Applied and Environmental Microbiology, Sept. 2002, p. 4534–4538 Vol. 68, No. 9
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.9.4534–4538.2002
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Fumarate-Mediated Inhibition of Erythrose Reductase, a Key Enzyme for Erythritol Production by *Torula corallina*

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Received 26 December 2001/Accepted 25 June 2002

***Torula corallina*, a strain presently being used for the industrial production of erythritol, has the highest erythritol yield ever reported for an erythritol-producing microorganism. The increased production of erythritol by *Torula corallina* with trace elements such as Cu^{2+} has been thoroughly reported, but the mechanism by which Cu^{2+} increases the production of erythritol has not been studied. This study demonstrated that supplemental Cu^{2+} enhanced the production of erythritol, while it significantly decreased the production of a major by-product that accumulates during erythritol fermentation, which was identified as fumarate by instrumental analyses. Erythrose reductase, a key enzyme that converts erythrose to erythritol in *T. corallina*, was purified to homogeneity by chromatographic methods, including ion-exchange and affinity chromatography. In vitro, purified erythrose reductase was significantly inhibited noncompetitively by increasing the fumarate concentration. In contrast, the enzyme activity remained almost constant regardless of Cu^{2+} concentration. This suggests that supplemental Cu^{2+} reduced the production of fumarate, a strong inhibitor of erythrose reductase, which led to less inhibition of erythrose reductase and a high yield of erythritol. This is the first report that suggests catabolite repression by a tricarboxylic acid cycle intermediate in *T. corallina*.**

Erythritol is a four-carbon polyol with properties similar to those of other polyols presently used as food ingredients (4). Erythritol has 60 to 70% of the sweetness of sucrose in a 10% (wt/vol) solution. Erythritol has very high negative heat when dissolved in solution, providing a strong cooling effect (10). Because it is a small molecule, erythritol has strong colligative properties, including freezing point depression, boiling point elevation, and high osmotic pressure. With its low hygroscopicity and viscosity in solution, it is very useful for reducing and controlling water activity in foodstuffs (9). Because erythritol cannot be used as a fermentation substrate for caries-producing bacteria, its use in food does not promote tooth decay.

Erythritol can be produced by microbial methods using osmophilic yeasts and some bacteria (1, 16, 17, 27, 30, 32, 33). Erythritol was found to be synthesized from erythrose-4-phosphate, an intermediate of the pentose-phosphate cycle, by dephosphorylation followed by reduction of the resultant erythrose. Erythrose reductase, catalyzing this last step, is well known as a key enzyme for the biosynthesis of erythritol (15, 31). It has been reported that polyols were produced by 43 of 1,753 osmophilic yeast strains isolated from honey and pollen (29). Three of these strains of *Trichosporon* and *Trichosporonoides* (1) produced only erythritol at good yields of 27.9 to 40.7% (wt/wt). Other microorganisms that have been reported to produce erythritol include *Pichia*, *Zygopichia*, *Candida*, *Torulopsis*, *Trigonopsis* (13, 28), and *Moniliella iomentosa* var. *pollis* (11). Erythritol production using this strain could not be applied on an industrial scale due to by-products such as glycerol and ribitol. Erythritol has been produced commercially by using a mutant of *Aureobasidium* that produced erythritol at a high concentration of 175 g/liter, with a high yield of 43.8% (wt/wt) after 4 days of cultivation in a jar fermentor (14).

Recently, a novel microorganism, which is able to produce erythritol, was isolated and identified as *Torula corallina* (16). A mutant of this strain produced erythritol at a high concentration of 196 g/liter with a high yield of 48.9% and did not produce by-products such as glycerol and ribitol, resulting in application on an industrial scale (16, 20). It was previously reported that erythritol production was improved by controlling glucose concentration in a fed-batch culture of *T. corallina* (26) and by providing inositol, phytic acid (19), Mn^{2+}, and Cu^{2+} supplements (20). The mechanisms by which Cu^{2+} increases erythritol production, however, have not yet been studied.

