ChemPubSoc Europe

DOI: 10.1002/cctc.201501170

CHEMCATCHEM Communications

A Novel (*R*)-Imine Reductase from *Paenibacillus lactis* for Asymmetric Reduction of 3*H*-Indoles

Hao Li, Guang-Xiang Zhang[+], Liu-Mei Li[+], Yu-Shi Ou[+], Ming-Yang Wang[+], Chun-Xiu Li, Gao-Wei Zheng,* and Jian-He Xu*[a]

A novel (*R*)-imine reductase (*Pl*RIR) from *Paenibacillus lactis* was heterologously overexpressed in *Escherichia coli*, purified and characterized. The purified *Pl*RIR exhibited relatively high catalytic efficiency ($k_{cat}/K_m = 1.58\ s^{-1}\ mM^{-1}$) towards 2,3,3-trimethylindolenine. A panel of 3*H*-indoles and 3*H*-indole iodides were reduced by *Pl*RIR to yield the corresponding products with good-to-excellent enantioselectivity (66–98% *ee*). In addition, *Pl*RIR also possesses good activities toward other types of imines such as pyrroline, tetrahydropyridine, and dihydroisoquinoline, indicating a reasonably broad substrate acceptance. In a 100 mg scale preparative reaction, 100 mM 2,3,3-trimethylindolenine was converted efficiently to afford (*R*)-2,3,3-trimethylindoline with 96% *ee* and 81% yield.

Chiral amines represent the core structure of numerous natural alkaloids, pharmaceuticals, and agrochemicals because of their exquisite biological activity, and thus are increasingly in demand in organic synthesis.[1] Traditionally, chiral amines are synthesized by kinetic resolution of a racemate by using a chiral acid as the resolving agent.[2] Compared with kinetic resolution, which has a maximum possible yield of 50%, asymmetric synthesis by chemical approaches has attracted considerable attention in recent years because of high atom efficiency, and includes asymmetric reduction of enamides, reductive amination of ketones, and asymmetric reduction of imines.[3] As an alternative method, biocatalysis has attracted attention for the preparation of chiral amines because of the high stereoselectivity and environmental friendliness of bioprocesses.[4] Despite the significant development of biocatalytic approaches,[5] there is strong interest in the asymmetric reduction of imines by using imine reductases (IREDs), which afford the product in 100% yield and with 100% *ee*.

IREDs, a rapidly emerging class of enzymes, catalyze the asymmetric hydrogenation of the prochiral C=N bond of imines (especially cyclic imines) to form chiral amine compounds.[6] Asymmetric reduction of aryl imines and β-carboline imines has been performed with *Candida parapsilosis* ATCC 7330 and *Saccharomyces bayanus*, respectively.[7] However, the two enzymes responsible for the reduction reactions have not been isolated, purified, and further studied. Recently, the Mitsukura group identified two useful wild-type strains from their stock microorganisms with *R*-selective (*Streptomyces* sp. GF 3587) and *S*-selective (*Streptomyces* sp. GF 3546) IRED activities by using 2-methyl-1-pyrroline (2-MPN) as the screening substrate.[8] Subsequently, both *R*- and *S*-selective IREDs were purified and characterized, and their DNA sequences were also reported.[9] Turner and co-workers overexpressed these two IREDs in *Escherichia coli*, and expanded their substrate scope to other cyclic imines, such as tetrahydropyridines, dihydroisoquinolines, and β-carboline imines.[10] Furthermore, the ability of IREDs to directly catalyze the reductive amination of ketones was demonstrated, even though the conversion was unusually low.[11] The X-ray crystal structures of *R*-selective and *S*-selective IREDs have been solved and revealed the active-site residues (Asp or Tyr).[11a,12]

Because of the tremendous potential in organic synthesis, the exploration of novel IREDs is very attractive. Hauer and co-workers constructed several IREDs by sequence alignment and characterized three representative IREDs.[13] Both reductive and oxidative activities of three other IREDs towards imines and amines were examined by the Höhne group.[14] Iding and co-workers identified 20 new IREDs by C-terminal domain clustering, further expanding the IRED toolbox.[15] Nonetheless, the exploration of novel IREDs with both a broad substrate scope and high enantioselectivity remains highly desirable for the synthesis of chiral amines.

