Identification and characterization of trans-3-hydroxy-1-proline dehydratase and Δ1-pyrroline-2-carboxylate reductase involved in trans-3-hydroxy-1-proline metabolism of bacteria

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A R T I C L E   I N F O

Article history:
Received 20 January 2014
Revised 18 February 2014
Accepted 19 February 2014

Keywords:
Hydroxypoline
trans-3-Hydroxy-1-proline metabolism
trans-3-Hydroxy-1-proline dehydratase
Δ1-Pyrroline-2-carboxylate reductase
Convergent evolution of enzyme

A B S T R A C T

trans-4-Hydroxy-1-proline (T4LHyp) and trans-3-hydroxy-1-proline (T3LHyp) occur mainly in collagen. A few bacteria can convert T4LHyp to α-ketoglutarate, and we previously revealed a hypothetical pathway consisting of four enzymes at the molecular level (J Biol Chem (2007) 282, 6685–6695; J Biol Chem (2012) 287, 32674–32688). Here, we first found that Azospirillum brasilense has the ability to grow not only on T4LHyp but also T3LHyp as a sole carbon source. In A. brasilense cells, T3LHyp dehydratase and NAD(P)H-dependent Δ1-pyrroline-2-carboxylate (Pyr2C) reductase activities were induced by T3LHyp (and D-proline and D-lysine) but not T4LHyp, and no effect of T3LHyp was observed on the expression of T4LHyp metabolizing enzymes: a hypothetical pathway of T3LHyp → Pyr2C → l-proline was proposed. Bacterial T3LHyp dehydratase, encoded to LhpH gene, was homologous with the mammalian enzyme. On the other hand, Pyr2C reductase encoded to Lhp1 gene was a novel member of ornithine cyclodeaminase/crystallin superfamily, differing from known bacterial protein. Furthermore, the Lhp enzymes of A. brasilense and another bacterium showed several different properties, including substrate and coenzyme specificities. T3LHyp was converted to proline by the purified LhpH and Lhp1 proteins. Furthermore, disruption of Lhp1 gene from A. brasilense led to loss of growth on T3LHyp, α-proline and α-lysine, indicating that this gene has dual metabolic functions as a reductase for Pyr2C and Δ1-piperidine-2-carboxylate in these pathways, and that the T3LHyp pathway is not linked to T4LHyp and l-proline metabolism.

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1. Introduction

Hydroxy-1-proline (l-Hyp) has been found in certain proteins, in particular collagen, and in some peptide antibiotics. In mammalian systems, l-proline residue is post-translationally hydroxylated to trans-4-hydroxy-l-proline (T4LHyp) or trans-3-hydroxy-l-proline (T3LHyp) by prolyl 4-hydroxylase (EC 1.14.11.2) and propyl 3-hydroxylase (EC 1.14.11.7), respectively [1]. Additionally, it is

Abbreviations: l-Hyp, hydroxy-1-proline; T4LHyp, trans-4-hydroxy-l-proline; T3LHyp, trans-3-hydroxy-l-proline; C4LHyp, cis-4-hydroxy-l-proline; C4DHyp, cis-4-hydroxy-2-carboxylate; Pyr4HIC2, Δ1-pyrroline-4-carboxylate dehydratase; C4DHypDH, C4DHyp dehydrogenase; Pyr2C, Δ1-pyrroline-2-carboxylate; Pip2C, Δ1-piperidine-2-carboxylate; OCD, ornithine cyclodeaminase; LCD, l-lysine cyclodeaminase

http://dx.doi.org/10.1016/j.fob.2014.02.010
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catalyzes the isomerization of T4LHyp to cis-4-hydroxy-D-proline (C4DHyp), and this is then oxidized to Δ¹-pyrroline-4-hydroxy-2-carboxylate (Pyr4H2C) by C4DHyp dehydrogenase (C4DHypDH; EC 1.4.99.-). Pyr4H2C is converted to α-ketoglutaric semialdehyde (αKGSA) by Pyr4H2C deaminase (EC 3.5.4.22; LhpC) and, in the fourth step, αKGSA is oxidized to α-ketoglutarate by the enzyme αKGSA dehydrogenase (EC 1.2.1.26; LhpG). Interestingly, there are two types of C4DHypDHs: Pseudomonas aeruginosa and...
Azospirillum brasilense, α4βκγ4-type enzyme encoded by LhpB (encoding to β-subunit), LhpE (α-subunit) and LhpF genes (γ-subunit); Pseudomonas putida; homodimeric-type enzyme encoded by LhpB gene. This finding strongly suggests that the T4LHyp pathway clearly evolved convergently in bacteria. LhpABCEFG genes are often clustered together with gene(s) encoding putative amino acid transporter on bacterial genomes (referred to as T4LHyp gene cluster) (Fig. 1D).

In the case of T3LHyp, small amounts (one amino acid per 1000 amino acids) have been shown to occur in different types of collagen, including collagen I, II and III, but it was found to be particularly abundant in collagen IV (as much as 10% of the total l-Hyp content). Although degradation by organism(s) is poorly understood, Visser et al. [8] recently reported that a human C14orf149 protein catalyzes the dehydration of T3LHyp to Δ1-pyrroline-2-carboxylate (Pyr2C) via a putative Δ2-pyrroline-2-carboxylate intermediate, although the mechanism of the ability to metabolize T3LHyp by human cells is unclear. Interestingly, in spite of different reactions, the T3LHyp dehydratase (EC 4.2.1.77) belongs to the proline racemase superfamily, in which the archetypical proline racemase (EC 5.1.1.4, Ref. [9]) and T4LHyp epimerase [10] are also contained (see Fig. S3B). It had been believed that the T3LHyp dehydratase is found only in animals and fungi [8].

