that are not harmful to the cells. ROS are now recognized as signaling molecules under subtoxic conditions, and they induce various biological processes such as gene expression by stimulating signal transduction components [18].

Bacteria such as *Escherichia coli* can develop resistance to normally lethal concentrations of H_2O_2 by induction of a series of defense and repair enzymes. This inducible set of genes is turned on by low doses of H_2O_2 [4]. Under similar conditions of H_2O_2 exposure, *Salmonella typhimurium* can also be induced to synthesize some proteins that are thought to be protective [2]. It was found in *E. coli* that glucose-6-phosphate dehydrogenase (G6PD) was induced with methyl viologen (paraquat) and menadione [7, 10, 20], and NAD(P)H-dehydrogenase (diaphorase) was induced with menadione [5]. *Ralstonia eutropha* cultivated in the presence of H_2O_2 or methyl viologen was induced to synthesize more active β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase, resulting in the increased biosynthesis of poly-β-hydrobutyrate (PHB) and poly(3-hydroxy-butyrate-3-hydroxyvalerate) [9]. These results suggest that the synthesis of some enzymes can be increased if cells are treated with oxidation-reduction active compounds. Since methyl viologen (MV^{2+}) is very toxic and represents an undesirable contaminant in substances prepared with MV^{2+}, alternative oxidants, especially biomolecular ones, would be desirable.

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Enterococcus faecalis has been found to intracellularly synthesize benzoylformate reductase which can reduce benzoylformic acid to (R)-(−)-mandelic acid [22]. (R)-(−)-Mandelic acid is well known as a side-chain modifier of penicillin and cephalosporin, and a precursor of antiobesity agents. The optically active acid is prepared by optical resolution of the racemate with chiral amines [6] or enzymatically with esterase [14], glyoxalase [15], and benzoylformate reductase [9, 22]. At present, (R)-(−)-mandelic acid is produced from the chemically synthesized racemic mixture by the optical resolution method. The enzymatic method has not been used for industrial production, because enzymes as well as coenzyme cannot be produced economically. Hence, enhanced production of the enzymes is necessary if there is to be industrial applications.

This paper reports the effect of some oxidants on the growth and activity of the benzoylformate reductase of *E. faecalis*. An attempt was made to enhance the biosynthesis of benzoylformate reductase by adding some natural electron carriers to the culture medium, since the biomolecules were expected to be nontoxic. α-lipoic acid, flavin adenine dinucleotide and ubiquinone were employed, and their effect was compared to those of H_2O_2 and MV^{2+}.

MATERIALS AND METHODS

Materials

Ubiquinone (Coenzyme Q_{10}, UQ), flavin adenine dinucleotide (FAD), α-lipoic acid (DL-6,8-thioctic acid, LA), methyl viologen (1,1'-dimethyl-4,4'-bipyridium dichloride, MV^{2+}), hydrogen peroxide, benzoylformic acid, and (R)-(−)-mandelic acid were purchased from Sigma (St. Louis, U.S.A.).

Bacterial Strain and Culture Conditions

E. faecalis (IFO 12964, ATCC10100, formerly *Streptococcus faecalis*) was obtained from the Korean Culture Center of Microorganisms (Seoul, Korea). Lyophilized bacteria in a vial from the supplier was suspended in 10 ml of the sterile nutrient medium with tryptic soy broth (TSB, Becton Dickinson, U.S.A.), and incubated at 37°C overnight. One milliliter of the fresh overnight culture was added to 10 ml of TSB medium and incubated under the same conditions, followed by repetitive subculture. The culture was streaked to grow at 37°C overnight on a tryptic soy broth agar plate. Starter cultures were prepared by inoculating 8 ml of TSB medium with a single colony and incubating this at 37°C for 24 h on a rotary shaker (180 rpm). One-hundred-milliliter portions of TSB medium were placed in a 250-ml conical flask. Eight milliliters of the starter culture were used to inoculate each portion of TSB medium and incubated at 37°C for 6 h on a rotary shaker (180 rpm). Separately, culturing with tomato-juice broth M-Co [22] was carried out.

Cells were counted using Bioscreen C (Labsystem, Oy, Helsinki, Finland) or harvested by centrifugation at 4°C. The harvested pellets were washed with 0.1 M phosphate buffer (pH 7.0) and suspended (1:2 w/v) in 15 M phosphate buffer (pH 6.3) containing 2 mM 2-mercaptoethanol, followed by disrupting with glass beads (150–212 microns). The suspensions were centrifuged and the resulting supernatants were used as a crude enzyme solution of benzoylformate reductase (BFR).