In our previous work, an *S*-selective IRED from *Paenibacillus lactis* (*Pl*SIR) was identified, and exhibited high activity and excellent enantioselectivity in the asymmetric reduction of 3*H*-indoles, yielding a series of (*S*)-chiral indolines.[16] 3*H*-indoles are a class of cyclic imine reported frequently in asymmetric reductions promoted by chemical catalysts.[17] Herein, an *R*-selective IRED from *P. lactis* (*Pl*RIR) was identified through a genome data-mining approach by using 2,3,3-trimethylindolenine as the model substrate (Scheme 1). The biochemical properties and substrate scope of this novel enzyme were investigated in detail. Furthermore, the enzymatic synthesis of (*R*)-2,3,3-trimethyl indoline was performed at a preparative scale to verify the practical capacity of *Pl*RIR.

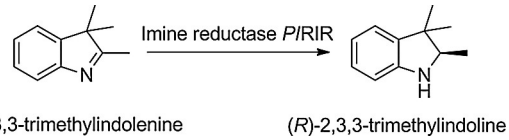
The *Pl*RIR-encoding gene (WP_007130043.1) inserted into plasmid pET-28a was successfully overexpressed in *E. coli* BL21 (DE3). The target protein was predominantly in the soluble

[a] H. Li, G.-X. Zhang,[+] L.-M. Li,[+] Y.-S. Ou,[+] M.-Y. Wang,[+] Dr. C.-X. Li, Dr. G.-W. Zheng, Prof. Dr. J.-H. Xu
State Key Laboratory of Bioreactor Engineering
Shanghai Collaborative Innovation Center for Biomanufacturing
East China University of Science and Technology
130 Meilong Road, Shanghai 200237 (P.R. China)
E-mail: gaoweizheng@ecust.edu.cn
jianhexu@ecust.edu.cn

[+] These authors contributed equally to this work.

Supporting Information for this article is available on the WWW under http://dx.doi.org/10.1002/cctc.201501170.

© 2016 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 1. Asymmetric reduction of 2,3,3-trimethylindolenine by imine reductase *Pl*RIR.

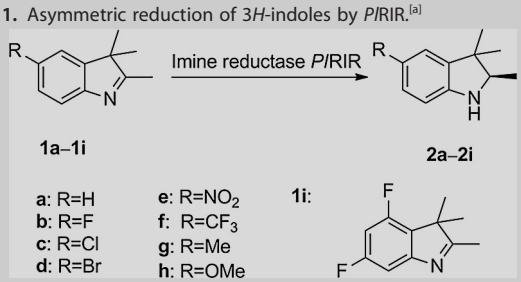
fraction. The N-terminal His-tagged *Pl*RIR was purified by Ni affinity chromatography and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S1 in the Supporting Information), indicating a subunit size of approximately 35 kDa. *Pl*RIR was shown to be a novel IRED because it shares only 35, 40, and 40% identities, respectively, with the reported *R*-selective IRED from *Streptomyces* sp. GF 3587, *S*-selective IRED from *Streptomyces* sp. GF 3546, and the above-mentioned *Pl*SIR from *P. lactis*.

The intrinsic properties of *Pl*RIR were characterized. The effects of temperature (20–60 °C) and pH (3.0–11.0) on *Pl*RIR activity are shown in Figure S2. *Pl*RIR showed the highest activity at 50 °C and 64% relative activity at 30 °C. The highest activity was obtained in potassium phosphate (KPB) buffer at pH 6.0, and was more than 2-fold higher than that in citrate buffer at the same pH. However, poor activity was observed at pH 8.0–9.0 in Tris-HCl buffer, and the activity was negligible at pH values <4.0 or >9.0. The kinetic parameters of purified *Pl*RIR for 2,3,3-trimethylindolenine were determined at 30 °C and pH 6.0 (Table S1). *Pl*RIR gave a K_m of 0.26 mM and a k_{cat} of 0.41 s^{-1}, resulting in a catalytic efficiency (k_{cat}/K_m) of 1.58 s^{-1} mM^{-1}. An extremely low K_m (merely 5.6 μM) was observed with the co-factor NADPH, indicating a very high affinity of *Pl*RIR towards NADPH. The thermostability of *Pl*RIR (Figure S3) showed half-lives of 165, 99, and 1.7 h, respectively, at 30, 40, and 50 °C, indicating that the enzyme has good stability, especially under mild reaction conditions. The influence of metal ions and ethylenediaminetetraacetic acid (EDTA) on *Pl*RIR activity is presented in Table S2. Among the ten metal ions and EDTA tested, no chemical was found to activate the enzyme significantly. In contrast, Ni^{2+} and Cu^{2+} ions showed modest inhibition of *Pl*RIR.

