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Differences in D-amino acid metabolism mediated by D-amino acid dehydrogenase in autotrophic and heterotrophic cultures of the Sulfur-Oxidizing Bacterium *Starkeya novella*

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The sulfur-oxidizing bacterium *Starkeya novella* (*S. novella*) is a facultative chemoautotroph. D-Amino acid dehydrogenase (DAD) is a membrane-bound flavoenzyme that dehydrogenates D-amino acids to produce 2-oxoacids, ammonia, and hydrogen to generate electrons. In *Helicobacter pylori*, these electrons are transferred to cytochromes in the respiratory chain. This study aimed to assess electron transfer from DAD to cytochromes in *S. novella*. Under heterotrophic conditions, when D-amino acids were used as substrates, the cytochrome system was reduced by the electrons produced by DAD, and oxygen consumption was observed. This suggests that D-amino acids are used as respiratory substrates and are involved in ATP synthesis. Under autotrophic conditions, NAD^+ was reduced by electrons dehydrogenated by DAD. This suggests that NAD^+ is used for carbonic acid fixation. These results suggest that the DAD of *S. novella* may play different roles under autotrophic and heterotrophic conditions.

Keywords: D-amino acid, D-amino acid dehydrogenase, Electron transfer system, *Starkeya novella*

The sulfur-oxidizing bacterium *Starkeya novella* is a gram-negative, facultative, autotrophic, chemosynthetic bacterium that was first isolated by Starkey[1], and can grow under neutral pH conditions in the presence of inorganic (*e.g.*, thiosulfate) or organic material[2-5]. D-Amino acid dehydrogenase (DAD) [EC 1.4.99.1] is a membrane-bound enzyme that uses FAD as a coenzyme to dehydrogenate D-amino acids into 2-oxo-acids and ammonia, concomitantly releasing protons and electrons[6]. This protein is expressed in obligate anaerobes, including *Pyrobaculum islandicum*, and those residing in microaerobic environments, including *Helicobacter pylori*[7-9]. D-Amino acid oxidase (DAO) [EC 1.4.3.3] is an enzyme that catalyzes the oxidation of D-amino acids to 2-oxo acids. This process utilizes FAD as a cofactor and produces ammonia and H_2O_2[10]. A notable similarity exists among the functions, amino acid sequences, and gene sequences of DAD and DAO. Previous assumptions posited that DAD was present in bacteria and that DAO was present in eukaryotes. The prevailing hypothesis, however, was that DAO was not present in prokaryotes. This notion was subsequently challenged by the discovery of DAO in *Arthrobacter protophormiae* by Geueke *et al*[11]. Subsequent studies by Takahashi *et al*[12] and Saito *et al*[13] further expanded the knowledge of DAO's distribution by isolating it from the hyperthermophilic bacterium *Rubrobacter xylanophilus* and *Streptomyces coelicolor*, respectively. Consequently, it became imperative to ascertain whether the enzyme in *S. novella* was DAD or DAO. DAD is reportedly involved in bioenergetic reactions in *Escherichia coli* and *H. pylori*, and the electrons released from DAD are transferred to the electron transport system[6,14]. In heterotrophic organisms such as *H. pylori*, the electrons produced when DAD dehydrogenates D-amino acids are transferred in the following order: quinone, cytochrome bc_1, cytochrome *c*, and cytochrome oxidase. Since it is thought that electron transfer from D-amino acids involving DAD is carried out via the quinone-cytochrome system, it is thought that the cytochrome system is also involved in the metabolism of D-amino acids *via* DAD in *S. novella* during heterotrophic respiration.

