Bull. Graduate School of Bioresources, Mie Univ.
No.35 : 47 − 53
December, 2008

Expression in *Escherichia coli* and Characterization of an Aldehyde Reductase from a Marine Bacterium, *Vibrio* sp. Strain XY-214

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Abstract

An aldehyde reductase gene (*alrA*) located between a β-1, 3-xylanase gene (*txyA*) and a β-1, 3-xylosidase gene (*xloA*) was cloned from a marine bacterium *Vibrio* sp. strain XY-214. The *alrA* gene consisted of a 975-bp nucleotide sequence encoding a protein of 324 amino acids with a predicted molecular weight of 36,367. The deduced protein (named AlrA) showed similarities to members of the aldo-keto reductase (AKR) superfamily. The intact coding region for AlrA was subcloned into pCold TF DNA vector and expressed in *Escherichia coli* BL21 (DE3). Recombinant AlrA (rAlrA), which was treated with thrombin protease to remove a Trigger Factor (TF) and a six-His tag at its N-terminal, was purified with HiTrap chelating HP and Resource Q column chromatographies. AlrA showed activity toward *p*-nitrobenzaldehyde, but no activities were observed toward D-xylose, D-arabinose, D-glucose, D-mannose, D-fructose, and D-maltose (all at 20 mM). These results suggested that AlrA of *Vibrio* sp. strain XY-214 is an aldehyde reductase of the AKR superfamily.

Key Words: aldehyde reductase • *Vibrio* sp. strain XY-214 • aldo-keto reductase (AKR) superfamily • β-1, 3-xylosidse (xloA)

Introduction

Aldehyde reductase (ALR) (EC 1.1.1.2), aldose reductase (EC 1.1.1.21), and carbonyl reductase (EC 1.1.1.184) catalyze the NAD (P) H-dependent reduction of a variety of carbonyl compounds and are widely distributed in mammals, plants, yeast, protozoa, and bacteria. The amino acid sequences of aldose reductases and ALRs show significant similarity, but that of carbonyl reductase does not show similarity to the other sequences. These enzymes are members of the aldo-keto reductase (AKR) superfamily[1], which includes prostaglandin F synthase[2], ρ-crystalline[3], δ-3-ketosteroid 5-β-reductase[4], a soybean reductase[5], and chlordecone reductase[6]. The physiological roles of the AKRs have not been established. It is suggested that under physiological conditions aldose reductase participates in osmoregulation, but under hyperglycaemic conditions it contributes to the onset and development of severe complications in diabetes[7].

Recently, we have cloned and sequenced *xloA* gene encoding a β-1,3-xylosidase, which is able to hydrolyze β-1,3-linked xylooligosaccharides to D-xylose, from a marine bacterium *Vibrio* sp. strain XY-214[8]. Furthermore, we have found that an operon encoding a putative maltose *o*-acetyltransferase and an aldehyde reductase (AlrA) genes, is located between the *xloA* gene and a β-1,3-xylanase gene (*txyA*).

2008年3月19日受理

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We deduced that these genes might be concerning in xylose metabolism. In this paper, we describe the characterization of AlrA from the strain XY-214 expressed in *Escherichia coli*.

Materials and Methods

Materials.

p-Nitrobenzaldehyde was obtained from Nacalai Tesque. The other chemicals were commercial special grade products from Wako Pure Chemical Industries.

Bacterial strains, plasmids and culture conditions.

Vibrio sp. strain XY-214 isolated from seaweed was grown as described previously[9] and used as the source of chromosomal DNA. *E. coli* XL1-Blue (Stratagene) and *E. coli* BL21 (DE3) (Novagen) were used as the hosts for derivative of pCold TF DNA (Takara, Kyoto, Japan). All *E. coli* strains were grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg ml^{-1}) when required.

Amplification of the *alrA* gene and construction of expression plasmid.

Chromosomal DNA from *Vibrio* sp. strain XY-214 was isolated by the method of Saito et al.[10]. Plasmid DNA was purified with the Wizard Plus miniprep DNA purification system (Promega). Agarose gel electrophoresis, transformation of *E. coli*, and ligation were done as described by Sambrook et al.[11]. The open reading frame of *alrA* gene was amplified from *Vibrio* sp. strain XY-214 genomic DNA with a combination of two synthetic oligonucleotide primers; primer 1 containing a *Nde* I recognition sequence, 5′-GGCATTCCATATGTCTAACATTACCGAT-3′, and primer 2 containing a *Xho* I recognition sequence, 5′-TATACTCGAGTTAAGCGACAGGTTCATCCC-3′. The amplified DNA fragment was digested with *Nde* I and *Xho* I, and ligated into a pCold TF DNA vector linearized with the same enzymes to construct *alrA*/pCold TF. The absence of undesired mutation in the amplified DNA fragment was verified by DNA sequencing. The plasmid, which provide the recombinant protein fused with a six-His tag and a Trigger Factor (TF) at the N-terminal, was transformed into *E. coli* BL21 (DE3) competent cells and used for the production of the rAlrA.