Subsequently, the specific activity and stereoselectivity of *Pl*RIR towards other 3*H*-indoles were investigated and are summarized in Table 1. *Pl*RIR showed good reduction activity towards substrates **1b**–**1e** and **1g**, and afforded >95% conversions. For substrates **1f**, **1h**, and **1i**, *Pl*RIR exhibited a moderate reactivity. Good-to-excellent enantiomeric excess (87–98%) was achieved for the majority of substrates. In all cases, the products obtained from the bioreduction were *R*-configuration, confirming the *R*-selectivity of *Pl*RIR, in contrast to the (*S*)-enantiomeric preference of *Pl*SIR.

The ability of *Pl*RIR to reduce iminium salts was also studied (Table 2). *Pl*RIR exhibited much higher specific activities toward 3*H*-indole iodides **3a**–**3f** than toward 3*H*-indoles, in line with the results of our previous study on *Pl*SIR.[16] All substrates, including bulky imines such as **3f**, could be completely reduced by *Pl*RIR into chiral amines with 82–98% *ee*. Furthermore, a pronounced improvement in the stereoselectivity was observed

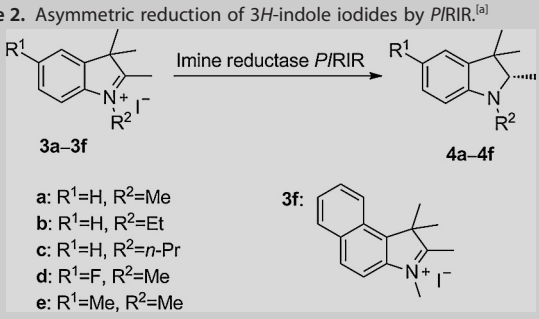
Table 1. Asymmetric reduction of 3*H*-indoles by *Pl*RIR.[a]



Entry	Substrate	Specific activity [U mg^{-1}][b]	Conversion [%][c]	*ee* [%][c]
1	**1a**	0.445	>99	96 (*R*)
2	**1b**	0.104	99	90 (*R*)
3	**1c**	0.151	99	96 (*R*)
4	**1d**	0.134	98	98 (*R*)
5	**1e**	0.035	96	83 (*R*)
6	**1f**	0.030	71	91 (*R*)
7	**1g**	0.126	97	96 (*R*)
8	**1h**	0.038	65	87 (*R*)
9	**1i**	0.118	82	66 (*R*)

[a] The reaction mixture (1 mL), comprising 10 mM 3*H*-indole substrate, 2 mg *Pl*RIR cell-free extract, 1 U *Bm*GDH (glucose dehydrogenase), 0.1 mM $NADP^+$, 50 mM glucose, 1% (v/v) DMSO, and 100 mM potassium phosphate buffer (pH 7.0), was shaken at 30 °C for 6 h. [b] Specific activity was determined at 30 °C and pH 7.0 under standard conditions using purified enzyme. [c] Conversion and enantiomeric excess were determined by chiral HPLC, and the absolute configuration of the products was assigned by comparing the HPLC peaks with those formed in the reduction catalyzed by *Pl*SIR.[16]

Table 2. Asymmetric reduction of 3*H*-indole iodides by *Pl*RIR.[a]