On the other hand, in the electron transport system of *S. novella* during autotrophic respiration, elemental sulfur and thiosulfate ions are used as respiratory

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substrates, and electrons are transferred in the order of sulfite-cytochrome *c* reductase, cytochrome *c*, and cytochrome aa_3. Since quinones are not present in this electron transport chain, it is unclear whether the electrons produced when DAD dehydrogenates D-amino acids are involved in the cytochrome system or whether they are transmitted through a completely different pathway. Therefore, considering that the metabolism of D-amino acids might vary according to culture conditions and to examine the differences in the electron transport system and its association with DAD, we analyzed the enzymes involved in the degradation of D-amino acids in *S. novella*. By investigating the metabolic mechanism of D-amino acids by DAD under autotrophic conditions, whose function is unknown, we may elucidate a new pathway for carbonic acid fixation and apply it to CO_2 reduction by autotrophic chemosynthetic bacteria.

Metabolism of D-amino acids using DAD has been studied for heterotrophs, but there is no knowledge of D-amino acid metabolism in autotrophs. This study investigated the metabolic pathway of D-amino acids by DAD in a facultative autotrophic chemosynthetic bacterium, *S. novella*, under independent nutrient conditions, and found that the metabolic pathway of D-amino acids is modified by different nutrient conditions during culture.

Materials and Methods

Bacterial culture

S. novella was obtained from the NITE Biological Resource Center (NBRC 12443, Kisarazu, Chiba, Japan) and cultured under autotrophic conditions for 7 d at 27°C, using a culture medium previously described by Santer *et al*[17]and under heterotrophic conditions for 3 d at 27°C in nutrient broth. In both cases, the Sakaguchi flask was used, and the shaking speed was 105 rpm. The strains were maintained in nutrient broth containing 3% agar. D-Amino acids were added to the cultures at a concentration of 50 mM. Cells were harvested via centrifugation at 4000 × g for 10 min at 4°C and washed once with 50 mM potassium phosphate buffer (pH 7.0), and then stored at −20°C.

Preparation of the cell-free extract

S. novella cells (approximately 3 g) were suspended in 5 volumes of 50 mM potassium phosphate buffer (pH 7.0), sonicated on ice at 50 W for 30 min using a sonicator (Sonifier 450; Branson, CT, USA), and centrifuged at 8000 × g for 10 min at 4°C. The pellet (intact cells) was discarded, and the supernatant (cell-free extract) was retained. The cell-free extract was ultra centrifuged (140,000 × g, 60 min, 4°C) to obtain a soluble fraction in the supernatant and a membrane fraction in the precipitate. The membrane fraction was solubilized by suspension in 50 mM potassium phosphate buffer (pH 7.0) containing 1% Tween 20, and the soluble membrane fraction was obtained by ultracentrifugation (140,000 × g, 60 min, 4°C). The cell-free extract and soluble membrane fraction were dialyzed (2 h at 4°C, 2 times) against 50 mM potassium phosphate buffer (pH 7.0). Protein concentrations were determined using the methods of Bradford[18] and Lowry *et al*[19] with bovine serum albumin as the standard.

Determination of DAD activity

DAD activity was measured using 2,6-dichlorophenolindophenol (DCIP) as the electron acceptor[9]. The reaction mixture (200 μL) consisted of the cell-free extract (10 μg), 50 mM potassium phosphate buffer (pH 7.0) (When measuring the optimal pH, 50 mM Britton Robinson buffer (pH 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, and 9.0) was used.)), 2 μM FAD, 1 μM phenazine methosulfate (PMS), and 0.75 mM DCIP, and was preincubated at 27°C. Immediately after adding 50 mM of amino acids (substrates), DCIP reduction was spectrophotometrically monitored at 600 nm (UltroSpec 4300; Amersham BioSciences, NJ, USA). The initial reaction velocity was determined using the millimolar extinction coefficient of DCIP of 21.6 $mM^{-1}cm^{-1}$ at 600 nm.