It has been demonstrated that many catabolic pathways are repressed in the presence of tricarboxylic acid (TCA) cycle intermediates (5, 23). In this study, we investigated the mechanism of Cu^{2+} enhancement of erythritol production. We determined that a major by-product of erythritol production was fumarate; we quantified its production during culture and examined its effect on the activity of erythrose reductase, a key enzyme in erythritol biosynthesis.

MATERIALS AND METHODS

Microorganisms, media, and chemicals. *T. corallina* was isolated from a 40% sucrose solution at the Bolak Co. R&D Center (Osan, Korea) (16). Growth medium (200 g of glucose and 10 g of yeast extract per liter) was used for initial shake-flask cultivation at 30°C. The production medium contained 400 g of glucose and 20 g of yeast extract per liter. Fermentor experiments were carried out with and without the addition of 2 μg of $CuSO_4 \cdot 5H_2O$/liter. Medium components were purchased from Difco (Detroit, Mich.) and Wako Pure Chemical Industries (Osaka, Japan). Organic acids such as oxalacetate, malate, succinate, fumarate, maleate, pyruvate, citrate, α-ketoglutarate, acetate, and lactate were purchased from Sigma Chemical Co. (St. Louis, Mo.).

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Culture conditions. A single colony of *T. corallina* was inoculated into a 20-mm-diameter test tube containing 5 ml of growth medium and was incubated at 30°C with agitation at 250 rpm for 48 h. Following agitation, 5 ml of broth was transferred into a 500-ml baffled flask containing 100 ml of growth medium and was then cultivated at 30°C and 250 rpm for 24 h. This seed culture was then transferred into a fermentor. Fermentor experiments were performed with 5-liter jar fermentors (KoBiotech Co., Inchon, Korea) containing 3 liters of production medium. The temperature and pH of the fermentor were controlled to 34°C and 5.5, respectively. To maintain the dissolved oxygen concentration above 20%, the agitation speed was adjusted to between 500 and 850 rpm. The aeration rate was 0.5 vol/vol/min (vvm) during fermentation (16).

Preparation of cell extracts. Cells from the culture broth were harvested by centrifugation. After being washed with 0.1 M Tris-HCl buffer (pH 7.8), cells were resuspended in disruption buffer (2) containing 20 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was disrupted by grinding with glass beads that were 0.5 mm in diameter (Sigma Chemical Co.). Cell extracts were obtained by removing the ruptured cells by centrifugation at 10,000 × *g* for 30 min.

Purification of erythrose reductase. Cell extracts were fractionated by ammonium sulfate precipitation. The fraction precipitated by between 40 and 70% saturation of ammonium sulfate was collected by centrifugation and dissolved in 50 mM Tris-HCl buffer (pH 7.8). Following removal of insoluble material by centrifugation at 10,000 × *g* for 1 h, the enzyme solution was dialyzed against the same buffer at 4°C for 24 h. Dialyzed enzyme solution was placed on a DEAE-Toyopearl 650S column (1.4 by 20 cm) equilibrated with 50 mM Tris-HCl buffer at pH 7.8. Protein was eluted by a linear 0 to 0.5 M NaCl gradient in the same buffer. Active fractions were pooled. The enzyme solution was put on a Cibacron Blue 3GA affinity column (1.4 by 20 cm) that had been equilibrated with the same buffer. The column was eluted by a linear gradient of 0 to 1 M NaCl in the same buffer. The active fractions were pooled, concentrated, and dialyzed against the same buffer and concentrated with Centricon (Amicon, Bedford, Mass.) and were then used as a purified enzyme in the following experiments.

Erythrose reductase activity assay. The activity of erythrose reductase was determined by monitoring the oxidation of NADPH in a spectrophotometric cuvette at 340 nm at 37°C (6). The cuvette contained 1.5 ml of 50 mM Tris-HCl buffer (pH 7.8), 0.1 ml of 0.1 M 2-mercaptoethanol, 0.2 ml of enzyme solution, and 0.2 ml of 3.4 mM NADPH. This reaction mixture was allowed to stand for 1 min to eliminate the endogenous oxidation of NADPH. The reaction was started by addition of 0.1 ml of 0.5 M D-erythrose. One unit of enzyme represents the amount of enzyme that caused an initial rate of decrease of 1 μmol of NADPH per min. Activities are expressed as units/milligram of protein.