Although the metabolic fate of Pyr2C may be conversion to l-proline by NAD(P)/H-dependent reductase (Fig. 1B), the corresponding gene has not yet been identified. On the other hand, reductase for Pyr2C from bacteria has been already studied. In Pseudomonas strains including P. putida [11] and Pseudomonas syringae [12], l-lysine is metabolized to α-ketoaspartate through the so-called “l-pipecolate pathway”, in which dPKA protein catalyzes the second step (conversion of Δ1-piperidine-2-carboxylate (Pip2C) to l-pipecolate) as a Pip2C reductase (EC 1.5.1.21) (Fig. 1B). Indeed, this gene also functions as a Pyr2C reductase (EC 1.5.1.1) involved in (hypothetical) l-proline metabolism, in which Pyr2C may be produced from l-proline by (an unknown) α-amino acid oxidase [11]. The bifunctional Pyr2C/Pip2C reductase belongs to a novel NAD(P)/H-dependent malate/α-ketoglutarate dehydrogenase (MDH/LDH) superfamily with no sequence homology to “orthodox” MDH/LDH, and shows strict NADPH dependence. It is unclear whether the dPKA-like) protein functions as Pyr2C reductase in T3LHyp metabolism, because there is no homolog on mammalian genomes, and P. putida cannot metabolize T3LHyp (see in text).

In this study, we first identified that A. brasilense, previously known as a T4LHyp-metabolizing bacterium [6], has the ability to grow on T3LHyp as a sole carbon source, and that the metabolic pathway actually contains T3LHyp dehydratase and Pyr2C reductase, as proposed in mammals. Interestingly, Pyr2C reductase is a novel member of the ornithine cyclodeaminase/μ-crystallin superfamily, different from known dPKA protein, and there are several significant differences in the enzymatic properties between A. brasilense and another bacteria: substrate and coenzyme specificities. Metabolic networks among T3LHyp, T4LHyp, α-proline and β-lysine are also discussed.

2. Results

2.1. Hypothetical metabolic pathway of T3LHyp in A. brasilense

First, we found that among three bacteria capable of metabolizing T4LHyp, only A. brasilense can grow on T3LHyp as a sole carbon source, not P. putida and P. aeruginosa; to our knowledge, this is the first report of T3LHyp metabolism by an organism(s) (Figs. 1E and S1). Next, we estimated whether the T4LHyp pathway is related to T3LHyp metabolism because of its structural similarity. However, all four enzymes involved in T4LHyp metabolism were induced only by T4LHyp (and C4DHyP), not by T3LHyp (Fig. 2A). On the other hand, significant activities of T3LHyp dehydratase and Pyr2C reductase with dual specificity between NADPH and NADH were found in cell-free extract prepared from A. brasilense cells grown not only on T3LHyp but also α-proline and β-lysine. Unexpectedly, although T4LHyp and C4DHyP also induced Pyr2C reductase, enzyme activity was clearly NADPH dependent. These results indicated that T3LHyp dehydratase and Pyr2C reductase are actually involved in the hypothetical T3LHyp pathway not only of mammalians but also bacteria, and that there are Pyr2C reductase isozymes with different inductivity by carbon sources and coenzyme specificity.

2.2. Candidates of T3LHyp dehydratase and Pyr2C reductase genes

Although the genome sequence of A. brasilense is unavailable, nucleotide sequences of several genes from this bacterium show very high similarity (>~98%) to those of Burkholderia lata, which was formerly described as Burkholderia sp. 183 [6]. Therefore, a homology search using the Protein-BLAST program was carried out against the genome sequence of B. lata using C14orf149 (T3LHyp dehydratase) as the probe protein sequence, although it had been believed that only animals and fungi possess this enzyme, not bacteria [8]. Among two homologous genes (proteins) annotated as putative proline racemases, Bcep18194_B1894 and Bcep18194_B1660 with sequence similarities of 29% and 44% to C14orf149, respectively, the former corresponded to T4LHyp epimerase (LhpA) (Fig. 1D), whereas the latter possessed two specific active sites for T3LHyp dehydratase (see below; Ref. [8]) (referred to as LhpH) (Fig. S3A). Therefore, we thought that the LhpH gene was the first candidate for a T3LHyp dehydratase.

B. lata (probably also A. brasilense) possessed one homologous protein (gene) to dPKA from P. putida (~40% identity; PP_3591). However, this gene (Bcep18194_B1898; referred to as LhpD) was contained within the T4LHyp gene cluster (Fig. 1D), which was up-regulated only by T4LHyp, not T3LHyp, as described above (Fig. 2A). On the other hand, further bioinformatics analysis revealed that a (putative) LhpF gene from other bacteria such as Colwellia psychrerythraea 34H (CPS_1453) is located within the T4LHyp gene cluster together with one function-unknown protein (gene) annotated as an ornithine cyclodeaminase (OCD; EC 4.3.1.12) (referred to as LhpI), instead of LhpD gene (Fig. 1D). A gene homologous to CplLhpI gene was also found within the flanking region of AbLhpH gene, and the enzyme reaction by OCD contained Pyr2C as an intermediate (see Fig. 5C). Based on these analysis, we selected LhpD and/or LhpI gene as candidates for Pyr2C reductase.