Determination of DAO activity

DAO activity was measured using the method described by Nagata *et al.*[20]with modifications. The reaction mixture consisted of cell-free extract (10 μg), 50 mM potassium phosphate buffer (pH 7.0), and substrates (50 mM D-alanine or 50 mM D-proline). After a 10-min incubation at 27°C, solution A, containing sodium3,5-dichloro-2-hydroxybenzensulfonic acid, 4-aminoantipyrine, and horseradish peroxidase in 10 mM Tris-HCl buffer (pH 8.2), was added, and the reaction was allowed to proceed at 27°C for 10 min and was terminated with a 100 mM sodium borate buffer (pH 10.0). After 2 min at 25°C, the resulting quinone imine dye was detected spectrophotometrically at 445 nm (UltroSpec 4300; Amersham BioSciences, NJ, USA).

Spectrophotometric detection of electron transfer from DAD to cytochromes

The genetic sequence of DAD of *S. novella* was examined using genome resolution, and its homology to the DAD of *E. coli* was low (approximately 40%). We conducted experiments using cell-free extracts containing all the components necessary for electron transfer to determine whether bacterial DAD is expressed and its physiological significance *in vivo*. Upon accepting electrons, the absorption spectrum of the cytochrome is converted from that of the oxidized to the reduced form. Electron transport from D-amino acids to cytochromes was investigated by monitoring spectral changes in cytochromes (UltroSpec 4300; Amersham BioSciences, NJ, USA). The reaction mixture consisted of the cell-free extract (0.156 or 0.84 mg upon culturing under autotrophic or heterotrophic conditions, respectively) and 10 μM of NaN_3 in 50 mM sodium phosphate buffer (pH 7.0) and 25% saturated oxygen. After a 5 min incubation at 37°C, the substrate (1 mM of $Na_2S_2O_3$, 50 mM of D-proline, or 50 mM of D-alanine) was added.

Polarimetric assay of respiration

Respiration was polarographically monitored with a Clark-type oxygen electrode (YSI Model 5300, Yellow Springs Instruments, OH, USA) in a semi-closed vessel containing 2 mL of a reaction mixture composed of cell-free extract (0.33 or 1.26 mg when cultured under autotrophic or heterotrophic conditions, respectively), and 25% saturated oxygen in 50 mM potassium phosphate buffer (pH 7.0). A saturated oxygen concentration of 25% was obtained by mixing a 50 mM potassium phosphate buffer saturated with oxygen (100%) in a buffer solution containing no oxygen. After a 5 min incubation at 27°C, the reaction was initiated with the substrate (20 mM of $Na_2S_2O_3$, 100 mM of D-proline, or 100 mM of D-alanine).

Assessment of NAD^+ reduction

Electron transport from D-amino acids to the NADH was assessed by monitoring NAD^+ reduction. The reaction mixture (200 μL) consisted of the cell-free extract (10 μg), 50 mM potassium phosphate buffer (pH 7.0), 1 μM of FAD, and 100 μM of NAD^+ (as an electron acceptor), and D-amino acids (50 mM) were added as substrates. The mixture was then incubated at 27°C for 10 min. The absorbance was measured at 340 nm using a spectrophotometer (UltroSpec 4300; Amersham BioSciences, NJ, USA). NAD^+ reduction was quantified using the millimolar extinction coefficient of NADH as 6.3 $mM^{-1}cm^{-1}$ at 340 nm.

Results and Discussion

Bacterial growth

When cultured under autotrophic conditions, growth was inhibited by adding 50 mM of D-alanine (Fig. 1A). D-Alanine is an essential component of peptidoglycan synthesis and is produced by alanine racemase in *E. coli*, which has high substrate specificity for D-alanine and can metabolize 50 mM of D-alanine. However, in *S. novella*, a substantial amount of D-alanine is considered to cause growth inhibition. However, when 50 mM D-proline was added, growth was delayed (Fig. 1A) but reached the same level as the control. It is thought that *S. novella* DAD is an enzyme with high substrate specificity for D-proline, similar to*H. pylori*[9] and *Pyrobaculum islandicum*[7] DAD under nutrient-deficient conditions and that *S. novella* DAD expression may be induced in the presence of D-proline.

When cultured under heterotrophic conditions, growth was inhibited when 50 mM of D-alanine was added (Fig. 1B), whereas growth was similar tothat of the control when 50 mM of D-proline was added (Fig. 1B). Regarding the heterotrophic medium, growth inhibition by D-proline may not have been observed because of the abundance of amino acids in the medium.

DAD activity

DAD activity was detected in the cell-free extracts (Fig. 2A) and soluble membrane fraction (Fig. 2B) of *S. novella*; DAO activity was not detected in either. DAD and DAO activity was not detected in the soluble fraction (data not shown). DAD and DAO are

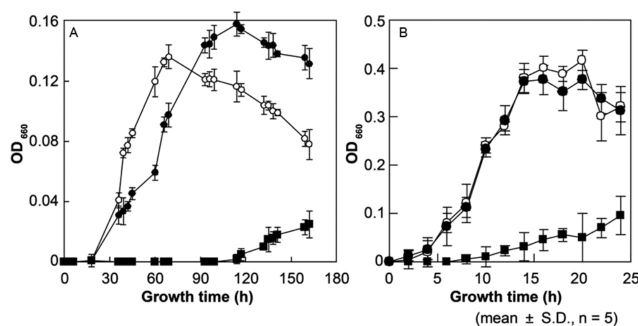

Fig. 1 — Effects of D-amino acids on the growth of *Starkeya novella.* D-Amino acids (50 mM) were added to the culture medium (50 mL) and the absorbance was measured at 600 nm (OD 660) throughout the reaction. "A" and "B" indicate autotrophic and heterotrophic conditions, respectively.
The white circle (○) indicates the control, the black square (■) indicates that 50 mM of D-alanine was added to the medium, and the black circle (●) indicates that 50 mM of D-proline was added.

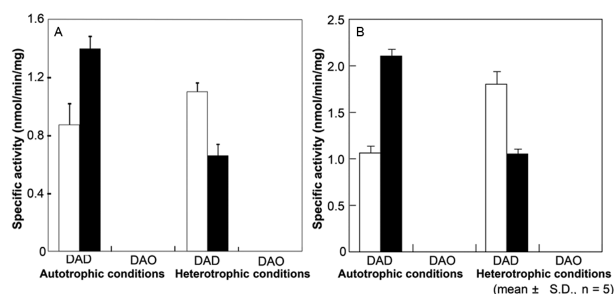

Fig. 2 — D-Amino acid dehydrogenase (DAD) and D-amino acid oxidase (DAO) activities of cell-free extracts of *Starkeya novella.* Amino acids (50 mM) were added to a mixture containing the sample (A: Cell-free extract, B: Soluble membrane fraction) (10 μg), 50 mM potassium phosphate buffer (pH 7.0), 1 μM of FAD, 2 μM of phenazinemethosulfate, and 0.75 mM of 2,6-dichlorophenolindophenol. The reaction mixture was incubated at 27°C for 10 min. White (□) and black (■) bars represent the activities of DAD and DAO, respectively, using D-alanine and D-proline as substrates

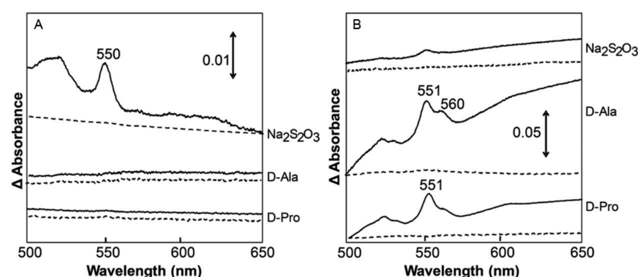

Fig. 3 — Difference spectrum of the cell-free extract of *Starkeya novella.* The reaction mixture (300 μL) containing the cell-free extract (under autotrophic conditions: 0.156 mg; under heterotrophic conditions: 0.84 mg), substrate (1 mM of $Na_2S_2O_3$, 50 mM of D-proline, or 50 mM of D-Ala) and 10 μM of NaN_3. "A" and "B" indicate the values obtained under autotrophic and heterotrophic conditions, respectively. The dashed line indicates the values obtained before substrate supplementation. The solid line indicates values obtained 10 min after substrate supplementation

genetically similar enzymes, but DAD is primarily a membrane surface protein[6,7,9], and DAO is a soluble protein[10-12]; DAO can also make DCIP an electron acceptor; however, since no activity was detected in the soluble fraction and activity was detected in the solubilized membrane fraction, it is likely that the *S. novella* D-amino acid metabolizing enzyme is DAD. In the autotrophic bacterial culture, enzyme activity was higher with D-proline than with D-alanine, whereas the opposite was evident in heterotrophic cultures (Fig. 2). However, the underlying cause of this phenomenon remains unknown. The optimum temperature for DAD cultured on an autotrophic culture medium was 27°C. The optimum temperature for DAD cultured in a heterotrophic medium was also 27°C. The optimum pH for DAD cultured in the autotrophic medium was 6.5. The optimum pH for DAD cultured in the heterotrophic medium was also 6.5. The optimum temperature and pH for DAD were the same regardless of the growth conditions (autotrophic or heterotrophic). This optimal pH was different from those reported for *E. coli* (pH 8.9)[6], *H. pylori* (pH 8.0)[9], *Pseudomonas fluorescens* (pH 7.0–8.0)[21], and *Pyrobaculum islandicum* (pH 6.5–9.0)[22]. These results suggest that the structure of *S. novella* DAD differs from that of other bacterial DADs.

Electron transfer from DAD to cytochromes

Upon culturing under autotrophic conditions and thiosulfate supplementation, the absorbance of the cell-free extract increased. The difference in the absorption spectrum upon thiosulfate supplementation yielded a cytochrome-type spectrum with an α-peak at 550 nm (Fig. 3A). However, upon supplementation with D-alanine and D-proline as substrates, no peaks corresponding to the cytochrome were detected (Fig. 3A). Conversely, upon culturing under heterotrophic conditions, cytochrome was reduced upon supplementation with D-alanine or D-proline as substrates (Fig. 3B). Regarding *E. coli* and *H. pylori*, electrons generated during the dehydrogenation of D-amino acids by DAD are transferred to the cytochromes through quinones[6,9]. Culturing *S. novella* under autotrophic conditions results in the conversion of thiosulfate to sulfurous acid by a thiosulfate-cleaving enzyme in the electron transfer system. The electrons generated when sulfite is oxidized by sulfite cytochrome *c* oxidoreductase are transferred to cytochrome *c* and cytochrome *c* oxidase, reducing oxygen to water[15]. Quinones are not required for this pathway. Therefore, our results suggest that when D-amino acids are dehydrogenated by DAD, the electrons transfer towards the quinones but are not transmitted to the cytochrome system. However, when bacteria are cultured under heterotrophic conditions, electrons are transferred to quinones, cytochrome *bc*, and terminal oxidases[16].

Respiration with the cell-free extract

Under autotrophic conditions, oxygen consumption was measured using sodium thiosulfate supplementation as the substrate. However, oxygen was not consumed upon supplementation with D-alanine or D-proline. Under heterotrophic conditions, the oxygen consumption rate was 0.74 ± 0.07 and

Table 1 — Oxygen consumption of cell-free extracts from *S. novella*

	Substrate	Specific activity (nmol/min/mg)
Autotrophic condition	20 mM $Na_2S_2O_3$	5.91 ± 0.02
	100 mMD-Ala	0
	100 mMD-Pro	0
Heterotrophic condition	20 mM $Na_2S_2O_3$	0
	100 mMD-Ala	7.40 ± 0.07
	100 mMD-Pro	6.20 ± 0.14

(mean ± S.D., n = 3)

Table 2 — NAD^+ reducing activity of cell-free extracts from *S. novella*

	Substrate	Specific activity (nmol/min/mg)
Autotrophic condition	50 mMD-Ala	8.36 ± 1.02
	50 mMD-Pro	11.63 ± 1.03
Heterotrophic condition	50 mMD-Ala	1.86 ± 0.31
	50 mMD-Pro	1.31 ± 0.35

