Alternative biotransformation of retinal to retinoic acid or retinol with cofactor switch by aldehyde dehydrogenase from *Bacillus cereus*

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ABSTRACT

A novel bacterial aldehyde dehydrogenase (ALDH) that converts retinal to retinoic acid has been first identified in *Bacillus cereus*. The amino acid sequence of ALDH from *B. cereus* (*Bc*ALDH) was more closely related to mammalian ALDHs than bacterial ALDHs. This enzyme converted not only small aldehydes to carboxylic acids but also the large aldehyde, all-*trans*-retinal to all-*trans*-retinoic acid with $NAD(P)^+$. We newly found that *Bc*ALDH and human ALDH (ALDH1A1) could reduce all-*trans*-retinal to all-*trans*-retinol with NADPH. The catalytic residues in *Bc*ALDH were Glu266 and Cys300, and the cofactor-binding residues were Glu194 and Glu457. The E266A and C300A variants showed no oxidation activity. The E194S and E457V variants showed 15- and 7.5-fold higher in the catalytic efficiency (k_{cat}/K_m) for the reduction of all-*trans*-retinal than the wild-type enzyme, respectively. The wild-type, E194S, and E457V variant enzymes with NAD^+ converted 400 μM all-*trans*-retinal to 210 μM all-*trans*-retinoic acid as the same amount for 240 min, while with NADPH they converted 400 μM all-*trans*-retinal to 20, 90, and 40 μM all-*trans*-retinol, respectively. These results indicate that *Bc*ALDH and its variants are efficient biocatalysts not only the conversion of retinal to retinoic acid but also the conversion to retinol with cofactor switch and that the retinol production could be increased by the variant enzymes. Therefore, *Bc*ALDH is a novel bacterial enzyme for the alternative production of retinoic acid and retinol.

Importance: Although mammalian ALDHs have catalyzed the conversion of retinal to retinoic acid with $NAD(P)^+$ as a cofactor, a bacterial ALDH involved in the conversion is first characterized. The biotransformation of all-*trans*-retinal to all-*trans*-retinoic acid by *Bc*ALDH and human ALDH was altered to that to all-*trans*-retinol by cofactor switch using NADPH. Moreover, the production of all-*trans*-retinal to all-*trans*-retinol was changed by mutations at 194 and 457 positions in *Bc*ALDH. The alternative biotransformation of retinoids was first performed in the present study. These results will contribute to the biotechnological production of retinoids, including retinoic acid and retinol.

INTRODUCTION

Retinoic acid can be formed in the body by two sequential oxidation steps, which convert retinol to retinaldehyde to retinoic acid. Retinol has been utilized in the manufacturing of food products, cosmetics, pharmaceuticals, nutraceuticals, and animal feed additives (8, 11, 31). Retinoic acid, a metabolite of vitamin A (retinol), is essential for a wide range of biological processes in mammals, including cell proliferation, differentiation, and morphogenesis (6, 29). Retinoic acid deficiency impairs vestibular functions (5, 30) and it is associated with Parkinson's disease (13). Retinoic acid is also one of the most important ingredients of skin care products in cosmetics because it is effective against UV radiation-induced damage on skin (2). Therefore, high demands for retinoic acid are arisen from the cosmetic and pharmaceutical industries. However, retinoic acid is commercially produced by only chemical synthesis. Biotechnological production of retinoic acid is limited yet because a selective production route is not developed.

In mammals, the biosynthesis of retinoic acid from retinol is occurred by two sequential enzyme reactions; Retinol is converted to retinal by the oxidation reaction of retinol dehydrogenase, and retinal is then converted to retinoic acid by the additional oxidation of retinal dehydrogenase (RALDH), which belongs to the aldehyde dehydrogenase (ALDH) family. ALDHs (enzyme classification (EC) number 1.2.1) have been well studied in mammalian for their biochemical properties and catalytic mechanisms (4, 9, 28). The oxidation of retinal to retinoic acid with $NAD(P)^+$ has been catalyzed by mammalian ALDHs (Fig. 1A), however, the reduction of retinal to retinol by these enzymes has not been reported (Fig. 1B) up to date. In spite of numerous studies on mammalian ALDHs, there are no reports of bacterial enzymes involved in the conversion of retinal to retinoic acid. Since the bacterial ALDHs showed diverse and versatile catalytic power for oxidation, we searched bacterial homologs of human ALDH (ALDH1A1) involved in the oxidation of retinal to retinoic acid. Among bacterial ALDHs, ALDH from *Bacillus cereus* (*Bc*ALDH) was selected because the enzyme exhibited the highest sequence identity with human ALDH.

In this study, the mammalian-like *aldh* gene from *B. cereus* was cloned and its purified enzyme was characterized. Mutation for the conserved catalytic and cofactor-binding residues involved in the

oxidation and reduction with cofactor selectivity of *Bc*ALDH was performed, and the variants with increased activity for oxidation and reduction were obtained. The alternative biotransformation of all-*trans*-retinal to all-*trans*-retinol or all-*trans*-retinoic acid by the wild-type and variant enzymes was first attempted with cofactor switch.

MATERIALS AND METHODS

Gene cloning and site-directed mutagenesis. The bcere0002_25940 gene (1,485 bp) encoding an ALDH from *B. cereus* and the *aldh1a1* gene (1,506 bp) encoding an ALDH1A1 from human were amplified by PCR using the genomic DNA isolated from *B. cereus* and human cDNA as a template respectively. The primer sequences used for gene cloning were based on the DNA sequence of *Bc*ALDH (GenBank accession No. ACLT01000061.1), and forward (5′-GGATCCGATGTTAAAGACAAATATTGAGCTG-3′) and reverse primers (5′-GCGGCCGCTTACTTTATTACTTTATATTTACCCAAACA-3′) were designed to introduce the underlined BamHI and NotI restriction sites, respectively. The sequences of the oligonucleotide primers used for gene cloning were based on the cDNA sequence of human ALDH (GenBank accession No. AAP35567.1), and forward (5′- GTCGACATGTCATCCTCAGGCACGCCA-3′) and reverse primers (5′-CTCGAGTGAGTTCTTCTGAGAGATTTTCAC-3′) were designed to introduce the underlined SalI and XhoI restriction sites, respectively. The amplified DNA fragments were purified using a QIAquik gel extraction kit (Qiagen, Germany), and the purified DNA fragments were ligated into the pRSFDuet-1 vector (Novagen, Diego, CA) digested with the same restriction enzymes. The resulting plasmid was transformed into *Escherichia coli* ER2566 (New England Biolabs, Hertfordshire, UK). Site-directed mutagenesis was performed using a QuikChange kit (Stratagene, La Jolla, CA).