2.3. Preparation of recombinant His6-tag proteins

After cloning all target genes into the vector pETDuet-1, the recombinant enzymes with attached His6-tags at their N-termini were expressed in Escherichia coli and purified with an Ni2+-chelating affinity column (PaLhpD was characterized instead of AbLhpD because of its successful expression in E. coli cells, and there is 59.8% identity between the proteins) (Fig. 1F). Apparent molecular masses of AbLhpH, CplLhpH, C14orf149, PaLhpD, AbLhpI, and CplLhpI, estimated by SDS–PAGE, were 40 (37,805.87), 40 (40,437.15), 44 (39,618.72), 40 (37,164.78), 37 (33,312.46), and 40 (36,181.76) kDa (values in parentheses indicate the calculated molecular mass of the enzyme with His6-tag), and those estimated by analytic gel filtration were 87, 98, 70, 62, 78 and 81 kDa, respectively (Fig. S2). Therefore, all of these enzymes appear to be dimeric.
2.4. Characterization of LhpH protein as bacterial T3LHyp dehydratase

Potential T3LHyp dehydratase activity in LhpH proteins were assayed by the colorimetric method based on the reaction of 2-aminobenzaldehyde with Pyr2C, described in Section 4. Specific activities with T3LHyp of AbLhpH, CpLhpH, and C14orf149 (as a reference) were 19.8, 19.3, and 7.22 U mg protein$^{-1}$, respectively. Optimum pH values was also determined by this method: common range of pH 8.0–9.5 (data not shown). This indicated that the LhpH gene encodes T3LHyp dehydratase (first example for bacteria), and that the biochemical properties are similar to the mammalian enzyme C14orf149.

2.5. Characterization of LhpD as Pyr2C reductase

The purified PaLhpD showed similar reductase activities for both Pyr2C and Pip2C in the presence of NADPH but not NADH: 42.3 and 32.8 U mg protein$^{-1}$, respectively (Table 1). Although both l-proline and l-pipecolate underwent NADP$^+$-dependent oxidation, their $k_{cat}/K_m$ values were ~500 and ~350-fold lower than those with Pyr2C and Pip2C, respectively (Table 2). Namely, the reaction equilibrium favors the direction toward NADPH-dependent reduction. Optimum pH values in reduction and oxidation using Pyr2C and l-proline were pH 7.0 and pH 10.0, respectively (Fig. 3A). These substrate and coenzyme specificities also corresponded to zymogram staining analysis (Fig. 3C). Overall, PaLhpD (probably also AbLhpD) showed similar enzymatic properties to dpkA [11,12]. Furthermore, it was likely that based on coenzyme specificity, LhpD corresponds to an enzyme induced by T4LHyp (and C4DHyp) in A. brasilense cells (Fig. 2A).

2.6. Characterization of LhpI as novel Pyr2C reductase

First, the LhpI proteins were assayed for OCD activity, with none detected (data not shown). Alternatively, when Pyr2C and NADPH was used as the substrate and coenzyme, respectively, significant

Table 1
Kinetic parameters of AbLhpI, CpLhpI, and PaLhpD in the forward direction.

| Enzymes | Substrates | pH$^a$ | Coenzymes | Specific activity (units/mg protein) | $K_m$ (mM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (min$^{-1}$ mM$^{-1}$) |
|---------|------------|--------|-----------|------------------------------------|-----------|----------------------|---------------------------------|
| AbLhpI  | Pyr2C      | 6.5    | NADPH     | 584                                | 0.628 ± 0.045 | 31400 ± 1300 | 50100 ± 1400 |
|         | NADH       |        | 600       | 0.837 ± 0.080                       | 36000 ± 2400 | 44100 ± 1400 |
|         | Pip2C      |        | NADPH     | 220                                | 0.474 ± 0.050 | 9950 ± 610   | 2110 ± 1010  |
|         | NADH       |        | 177       | 0.600 ± 0.005                       | 9690 ± 380  | 16200 ± 680 |
| CpLhpI  | Pyr4SH2C   |        | NADPH     | 12.0                               | 0.491 ± 0.063 | 621 ± 58    | 1270 ± 45   |
|         | Pyr2C      |        | NADPH     | 30.2                               | 5.50 ± 0.36  | 7470 ± 470  | 1270 ± 2    |
|         | NADH       |        | 4.76      | 2.79 ± 0.42                        | 621 ± 34    | 225 ± 24    |
|         | Pip2C      |        | NADH      | 0.291                              | 7.26 ± 1.22  | 82.9 ± 13.7 | 11.4 ± 0.2  |
|         | Pip4SH2C   |        | NADPH     | 0.0464                             | 2.10 ± 0.17  | 5.27 ± 0.24 | 2.51 ± 0.09 |
| PaLhpD  | Pyr2C      | 7.0    | NADPH     | 42.3                               | 0.447 ± 0.040 | 2500 ± 120 | 5600 ± 220 |
|         | Pip2C      |        | 38.3      | 1.57 ± 0.3                         | 2120 ± 290  | 1350 ± 68   |
|         | Pip4SH2C   |        | 12.1      | 0.835 ± 0.109                      | 868 ± 71    | 1043 ± 48   |

$^a$ pH of potassium phosphate buffer for assay.

$^b$ Illustrated in Fig. 3D.
reduction of activity was observed. Furthermore, \( \text{L-proline} \) was the active substrate for the NADP\(^{+}\)-dependent oxidation reaction, and ratios of Pyr2C to \( \text{L-proline} \) in \( k_{\text{cat}}/K_{\text{m}} \) were 771 and 356 for AbLhpI, respectively. To estimate more detailed sub-value for Pyr2C of AbLhpI, \( k_{\text{cat}}/K_{\text{m}} \) was 161. Third, AbLhpI could utilize Pip2C and \( \text{L-pipecolate} \). The ratio of Pyr2C to \( \text{L-pipecolate} \) of \( k_{\text{cat}}/K_{\text{m}} \) was 70-Arg137-Thr-Gly-Lys-Gln-Ala, respectively (Fig. 3A). These properties as a Pyr2C reductase were similar to PaLhpD.

On the other hand, there were also several significant differences in enzymatic properties between AbLhpI and CpLhpI (and PaLhpD). First, the \( k_{\text{cat}}/K_{\text{m}} \) value for Pyr2C of AbLhpI (50,100 min\(^{-1}\) mM\(^{-1}\)) was ~350- and 8.9-fold higher than those of CpLhpI and PaLhpD, respectively, commonly caused by the higher \( k_{\text{cat}} \) values. Second, there was no preference for coenzyme utilization between NADPH and NADH in AbLhpI, whereas the \( k_{\text{cat}}/K_{\text{m}} \) value for Pyr2C of CpLhpI in the presence of NADH was 5.6-fold lower than that in the presence of NADPH, mainly caused by the 12-fold lower \( k_{\text{cat}} \) value. Much higher preference for coenzymes was found in the oxidation of \( \text{L-proline} \): the ratio of NADPH to NADH was 5.6-fold in AbLhpI, whereas the \( k_{\text{cat}}/K_{\text{m}} \) value for Pyr2C of CpLhpI was 1.28 ± 0.04, 0.016 ± 0.001, and 0.11 ± 0.03 activity for T3LHyp, \( \text{D-proline} \), and \( \text{D-lysine} \) in the presence of NADPH, NADPH-preference Pyr2C reductase, and monofunctional NADPH-preference Pyr2C reductase, respectively, and that Pyr2C reductase activities induced by T3LHyp, \( \text{L-proline} \), and \( \text{L-lysine} \) in \( \text{A. brasilense} \) cells may be derived from LhpD but not LhpD (Fig. 2A).

In HPLC analysis (Fig. 4), a peak corresponding to T3LHyp was completely eliminated by incubation with purified AbLhpH (or CplhpH), probably caused by no reaction of Pyr2C with labeling reagent for amino acid analysis. On the other hand, when T3LHyp was incubated together with purified AbLhpI and AbLhpI (or CplhpI and CplhpI) in the presence of NADPH, a novel peak corresponding to \( \text{L-proline} \) appeared. This suggested that T3LHyp is converted to \( \text{L-proline} \) by continuous reactions with LhpH and LhpH under physiologically neutral conditions (pH 7.0).

### 2.7. Amino acid sequence analysis of LhpI

As expected from preliminary annotation, LhpI belongs to the OCD/\( \mu \)-crystallin superfamily including the archetype OCD [13], \( \mu \)-crystallin [14], \( \text{L-alanine dehydrogenase} \) (EC 1.4.1.1) [15], \( \text{L-arginine dehydrogenase} \) [16], \( \text{L-lysine cyclodeaminase} \) (LCD; EC 4.3.1.28) [17] and \( \text{tauropine dehydrogenase} \) (EC 1.5.1.23) [18] (Fig. 5A). On the other hand, phylogenetic analysis revealed that AbLhpI and CplhpI have poor relationship not only to any subclasses of the other members but also with each other. Putative amino acid sequences of the LhpI proteins contained essential amino acid residues for coenzyme binding (Rossmann-fold motif consisting of Gly-X-Gly-X-X-[Ala/Ser], where X indicates any amino acid) and the catalytic triad for binding to a carboxyl group of substrate (Lys-Arg-Gsp; Gly\(^{137}\)-Thr\(^{-}\)-Gly\(^{-}\)-Gln-Ala\(^{142}\) and Lys\(^{150}\)-Arg\(^{131}\), Asp\(^{224}\) in AbLhpI, respectively (Fig. 5B and C). On the other hand, putative amino acid residues, responsible for discrimination between NAD\(^{+}\) and NADPH, were different not only from other OCD/\( \mu \)-crystallin members but also within LhpI proteins: AbLhpI, Gly\(^{162}\)-Thr\(^{161}\), CplhpI, Gly\(^{158}\)-Arg\(^{158}\).

### 2.8. Gene regulation and disruptant analysis

It is likely that \( \text{AbLhpI} \) and \( \text{AbLhpI} \) genes are clustered together with the putative amino acid transporter gene on the genome of \( \text{A. brasilense} \) (Fig. 1D). Northern blot analysis revealed that \( \text{AbLhpI} \) gene was induced by T3LHyp, \( \text{L-proline} \), and \( \text{L-lysine} \), but not T4LHyp (and C4DHyp) (Fig. 2B). To further estimate the physiological roles of LhpI gene, we carried out gene disruption experiments by introducing a kanamycin-resistant gene (Km\(^{\text{mutant}}\)) into AbLhpI gene. The obtained \( \text{AbLhpI} \) mutant strain was distinct from the wild-type strain in that T3LHyp, \( \text{L-proline} \), and \( \text{L-lysine} \) did not support growth as a sole carbon source. On the other hand, there was no difference in growth on other carbon sources, including T4LHyp, between the two strains. Although we did not analyze \( \text{AbLhpD} \) in this study, it has been reported that disruption of LhpD gene from \( \text{Sinorhizobium meliloti} \) had no effect on growth on T4LHyp, in spite of transcriptional induction by T4LHyp (Fig. 1D) [19]. Overall, these results suggested clearly that LhpI gene is a Pyr2C/Pip2C reductase.

### Table 2

Kinetic parameters of AbLhpI, CpLhpI and PaLhpD in the reverse direction.

| Enzymes | Substrates | pH | Coenzymes | Specific activity (units/mg protein) | \( k_{\text{m}} \) (mM) | \( k_{\text{cat}} \) \( (\text{min}^{-1}) \) | \( k_{\text{cat}}/K_{\text{m}} \) \( (\text{min}^{-1} \text{mM}^{-1}) \) |
|---------|------------|----|-----------|-------------------------------------|------------------|------------------|------------------|
| AbLhpI  | 1-Proline  | 10.5| NADP\(^{+}\) | 5.18 | 3.63 ± 0.31 | 235 ± 7 | 65.0 ± 3.7 |
|         | 1-Proline  | 10.5| NAD\(^{+}\)  | 5.50 | 3.99 ± 0.18 | 254 ± 3 | 63.6 ± 2.1 |
|         | Pip2C      | 9.0 | NADP\(^{+}\) | 2.92 | 6.54 ± 0.96 | 150 ± 14 | 23.0 ± 1.2 |
|         | Pip2C      | 9.0 | NAD\(^{+}\)  | 2.72 | 14.8 ± 2.1 | 222 ± 24 | 15.0 ± 0.5 |
| CpLhpI  | 1-Proline  | 10.0| NADP\(^{+}\) | 0.630 | 18.3 ± 2.4 | 653.5 ± 63 | 3.57 ± 0.09 |
|         | 1-Proline  | 10.0| NAD\(^{+}\)  | 0.00451 | 18.8 ± 3.8 | 0.415 ± 0.077 | 0.0222 ± 0.0004 |
|         | Pip2C      | 9.0 | NADP\(^{+}\) | 0.00435 | 80.1 ± 2.8 | 1.28 ± 0.04 | 20.7 ± 0.01 |
|         | Pip2C      | 9.0 | NAD\(^{+}\)  | 0.00457 | N.D. | N.D. | N.D. |
| PaLhpD  | 1-Proline  | 10.0| NADP\(^{+}\) | 1.98 | 18.5 ± 2.1 | 205 ± 18 | 11.1 ± 0.3 |
|         | 1-Proline  | 10.0| NAD\(^{+}\)  | 0.679 | 34.8 ± 4.0 | 135 ± 14 | 3.88 ± 0.05 |
|         | Pip2C      | 9.0 | NADP\(^{+}\) | 0.490 | 132 ± 26 | 272 ± 53 | 2.07 ± 0.01 |
|         | Pip2C      | 9.0 | NAD\(^{+}\)  | N.D. | N.D. | N.D. | N.D. |

\( ^{a} \) pH of glycine–NaOH buffer for assay.  
\( ^{b} \) Illustrated in Fig. 3E.  
\( ^{c} \) Not determined due to trace activity.

\( K_{\text{m}} \): Michaelis constant; \( k_{\text{cat}} \): catalytic constant; \( k_{\text{cat}}/K_{\text{m}} \): catalytic efficiency; N.D.: Not determined.
involved in T3LHyp, D-proline and D-lysine metabolism, and that LhpD gene is not related directly to T3LHyp and T4LHyp metabolism (see below).

3. Discussion

In this study, we identified T3LHyp pathway consisting of T3LHyp dehydratase and Pyr2C reductase of bacteria. A similar T3LHyp metabolic pathway may exist in mammals [8], however, this contrasts with T4LHyp metabolic pathways as there are complete different in bacteria and mammals [5–7].

On the basis of two specific residues at the active sites, proline racemase-like enzymes have been classified into three types: Cys-Cys type (proline racemase and T4LHyp epimerase); Cys-Thr type (T3LHyp dehydratase); Ser-Cys type (function-unknown) (Fig. S3A). Interestingly, a mutant enzyme of T3LHyp dehydratase
(C14orf149) with a substitution of threonine to cysteine shows “T3LHyp epimerase” activity [8]. In the phylogenetic tree (Fig. S3B), T4LHyp epimerase and T3LHyp dehydratase in the same bacteria (LhpA and LhpH, respectively) belong to different subfamilies, indicating that dehydratase activity with T3LHyp in this superfamily was acquired once at an early evolutionary stage.

In mammalians, Pyr2C is one of the substrates for ketimine reductase (EC 1.5.1.25), in which Pip2C and several ketimine compounds with neurological functions are also contained [20]. It was recently reported that μ-crystallin, belonging to the same protein family as LhpI, has ketimine reductase activity [21]: this protein was previously known as an NADPH-dependent thyroid hormone-binding protein without enzymatic function [14]. However, the purified μ-crystallin accounted for only 0.19% of total enzyme activity measured in a cell-free extract of the lamb forebrain, and enzyme activity was optimal at acidic pH 4.5–5.0, in contrast with activity measured in a cell-free extract of the lamb forebrain, and requires expensive and large apparatus and organic solvent, the final product of T3LHyp metabolism is L-proline, these pathways might have evolved by duplication and divergence of a common ancestor. Then, what is the physiological role of LhpD? This enzyme can efficiently utilize Pyr4S2H2C (and C4DHyp) as a substrate (Table 1 and Fig. 3B, D and E). Furthermore, we recently found that C4LHyp and T4DHyp are also substrates of T4LHyp epimerase and C4DHypDH, respectively, by which C4LHyp is converted to T4DHyp (via Pyr4S2H2C) (Fig. 1C) [26]. Such l- to d-epimerization (racemization) of amino acids consisting of two distinct FAD− and NAD(P)−dependent dehydrogenase (oxidase) is also found in arginine, lysine and arginine metabolism from bacteria (Fig. 5A). Therefore, it is likely that LhpD is involved in the degradation of T4LHyp, a compound which is generated by the hydroxylation of L-proline by bacteria [3].

A. brasilense possesses two separated gene clusters for T4LHyp and T3LHyp metabolism (Fig. 1D), which are up-regulated only by each carbon source (Fig. 2). These properties may be suitable for the metabolism of T4LHyp and T3LHyp (and C4LHyp) produced by direct hydroxylation of free l-proline, as described in Section 1: in fact, the hydroxylase is often found in soil bacteria that fix nitrogen (so-called rhizobia; Ref. [3]), similar to A. brasilense. In contrast, the homologous gene clusters of C. psychrerythraea, a marine bacteria, are combined (Fig. 1D); this gene cluster may be induced by both T4LHyp and T3LHyp. If this hypothesis is true, it is likely that gene regulation is advantageous for the utilization of much marine collagen, because of the co-production of T4LHyp and T3LHyp.

Since post-translational hydroxylation of l-proline residues is almost specific to collagen protein, l-Hyp(s) provides an important marker to directly measure collagen content in several sample types, including foods and tissue fibrosis. Furthermore, l-Hyp(s) in urine and serum has been focused on as a significant biomarker for bone resorption and many human diseases (urinal T3LHyp for cancer; Ref. [27]). On the other hand, the most popular HPLC method for determination of l-Hyp(s) is time-consuming and requires expensive and large apparatus and organic solvent.
Fig. 5. (A) Phylogenetic tree of OCD/μ-crystallin protein family. The number on each branch indicates the bootstrap value. Proteins with asterisks were used for B. (B) Partial multiple sequence alignment of deduced amino acid sequences of Pyr2C reductases. Binding sites for carboxyl group of substrate, lysine, arginine and aspartate, are shaded in green, yellow and light-green, respectively. Aspartate and glutamate residues, related to the cyclization by OCD and LCD, are shaded in red and blue, respectively. Amino acid residues interacting with 2'- and 3'-functional groups in the ribosyl moiety of NAD(P)H and NADP(H) are shaded in pink. Coenzyme-binding motif of Rossman-fold are shown as white letters in black boxes. Gray-shaded letters indicate highly conserved amino acid residues. (C) Schematic diagram showing the interactions of s-ornithine, Pyr2C, and nearby residues in OCD. Color of residues correspond to B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
and l-Hyp(s) (and l-proline), a cyclic amino acid, cannot react with a general labeling reagent for amino acids. Alternatively, an enzymatic method for the T3LHyp dehydratase assay, described in Section 4, would be helpful for conventional detection of T3LHyp in in vivo samples. This line of study is in progress in our laboratory [28].

4. Experimental procedures

4.1. Materials

T3LHyp was purchased from Kanto Chemical (Tokyo, Japan). C4LHyp, l-pipecolate, and d-pipecolate were obtained from Tokyo Chemical Industry (Tokyo, Japan). T4DHyp and C3LHyp were from Sigma Aldrich (USA). T4LHyp, C4DHyp, l-proline, and d-proline were from Wako Pure Chemical Industries (Osaka, Japan).

4.2. General procedures

Basic recombinant DNA techniques were performed as described by Sambrook et al. [29]. Bacterial genomic DNA was prepared using a DNeasy Tissue Kit (Qiagen). PCR was carried out using a GeneAmp PCR System 2700 (Applied Biosystems) for 30 cycles in 50 μl reaction mixture containing 1 U of KOD FX DNA polymerase (TOYOBO), appropriate primers (15 pmol) and template DNA under the following conditions: denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s and extension at 68 °C for time periods calculated at an extension rate of 1 kbp min⁻¹. DNA sequencing was carried out using the BigDye Cycle Sequencing Kit ver.3.1 (Applied Biosystems) and appropriate primers with the Genetic Analyzer 3130 (Applied Biosystems). Protein concentrations were determined by the method of Lowry et al. [30] with tricine SDS–PAGE (Laemmli [31]). Amino acids were identified using an amino acid analyzer (L-8900; Hitachi, Tokyo, Japan) using commercial standards (Wako).

4.3. Substrates

Pyr2C was enzymatically synthesized from T3LHyp with C14orf149. The reaction mixture (10 mL) consisted of 50 mM potassium phosphate buffer (pH 7.0) and 10 mM T3LHyp. After the addition of ~20 mg of purified C14orf149, the mixture was left at 30 °C overnight. For enzymatic synthesis of Pip2C and Pyr4SH2C, a reaction mixture (10 mL) consisting of 50 mM Tris-HCl buffer (pH 9.0), 10 mM d-pipecolate (for Pip2C) or T4DHyp (for Pyr4SH2C), 0.02 mM phenazine methosulfate (PMS), and ~20 mg purified AbLhpBEF (C4DHypDH) was incubated with shaking at 30 °C overnight in the dark. Pyr2C, Pip2C, and Pyr4SH2C in each reaction mixture were purified using a Dowex 1 × 2 Cl⁻ form (100–200 mesh) resin column, described previously [11].

4.4. Bacterial strain, culture conditions and preparation of cell-free extracts

A. brasilense ATCC29145 and P. aeruginosa PAO1 were cultured aerobically with vigorous shaking at 30 °C in minimal medium [7] supplemented with 30 mM carbon source. C. psychrerythraea 34H was grown at 8 °C in Marine Broth (Difco 2216). The grown cells were harvested by centrifugation at 30,000g for 20 min, suspended in 50 mM Tris-HCl (pH 8.0), and disrupted by sonication for 20 min at appropriate intervals on ice using Ultra Sonic Disruptor Model UR-200P (TOMY SEIKO Co., Ltd., Tokyo, Japan) and then centrifuged at 108,000g for 20 min at 4 °C to obtain cell-free extracts.

4.5. Plasmid construction for expression of recombinant proteins

Primer sequences used in this study are shown in Table S1. In this report, the prefixes Ab (A. brasilense), Pa (P. aeruginosa), Cp (C. psychrerythraea) and Pp (P. putida) have been added to gene symbols or protein designations when required for clarity. PaLhpD (PA1252), CplhpH (CPS_1453), and CplhpI genes (CPS_1455) were amplified by PCR using primers containing appropriate restriction enzyme sites at the 5’- and 3’-ends and genome DNA of P. aeruginosa or C. psychrerythraea as a template. C14orf149 gene was obtained from Human cDNA clone AK058165 (NITIE Biological Resource Center (NBRC), Chiba, Japan). AbLhpH and AbLhpI genes were amplified by PCR using primers designed from putative proline racemase (Bcep18194_181660) and OCD genes (Bcep18194_181663) from B. lata and genome DNA of A. brasilense as a template, and the amplified products were sequenced. The nucleotide sequences of AbLhpH and AbLhpI genes were submitted to GenBank with accession numbers GenBank: AB894494 and GenBank: AB894495, respectively.

Each amplified DNA fragment was introduced into BamHI-HindIII sites (for PaLhpD, AbLhpH, AbLhpI and CplhpI genes) or BamHI-PstI (for C14orf149 and CplhpI genes) in pETDuet−1 (Novagen), a plasmid vector for conferring N-terminal His₆-tag on expressed proteins, to obtain pET/PaLhpD, pET/AbLhpH, pET/AbLhpI, pET/CplhpH, pET/CplhpI, and pET/C14orf149. The five former and pET/C14orf149 were transformed into E. coli strains BL21(DE3) and BL21(DE3)-RIL (Novagen), respectively.

4.6. Expression and purification of His₆-tagged recombinant proteins

E. coli harboring the expression plasmid for His₆-tagged proteins was grown at 37 °C to a turbidity of 0.6 to 600 nm in Super broth medium (pH 7.0, 12 g tryptone, 24 g yeast extract, 5 mL glycercol, 3.81 g KH₂PO₄, and 12.5 g K₂HPO₄ per liter) containing 50 mg/liter ampicillin. After the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the culture was further grown for 6 h at 37 °C (for C14orf149 and AbLhpH) or for 18 h at 18 °C (for PaLhpD, AbLhpI, CplhpH and CplhpI) to induce the expression of His₆-tagged protein. Cells were harvested and resuspended in Buffer A (50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole). The cells were then disrupted by sonication, and the solution was centrifuged. The supernatant was loaded onto a Ni-NTA Superflow column (Qiagen) equilibrated with Buffer A linked to the BioAssist eZ system (TOSOH). The column was washed with Buffer B (50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 10% (v/v) glycerol, and 50 mM imidazole). The enzymes were then eluted with Buffer C (pH 8.0, Buffer B containing 250 mM imidazole instead of 50 mM imidazole), concentrated by ultrafiltration with Centriplus YM-30 (Millipore), dialyzed against 50 mM Tris–HCl buffer (pH 8.0) containing 50% (v/v) glycerol, and stored at −35 °C until use.

The native molecular mass of recombinant proteins was estimated by gel filtration, which was carried out using HPLC with a Multi-Station LC-8020 model II system (TOSOH) at a flow rate of 1 mL min⁻¹. The purified enzyme (~10 mg mL⁻¹) was loaded onto a TSKgel G3000SWXL column (TOSOH) equilibrated with 50 mM Tris–HCl buffer (pH 8.0). A high molecular weight gel filtration calibration kit (GE Healthcare) was used as a molecular marker.

4.7. Enzyme assay

All enzyme assays were performed at 30 °C. T3LHyp dehydratase was assayed spectrophotometrically in the coupling system with PaLhpD (NADPH-dependent Pyr2C reductase) using a Shimadzu UV-1800 spectrophotometer (Shimadzu GLC Ltd., Tokyo, Japan). The reaction mixture consisted of 50 mM
Tris–HCl (pH 8.0), 0.15 mM NADPH and 10 μg purified PaLhpD. The reaction was started by the addition of 100 mM T3LHyp (100 μL) with a final reaction volume of 1 mL. One unit of enzyme activity refers to 1 μmol NADPH produced/min. \( k_m \) and \( k_{cat} \) values were calculated by a Lineweaver–Burk plot. The enzyme was alternatively assayed by the colorimetric method based on the reaction of 2-aminobenzaldehyde with Pyr2C, which yields a yellow reaction product [8]. This method was used for the determination of optimum pH for the activity.