0.62 ± 0.14 nmol/min/mg when D-alanine and D-proline were used as the substrate, respectively. However, upon thiosulfate supplementation, the oxygen consumption rate was low (Table 1). In the case of heterotrophic nutrition, electrons are transferred from NADH and other substances to quinone, which is then transferred to cytochrome bc_1 and the cytochrome system. This process enables the organism to generate a greater amount of ATP compared to the autotrophic nutrition pathway. It is hypothesized that this pathway is preferentially expressed, which might explain the observation that oxygen consumption activity is greater from D-amino acids than from thiosulfate.

NAD^+ reduction

NAD^+ reduction upon culturing under autotrophic conditions was 4.5-fold that of under heterotrophic conditions when D-alanine was used as the substrate and approximately 9-fold when D-proline was used as the substrate (Table 2); this suggests that under autotrophic conditions, the electrons generated during DAD-mediated dehydrogenation are transferred to the quinones and subsequently to NAD^+ reductase, thus reducing NAD^+ and producing NADH. A similar mechanism has been observed in *Acidithiobacillus ferrooxidans*, wherein Fe^{2+}oxidation is accompanied by a reversal in the order of the quinones and the production of NADH[23].

Conclusion

In the present study, *S. novella* was cultured under heterotrophic conditions, and electrons were transferred

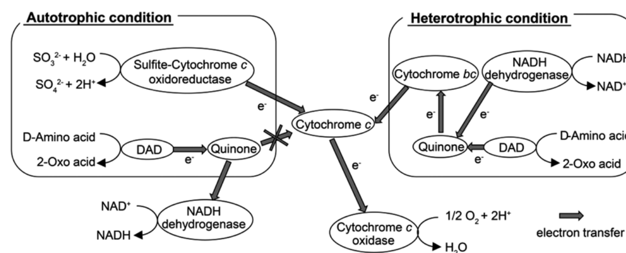

Fig. 4 — Possible mechanism underlying respiration with D-amino acids in *Starkeya novella.* In the metabolism of D-amino acids by DAD in *S. novella*, electrons generated by the dehydrogenation of D-amino acids are transferred to quinone in autotrophic culture, and NAD^+ is theorized to act as the terminal electron acceptor. In heterotrophic cultures, the electrons generated by the dehydrogenation of D-amino acids are transferred to quinone and then to the cytochrome system, where they are coupled with this electron transfer system to synthesize ATP

from both D-proline and D-alanine to the respiratory chain. However, upon culturing under autotrophic conditions, no electron transfer from D-alanine or D-proline to the respiratory chain was observed. When cultured under autotrophic conditions, electrons were used for NAD^+ reduction. These results suggest that D-amino acids metabolized by DAD in *S. novella* are used to reduce NAD^+, *i.e.*, for CO_2 fixation in autotrophic cultures, and are passed to the cytochrome system and used for ATP production in heterotrophic cultures (Fig. 4). It was assumed that, in the case of heterotrophic nutrition, the electrons produced when D-amino acids are metabolized in *S. novella* are transferred to the respiratory chain for ATP production, as in *H. pylori*[14] and *E. coli*[6]. However, in the process of autotrophy, quinone does not participate in the electron transport chain involved in ATP synthesis[16]. The electrons produced when D-amino acids were dehydrogenated reduced NAD^+ *via* quinone. In contrast to soluble DAD, this enzyme exhibits no activity when NADPH is utilized as a substrate or electron acceptor (data not shown). This is a novel finding that the metabolic mechanism of D-amino acids exhibits a distinction between heterotrophic and autotrophic metabolism. It is possible that the mechanism of carbon dioxide fixation occurs by using D-amino acids, which inhibit growth when they are present in high concentrations during autotrophic metabolism, for NADH production, and that D-amino acids can be used effectively without causing growth inhibition.

Conflicts of interest

All authors declare no conflicts of interest.

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