Protein purification and molecular mass determination. *Bc*ALDH protein and human ALDH was expressed and purified as described by Ngo *et al* (27) and Morgan *et al* (25), respectively. The subunit molecular mass of *Bc*ALDH was determined by SDS-PAGE under denaturing conditions, using prestained protein makers as reference proteins. The molecular mass of the native enzyme was

determined using Sephacryl S-300 high resolution gel filtration chromatography (GE Healthcare, Pittsburgh, PA). The purified enzyme solution was applied to the gel filtration column and eluted at a flow rate of 0.3 ml min^{-1} with 50 mM PIPES buffer (pH 7.0) containing 150 mM NaCl. The column was calibrated using thyroglobulin (689 kDa), ferritin (453 kDa), aldolase (158 kDa), and conalbumin (75 kDa) as reference proteins. The mass of the native enzyme was calculated by comparing the migration time of the enzyme with those of the reference proteins.

Enzyme assay. Unless otherwise stated, the enzymatic reaction of *Bc*ALDH for the conversion of all-*trans*-retinal to all-*trans*-retinoic acid or all-*trans*-retinol was performed at 37 °C in 50 mM PIPES buffer (pH 7.0) or at 40 °C in 50 mM HEPPS buffer (pH 7.5), respectively, containing 0.2 mM all-*trans*-retinal, 0.4 unit ml^{-1} (1 mg ml^{-1}) enzyme, 5% (v/v) methanol, 4 mM 2-mercaptoethanol (2-ME), and 1 mM NAD^+ or NADPH, respectively, for 30 min. Methanol at 5% (v/v) was used to solubilize all-*trans*-retinal (12). One unit was defined as the amount of enzyme required to produce 1 pmol of all-*trans*-retinoic acid or all-*trans*-retinol per min from all-*trans*-retinal at 37 °C and pH 7.0 or at 40 °C and pH 7.5, respectively, for *Bc*ALDH activity and at 25 °C and pH 7.0 for human ALDH. The K_m (μM) and k_{cat} (min^{-1}) were determined by the Lineweaver-Burk plot derived from the Michaelis-Menten equation. To determine the kinetic parameters for aldehyde substrates, the concentrations of retinoids were in the range of 2 to 40 μM and those of acetaldehyde and benzaldehyde were in the range of 20 to 1,000 μM with 1 mM NAD^+. To determine the kinetic parameters for cofactors, the concentrations of cofactors were in the range of 1 to 200 μM with 200 μM all-*trans*-retinal.

Conversion of all-*trans*-retinal into all- *trans*-retinoic acid or all-*trans*-retinol. The conversion of all-*trans*-retinal to all-*trans*-retinoic acid or all-*trans*-retinol by the wild-type and variant enzymes of *Bc*ALDH was performed under the same conditions of enzyme assay except for 0.4 mM all-*trans*-retinal, 4 mM NAD^+ or NADPH for 240 min, respectively. The conversion by human ALDH was performed in 50 mM EPPS buffer (pH 8.0) containing 0.4 mM all-*trans*-retinal, 0.4 unit/ml enzyme, 1 mM dithiothreitol (DTT), and 4 mM NAD^+ or NADPH at 25°C for 80 min.

Analytical methods. The same volume of methanol was added to the reaction solution, and the resulting solution was mixed with hexane and kept on ice for 5 min. After centrifugation at 10,000 × *g*

for 30 min at 4°C, substrates and products in the supernatant were analyzed by an HPLC system (Agilent 1200, Santa Clara, CA) equipped with an Atlantis dC18 column (4.6 × 150 mm, Waters, Milford, MA) and a UV detector at 350 nm for retinal and retinoic acid, at 210 nm for acetaldehyde and acetic acid, and at 250 nm for benzaldehyde and benzoic acid. The column was eluted with a mixture of acetonitrile and 1% ammonium acetate in water with 80:20 (v/v) as the mobile phase at a flow rate of 1.2 ml min^{-1} at 30°C.

RESULTS

Sequence alignment of ALDHs. The amino acid sequence of *Bc*ALDH was compared with those of mammalian and bacterial ALDHs using ClustalX2 (21). The proposed catalytic residues Glu266 and Cys300, were completely conserved for all of the ALDHs. The Glu194, Glu246, Ser247, and Glu457 were conserved for all of the ALDHs among the proposed cofactor-binding residues Glu194, Gly246−Lys252, and Glu457 (32) (Fig. 2A). The amino acid sequence of *Bc*ALDH had 54.2, 54.0, 51.1, 50.9, 49.9, 49.7, 48.9, and 48.6 % identity with ALDHs from eukaryotes, including orangutan, *Aspergillus nidulans*, human, sheep, monkey, bovine, rat, and mouse, respectively, and had 42.2, 41.7, 41.6, 41.2, 40.4, and 22.1 % identity with ALDHs from bacteria, including *Cronobacter sakazakii*, *E. coli*, *Shigella flexneri*, *Serratia proteamaculans*, *Klebsiella pneumoniae*, and *Crocosphaera watsonii* respectively. A phylogenic tree was constructed based on the genetic characteristics using CLUSTALW and MEGA6 (33), and *Bc*ALDH showed more closed relation to mammalian ALDHs than bacterial ALDHs in the phylogenic tree (Fig. 2B).

Molecular mass of *Bc*ALDH and identification of all-*trans*-retinoic acid. The purified *Bc*ALDH showed a single band in SDS-PAGE with a molecular mass of approximately 54 kDa (Fig. S1A), which is consistent with the calculated value of 54 kDa based on the 494 amino acids plus the hexa-histidine tag. The native protein existed as a tetramer with a molecular mass of 216 kDa (Fig. S1B), as determined by gel filtration chromatography based on the masses of the reference proteins. LC-MS analysis was performed to identify the product obtained from all-*trans*-retinal by the reaction of *Bc*ALDH (Fig. S6A). The total molecular mass of the product was shown as a peak at *m/z* 301.19,

corresponding to the exactly same molecular mass of the all-*trans*-retinoic acid standard (Fig. S6B) (23), indicating that the peak was all-*trans*-retinoic acid.

