Journal of Molecular Catalysis B: Enzymatic 60 (2009) 93–95

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Letter

Asymmetric reduction of a ketone by knockout mutants of a cyanobacterium

ARTICLE INFO

Keywords:
Asymmetric reduction
Knockout mutant
Stereochemical control
Cyanobacteria
Synechocystis sp. PCC 6803

ABSTRACT

Enantioselectivity in the asymmetric reduction of a ketone by cyanobacteria can be controlled by deleting an alcohol dehydrogenase gene from the microbe.

1. Introduction

Microbial reductions have been widely used for synthesis of chiral alcohols. However, the reductions could not always afford desired alcohols in excellent enantioselectivities and several methods such as screening of microbes, modification of reaction conditions, overexpression of alcohol dehydrogenase genes in *E. coli* have been developed to increase enantioselectivity [1]. Although these methods were effective for stereochemical control, they were not applied to the asymmetric reduction by microalgae until now.

Cyanobacteria play important roles on earth in carbon dioxide absorption as well as in utilization of solar energy by photosynthesis. Thus, the use of cyanobacteria as sustainable biocatalysts is of great significance from the standpoint of green chemistry.

However, algae, including cyanobacteria, are rarely used as biocatalysts because of lack of knowledge about how to control enantioselectivities in asymmetric reactions compared to other microbes [2]. Previously, we have reported that the selectivity of asymmetric reduction could be controlled by illumination [3,4] and now, we would like to report a novel method for controlling the stereoselectivity of reduction with cyanobacteria.

Our strategy is as follows. If a low enantioselectivity is due to the result of participating multiple active dehydrogenases (i.e. *R* and *S* enzymes), then deletion of a gene of the microbe will change stereoselectivity of the reduction. Hopefully, reduction by the mutant microbe will improve the enantioselectivity of the product alcohol.

Synechocystis sp. PCC 6803 was chosen as the microbe because all gene sequences of the microbe can be obtained from "cyanobase" [5] and we prepared several dehydrogenase-gene-deleted mutants and the effect of deletion on asymmetric reduction of ketones was investigated.

2. Experimental

Synechocystis sp. PCC 6803 Williams was used as the host microbe. Nine dehydrogenase and oxidoreductase genes, i.e. open reading frames (ORF's), shown in Table 1 were selected to be target genes. For example, *slr0942* was chosen as the target gene since the gene is classified as a gene for short chain alcohol dehydrogenase (ADH). It is known that several short chain ADHs could reduce artificial ketones and were used as catalysts for biotransformations [6]. Another gene, *slr0315*, was also chosen because the gene is categorized to 3-oxyacyl-(acyl-carrier protein) reductase.

Generation of insertion mutants. Mutants impaired in selected genes were generated by reverse genetics. The encoding sequences and neighboring sequences were amplified by PCR. The approximately 2 kb PCR products were cloned into pUC19 (TOYOBO). The primers for amplification were designed using the complete genome sequence of *Synechocystis* [7]. Sequences were selected which contained appropriate restriction sites to improve cloning of the fragments. The *aphII* gene [aminoglycoside phosphotransferase II conferring kanamycin (Km) resistance] isolated from plasmid pUC4K (Pharmacia) was inserted into unique restriction sites of the encoding sequences. Transformation of *Synechocystis* has been described previously [7]. Transformants were initially selected on a medium containing 10 μg Km ml^{-1} (Wako), whilst the segregation of clones was performed by numerous restreaking (at least three transfers) of primary clones on plates supplemented with 50 μg Km ml^{-1}. During the cultivation of mutants, 50 μg Km ml^{-1} was added to the liquid media. Deletion of *slr0942* gene and other genes was checked by PCR analysis using DNA of wild-type and the mutant cells as shown in Fig. 1.

Cultivation and reaction. The mutant microbe was cultivated under 25–40 μmol photon m^{-1} s^{-2} and for biotransformation, concentrations of the microbe was set to be OD_{730} = 0.6. The mutant microbes grew almost the same rate with the original microbe. The reaction was conducted by adding 10% solution in DMSO of *t*-butyl acetoacetate (1.4 mM) to the suspension of the microbe under 49 μmol photon m^{-1} s^{-2} of fluorescent light at 30 °C for 1 day. The reaction mixtures were analyzed by gas chromatography (GC); Column: Chirasil-DEX-CB, 25 m, Temp. 90 °C, He, 0.5 ml min^{-1}, *S*, 10.9 min, *R*, 11.7 min and the results are listed in Table 2.