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (18). Native PAGE was performed on a 10% polyacrylamide gel without SDS. Protein bands were stained with Coomassie brilliant blue R250. Erythrose reductase activity staining on a polyacrylamide gel was done by use of a modification of the procedure of Birken and Pisano (5). The staining mixture used for the detection of NADP-erythritol activity consisted of 40 ml of 0.1 M Tris-HCl buffer (pH 7.8), 25 mg of nitroblue tetrazolium, 3 mg of phenazine methosulfate, 30 mg of NADP, and 2 ml of 0.5 M erythritol. Gels were incubated in appropriate activity staining solution for 15 min, washed in water, and stored in 7% acetic acid.

Analytical methods. Dry cell weight was estimated by using a calibration curve derived from the relationship between the absorbance at 600 nm and the dry cell weight. Protein was determined by the Lowry method using bovine serum albumin as a standard. Concentrations of erythritol and glucose were determined by high-performance liquid chromatography (HPLC) coupled to a refractive index detector (model 2410; Waters, Milford, Mass.) and a KR100-10NH_2 column (4.6 by 250 mm; Kromasil, Bohus, Sweden). The mobile phase was acetonitrile/water (80:20 [vol/vol]), and the flow rate was 1.5 ml/min. For identification of individual organic acids by HPLC, culture broths were compared with standard solutions of organic acids, such as oxalacetate, malate, succinate, fumarate, pyruvate, citrate, α-ketoglutarate, acetate, and lactate, on a Capcell Pak C_{18} MG column (4.6 by 250 mm; Shiseido, Tokyo, Japan) with a UV detector (Waters model 486). The mobile phase was 2.5% aqueous CH_3CN (pH 2.5 with H_3PO_4), and the flow rate was 0.7 ml/min.

Mass and ^{1}H-NMR spectroscopy. Atmospheric pressure chemical ionization was carried out in the negative ion mode on a JEOL AX505WA mass spectrometer (JEOL Inc., Peabody, Mass.) at a 5-kV needle voltage, 250°C, 5-mA corona discharge current, and 500°C capillary temperature. ^{1}H-nuclear magnetic resonance (NMR) measurements were recorded in the pulsed Fourier transform mode on a Bruker ARX Fourier transform spectrometer (Bruker Instruments Inc., Billerica, Mass.) operating at 400 MHz.

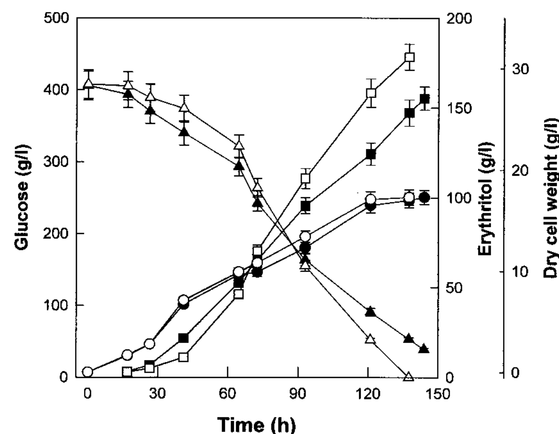

FIG. 1. Glucose consumption and erythritol production in a culture without and with 2.0 μg of $CuSO_4 \cdot 5H_2O$/ml by *T. corallina*. Glucose consumption without (▲) and with (△) $CuSO_4 \cdot 5H_2$ is shown, as is erythritol production without (■) and with (□) $CuSO_4 \cdot 5H_2O$; dry cell weight without (●) and with (○) $CuSO_4 \cdot 5H_2O$ is also shown. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