Effects of metal ions, temperature, pH, and antioxidants on the *Bc*ALDH activity. The effect of metal ions on the activity of *Bc*ALDH was evaluated in the presence of metal ions at 1 mM. The oxidation activity of *Bc*ALDH that produces all-*trans*-retinoic acid (Fig. S2A) and reduction activity (Fig. S3A) that produces all-*trans*-retinol from all-*trans*-retinal were not activated by divalent cations. The maximum activity was observed at 37°C and pH 7.0 for oxidation (Fig. S2B and S2C) and 40°C and pH 7.5 for reduction (Fig. S3B and S3C). The effect of antioxidants to prevent the disruption of retinal and to restore the catalytic residue cysteine (7, 26), such as butylated hydroxytoluene (BHT), DTT, hydroquinone (HQ), and 2-ME on the activity of *Bc*ALDH was investigated. Enzyme activity both for the oxidation and reduction was the highest when 2-ME was used as the antioxidant (Fig. S4A), and its optimal concentration was 4 mM (Fig. S4B). Thus, 4 mM 2-ME was used for the enzymatic reactions. Cysteine residue in ALDH with a thiol group as a functional group has been involved in the catalytic activity. The addition of DTT or 2-ME reduces the thiol group of cysteine to a thiolate group by changing the antioxidant to the oxidized form (7, 26). 2-ME has a reducing power and increased enzyme solubility, which may result in the increased activity for ALDH by 2-ME.

Substrate- and cofactor-specificity of *Bc*ALDH. The oxidation activity for aldehyde substrates was measured in the presence of 1 mM NAD^+. The specific activity followed the order benzaldehyde, acetaldehyde, and all-*trans*-retinal, and no activity was observed for 9-*cis*-retinal and 13-*cis*-retinal (Table 1). All-*trans*-retinal showed the lowest K_m, which was 14- and 29-fold lower than the K_m values of acetaldehyde and benzaldehyde, respectively. The kinetic parameters of *Bc*ALDH with cofactor were determined using all-*trans*-retinal (Table 2). All-*trans*-retinal was oxidized to all-*trans*-retinoic acid with $NADP^+$ as well as NAD^+. The K_m and k_{cat} of NAD^+ for the oxidation of all-*trans*-retinal were similar to those of $NADP^+$. *Bc*ALDH reduced all-*trans*-retinal to all-*trans*-retinol with NADPH, although the reduction activity was much lower than the typical oxidation activity. However, the reduction activity for all-*trans*-retinal was not detected with NADH. For other substrates, including benzaldehyde, acetaldehyde, 9-*cis*-retinal, and 13-*cis*-retinal, the atypical reduction activity

of *Bc*ALDH was not found with both of NADH and NADPH.

The production of all-*trans*-retinoic acid and all-*trans*-retinol was investigated by supplementing NADH to NAD^+ or $NADP^+$ to NADPH at the ratios ranging from 0:4 to 4:0 mM, to make up a total concentration of 4 mM. All-*trans*-retinoic acid increased as increasing the ratio of NAD^+ to NADH. However, all-*trans*-retinoic acid decreased and all-*trans*-retinol increased as increasing the ratio of NADPH to $NADP^+$ (Fig. S7A and S7B). When NADH was added to 4 mM NAD^+, all-*trans*-retinoic acid decreased as increasing the concentration of additional NADH (Fig. S7C). However, all-*trans*-retinoic acid increased and all-*trans*-retinol decreased as increasing the concentration of additional $NADP^+$ supplemented with 4 mM NADPH (Fig. S7D). Thus, all-*trans*-retinoic acid production is stimulated by decreasing the ratio of NADPH to $NADP^+$ whereas all-*trans*-retinol production is stimulated by increasing the ratio.

The effect of cofactor concentration on the conversion of all-*trans*-retinoic acid or all-*trans*-retinol from all-*trans*-retinal to by *Bc*ALDH was performed by varying the concentration of NAD^+ or NADPH, respectively (Fig. 3). The conversion of all-*trans*-retinal to all-*trans*-retinoic acid or all-*trans*-retinol was increased up to approximately 5 mM cofactor. However, above this concentration, the formation of products did not increase. Thus, the optimal concentration for the conversion of all-*trans*-retinal to all-*trans*-retinoic acid or all-*trans*-retinol was 5 mM.

Catalytic and cofactor-binding residues of *Bc*ALDH involved in the oxidation and reduction of all-*trans*-retinal. Site-directed mutagenesis for the catalytic and cofactor-binding residues involved in the oxidation and reduction of all-*trans*-retinal was performed, and kinetic parameters of the wild-type and variant enzymes were determined (Table 3). The E266A and C300A variants for the catalytic residues abolished the oxidation activity, and the catalytic efficiency (k_{cat}/K_m) of the C300S variant for the oxidation of all-*trans*-retinal with NAD^+ was 23.5% of that of the wild-type enzyme. The catalytic efficiencies of the E194S and E457V variants for the cofactor-binding residues for the reduction of all-*trans*-retinal with NADPH were 15- and 6.2-fold higher than that of the wild-type enzyme, respectively. The catalytic efficiency of the E194S variant for the oxidation with NAD^+ was 1.3-fold lower than that of the wild-type enzyme, whereas the catalytic efficiency of the E457V variant for the

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oxidation was 7.5-fold higher.

Conversion of all-*trans*-retinal to all-*trans*-retinoic acid or all-*trans*-retinol by the wild-type, E194S, and E457V variant enzymes of *Bc*ALDH with cofactor switch. Time-course reactions for the production of all-*trans*-retinoic acid or all-*trans*-retinol were performed from 400 μM all-*trans*-retinal in the presence of 5 mM NAD^+ using the wild-type, E194S, and E457V variant enzymes of *Bc*ALDH. After 240 min, the concentrations of all-*trans*-retinoic acid produced from 400 μM all-*trans*-retinal by the wild-type E194S and E457V variant enzymes were the same as 210 μM, with a molar conversion yield of 53% (Fig. 4A). The concentrations of all-*trans*-retinol produced from 400 μM all-*trans*-retinal by the wild-type, E194S, and E457V variant enzymes were 20, 90, and 40 μM, respectively, with molar conversions yield of 5, 24, and 11%, respectively (Fig. 4B). Glu194 of *Bc*ALDH was corresponded to Glu196 of human ALDH. The production of all-*trans*-retinoic acid or all-*trans*-retinol from all-*trans*-retinal by the wild-type and E196S variant enzymes of human ALDH, the production of all-*trans*-retinoic acid or all-*trans*-retinol was performed. After 80 min, the produced concentration of all-*trans*-retinoic acid by the wild-type enzyme was almost the same (5 μM) with that by the E196S variant (Fig. S5A). However, the concentration of all-*trans*-retinol produced by the E196S variant (13 μM) was 4.6-fold higher than that by the wild-type enzyme (3 μM) (Fig. S5B). Therefore, the effect of the E194S variant of *Bc*ALDH on the production of all-*trans*-retinol or all-*trans*-retinoic acid was the similar to that of the E196S variant of human ALDH.