3. Results and discussion

GC analysis of the reaction mixtures revealed that *slr0942* knockout mutant afforded the corresponding alcohol in 30% yield with 53% ee (*R*), while the wild-type microbe gave the alcohol in 32%

1381-1177/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2009.03.017

Table 1
Target genes in *Synechocystis* sp. PCC 6803 and homology of these genes.

ORF	Similar dehydrogenase
sll0990	Glutathione dehydrogenase
sll1825	Short-chain dehydrogenase/reductase
slr0315	3-Oxyacyl-(acyl-carrier protein) reductase
slr0506	NADPH-protochlorophyllide oxidoreductase
slr0886	3-Oxoacyl-[acyl-carrier protein] reductase
slr0942	Alcohol dehydrogenase; aldo-keto reductase
slr1192	Alcohol dehydrogenase
slr1994	Acetoacetyl-CoA reductase
slr2124	3-Oxoacyl-[acyl-carrier-protein] reductase

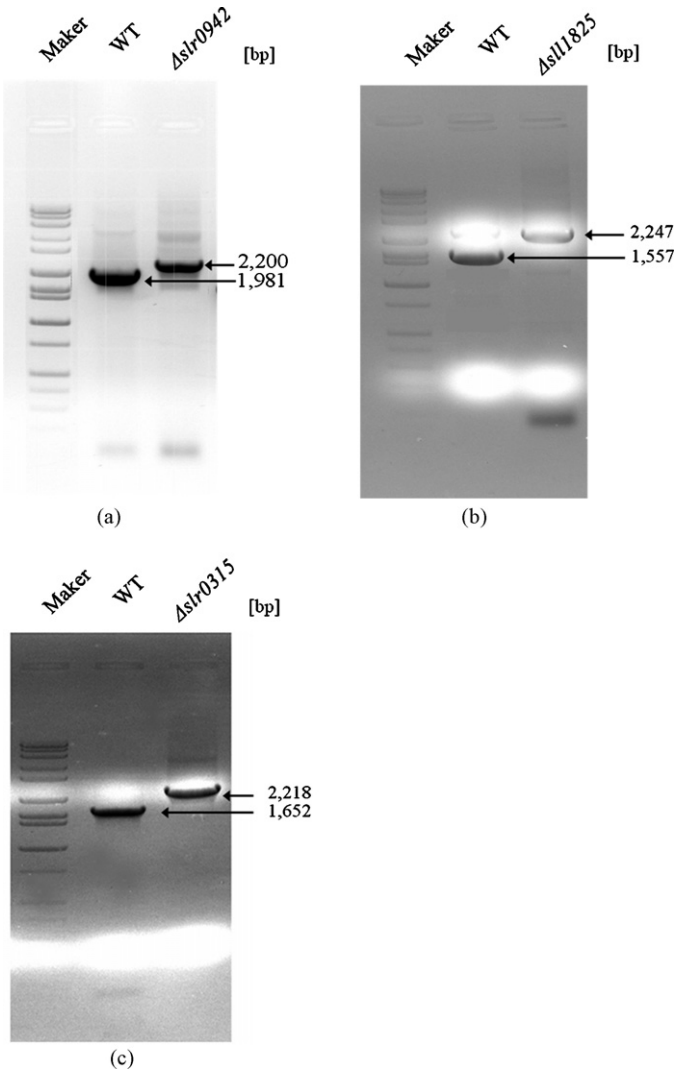
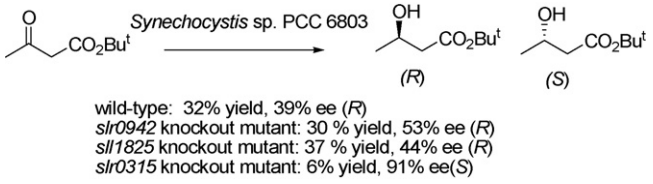


Fig. 1. Agarose-gel electrophoresis analyses of DNA extracted from wild-type and knockout mutant cells, each right lane showing deletion of (a) *slr0942*, (b) *sll1825*, and (c) *slr0315* respectively.

Table 2
Reduction of *t*-butyl acetoacetate with the mutants of *Synechocystis* sp. PCC 6803[a].

Strain[b]	Conv., %	ee, % (config.)
WT	32	39 (*R*)
Δ*slr094*	30	53 (*R*)
Δ*sll182*	37	44 (*R*)
Δ*slr0315*	6	91 (*S*)

[a] Reaction conditions were described in the experimental section.
[b] Deletion of the other genes cited in Table 1 gave similar results to the wild-type microbe. Delta means deletion of the gene.