DNA sequencing and bioinformatics approaches.

Nucleotide sequence analysis of the DNA fragment inserted into the vector was carried out on a Beckman CEQ2000XL sequencer (Beckman Coulter) using a GenomeLab™ DTCS-Quick Start Kit. Oligonucleotide primers designed on the basis of the known sequence were also used for DNA sequencing. The nucleotide sequence data were analyzed with GENETIX-WIN computer software (Software Development, Tokyo). Similarity searches were performed using the basic local alignment search tool algorithm at the National Center for Biotechnology Information Server[12]. Molecular masses of polypeptide products were estimated using the peptide mass tool at the ExPASy server of the Swiss Institute of Bioinformatics[13].

Expression and purification of rAlrA.

E. coli BL21 (DE3) transformants carrying *alrA*/pCold TF were cultivated at 37℃ in 800 ml of LB medium in the presence of ampicillin (100 μg ml^{-1}). When the optical density at 600 nm of the culture reached about 0.5, the temperature was changed to 15℃ and isopropyl-β-D-galactopyranoside (IPTG) was added to the culture to give a final concentration of 1 mM for induction of gene expression. After an

additional incubation of 20 h at 15℃, the cells harvested by centrifugation were suspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4), and disrupted on ice by sonication. The supernatant of the cell lysate collected by centrifugation was applied to a HiTrap chelating HP column (GE Healthcare) equilibrated with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl and 10 mM imidazole. After being washed with the same buffer, the column was eluted with a linear gradient of imdazole (10 to 500 mM). After the removal of imidazole by dialysis against 20 mM sodium phosphate buffer (pH 7.0), the purified protein was treated with thrombin protease (10 units per mg of protein) at 19℃ for 16 h to remove the TF and a six-His tag fused to its N-terminal. To purify the recombinant AlrA from digested peptide fragments, the mixtures were again fractionated by a HiTrap chelating HP column precharged with Ni^{2+}, after the mixtures were dialyzed against binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4). The eluted recombinant protein was further purified with a 1 ml Resource Q column (GE Healthcare) according to the manufacturer's instructions, after the eluted solutions were dialyzed against 20 mM sodium phosphate buffer (pH7.0). The final preparation of purified rAlrA was used in all of the experiments in this study.

Enzyme assays.

The activity of AlrA was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm upon oxidation of NADPH at 25℃. Unless indicated otherwise, the AlrA assay mixture (2.0 ml) for reduction contained 20 mM sodium phosphate (pH 7.0), 0.25 mM NADPH, 1 mM *p*-nitrobenzaldehyde, and enzyme solution (0.2 ml). The reaction was started by the addition of 0.5 ml of substrate. To examine the substrate specificity of rAlrA, various substrates such as D-xylose, D-arabinose, D-glucose, D-mannose, D-fructose, and D-maltose (all at 20 mM) were used instead of *p*-nitrobenzaldehyde.

SDS-PAGE.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12.5% polyacrylamide gel by the method of Laemmli[14]. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. A low molecular weight SDS calibration kit (GE Healthcare) was used as a standard.

Results and Discussion

The *alrA* gene consisted of 975-bp nucleotides encoding a protein of 324 amino acids with a predicted molecular weight of 36,367. The deduced amino acid sequence of AlrA was compared with other protein sequences in the GenBank database. A high level of identity was found for proteins belonging to the AKR superfamily. Identities among ALRs from *Alcanivorax borkumensis* SK2, *Cyanothece* sp. CCY0110, *Salinibacter ruber* DSM 13855, *Mus musculus*, and *Homo sapiens* were 59, 52, 51, 46, and 46%, respectively. These enzymes, which showed high similarities to AlrA of strain XY-214, belonged to family 1 of the AKR superfamily (AKR1). It indicated that AlrA should be classified into the AKR1. Furthermore, the AlrA exhibited the high similarities to the xylose reductases (identity percentages are in parentheses) from *Candida tropicalis* (44%), *Pichia guilliermondii* (43%), and *Candida shehatae* (43%), which have been classified into AKR2. In general, the AKRs have greater similarity at their N-terminal regions than at their C-terminal regions (Fig. 1). Structural models of human aldose reductase suggested that Tyr-49 acts as the acid base catalyst and Asp-44, Lys-79, and His-112 play an important role in facilitating the hydride

transfer[15, 16]. All four of these amino acid residues are conserved throughout the superfamily, including the AlrA from *Vibrio* sp. strain XY-214. The tetra-amino acid motif IPKS is conserved among these NADPH-dependent reductases, and the lysine residue in this motif is involved in NADPH binding[17] (Fig. 1). Although the motif is present in *Vibrio* sp. strain XY-214 AlrA, some amino acid residues were not conserved around the motif. The purified enzyme was ascertained to be a single band on SDS-PAGE (Fig. 2). Its relative molecular mass was estimated to be approximately 32 kDa. The molecular mass is in good agreement with that deduced from the amino acid sequence (36,367 Da). Although the majority of known AKRs are monomeric, and most xylose reductases function as noncooperative, tightly associated dimers with a subunit molecular mass of 33 to 40 kDa[18, 19], the subunit structure of AlrA has not yet been determined.

The *Vibrio* sp. strain XY-214 AlrA showed highest activity toward *p*-nitrobenzaldehyde (Fig. 3). The high affinity of the enzyme for *p*-nitrobenzaldehyde is typical for all AKRs[20]. On the other hand, AlrA did not show any activity towards D-xylose, D-arabinose, D-glucose, D-mannose, D-fructose, and D-maltose (all at 20 mM), typical substrates used to characterize aldose reductases of the AKR superfamily. These results suggested that *Vibrio* sp. strain XY-214 AlrA is an aldehyde reductase of the AKR superfamily. Although the operon encoding the *alrA* gene and a maltose *o*-acetyltransferase gene is located between the β-1,3-xylan utilization genes, it might not concern with the utilization of β-1,3-xylan.

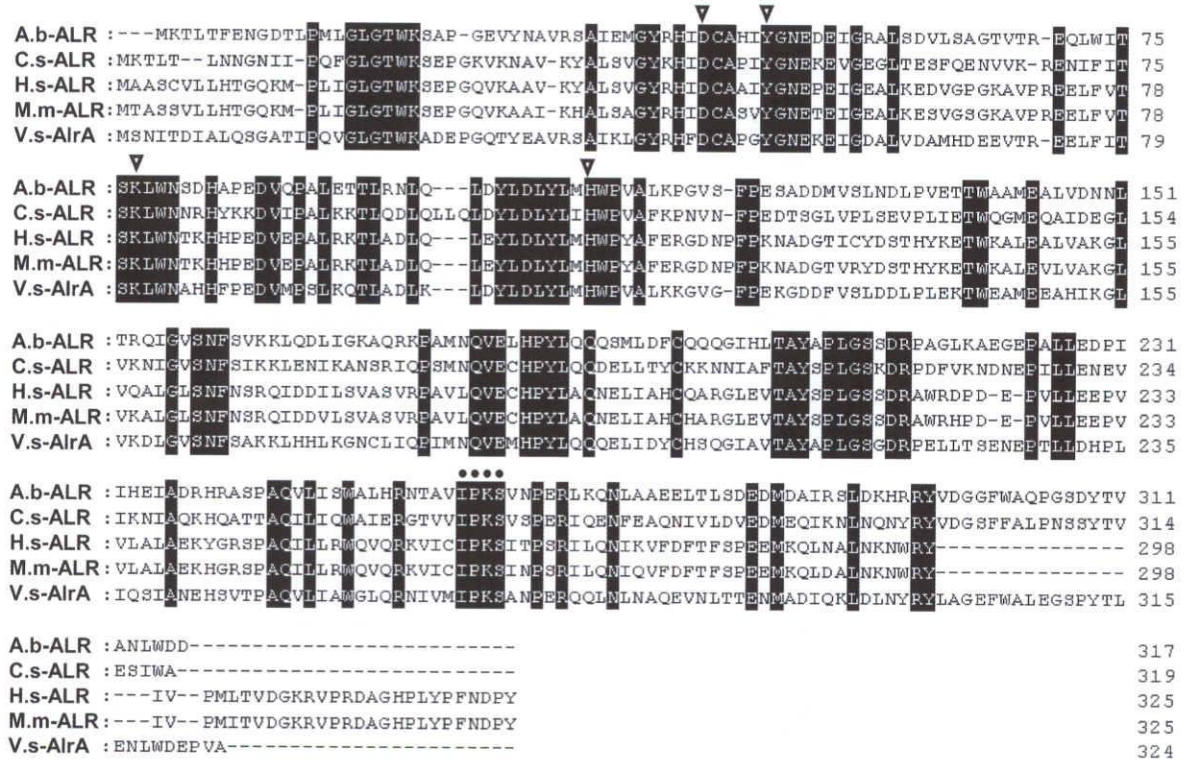

Fig. 1. Comparison of the deduced amino acid sequences of aldehyde reductases.
The sequences of ALR from *Alcanivorax borkumensis* SK2 (A.b-ALR), *Cyanothece* sp. CCY0110 (C.s-ALR), *Homo sapiens* (H.s-ALR), *Mus musculus* (M.m-ALR), and *Vibrio* sp. strain XY-214 (V.s-AlrA) are aligned. The amino acid residues conserved in the five sequences are highlighted. The putative active site residues are indicated by triangles. Dots represent the predicted NADPH-binding motifs, IPKS.

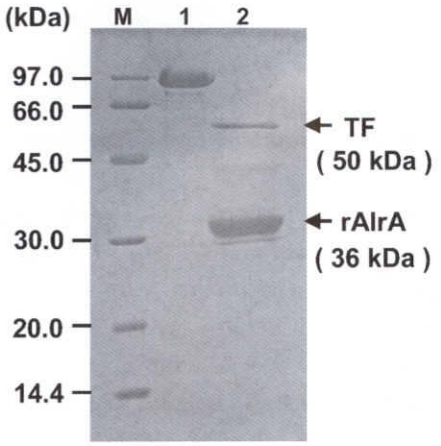

Fig. 2. SDS-PAGE of the rAlrA.
Lane M, standard markers; lane 1, rAlrA fused with a Trigger Factor (TF) and a six-His tag which was purified with HiTrap chelating HP column; lane 2, rAlrA which was purified with HiTrap chelating and Resource Q columns after the removal of a TF and a six-His tag by thrombin protease.

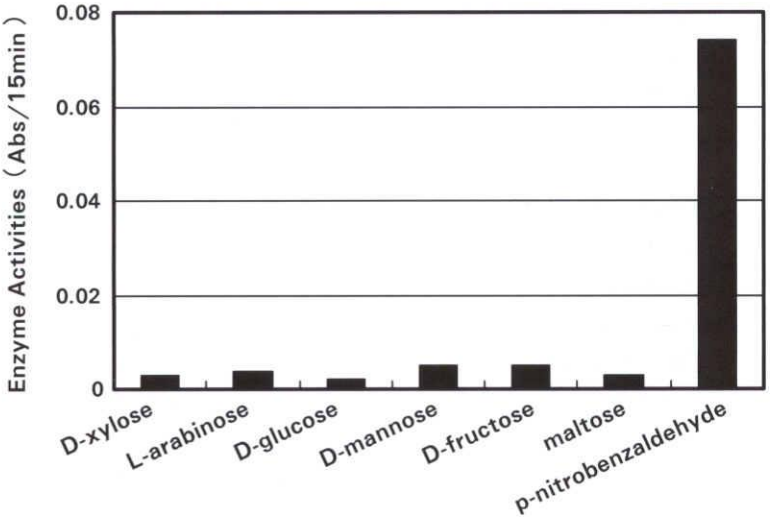

Fig. 3. The NADPH-dependent reduction of various carbonyl compounds by rAlrA.
The purified enzyme was assayed in the standard assay condition for reduction with various substrates. Each value represents the mean of triplicate measurements.

Acknowledgment

This study was supported by a grant program of Japan Science and Technology Agency (Research for Promoting Technological Seeds, 2007).

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海洋細菌 *Vibrio* sp. XY-214 株由来アルデヒドレダクターゼの発現と性質

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要　約

海洋細菌 *Vibrio* sp. XY-214 株からβ-1, 3-キシラナーゼ遺伝子（*txyA*）とβ-1, 3-キシロシダーゼ遺伝子（*xloA*）の間に位置する推定アルデヒドレダクターゼ遺伝子（*alrA*）をクローニングした。*alrA* 遺伝子は，324 アミノ酸残基をコードする 975 塩基対のオープンリーディングフレームから構成されており，その翻訳タンパク質の推定分子量は 36,367 Da であった。本アルデヒドレダクターゼ（AlrA）は，そのアミノ酸配列からアルド-ケトレダクターゼ（AKR）スーパーファミリーに分類された。*alrA* 遺伝子を pCold TF DNA ベクターにサブクローニングし，大腸菌（BL 21）において組換えタンパク質を過剰発現させた。組換え体 AlrA（rAlrA）の N 末端に付加された可溶化タグである Trigger Factor（TF）およびヒスチジンタグをトロンビンにより切断し，その後 HiTrap chelating HP カラムと Resource Q カラムを用いて rAlrA を精製した。AlrA は *p*-ニトロベンズアルデヒドに対しては活性を示したが，D-キシロースや D-アラビノース，D-グルコース，D-マンノース，D-フルクトース，D-マルトース（最終濃度 20 mM）などの基質には作用しなかった。これらの結果から，*Vibrio* sp. XY-214 株由来 AlrA が AKR スーパーファミリーに属するアルデヒドレダクターゼであることが示唆された。