DISCUSSION

ALDHs catalyze the oxidation of aldehydes to carboxylic acids, which are given from EC 1.2.1.1 to EC 1.2.1.97. EC 1.2.1.1 and EC 1.2.1.2 are *S*-(hydroxymethyl)glutathione dehydrogenase and formate dehydrogenase respectively. EC 1.2.1.3, EC 1.2.1.4, and EC 1.2.1.5 are depending on NAD^+, $NADP^+$, and $NAD(P)^+$, respectively. Other fourth digits are specified by preferred aldehydes. Among them, RALDH (EC 1.2.1.36) catalyzes the oxidation of retinal to retinoic acid. *Bc*ALDH was identified as EC 1.2.1.5 (3) because it converted aldehydes to carboxylic acids using both NAD^+ and $NADP^+$. *Bc*ALDH also exhibited RALDH activity with a high sequence identity to RALDH. Although retinol

dehydrogenase (EC 1.1.1.300) catalyzes the conversion of retinal to retinol using NAD(P)H, the enzyme is belonged to other subclass groups (EC 1.1) as alcohol dehydrogenases.

Retinoic acid has been synthesized by the oxidation activity of mammalian ALDHs from retinal (Fig. 1A). Retinoic acid plays a key role in embryonic development and the formation of retinoic acid is generally considered as a mammalian-specific reaction (14). Bacterial ALDHs are known for enzymes that transform small aldehydes such as acetaldehyde and benzaldehyde to their corresponding carboxylic acids for detoxification (16). Thus far, no evidence for the formation retinoic acid by bacterial ALDHs has been provided. To find a bacterial ALDH that converts retinal to retinoic acid, the sequences of bacterial ALDHs were aligned with those of mammalian ALDHs showing all-*trans*-retinal conversion (Fig. 2A), and a phylogenic tree with bacterial and mammalian ALDHs was constructed (Fig. 2B). As a result, human ALDH1 and *B. cereus* ALDHs were grouped in the same branch of ALDH1 family in the phylogenic tree. ALDH1 family has been known to involve in the conversion of the large aldehyde, all-*trans*-retinal to all-*trans*-retinoic acid (32), suggesting that *Bc*ALDH can transform retinal to retinoic acid. This suggestion was verified by determining the converting activity of *Bc*ALDH for retinal. Therefore, this enzyme is the first retinoic acid-producing enzyme among bacterial ALDHs.

Glu266, Cys300, Glu194, and Glu457 of *Bc*ALDH were selected based on sequence alignment with mammalian ALDHs (Fig. 2A) because the Glu266 and Cys300 residues have been known as catalytic residues for oxidation activity for all-*trans*-retinal (10, 34) and the Glu194 and Glu457 residues were the cofactor-binding residues in mammalian ALDHs (1, 24, 27, 34). The E266A and C300A variants of *Bc*ALDH completely lost the oxidative activity (Table 3). ALDHs favor NADPH if the residue at 194 position has a short polar side chain like Ser or Thr and prefer NAD^+ if the residue at 194 position has a longer side chain like Glu (35). The substitution of smaller residue at 194 position could help the reduction of *Bc*ALDH by the higher usage of NADPH. Thus, the reduction activity of the E194S variant with NADPH was significantly increased, compared to that of the wild-type enzyme (Table 3). The acidic residue of Glu at 457 position of *Bc*ALDH is uncommon in mammalian ALDHs, which have the hydrophobic residue Val or Leu at the corresponding position

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(Fig. 2A). The residue at 457 position is located in the substrate binding channel for the long hydrophobic chain of all-*trans*-retinal (32). The activity of the E457V variant increased in both oxidation and reduction of all-*trans*-retinal (Table 3). Thus, we speculate that the increased hydrophobicity via the substitution of Glu to Val at 457 position may facilitate the substrate binding.

Mammalian-like *Bc*ALDH catalyzes not only the oxidation of all-*trans*-retinal to all-*trans*-retinoic acid but also the reduction of all-*trans*-retinal to all-*trans*-retinol with cofactor switch. Although the production of all-*trans*-retinoic acid by the E457V and E194S variants of *Bc*ALDH was the same as that by the wild-type enzyme (Fig. 4A), the production of all-*trans*-retinol by the E457V and E194S variants was higher (Fig. 4B). The results imply that we may engineer *Bc*ALDH more suitable for the specific production of all-*trans*-retinol. All-*trans*-retinal has been produced from *β*-carotene by *β*-carotene 15,15′-oxygenases (18-20) and from glycerol by metabolically engineered *E. coli* expressing carotenoid synthesizing enzymes and carotenoid oxygenase (15, 22). However, the biotechnological production of retinoic acid from retinal has not been attempted yet because convincing metabolic pathway or specific enzyme related to retinoic acid production in prokaryotes has not been identified. We first found *Bc*ALDH as an effective enzyme for the conversion of retinal to retinoic acid. *Bc*ALDH can be used with carotenoid oxygenase to the production of all-*trans*-retinoic acid from *β*-carotene by enzymatic reactions or whole cell production with cofactor regeneration system. Its gene can be introduced into all-*trans*-retinal-producing metabolic engineered cells for the production of all-*trans*-retinoic acid from relevant substrate such as glucose or glycerol for the cost effective industrial production.

In summary, *Bc*ALDH, which was found as a mammalian-like ALDH in the phylogenic tree with mammalian and bacterial ALDHs, was first identified as a bacterial retinoic acid-producing enzyme from retinal. We newly found that *Bc*ALDH and human ALDH also could reduce retinal to retinol with cofactor switch. The reduction activity of *Bc*ALDH was increased by its E457V and E194S variants. The wild-type *Bc*ALDH and its variants can be used for the alternative biotransformation of retinal to retinoic acid or retinol and the genes can be introduced in retinal-producing metabolically engineered cells for retinoic acid or retinol production. Our work is an initiative for the

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biotechnological production of retinoids, including retinoic acid and retinol, using a specific bacterial enzyme. Our approach can also contribute to find a novel biocatalyst that has not been discovered yet from prokaryotic origins based on the information of eukaryotic biocatalysts.

The authors declare no conflict of interest

296 **REFERENCES**

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298 1. **Abriola, D. P., R. Fields, S. Stein, A. D. MacKerell, Jr., and R. Pietruszko.** 1987. Active
299 site of human liver aldehyde dehydrogenase. Biochemistry **26:**5679-84.

300 2. **Ascenso, A., H. Ribeiro, H. C. Marques, H. Oliveira, C. Santos, and S. Simoes.** 2014. Is
301 tretinoin still a key agent for photoaging management? Mini Rev Med Chem **14:**629-41.

302 3. **Barthelmes, J., C. Ebeling, A. Chang, I. Schomburg, and D. Schomburg.** 2007. BRENDA,
303 AMENDA and FRENDA: the enzyme information system in 2007. Nucleic Acids Res
304 **35:**D511-4.

305 4. **Bhat, P. V., L. Poissant, and X. L. Wang.** 1996. Purification and partial characterization of
306 bovine kidney aldehyde dehydrogenase able to oxidize retinal to retinoic acid. Biochem Cell
307 Biol **74:**695-700.

308 5. **Chanda, B., A. Ditadi, N. N. Iscove, and G. Keller.** 2013. Retinoic acid signaling is
309 essential for embryonic hematopoietic stem cell development. Cell **155:**215-27.

310 6. **Chytil, F., and H. Riaz ul.** 1990. Vitamin A mediated gene expression. Crit Rev Eukaryot
311 Gene Expr **1:**61-73.

312 7. **Daiber, A., M. Oelze, M. Coldewey, M. Bachschmid, P. Wenzel, K. Sydow, M. Wendt, A.**
313 **L. Kleschyov, D. Stalleicken, V. Ullrich, A. Mulsch, and T. Munzel.** 2004. Oxidative stress
314 and mitochondrial aldehyde dehydrogenase activity: a comparison of pentaerythritol
315 tetranitrate with other organic nitrates. Mol Pharmacol **66:**1372-82.

316 8. **De Luca, L. M.** 1991. Retinoids and their receptors in differentiation, embryogenesis, and
317 neoplasia. FASEB J **5:**2924-33.

318 9. **Emsley, P., B. Lohkamp, W. G. Scott, and K. Cowtan.** 2010. Features and development of
319 Coot. Acta Crystallogr D Biol Crystallogr **66:**486-501.

320 10. **Farres, J., T. T. Wang, S. J. Cunningham, and H. Weiner.** 1995. Investigation of the active
321 site cysteine residue of rat liver mitochondrial aldehyde dehydrogenase by site-directed
322 mutagenesis. Biochemistry **34:**2592-8.

11. **Fisher, G. J., and J. J. Voorhees.** 1996. Molecular mechanisms of retinoid actions in skin. FASEB J **10:**1002-13.

12. **Hong, S. H., H. K. Nam, K. R. Kim, S. W. Kim, and D. K. Oh.** 2013. Molecular characterization of an aldo-keto reductase from *Marivirga tractuosa* that converts retinal to retinol. J Biotechnol **169:**23-33.

13. **Jacobs, F. M., S. M. Smits, C. W. Noorlander, L. von Oerthel, A. J. van der Linden, J. P. Burbach, and M. P. Smidt.** 2007. Retinoic acid counteracts developmental defects in the substantia nigra caused by Pitx3 deficiency. Development **134:**2673-84.

14. **Janesick, A., S. C. Wu, and B. Blumberg.** 2015. Retinoic acid signaling and neuronal differentiation. Cell Mol Life Sci **72:**1559-76.

15. **Jang, H. J., B. K. Ha, J. Zhou, J. Ahn, S. H. Yoon, and S. W. Kim.** 2015. Selective retinol production by modulating the composition of retinoids from metabolically engineered *E. coli*. Biotechnol Bioeng **112:**1604-12.

16. **Jo, J. E., S. Mohan Raj, C. Rathnasingh, E. Selvakumar, W. C. Jung, and S. Park.** 2008. Cloning, expression, and characterization of an aldehyde dehydrogenase from *Escherichia coli* K-12 that utilizes 3-hydroxypropionaldehyde as a substrate. Appl Microbiol Biotechnol **81:**51-60.

17. **Jones, J. H., J. E. Lennard-Jones, B. C. Morson, M. Chapman, M. J. Sackin, P. H. Sneath, C. C. Spicer, and W. I. Card.** 1973. Numerical taxonomy and discriminant analysis applied to non-specific colitis. Q J Med **42:**715-32.

18. **Kim, N. H., Y. S. Kim, H. J. Kim, and D. K. Oh.** 2008. Optimized formation of detergent micelles of beta-carotene and retinal production using recombinant human beta,beta-carotene 15,15'-monooxygenase. Biotechnol Prog **24:**227-31.

19. **Kim, Y. S., N. H. Kim, H. J. Kim, J. K. Lee, S. W. Kim, and D. K. Oh.** 2007. Effective production of retinal from beta-carotene using recombinant mouse beta-carotene 15,15'-monooxygenase. Appl Microbiol Biotechnol **76:**1339-45.

20. **Kim, Y. S., C. S. Park, and D. K. Oh.** 2010. Retinal production from beta-carotene by beta-

carotene 15,15'-dioxygenase from an unculturable marine bacterium. Biotechnol Lett **32:**957-961.

21. **Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins.** 2007. Clustal W and Clustal X version 2.0. Bioinformatics **23:**2947-8.

22. **Lee, J. H., J. G. Choi, Y. S. Kim, K. R. Kim, S. W. Kim, and D. K. Oh.** 2012. Enhancement of retinal production by supplementing the surfactant Span 80 using metabolically engineered *Escherichia coli*. J Biosci Bioeng **113:**461-6.

23. **Lee, S., D. G. Oh, G. R. Kim, J. S. Lee, Y. K. Son, C. H. Bae, J. Yeo, and C. H. Lee.** 2015. Chemotaxonomic Metabolite Profiling of 62 Indigenous Plant Species and Its Correlation with Bioactivities. Molecules **20:**19719-34.

24. **Moore, S. A., H. M. Baker, T. J. Blythe, K. E. Kitson, T. M. Kitson, and E. N. Baker.** 1998. Sheep liver cytosolic aldehyde dehydrogenase: the structure reveals the basis for the retinal specificity of class 1 aldehyde dehydrogenases. Structure **6:**1541-51.

25. **Morgan, C. A., and T. D. Hurley.** 2015. Characterization of two distinct structural classes of selective aldehyde dehydrogenase 1A1 inhibitors. J Med Chem **58:**1964-75.

26. **Mukerjee, N., and R. Pietruszko.** 1994. Inactivation of human aldehyde dehydrogenase by isosorbide dinitrate. J Biol Chem **269:**21664-9.

27. **Ngo, H. P., S. H. Hong, D. K. Oh, and L. W. Kang.** 2013. Expression, crystallization and preliminary X-ray crystallographic analysis of aldehyde dehydrogenase (ALDH) from Bacillus cereus. Acta Crystallogr Sect F Struct Biol Cryst Commun **69:**528-31.

28. **Penzes, P., X. Wang, and J. L. Napoli.** 1997. Enzymatic characteristics of retinal dehydrogenase type I expressed in *Escherichia coli*. Biochim Biophys Acta **1342:**175-81.

29. **Raifen, R., Y. Altman, and Z. Zadik.** 1996. Vitamin A levels and growth hormone axis. Horm Res **46:**279-81.

30. **Romand, R., W. Krezel, M. Beraneck, L. Cammas, V. Fraulob, N. Messaddeq, P. Kessler, E. Hashino, and P. Dolle.** 2013. Retinoic acid deficiency impairs the vestibular function. J

Neurosci **33:**5856-66.

31. **Semba, R. D.** 1999. Vitamin A as "anti-infective" therapy, 1920-1940. J Nutr **129:**783-791.

32. **Sobreira, T. J., F. Marletaz, M. Simoes-Costa, D. Schechtman, A. C. Pereira, F. Brunet, S. Sweeney, A. Pani, J. Aronowicz, C. J. Lowe, B. Davidson, V. Laudet, M. Bronner, P. S. de Oliveira, M. Schubert, and J. Xavier-Neto.** 2010. Structural shifts of aldehyde dehydrogenase enzymes were instrumental for the early evolution of retinoid-dependent axial patterning in metazoans. Proc Natl Acad Sci U S A **108:**226-31.

33. **Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar.** 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol **30:**2725-9.

34. **Wang, X., and H. Weiner.** 1995. Involvement of glutamate 268 in the active site of human liver mitochondrial (class 2) aldehyde dehydrogenase as probed by site-directed mutagenesis. Biochemistry 34:237-43.

35. Yuan, Z., B. Yin, D. Wei, and Y. R. Yuan. 2013. Structural basis for cofactor and substrate selection by cyanobacterium succinic semialdehyde dehydrogenase. J Struct Biol 182:125-35.

393 **Figure Legends**

394

395 **FIG 1** Schematic diagrams of reactions catalyzed by aldehyde dehydrogenase. (A) Oxidation reaction
396 of all-*trans*-retinal to all-*trans*-retinoic acid reported by mammalian ALDH. (B) Oxidation reaction of
397 all-*trans*-retinal to all-*trans*-retinoic acid as well as reduction reaction of all-*trans*-retinal to all-*trans*-
398 retinol identified by *Bc*ALDH and human ALDH in this study.

399

400 **FIG 2** (A) Sequence alignment of bacterial and mammalian ALDHs. The amino acid sequences of
401 ALDHs from *B. cereus*, *E. coli*, *Shigella flexneri*, *Klebsiella pneumonia*, *Cronobacter sakazakii*,
402 *Serratia proteamaculans*, *Crocosphaera watsonii*, bovin ALDH1A1, sheep ALDH, human ALDH1A1,
403 mouse ALDH1A1, rat ALDH1A1, human ALDH1A2, human ALDH1A3, and human ALDH8A1 are
404 aligned. The GenBank accession numbers of these ALDHs are KFL74159.1, BAA14869.1,
405 ABF03518.1, ABR76453.1, ABU77332.1, ABV41168.1, CCQ55365.1, AAI05194.1, AAA85435.1,
406 AAP35567.1, AAH54386.1, AAC53305.1, BAG54714.1, AAH69274.1, and BAD96568.1,
407 respectively. The proposed catalytic and active-site residues are highlighted with black box. Cofactor
408 binding residues are presented with white letters in black backgrounds. Asterisk, colon, and dot
409 present identity, conserved substitutions, and semi-conserved substitution, respectively. (B)
410 Phylogenetic tree of mammalian and bacterial ALDHs. The evolutionary history was inferred using
411 the UPGMA method (17). The optimal tree with the sum of branch length = 3.73188906 is shown.
412 The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the
413 evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed
414 using the Poisson correction method and are in the units of the number of amino acid substitutions per
415 site. The analysis involved 18 amino acid sequences. All positions containing gaps and missing data
416 were eliminated. There were a total of 421 positions in the final dataset. Evolutionary analyses were
417 conducted in MEGA6 (33).

418

419 **FIG 3** Effect of cofactor concentration on the conversion of all-*trans*-retinal to all-*trans*-retinoic acid

or all-*trans*-retinol. The cofactor concentrations used for investigating the effect were significant higher than K_m values. The conversion of all-*trans*-retinal to all-*trans*-retinoic acid using NAD^+ (●) or $NADP^+$ (□) was performed in 50 mM PIPES buffer (pH 7.0) containing 0.4 mM all-*trans*-retinal, 0.4 unit/ml enzyme, and 1 mM 2-ME at 37 °C for 30 min. The conversion of all-*trans*-retinal to all-*trans*-retinol using NADPH (■) was performed in 50 mM EPPS buffer (pH 7.5) containing 0.4 mM all-*trans*-retinal, 0.4 unit/ml enzyme, and 1 mM 2-ME at 40 °C for 30 min. Data represent the means of three experiments and error bars represent standard deviation.

FIG 4 Biotransformation of all-*trans*-retinal to retinoic acid or retinol by the wild-type, E194S, and E457V variant enzymes of *Bc*ALDH with cofactor switch. (A) Time course reactions for the conversion of all-*trans*-retinal to all-*trans*-retinoic acid by the wild-type (●), E194S (○), and E457V (▲) variant enzymes of *Bc*ALDH using NAD^+. The reactions were performed in 50 mM PIPES buffer (pH 7.0) containing 0.4 mM all-*trans*-retinal, 0.4 unit/ml enzyme, 4 mM 2-ME, and 5 mM NAD^+ at 37°C for 240 min. (B) Time course reactions for the conversion of all-*trans*-retinal to all-*trans*-retinol by the wild-type (●), E194S (○), and E457V (▲) variant enzymes of *Bc*ALDH using NADPH. The reactions were performed in 50 mM EPPS buffer (pH 7.5) containing 0.4 mM all-*trans*-retinal, 0.4 unit/ml enzyme, 4 mM 2-ME, and 5 mM NADPH at 40°C for 240 min.

TABLE 1 Kinetic parameters for aldehyde substrates of *Bc*ALDH in the oxidation activity using NAD^+ as a co-substrate[a]

Substrate	Co-substrate (mM)	Product	Specific activity (U/mg)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
All-*trans*-retinal	NAD^+ (1)	All-*trans*-retinoic acid	461±10	7±0.1	0.28±0.03	40±0.2
9-*cis*-Retinal	NAD^+ (1)	9-*cis*-Retinoic acid	NC[b]	NC	NC	NC
13-*cis*-Retinal	NAD^+ (1)	13-*cis*-Retinoic acid	NC	ND[c]	ND	ND
Acetaldehyde	NAD^+ (1)	Acetic acid	2,550±10	95±2	11±0.4	111±5
Benzaldehyde	NAD^+ (1)	Benzoic acid	3,370±16	200±5	13±0.8	65±4

[a] Data represent the mean of three experiments and ± values represent standard deviations.

[b] NC, not calculated by the analytical methods used in this study due to limits of sensitivity.

[b] ND, not detected by the analytical methods used in this study.

TABLE 2 Kinetic parameters for cofactors of *Bc*ALDH in the oxidation and reduction reactions using all-*trans*-retinal as a cosubstrate[a]

Substrate	Co-substrate (mM)	Reaction	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
NAD^+	All-*trans*-retinal (0.2)	Oxidation	4.0±0.2	0.10±0.01	25±0.1
$NADP^+$	All-*trans*-retinal (0.2)	Oxidation	4.2±0.3	0.11±0.02	26±0.2
NADPH	All-*trans*-retinal (0.2)	Reduction	40±1.0	0.033±0.00	0.83±0.05
NADH	All-*trans*-retinal (0.2)	Reduction	ND[b]	ND	ND

[a] Data represent the mean of three experiments and ± values represent standard deviations.

[b] ND, not detected by the analytical methods used in this study.

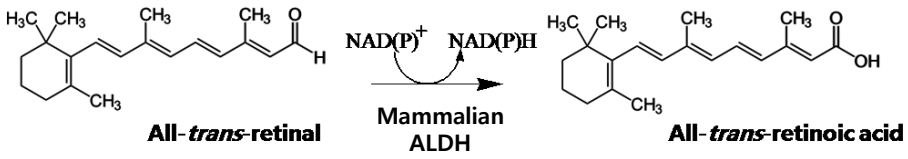
TABLE 3 Kinetic parameters of the wild-type and variant enzymes of *Bc*ALDH related to catalytic and cofactor-binding residues for all-*trans*-retinal in the oxidation and reduction activities [a]

Enzyme	Cofactor	Specific activity (U mg^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
Wild-type	NAD^+	461±10	7.0±0.13	0.28±0.03	40±0.2
	NADPH	136±6	130±5	0.12±0.01	0.90±0.08
E194S	NAD^+	493±3	10±0.5	0.30±0.01	30±0.1
	NADPH	408±11	29±0.9	0.39±0.01	14±0.1
E266A	NAD^+	ND[b]	ND	ND	ND
	NADPH	80±2.0	920±5	0.07±0.003	0.07±0.005
C300A	NAD^+	ND	ND	ND	ND
	NADPH	68±2.0	38±2.0	0.04±0.003	1.1±0.05
C300S	NAD^+	38.6±2	3.70±0.05	0.04±0.002	9.4±0.05
	NADPH	12±0.5	420±3	0.04±0.002	0.08±0.01
E457V	NAD^+	2,536±11	6.25±0.5	1.54±0.1	246±6
	NADPH	260±2	34±0.5	0.23±0.02	6.76±0.2

[a] Data represent the mean of three experiments and ± values represent standard deviations.

[b] ND, not detected by the analytical methods used in this study.