Scheme 1.

yield with 39% ee (*R*). The other *sll1825* knockout mutant gave (*R*)-alcohol in 37% yield with 44% ee. Chemical yields of the product alcohols increased with the reaction time. The enantioselectivity, however, was not influenced by the reaction time for 24 h in each case. Another *slr0315* knockout mutant gave the antipode (*S*)-alcohol in 6% yield with 91% ee, although the reduction scarcely proceeded (Scheme 1).

Thus, control of stereoselectivities was observed to our surprise. Deletion of the *slr0942* and *sll1825* dehydrogenase genes from cyanobacteia increased the (*R*)-enantioselectivity and deletion of the *slr0315* oxidoreductase gene reversed the stereochemistry to give (*S*)-alcohol. Thus, a novel method for stereochemical control was developed. Until now, improvement of enantioselectivity in microalgae utilized asymmetric reduction was not reported except for that of using light [3,4].

Unfortunately, other knockout mutants did not affect the stereoselectivity of the reduction of *t*-butyl acetoacetate. Other substrates such as trifluoroacetophenone and pentafluoroacetophenone were also subjected to the reaction. However, the stereoselectivities of their reductions were scarcely changed by the use of the mutant microbes. Therefore, the enzymes encoded by *slr0942*, *sll1825* or *slr0315* most likely do not significantly participate in the reduction of these substrates. To improve this low enantioselectivity, preparation of multiple knockout mutants and use of selective inhibitor are necessary.

Although the effectiveness of this method for novel stereochemical control is restricted to a β-keto ester substrate, other knockout mutants of cyanobacteria will be applicable for increasing the enantioselectivities of other alcohol-generating microbial reductions.

4. Conclusion

We have developed a novel method for microalgae-mediated asymmetric reduction of ketones. The reduction with the *Synechocystis* sp. PCC 6803 *slr0942* and *sll1825* knockout mutants increased the enantioselectivity compared to the wild-type microbe, and the deletion of *slr0315* reversed the stereochemistry to give the antipode alcohol by reduction.

Further research on stereochemical control using knockout mutants are under investigation in our laboratories.

References

[1] K. Nakamura, T. Matsuda, in: V. Gotor, I. Alfonsa, E. Garcia-Uradiales (Eds.), Asymmetric Organic Synthesis with Enzymes, Wiley–VCH Verlag GmbH & Co. KGaA, Weinheim, 2008, pp. 193–228.
[2] K. Nakamura, in: T. Matsuda (Ed.), Future Directions in Biocatalysis, Elsevier, Amsterdam, 2007, pp. 51–58.
[3] K. Nakamura, R. Yamanaka, J. Chem. Soc. Chem. Commun. (2002) 1782–1783.
[4] K. Nakamura, R. Yamanaka, Tetrahedron: Asymm. 13 (2002) 2529–2533.
[5] Y. Nakamura, T. Kaneko, M. Hirosawa, N. Miyajima, S. Tabata, Nucleic Acids Res. 26 (1998) 63–67.
[6] Y. Nie, Y. Xu, X.Q. Mu, H.Y. Wang, M. Yang, R. Xiao, Appl. Environ. Microbiol. 73 (2007) 3759–3764.
[7] H. Ohta, Y. Shibata, Y. Haseyama, Y. Yoshino, T. Suzuki, I. Enami, M. Ikeuchi, Photosyn. Res. 91 (2007) 311.

Tetsuo Takemura[a,*], Kaori Akiyama[a], Nobuaki Umeno[a], Yukiko Tamai[a], Hisataka Ohta[b], Kaoru Nakamura[c]

[a] *Department of Chemistry, Faculty of Science, Tokyo University of Science, Kagurazaka 1-3, Shinjuku-ku, Tokyo 162-8601, Japan*

[b] *Department of Biology, Faculty of Science, Tokyo University of Science, Kagurazaka 1-3, Shinjuku-ku, Tokyo 162-8601, Japan*

[c] *Institute for Chemical Research, Kyoto University, Uji Kyoto 611-0011, Japan*

[*] Corresponding author. Tel.: +81 3 5228 8273; fax: +81 3 5261 4631.
E-mail address: ttakemur@rs.kagu.tus.ac.jp (T. Takemura)

5 January 2009
Available online 5 April 2009