Entry	Substrate	Specific activity [U mg^{-1}][b]	Conversion [%][c]	*ee* [%][c]
1	**3a**	1.18	>99	84 (*S*)
2	**3b**	1.64	>99	92 (*S*)
3	**3c**	1.23	>99	98 (*S*)
4	**3d**	1.44	>99	89 (*S*)
5	**3e**	0.375	>99	90 (*S*)
6	**3f**	0.118	>99	82 (–)

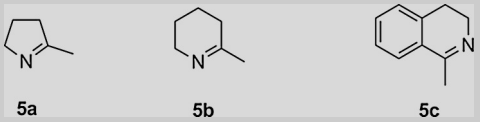
[a] The reaction mixture (1 mL), comprising 10 mM 3*H*-indole substrate, 2 mg *Pl*RIR cell-free extract, 1 U *Bm*GDH, 0.1 mM $NADP^+$, 50 mM glucose, 1% (v/v) DMSO, and 100 mM potassium phosphate buffer (pH 7.0), was shaken at 30 °C for 6 h. [b] Specific activity was determined at 30 °C and pH 7.0 under standard conditions using purified enzyme. [c] Conversion and enantiomeric excess were determined by chiral HPLC or GC. The absolute configuration of the products was assigned by comparing the LC/GC peaks with those of *Pl*SIR.[16]

© 2016 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

with the increase of the size of the substituent group on the N atom (Table 2, entries 1–3), and the highest (98%) *ee* value was obtained for substrate **3c**, which has the largest substituent on the N atom. Interestingly, tertiary amines of a contrary (*S*)-configuration were formed, indicating a peculiar property of *Pl*IRIR.

In addition to indole imines, the activity and stereoselectivity of *Pl*IRIR towards other types of imine substrates were measured (Table 3). *Pl*IRIR also showed good activity towards 2-methyl-1-pyrroline (**5a**), 6-methyl-2,3,4,5-tetrahydropyridine (**5b**), and 1-methyl-3,4-dihydroisoquinoline (**5c**), giving 0.074, 0.88 and 0.14 U mg^{-1} protein, respectively. These results demonstrated that *Pl*IRIR possesses a broad substrate acceptance scope. The three corresponding products formed in the bioreductions are (*S*)-configuration with 93–99% *ee*, which is similar to previously reported *S*-selective IREDs.[10a]

Table 3. Activity and stereoselectivity of *Pl*IRIR towards other types of imines.[a]



Entry	Substrate	Specific activity [U mg^{-1}][b]	Conversion [%][c]	*ee* [%][c]
1	**5a**	0.074	74	93 (*S*)
2	**5b**	0.88	>99	96 (*S*)
3	**5c**	0.14	98	99 (*S*)

[a] The reaction mixture (1 mL), comprising 10 mM 3*H*-indole substrate, 2 mg *Pl*IRIR cell-free extract, 1 U *Bm*GDH, 0.1 mM NADP$^+$, 50 mM glucose, 1% (v/v) DMSO, and 100 mM potassium phosphate buffer (pH 7.0), was shaken at 30 °C for 6 h. [b] Specific activity was determined at 30 °C and pH 7.0 under standard conditions using purified enzyme. [c] Conversion and enantiomeric excess were determined by chiral HPLC or GC.

To evaluate the practical capability of *Pl*IRIR for synthetic reactions, the bioreduction of 2,3,3-trimethylindolenine was performed on a 100 mg scale (100 mM substrate dissolved in 10 mL reaction volume). The reaction mixture was composed of 1 mmol 2,3,3-trimethylindolenine, 1.5 mmol glucose, 1 μmol NADP$^+$, 0.5 g wet *E. coli* cells containing *Pl*IRIR, 10 mg cell-free extract of *Bm*GDH (100 U), 0.5 mL DMSO, and 9.5 mL potassium phosphate buffer (100 mM, pH 7.0). The substrate was almost completely reduced into (*R*)-2,3,3-trimethylindoline (>99% conversion) within 15 h. After treatment according to the protocol described in the Experimental Section, (*R*)-2,3,3-trimethylindoline (yellow oil, 130 mg) was isolated in 81% yield and with 96% *ee*.