Zone of Inhibition Testing

Petri dishes (87×15 mm) containing 20 ml TSB agar were prepared. A cotton tipped swab was dipped into the starter culture and inoculum was spread over the surface of the TSB agar plates in three directions in order to obtain a uniform lawn of growth. Filter paper discs of 6 mm diameter were saturated with 0.15 ml of each oxidant solution to give a final concentration of 4 or 10 μM and placed on the inoculated TSB agar surface. Plates were incubated at 30°C aerobically. After 24 h of incubation, horizontal and vertical diameters of the zones of inhibition were measured in mm.

The Enzymatic Activity

The activity of BFR was evaluated from the decrease of absorbance at 340 nm, due to oxidation of NADH determined by a spectrophotometer (Shimadzu UV-1601; Shimadzu Co., Japan). Assay mixtures were prepared by mixing 2.5 ml of 0.1 M phosphate buffer (pH 7.5), 0.2 ml of 83 mM sodium benzoylformate, and 100 μl of the crude enzyme solution. The mixture was kept at 30°C for 5 min, and then the enzyme reaction was started by adding 50 μl of 13 mM NADH. The activity was calculated using the following equation:

$$IU/ml=(\Delta A/min)\ VD/\varepsilon dv$$

where (ΔA/min) is the linear slope of decreasing absorption, V the total volume in cuvette, D the dilution of crude enzyme solution, ε the molar extinction coefficient for NADH (6220 l mol^{-1} cm^{-1}), d the pathlength of light through cuvette, and v the volume of crude enzyme solution. One unit of enzymatic activity was defined as the amount of enzyme to reduce 1 μmol of benzoylformic acid.

Sensitivity to Oxidizing Agents

E. faecalis was grown overnight on a TSB agar plate, then inoculated into 8 ml of fresh TSB medium to incubate for 24 h. Eight milliliters of the starter culture were inoculated into 100 ml of TSB medium placed in a 250-ml conical flask. The culture was exposed to oxidative stress by the addition of H_2O_2, MV^{2+}, UQ, FAD, or LA (0.5 to 10 μM) to the culture medium after 1 h of cultivation.

RESULTS AND DISCUSSION

Effect of Culture Media on Cell Growth and Enzymatic Activity

TSB medium was used to cultivate *E. faecalis* and compared with tomato-juice broth M-Co medium which was reported to be effective in producing more active benzoylformate reductase (BFR) [22]. Figure 1 shows the growth characteristics and the activity profiles of the BFR of *E. faecalis* which was cultivated with TSB and tomato-juice broth M-Co media. There was a remarkable difference in cell growth and enzyme production between TSB and tomato-juice broth M-Co culture media. In the TSB medium, the cells grew rapidly and reached the stationary phase in about 4 h of cultivation, while the cells divided slowly in the tomato-juice broth M-Co medium and the density of the cells was low. The density of the cells grown in the TSB medium was about 40% higher than that in the tomato-juice M-Co broth medium.

The activity of BFR in the bacterial extracts increased with cultivation time and then decreased after reaching the maximum value at 6 h of cultivation in both tomato-juice broth M-Co medium and TSB medium. The maximum enzymatic activity of the cells grown in the TSB medium was about 50% higher than that in the tomato-juice broth M-Co medium, indicating that the high enzymatic activity of the cells grown in the TSB medium resulted from the high density of the cells in the TSB medium.

Since tomato-juice broth M-Co medium was less effective in cell growth and enzymatic activity in the cell extracts than the TSB medium, the latter was used in the next set of experiments.

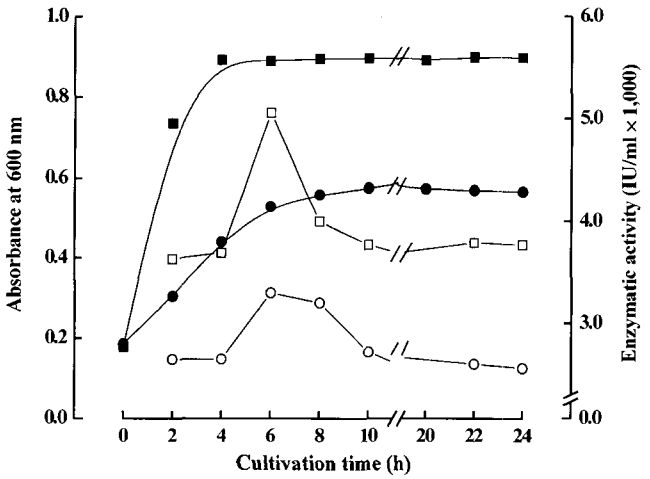

Fig. 1. Effect of culture media on cell growth and benzoylformate reductase activity of *E. faecalis*.
Symbols: ■, cell growth in TSB; ●, cell growth in tomato-juice broth M-Co; □, enzymatic activity of cells grown in TSB; ○, enzymatic activity of cell grown in tomato-juice broth M-Co.

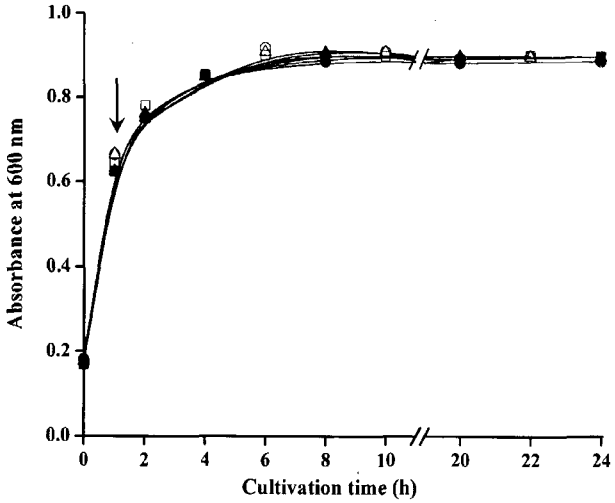

Fig. 2. Effect of some oxidants (4 μM) on cell growth during cultivation of *E. faecalis*.
Arrow indicates the addition of oxidants. Symbols: ○, control; △, methyl viologen; □, hydrogen peroxide; ▲, α-lipoic acid; ●, flavin adenine dinucleotide; ■, ubiquinone.

Effect of Oxidants on Cell Growth

Figure 2 shows cell growth under stress caused by 4 μM oxidants. Each oxidant was added to the culture medium after 1 h of cultivation, because the cells seemed to be more sensitive to environmental stress during the exponential phase than during the stationary phase as were *E. coli* and *S. typhimurium* [3]. The oxidants did not inhibit the growth of *E. faecalis*, implying that the oxidants did not have any cytotoxic effect on the cell when the concentration of oxidants was as low as 4 μM. This was confirmed by the zone diameters of bacterial growth inhibition due to oxidants as shown in Fig. 3. Inhibition of cell growth was not observed during 24 h of cultivation, when 4 μM oxidants were added to the bacteria after 1 h of cultivation. LA, FAD, and UQ did not inhibit cell growth even though the concentration was as high as 10 μM. However, H_2O_2 and MV^{2+} inhibited the bacterial growth at the concentration

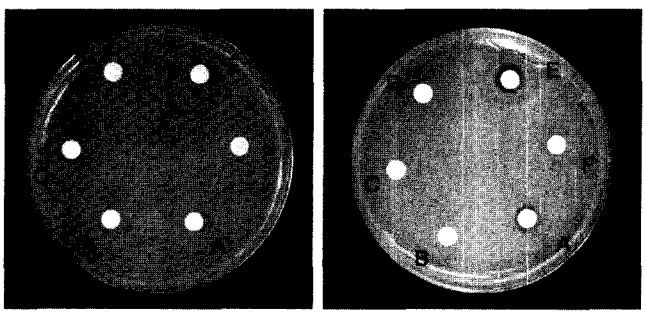

Fig. 3. Zone diameters of inhibition of *E. faecalis* growth by 4 (left plate) and 10 μM (right plate) of methyl viologen (A), α-lipoic acid (B), ubiquinone (C), flavin adenine dinucleotide (D), hydrogen peroxide (E), and water (F).

of 10 μM. H_2O_2 was found to be more inhibitory to the cell culture than MV^{2+}, because H_2O_2 resulted in a more distinct zone perimeter (1.9 mm) than MV^{2+} (1.0 mm). The reason for the difference in bactericidal effectiveness between MV^{2+} and H_2O_2 is not known. The cytotoxic effect of MV^{2+} and H_2O_2 is considered to be resulted from the generation of superoxide anions and hydroxyl radicals, respectively, to the levels that are harmful to the cells. The superoxide anions can be generated simply by treating cells with MV^{2+}, and the extremely reactive hydroxyl radical is converted from H_2O_2 in the presence of metals [17]. *E. faecalis* appears to be more tolerant to oxidants than *R. eutropha*, since the latter morphologically changed by 3 h of exposure to 4 μM of MV^{2+} [9]. The exposure of *Streptococcus thermophilus* to hydrogen peroxide concentrations (0.25–30 mM) led to a depletion of cell viability [19].

Effect of Oxidants on Production of Benzoylformate Reductase

Production of BFR by *E. faecalis* under the oxidative stress established by some oxidants was investigated by measuring the activity of BFR in bacterial extracts. Production of BFR was significantly enhanced by the addition of oxidants. Figure 4 shows activity profiles of BFR when each oxidant of 4 μM was introduced into the growth medium after 1 h of cultivation. Enzyme biosynthesis was significantly stimulated under the oxidative cultivations, although productivity of BFR varied depending on the kinds of oxidant used. In the absence of oxidant, BFR activity increased with time and then decreased after reaching the highest enzymatic activity after about 6 h of cultivation. It appeared that BFR was autolysed during the longer cultivation period after the enzyme was induced at the earlier stationary phase, but the exact reason for this is not clear. BFR activity increased when treating the cells with MV^{2+} at an elevated rate to reach the highest enzymatic activity after about 5 h of cultivation, and then decreased rapidly with further cultivation. The maximum enzymatic activity of the cells treated with MV^{2+} was 40% higher than that of the control, although the activity profile in the presence of MV^{2+} was similar to that in the absence of MV^{2+}. Under H_2O_2 exposure, BFR activity of the bacterium increased and then reached the maximum value at about 5 h of cultivation, followed by a slow decrease of activity. BFR activity of both exponentially growing and stationary-phase cells treated with H_2O_2 was higher than that with MV^{2+}, except that the maximum activities were almost the same with each other. Interestingly, BFR activity increased rapidly after 1 h of cultivation after exposure of the cells to LA, FAD, and UQ, exceeding the level of maximum activity under MV^{2+} and H_2O_2 treatments, however, the level was unchanged during further cultivation. It seemed that the biomolecular oxidants stimulated the production of BFR without exerting any destabilizing influence on the enzyme.

The cells treated with MV^{2+} exhibited a pronounced lag during the stimulation of enzyme production in the first 3 h, compared to the cells with H_2O_2. Lower BFR activity under treatments with MV^{2+} and H_2O_2 at the late stationary phase might be ascribed to a lack of enzyme stabilization effect by the oxidants, and this requires further experimental study.

Figure 5 shows the effect of the concentration of oxidants on the activity of BFR. A range of oxidant concentrations (0–10 μM) was added to the growth medium 1 h after cultivation, and then was cultivated for further 5 h. The activity of BFR in the bacterial extracts increased between

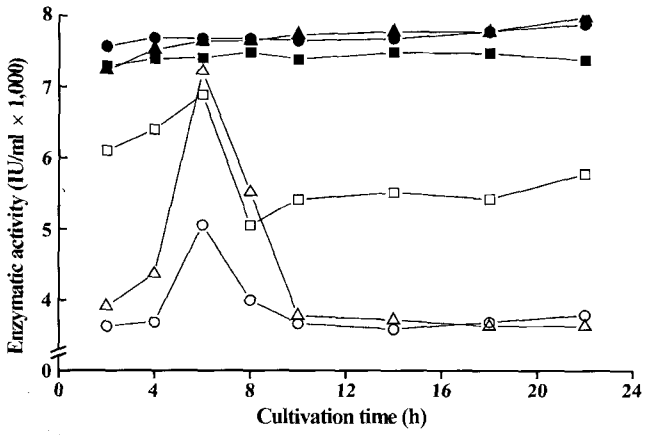

Fig. 4. Effect of some oxidants on the activity of benzoylformate reductase during cultivation of *E. faecalis*.
Each oxidant of 4 μM was added after 1 h of cultivation. Arrow indicates the addition of oxidants. Symbols: ○, control; △, methyl viologen; □, hydrogen peroxide; ▲, α-lipoic acid; ●, flavin adenine dinucleotide; ■, ubiquinone.

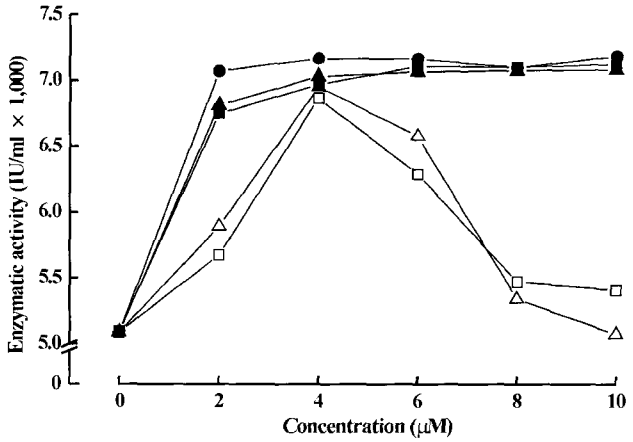

Fig. 5. Effect of the concentrations of oxidants on the activity of benzoylformate reductase activity.
Each oxidant was added after 1 h of cultivation, and then the culture was further incubated for 5 h. Symbols: △, methyl viologen; □, hydrogen peroxide; ▲, α-lipoic acid; ●, flavin adenine dinucleotide; ■, ubiquinone.

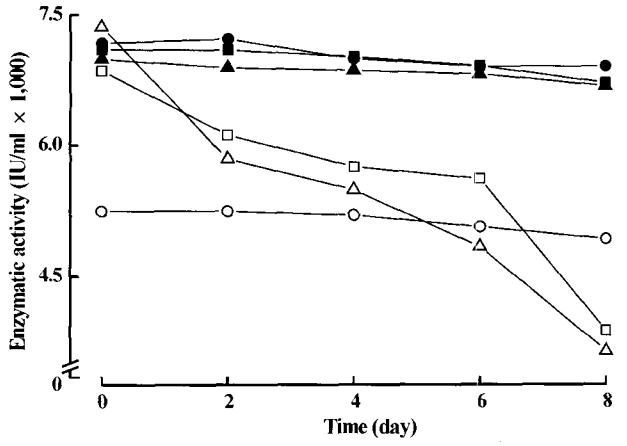

Fig. 6. Effect of some oxidants on the stability of benzoylformate reductase.

The enzyme solution was incubated at 30°C, to which each oxidant was added to give a final concentration of 4 μM. Symbols: ○, control; △, methyl viologen; □, hydrogen peroxide; ▲, α-lipoic acid; ●, flavin adenine dinucleotide; ■, ubiquinone.

0 and 4 μM H_2O_2 and MV^{2+}, but addition of H_2O_2 and MV^{2+} above 4 μM resulted in a loss of activity: High concentrations of H_2O_2 and MV^{2+} exerted a harmful influence on the cells, although they stimulated the biosynthesis of BFR in low doses. However, the BFR activity of the cells treated with LA, FAD, and UQ increased at an elevated rate, as the concentration of the oxidants increased to 2 μM, and then continued to increase slightly with amounts of the oxidants over 2 μM, indicating that the natural electron carriers are nontoxic to the cells, as shown in Fig. 3.

It seems that the increased level of superoxide anions, the product of a one-electron reduction of oxygen, can be generated by treating cells with the biomolecular oxidants, similar to the generation with oxidation-reduction cycling agents [17] such as paraquat (MV^{2+}), menadione, and plumbagin. However, the exact molecular target of the oxidants for the induction of sinal transduction have yet to be elucidated.

Effect of Oxidants on the Stability of Benzoylformate Reductase

The effect of oxidants on the stability of BFR was examined by measuring the enzymatic activity after incubating crude BFR with oxidants at 30°C. As shown in Fig. 6, LA, FAD, and UQ hardly affected the activity of BFR, while H_2O_2 and MV^{2+} decreased the enzymatic activity. The fact that in the absence of an oxidant the enzymatic activity barely decreased during 8 days of incubation indicates that the enzyme can be considered to be very stable at mild temperatures. Therefore, a decrease in the enzymatic activity in the presence of H_2O_2 and MV^{2+} might have been due the to inactivation of the enzyme by the oxidants.

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