A



B

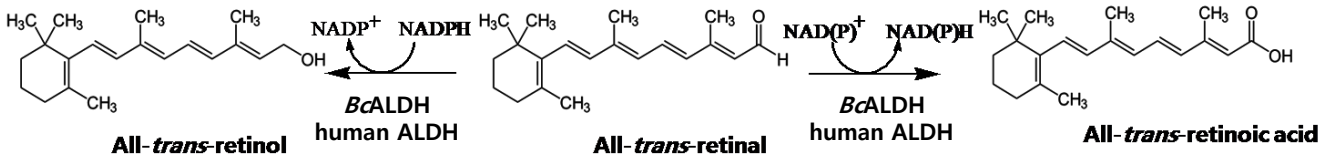


FIG 1

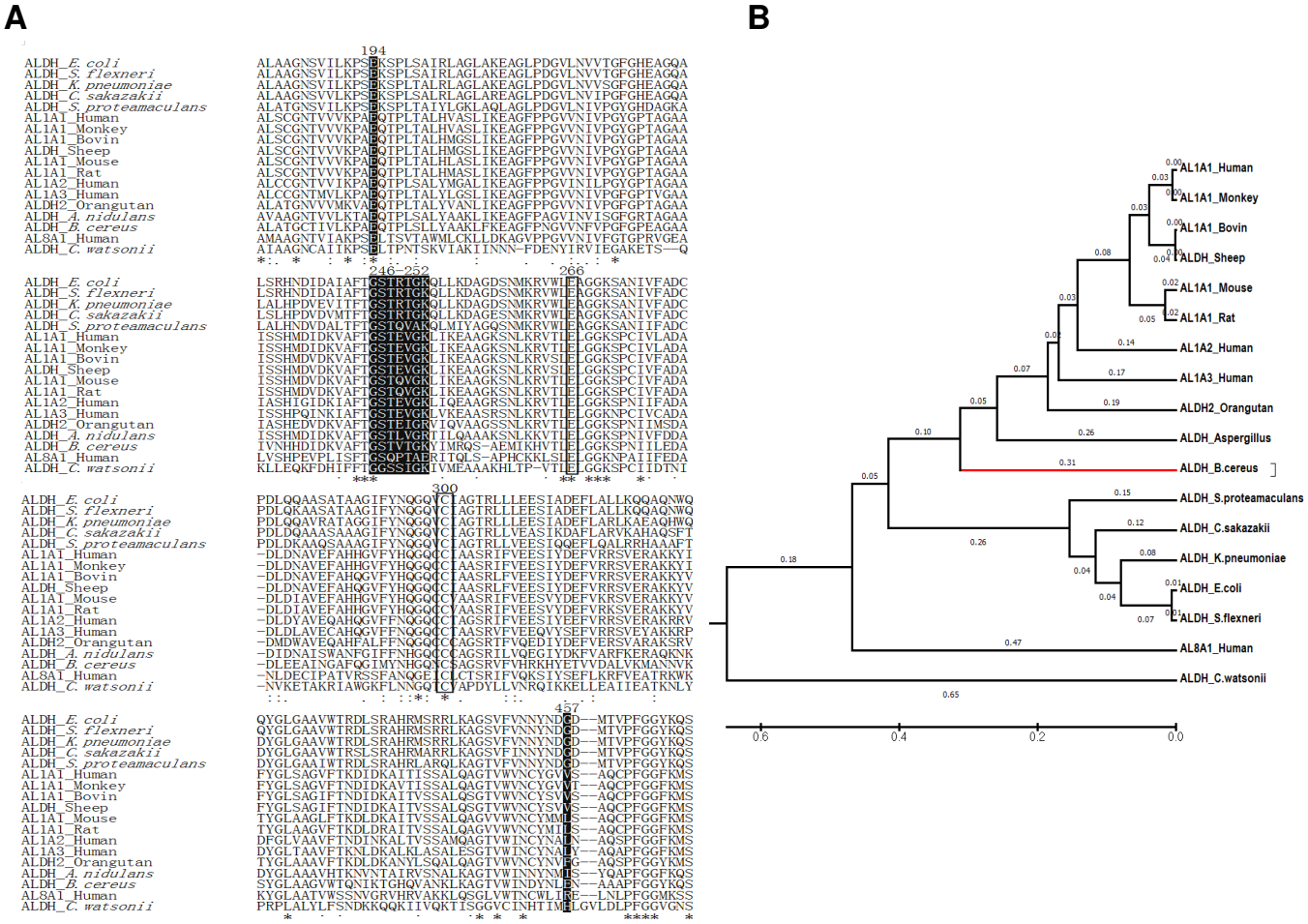


FIG 2

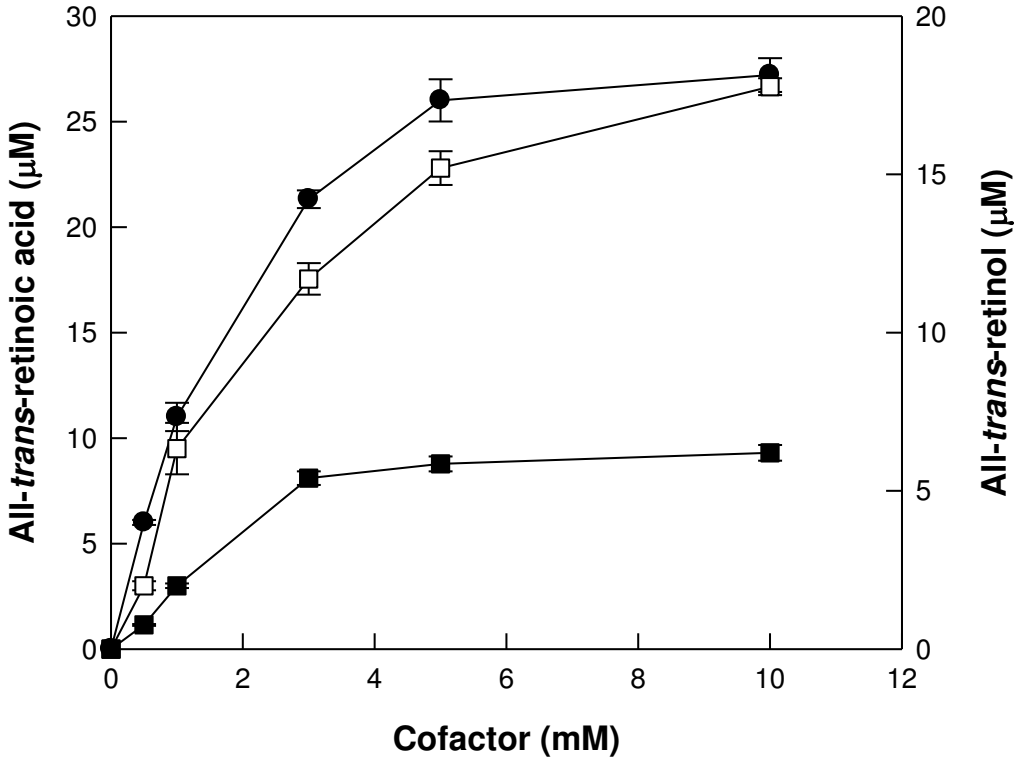
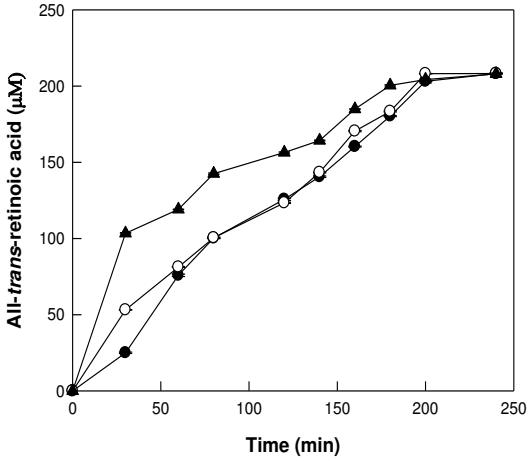


FIG 3

A



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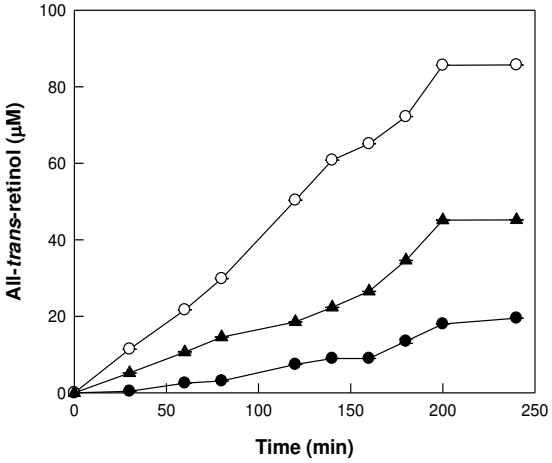

FIG 4