Pyr2C/Pip2C reductase was assayed routinely in the direction of Pyr2C reduction by measuring the oxidation of NAD(P)H at 340 nm. The standard assay mixture contained 10 mM Pyr2C (or Pip2C) in 50 mM potassium phosphate (pH 6.5 for AbLhp and CpLhp, or pH 7.0 for PaLhp) buffer. The reactions were started by the addition of 100 μL of a 1.5 mM NAD(P)H solution to a final volume of 1 mL. To assay the reverse reaction, the reaction mixture consisted of 50 mM Glycine–NaOH (pH 10.0 for PaLhpD or pH 10.5 for AbLhp and CpLhp) and 10 mM l-proline (or l-pipeolate). The reaction was started by the addition of 15 mM NAD(P)H (100 μL) with a final reaction volume of 1 mL. One unit of enzyme activity refers to 1 μmol NAD(P)H produced/min. Potential ODC activity in LhpI was assayed by the method described previously [23].

T4LHyp epimerase, C4DHypDH, Pyr4RH2C deaminase, and αKCSA dehydrogenase, involved in T4LHyp metabolism, were assayed by the method described previously [7]. If necessary, recombinant AbLhpBEF [26] was used as a coupling enzyme of the epimerase assay as C4DHypDH, instead of recombinant PaLhpBEF.

### 4.8. Reaction product analysis

Purified AbLhpH and AbLhpC or CpLhpH and CpLhpC (each 10 μg) were added to 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM T3LHyp and 0.15 mM NADPH (1 mL). After incubation at 30 °C overnight, each enzyme product was then analyzed by Hitachi L-8900 amino acid analyzer (Tokyo, Japan), using ion exchange chromatography followed by post-column derivatization with ninhydrin. Retention times of T3LHyp and l-proline (potential product) were appropriately 8.6 and 33 min, respectively.

### 4.9. Zymogram staining analysis

Purified PaLhpD, AbLhpH and CpLhpH were separated at 4 °C on non-denaturing PAGE with 10% (w/v) gel, which was performed by omitting SDS and 2-mercaptoethanol from the solution used in SDS–PAGE. The gels were then soaked in 10 mL staining solution consisting of 50 mM Glycine–NaOH (pH 10), 0.25 mM nitroblue tetrazolium (NBT), 0.06 mM PMS, 10 mM substrate (l-proline or l-pipeolate), and 15 mM NAD(P)⁺ at room temperature for 15 min in the dark. Dehydrogenase activity appeared as a dark blue band.

### 4.10. Northern blot analysis

*A. brasilense* cells were cultured at 30 °C to the mid-log phase (OD600 = 0.6–0.8) in minimal medium containing 30 mM carbon source, and harvested by centrifugation. Total RNA preparation was isolated using the SV Total RNA Isolation Kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The isolated RNA (4 μg) was subjected to electrophoresis on 1.2% (w/v) agarose gel containing 0.66 M formaldehyde, and hybridized to Blot-bond–N (GE Healthcare) by capillary transfer using 10× SSC as a transfer buffer (1× SSC is 15 mM sodium citrate (pH 7.0), and 0.15 M NaCl). The blotted filter was cross-linked in a UV cross-linker CX-2000 (Ultra-Violet Products, Ltd.). A double-stranded probe DNA was labeled with digoxigenin-11-dUTP and hybridized using a DIG–High Prime DNA labeling and detection starter kit (Roche Applied Science). Membrane was visualized using a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate reagent detection system (Roche Applied Science).

### 4.11. Target disruption of AbLhpI gene

The Tn5-derived SacII 1.3-kbp kanamycin resistance (Km⁺) cassette was amplified by PCR using PUC4K (GE Healthcare) as a template and two primers P13 and P14 (Table S1), and inserted into the single SacII site in the coding sequence of AbLhpI gene of pET/AbLhp to yield pLhpI::Km. To introduce the restriction site for MfeI at the 5’- and 3’-end of the DNA fragment containing the Km⁺ gene in the AbLhpI gene, PCR was carried out using pLhpI::Km as a template and two primers P15 and P16. The 2.2-kbp MfeI DNA fragment was subcloned into EcoRI site in a chloramphenicol resistance (Cam⁺) cassette of the suicide vector pSUP202 [32] to yield pSUP/LhpI::Km. *E. coli* S17-1 [32] was transformed with pSUP/LhpI::Km, and then the transformant was further mobilized to A. brasilense by biparental mating. The transconjugants were selected on a minimal medium agar plate supplemented with 5 g sodium malate and 25 μg kanamycin per liter using Km⁺ (the presence of Km⁺ cassette) and Tc⁺ (loss of pSUP202) phenotypes. The construction was confirmed by genomic PCR.

### 4.12. Amino acid sequence alignment and phylogenetic analysis

Protein sequences were analyzed using the Protein-BLAST and Clustal W program distributed by DDBJ (DNA Data Bank of Japan) (www.ddbj.nig.ac.jp). The phylogenetic tree was produced using the TreeView 1.6.1. program.

### 5. Database

Nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession number(s) GenBank: AB894494 and GenBank: AB894495 for T3LHyp dehydratase and Pyr2C reductase genes from *A. brasilense*.

### Acknowledgements

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan (25440049) (to S.W.), the A-STEP feasibility study program (AS242Z00554M) from the Japan Science and Technology Agency (JST) (to S.W.), and Hokuto Foundation for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan (25440049) (to S.W.), and Hokuto Foundation for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan (25440049) (to S.W.).

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2014.02.010.

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