RESULTS

Increased erythritol production by Cu^{2+} supplementation. To investigate the role of Cu^{2+} in the metabolism of *T. corallina* and its stimulatory action on erythritol production, a control culture without Cu^{2+} and an experimental culture with 2 μg of $CuSO_4 \cdot 5H_2O$/ml were established, as shown in Fig. 1. Maximal cell growth required about 1 μg of $CuSO_4 \cdot 5H_2O$/ml, whereas maximal erythritol production required about 2 μg of $CuSO_4 \cdot 5H_2O$/ml. Inhibition of cellular growth was observed with 10-μg/ml and higher concentrations of $CuSO_4 \cdot 5H_2O$. There were no large differences between the cultures for approximately 70 h. After 70 h, cell growth, glucose consumption, and erythritol production rates (in grams/liter-hour) of cultures containing Cu^{2+} were higher than those of control cultures. The supplemental Cu^{2+} increased cellular growth slightly and significantly enhanced erythritol production. After culture for 144 h, the final concentration of erythritol produced from 400 g of glucose/liter in the control culture without Cu^{2+} was 155 g/liter. The concentration increased to 178 g/liter at 138 h with supplemental Cu^{2+}. In the control culture the volumetric rate of erythritol production was 1.08 g/liter-h, and the erythritol yield from glucose was 38.3% (wt/wt). With the addition of Cu^{2+}, these values increased to 1.29 g/liter-h and 44.5% (wt/wt), respectively.

Identification of a major by-product in *T. corallina* culture. The by-products of cultures with and without Cu^{2+} were analyzed by using HPLC coupled to a UV detector and a Shiseido Capcell Pak C_{18} MG column at 215 nm (Fig. 2).

Separation of individual organic acids by HPLC revealed that an unknown compound that accumulated in cultures of *T. corallina* coeluted with fumarate. This major by-product was detected at a retention time of approximately 9 min, identical to that of fumarate under conditions described in Materials and Methods. To confirm that this by-product was fumarate, we purified it by preparative HPLC. Eluates containing the by-product were subjected to atmospheric pressure chemical

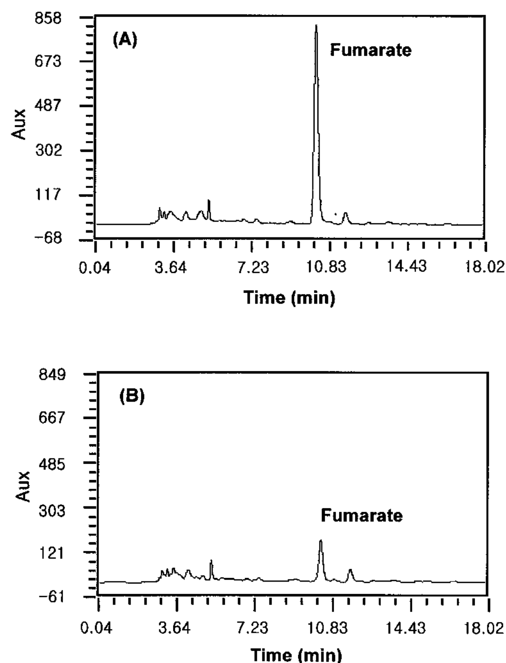

FIG. 2. HPLC pattern of erythritol fermentation broth in a culture of *T. corallina* without (A) and with (B) 2.0 μg of $CuSO_4 \cdot 5H_2O$/ml. A major by-product was identified as fumarate by instrumental analyses.