In summary, a novel (*R*)-IRED, *Pl*IRIR, from *P. lactis* has been identified and characterized. This enzyme exhibited high activity and good-to-excellent enantioselectivity towards various 3*H*-indoles, 3*H*-indole iodides, and other types of imines. Moreover, (*R*)-2,3,3-trimethylindoline was facilely synthesized on a preparative scale by using the novel (*R*)-IRED, affording the product in 81% yield and with 96% *ee*. Ongoing work is focused on the engineering of *Pl*IRIR to identify more-active IRED variants with higher activity and broader substrate scope.

Experimental Section

Enzyme activity was assayed by monitoring the decrease in the absorbance of NADPH ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$) at 340 nm by using a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NADPH per minute. The standard assay mixture (1 mL) contained 2 mM substrate, 0.1 mM NADPH, 100 mM potassium phosphate buffer (pH 7.0), and an appropriate amount of enzyme sample. For 2-methyl-1-pyrroline and 6-methyl-2,3,4,5-tetrahydropyridine, the substrate concentration was adjusted to 10 mM.

The reaction mixture (1 mL), comprising 3*H*-indole substrate (10 mM), *Pl*IRIR cell-free extract (2 mg), *Bm*GDH (1 U), NADP$^+$ (0.1 mM), glucose (50 mM), 1% (v/v) DMSO, and potassium phosphate buffer (100 mM, pH 7.0), was shaken at 30 °C for 6 h. The resultant reaction mixture was adjusted to pH 13 by addition of 2 M NaOH, and extracted with ethyl acetate. The extract was analyzed by HPLC or GC on a chiral column to determine the enantiomeric excess of the product.

The preparative reaction mixture was composed of 2,3,3-trimethylindolenine (1 mmol), glucose (1.5 mmol), NADP$^+$ (1 μmol), wet *E. coli* cells containing *Pl*IRIR (0.5 g), 10 mg cell-free extract of *Bm*GDH (100 U), DMSO (0.5 mL) and potassium phosphate buffer (9.5 mL, 100 mM, pH 7.0). The reaction was performed at 30 °C and the pH was adjusted to 7.0 by titration with a 1 M NaOH solution. After 15 h, the reaction was terminated by addition of NaOH (0.5 mL, 2 M), and the mixture was extracted with ethyl acetate. The organic phase was dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was further purified by column chromatography eluting with petroleum ether and ethyl acetate (10:1) to obtain pure (*R*)-2,3,3-trimethylindoline.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (No. 31200050, 21472045 and 21536004) and the Fundamental Research Funds for the Central Universities (22A201514043).

Keywords: asymmetric reduction · biocatalysis · imine reductase · indoles · indolines

[1] D. Ghislieri, N. J. Turner, *Top. Catal.* **2014**, *57*, 284–300.
[2] M. Höhne, U. T. Bornscheuer, *ChemCatChem* **2009**, *1*, 42–51.
[3] T. C. Nugent, M. El-Shazly, *Adv. Synth. Catal.* **2010**, *352*, 753–819.
[4] a) G. W. Zheng, J. H. Xu, *Curr. Opin. Biotechnol.* **2011**, *22*, 784–792; b) Y. Ni, J. H. Xu, *Biotechnol. Adv.* **2012**, *30*, 1279–1288.
[5] H. Kohls, F. Steffen-Munsberg, M. Höhne, *Curr. Opin. Chem. Biol.* **2011**, *22*, 784–792.
[6] a) D. Gamenara, P. D. de María, *Org. Biomol. Chem.* **2014**, *12*, 2989–2992; b) J. H. Schrittwieser, S. Velikogne, W. Kroutil, *Adv. Synth. Catal.* **2015**, *357*, 1655–1685.
[7] a) T. Vaijayanthi, A. Chadha, *Tetrahedron: Asymmetry* **2008**, *19*, 93–96; b) M. Espinoza-Moraga, T. Petta, M. Vasquez-Vasquez, V. F. Laurie, L. A. B. Moraes, L. S. Santos, *Tetrahedron: Asymmetry* **2010**, *21*, 1988–1992.
[8] K. Mitsukura, M. Suzuki, K. Tada, T. Yoshida, T. Nagasawa, *Org. Biomol. Chem.* **2010**, *8*, 4533–4535.
[9] a) K. Mitsukura, M. Suzuki, S. Shinoda, T. Kuramoto, T. Yoshida, T. Nagasawa, *Biosci. Biotechnol. Biochem.* **2011**, *75*, 1778–1782; b) K. Mitsukura, T. Kuramoto, T. Yoshida, N. Kimoto, H. Yamamoto, T. Nagasawa, *Appl. Microbiol. Biotechnol.* **2013**, *97*, 8079–8086.