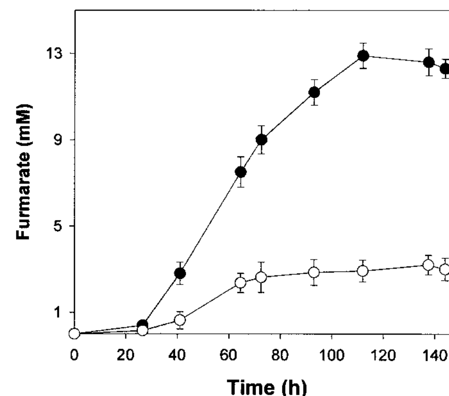

FIG. 3. Time course of fumarate production in cultures without (●) and with (○) 2.0 μg of $CuSO_4 \cdot 5H_2O$/ml during erythritol fermentation. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

ionization mass spectroscopy in the negative ion mode. In the total ion current chromatogram, one major peak was detected at a retention time of 2.27 min, corresponding to HPLC UV detection. In deprotonated form, this peak represented a molecular mass of 115 Da. This value corresponded well with the known mass of the fumarate pseudomolecular ion, $[M-H]^+$. The ions with an *m/z* of 71 corresponded to $[M-H-COOH]^+$. ^{1}H-NMR analysis was also performed, and the result was identical to that for fumarate (data not shown). Taken together, these results indicate that the by-product accumulated in *T. corallina* culture was indeed fumarate.

Effect of Cu^{2+} on by-product production and its quantification. The effect of Cu^{2+} on fumarate production was studied by performing fumarate production time course experiments during erythritol fermentation in cultures with and without 2 μg of $CuSO_4 \cdot 5H_2O$/ml (Fig. 3). HPLC analysis was carried out to examine the accumulation of fumarate. In the absence of exogenous Cu^{2+}, the highest fumarate level reached was 12.9 mM. In the presence of exogenous Cu^{2+}, the levels of fumarate accumulation significantly decreased at $CuSO_4 \cdot 5H_2O$ concentrations exceeding 1 μg/ml. The decrease in erythritol production at $CuSO_4 \cdot 5H_2O$ concentrations above 2 μg/ml was due to the inhibition of cellular growth by Cu^{2+}. Following supplementation with 2 μg of $CuSO_4 \cdot 5H_2O$/ml, a stimulator of erythritol production, fumarate production was reduced to about 25%. The maximum concentrations of fumarate found were 12.9 mM without Cu^{2+} and 3.2 mM with Cu^{2+}. Cu^{2+} was found to strongly inhibit the production of fumarate, a major by-product during erythritol fermentation.

Purification of erythrose reductase. Crude extracts of *T. corallina* were obtained from culture at 4 days, when the enzymatic activity of erythrose reductase is high. Fractionation with ammonium sulfate increased the specific activity twofold, with 76% recovery of the erythrose reductase activity. The active fractions were applied to a DEAE-Toyopearl 650S column, and erythrose reductase was eluted by a 0 to 0.5 M NaCl linear gradient. Erythrose reductase activities were recovered in approximately 0.2 M NaCl-containing fractions. On Cibacron Blue 3GA affinity column chromatography, three peaks with protein content and one peak with erythrose reductase activity were resolved (data not shown). The first large peak and the third peak did not show erythrose reductase activity, whereas the second small peak that eluted as a single peak at approximately 0.7 M NaCl did show erythrose reductase activity. The active fractions were collected, concentrated, and dialyzed with a stirred cell (Amicon). The chromatographic procedure resulted in a 53.5-fold purification of erythrose reductase with a recovery of 10.8% and a specific activity of 1.8 U/mg. At this purification step, erythrose reductase was found to be homogeneous by SDS-PAGE (Fig. 4A). And this enzyme solution was used for an activity assay of erythrose reductase.

Effect of Cu^{2+} and fumarate on erythrose reductase. In order to investigate in detail the role of Cu^{2+} in erythritol production, erythrose reductase from *T. corallina* was purified and its in vitro activity was assayed with increasing Cu^{2+} concentration. Cu^{2+} was added as $CuSO_4 \cdot 5H_2O$, and the Cu^{2+} concentration was measured by atomic absorption spectrophotometry. Cu^{2+} did not have a great effect on erythrose reductase activity (Fig. 5). Divalent metals such as Cu^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} did not affect enzyme activity at concentrations of 1 to 10 μg/ml. These results suggest that supplementation with Cu^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} did not directly affect the activity of erythrose reductase. The effect of fumarate on erythrose reductase was also investigated. When the fumarate concentration in purified enzyme solution was increased, the activity of erythrose reductase significantly decreased (Fig. 5). Fumarate was found to be a strong inhibitor for erythrose reductase.

The kinetic parameters of erythrose reductase were determined by using initial rate determination followed by Lineweaver-Burk plotting. The K_m and V_{max} for the reduction of erythrose in the plot were determined to be about 7.12 mM

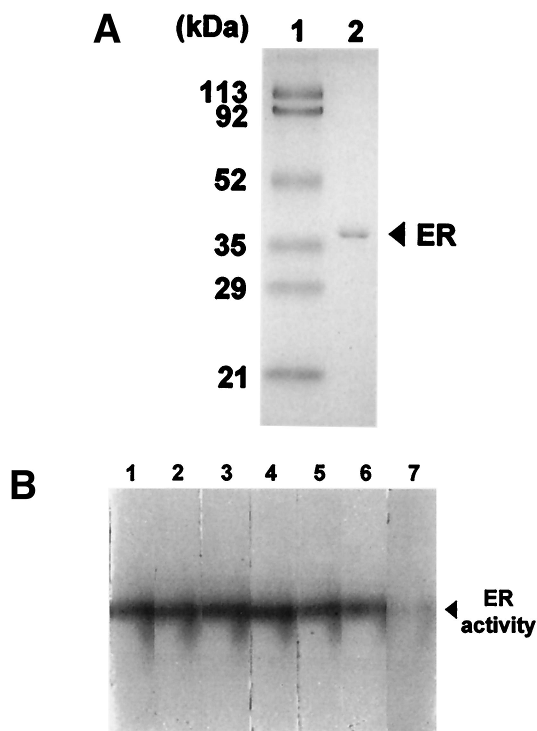

FIG. 4. (A) SDS–10% PAGE shows a homogeneous enzyme preparation after the final purification step. Lane 1, molecular weight marker; lane 2, purifed erythrose reductase. (B) In vitro activity staining assay demonstrating that fumarate specifically inhibits the activity of erythrose reductase. Lane 1 contains no TCA cycle intermediates. Lanes 2 to 7 contain TCA cycle intermediates as follows: lane 2, 10 mM citrate; lane 3, 10 mM α-ketoglutarate; lane 4, 10 mM succinate; lane 5, 10 mM malate; lane 6, 10 mM oxalacetate; and lane 7, 10 mM fumarate. ER, erythrose reductase.

and 26 mmol/min · mg of protein, respectively. The effect of fumarate concentration on erythrose reductase inhibition was studied as a Lineweaver-Burk plot. The inhibition was found to be noncompetitive, and the K_i for fumarate was about 3.9 mM. In vitro assays demonstrated that fumarate specifically inhibited erythrose reductase in a noncompetitive manner against an enzyme-substrate (erythrose reductase-erythrose) complex (Fig. 6).

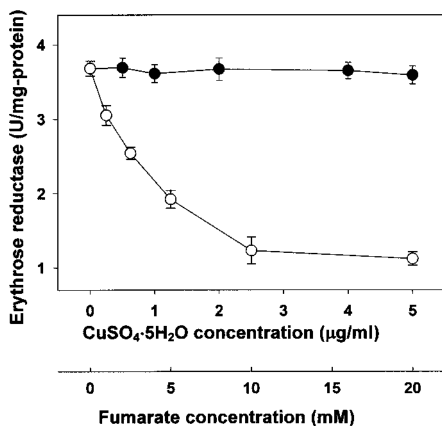

FIG. 5. Effect of Cu^{2+} (●) and fumarate (○) on the activity of purified erythrose reductase from *T. corallina*. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

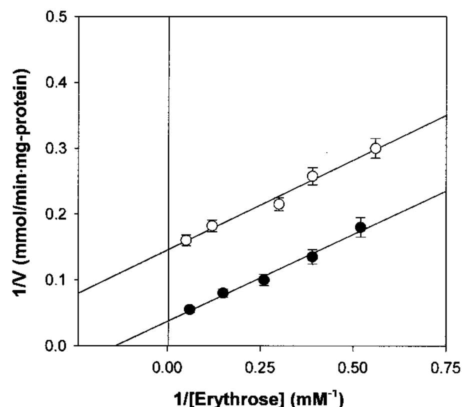

FIG. 6. Lineweaver-Burk plot of the inhibition of erythrose reductase by fumarate in *T. corallina*. The rate of decrease in the absorbance of NADPH at 340 nm was measured as reported in Materials and Methods. Symbols: ●, without fumarate; ○, with 10 mM fumarate. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

To determine if any TCA cycle intermediates directly inhibited erythrose reductase, in vitro assays were performed with activity staining (5) and either 10 mM citrate (Fig. 4B, lane 2), 10 mM α-ketoglutarate (lane 3), 10 mM succinate (lane 4), 10 mM malate (lane 5), 10 mM oxalacetate (lane 6), or 10 mM fumarate (lane 7). Lanes 2 to 6 demonstrated that samples with organic acids other than fumarate had full erythrose reductase activity. Fumarate (lane 7) was the only metabolite among tested organic acids that caused specific inhibition of erythrose reductase.

DISCUSSION

Many organisms produce small amounts of fumarate, malate, lactate, and other organic acids as metabolic by-products during oxidative metabolism. In some instances, certain fungi produce significant quantities of fumarate from glucose and carbon dioxide (8, 21, 25). Catabolite inhibition is a general phenomenon, even though the inhibitory effects vary widely from metabolite to metabolite. Interest in the inhibition of by-product production during the biosynthesis process as a way to minimize the effect of catabolite repression has been strong. During the culture of *T. corallina* for the production of erythritol, large amounts of a by-product were accumulated and instrumental analyses determined that it was fumarate. During erythritol fermentation, the levels of fumarate declined in Cu^{2+}-supplemented cultures compared to those in Cu^{2+}-deficient cultures.

It has been demonstrated that Cu^{2+} ions stimulate the synthesis of erythritol in *T. corallina* (20), and we propose that the stimulatory effect of Cu^{2+} on erythritol synthesis is mediated by the inhibition of fumarate, a strong inhibitor of erythrose reductase. Regulation by intracellular fumarate levels is not unique to this system. Alginate production in *Pseudomonas aeruginosa* (12) and phosphorylation-independent flagellar

motor switching in *Escherichia coli* (3, 23) are both regulated by intracellular fumarate levels.

Growth on TCA cycle intermediates has been reported to cause carbon catabolite repression and to repress the expression of many operons at the transcriptional level in *Pseudomonas* (7, 24, 34). McFall et al. (22) suggested that the growth and metabolism of some bacteria and yeasts were repressed during growth with TCA cycle intermediates and that this repression was found at the transcriptional level.

The mechanism by which fumarate inhibits erythrose reductase is unknown. To determine whether related compounds could play either an activating or a repressing role in the activity of erythrose reductase, many analogous organic acids, including the *cis* isomer of fumarate, maleic acid, were added to in vitro assay mixtures. None of these compounds modulated the activity of erythrose reductase. Fumarate has a *trans* double bond that is unique among the TCA cycle intermediates. Although this difference may allow fumarate to act as a key signaling molecule for the metabolic status of the cell, the mechanism by which fumarate inhibits erythrose reductase in vivo has still to be investigated.

In conclusion, supplementation with Cu^{2+} in cultures of *T. corallina* reduced the production of fumarate, a strong inhibitor of erythrose reductase. As a result, erythrose reductase activity became less inhibited and a high yield of erythritol was produced. To our knowledge this is the first report that proposes the catabolite repression of a key enzyme, erythrose reductase, in the biosynthesis of a sugar alcohol by a TCA cycle intermediate, fumarate.

ACKNOWLEDGMENT

This research was supported by a grant from the Korean Ministry of Science & Technology.

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