© 2016 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[10] a) F. Leipold, S. Hussain, D. Ghislieri, N. J. Turner, *ChemCatChem* **2013**, *5*, 3505 – 3508; b) S. Hussain, F. Leipold, H. Man, E. Wells, S. P. France, K. R. Mulholland, G. Grogan, N. J. Turner, *ChemCatChem* **2015**, *7*, 579 – 583.

[11] a) T. Huber, L. Schneider, A. Präg, S. Gerhardt, O. Einsle, M. Müller, *ChemCatChem* **2014**, *6*, 2248 – 2252; b) P. N. Scheller, M. Lenz, S. C. Hammer, B. Hauer, B. M. Nestl, *ChemCatChem* **2015**, *7*, 3239 – 3242.

[12] a) M. Rodríguez-Mata, A. Frank, E. Wells, F. Leipold, N. J. Turner, S. Hart, J. P. Turkenburg, G. Grogan, *ChemBioChem* **2013**, *14*, 1372 – 1379; b) H. Man, E. Wells, S. Hussain, F. Leipold, S. Hart, J. P. Turkenburg, N. J. Turner, G. Grogan, *ChemBioChem* **2015**, *16*, 1052 – 1059.

[13] P. N. Scheller, S. Fademrecht, S. Hofelzer, J. Pleiss, F. Leipold, N. J. Turner, B. M. Nestl, B. Hauer, *ChemBioChem* **2014**, *15*, 2201 – 2204.

[14] M. Gand, H. Müller, R. Wardenga, M. Höhne, *J. Mol. Catal. B* **2014**, *110*, 126 – 132.

[15] D. Wetzl, M. Berrera, N. Sandon, D. Fishlock, M. Ebeling, M. Müller, S. Hanlon, B. Wirz, H. Iding, *ChemBioChem* **2015**, *16*, 1749 – 1756.

[16] H. Li, Z. J. Luan, G. W. Zheng, J. H. Xu, *Adv. Synth. Catal.* **2015**, *357*, 1692 – 1696.

[17] a) G. X. Zhu, X. M. Zhang, *Tetrahedron: Asymmetry* **1998**, *9*, 2415 – 2418; b) R. Giernoth, M. S. Krumm, *Adv. Synth. Catal.* **2004**, *346*, 989 – 992; c) D. Liu, W. G. Li, X. M. Zhang, *Tetrahedron: Asymmetry* **2004**, *15*, 2181 – 2184; d) L. Q. Qiu, F. Y. Kwong, J. Wu, W. H. Lam, S. S. Chan, W. Y. Yu, Y. M. Li, R. W. Guo, Z. Y. Zhou, A. S. C. Chan, *J. Am. Chem. Soc.* **2006**, *128*, 5955 – 5965; e) J. W. Faller, S. C. Milheiro, J. Parr, *J. Organomet. Chem.* **2006**, *691*, 4945 – 4955; f) M. Rueping, C. Brinkmann, A. P. Antonchick, I. Atodiresei, *Org. Lett.* **2010**, *12*, 4604 – 4607; g) A. M. Kluwer, R. J. Detz, Z. Abiri, A. M. Burg, J. N. H. Reek, *Adv. Synth. Catal.* **2012**, *354*, 89 – 95; h) I. Podolean, C. Hardacre, P. Goodrich, N. Brun, R. Backov, S. M. Coman, V. I. Parvulescu, *Catal. Today* **2013**, *200*, 63 – 73; i) A. D. Lackner, A. V. Samant, F. D. Toste, *J. Am. Chem. Soc.* **2013**, *135*, 14090 – 14093.

Received: October 28, 2015
Published online on January 26, 2016

© 2016 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim