Journal of Biochemistry and Molecular Biology, Vol. 32, No. 1, January 1999, pp. 72–75

JOURNAL OF
Biochemistry and Molecular Biology

© BSRK & Springer-Verlag 1999

The Function of *ery*BVII Gene is to Epimerize TDP-6-Deoxy-L-threo-D-glycero-4-hexulose in the Biosynthesis of Erythromycin A

Wonyoung Kim, Choonkeun Kim and Oksoo Han*

Department of Genetic Engineering, Institute of Biotechnology, Institute of Agricultural Science and Technology, and Hormone Research Center, Chonnam National University, Kwangju, 500–757, Korea

Received 21 September 1998, Accepted 4 November 1998

In an effort to understand the function of the *ery*BVII gene in the erythromycin biosynthetic gene cluster, we overexpressed the *ery*BVII gene in *E. coli* and TDP-6-deoxy-L-threo-D-glycero-4-hexulose was used as a substrate of the overexpressed EryBVII enzyme. The enzymatic reaction product was chemically modified by reduction and peracetylation. Structural analysis of the derivatized enzymatic products by GC-Mass Spectrophotometry indicated that TDP-6-deoxy-L-threo-D-glycero-4-hexulose could be converted into its epimer by EryBVII enzyme. Based on this result, TDP-6-deoxy-L-threo-D-glycero-4-hexulose was indeed the substrate of EryBVII enzyme and the function of the *ery*BVII gene was confirmed.

Keywords: Biosynthesis, Deoxysugar, Erythromycin, TDP-L-mycarose.

Introduction

Erythromycin A, a clinically valuable anti-infective drug, is produced by *Saccharopolyspora erythrea*. Structurally, it consists of a 14-membered macrolactone ring and two deoxysugars, L-cladinose and D-desosamine. Because of its medical and commercial importance, its biosynthetic genes have been extensively studied, including the genes of polyketide synthase (Cortes *et al.*, 1990; Jacobson *et al.*, 1997) and 6-deoxyerythronolide B hydroxylase (Haydock *et al.*, 1991; Cupp-Vickery *et al.*, 1996). The nucleotide sequences of some putative genes involved in the biosynthesis of the two deoxysugars have also been determined (Dhilson *et al.*, 1989; Summers *et al.*, 1997; Gaisser *et al.*, 1998; Sohng *et al.*, 1998). However, the biosynthetic pathway for the deoxysugars of erythromycin A is still not clearly understood. Based on the nucleotide sequences of *ery*B genes, the *ery*BVII gene seems to be involved in the biosynthesis of L-mycarose, a precursor of L-cladinose (GenBank Accession Number U77459). From the sequence homology of *ery*BVII gene to other sugar biosynthetic genes, the *ery*BVII gene could be involved in an epimerization of deoxysugars (Summers *et al.*, 1997; Gaisser *et al.*, 1998).

In an effort to identify the function of the *ery*BVII gene, we heterologously overexpressed the gene in *E. coli* and the crude enzyme was able to convert TDP-6-deoxy-L-threo-D-glycero-4-hexulose into the epimerized product. Based on this function of the *ery*BVII gene, the previously proposed biosynthetic pathway of TDP-L-mycarose (Summers *et al.*, 1997; Gaisser *et al.*, 1998) was confirmed.

Materials and Methods

Materials The recombinant plasmids containing the *ery*BVII gene cluster, pUC19 (orf13-orf20) and TDP-D-glucose dehydratase gene, pRSJ462-B, were generous gifts of Professors C. R. Hutchinson (University of Wisconsin, USA) and J. Yoo (Chosun University, Kwangju, Korea), respectively. *E. coli* DH5α MCR was used for cloning and expression of the *ery*BVII gene and *E. coli* BL21 (DE3) pLysS was used for expression of the TDP-D-glucose dehydratase gene. PCR kits (PreMix-Top) and sequencing kits (Sequenase version 2.0) were purchased from Bioneer (Taejeon, Korea) and United States Biochemicals (Cleveland, USA), respectively. QIAEX II Extraction kit was from QIAGEN (Dusseldorf, Germany). All other reagents were purchased from Amersham (Seoul, Korea) or Sigma (St. Louis, USA) and were of the highest grade available.

DNA manipulation and overexpression pUC19(orf13-orf20) containing *ery*BVII was digested with *Eco*RI. The resulting linear DNA was used as a template in the PCR reaction. The PCR reaction mixture contained 2 units of Taq polymerase, 1.5 mM $MgCl_2$, 1 mM dNTPs, template DNA (50 ng), and 2.5 μM of primers in a final volume of 20 μl. The sense primer used was

* To whom correspondence should be addressed.
Tel: 82-62-530-2163; Fax: 82-62-530-2169
E-mail: oshan@chonnam.chonnam.ac.kr

5′-CGT aag ctt CCC ATG GCT GGT GGT-3′ (24 mer). The unique restriction sites of *Hin*dIII (lower case) and *Nco*I (underlined) were placed and bases were changed according to a codon usage of *E. coli* to facilitate an expression in *E. coli*. The antisense primer used was 5′-AGA CGA TGT GGT TCA TCT-3′ (18 mer). The PCR reaction was run at 94°C for 1 min, 53°C for 2 min, 72°C for 1 min for 30 cycles, and a final extension at 72°C for 5 min. The PCR product was purified by the QIEAX II DNA extraction kit and digested with *Hin*dIII and *Pst*I. The resulting recombinant PCR fragment (148 bp) substituted 5′-end of *ery*BVII gene in pUC19 (orf13-orf20) and the DNA sequence of the PCR fragment was confirmed by the dideoxy sequencing method with a Sequenase version 2.0 sequencing kit. The resulting pUC19 (orf13-0rf20) containing the recombinant *ery*BVII gene was digested with *Nde*I/*Eco*RI and the *ery*BVII gene was inserted into the pTrc99A vector to produce pTrc(*ery*BVII), which was transformed into *E. coli* DH5αMCR. Transformed cells were grown in 50 ml of LB containing ampicillin (50 μg/ml) and IPTG (1 mM) at 30°C for 12 h with shaking (150 rpm). *E. coli* culture (50 ml) of the overexpressed EryBVII enzyme was centrifuged (6000 × *g*, 4°C, 15 min) and pellets were suspended in a minimal volume of buffer A (50 mM Tris, 1 mM EDTA, pH 7.5), and phenylmethanesulfonyl fluoride was added (0.2 mM). The resulting cell suspension was sonicated (0°C, 1 min, 5 times), centrifuged (4°C, 39,000 × *g*, 30 min), and subjected to SDS-PAGE analysis. For the overexpression of dehydratase enzyme (Sohng and Yoo, 1996), the pRSJ462-B was transformed into *E. coli* BL21 (DE3) pLysS and transformed cells were grown in 50 ml of LB containing ampicillin (50 μg/ml) and IPTG (1 mM) at 37°C for 18 h with shaking (150 rpm). Crude extract of the overexpressed dehydratase enzyme was prepared following the same procedure as for the EryBVII enzyme.

Enzymatic reaction and chemical modification of enzymatic products The dehydratase reaction mixture contained TDP-D-glucose (1 mM), NAD^+ (1 pM), Tris buffer (50 mM, pH 7.5), and crude dehydratase (1 mg) in a total volume of 500 μl. The reaction mixture was incubated at 25°C for 20 min, filtered through Amicon Centricon (MWCO = 10,000, Bevery, USA) to remove proteins, and the filtrate was purified by Mono-Q HR 5/5 FPLC (Pharmacia, Uppsala, Sweden) with a linear salt gradient of 24 – 30 mM Tris, pH 7.5. TDP-6-Deoxy-L-threo-D-glycero-4-hexulose was collected in a single fraction of 500 μl. The resulting sugar nucleotide was converted to aldoses by slight modification of the procedure reported by Weigel *et al.* (1992). Specifically, TDP-6-deoxy-L-threo-D-glycero-4-hexulose was immediately reduced with excess sodium borohydride (1.4 mg) at room temperature for 30 min. The reaction mixture was boiled for 1 min and centrifuged. The supernatant solution was acidified to pH 2.0 by adding concentrated HCl and boiled for 10 min to hydrolyze TDP. The mixture was neutralized with concentrated KOH to pH 7.0, completely lyophilized, and dissolved in 0.6 ml of 0.5 M ammonium hydroxide. The resulting aldose was converted to the peracetylated alditols by slight modification of the procedure reported by Blakeney *et al.* (1983). Briefly, the aldose solution was mixed with sodium borohydride (21 mg in anhydrous DMSO), stirred at 40°C for 90 min, and the reaction was quenched by slowly adding 0.1 ml of glacial acetic acid. To the reaction mixture containing alditols was added 0.2 g of 1-methyl-imidazole and 6 ml of acetic anhydride. The mixture was stirred at room temperature for 1 h and the reaction was stopped by adding 5 ml of methanol at 0°C. Products were extracted with chloroform, washed with water, dried with anhydrous sodium sulfate, and solvent was evaporated. For the EryBVII enzymatic reaction, exactly the same components and conditions as the dehydratase reaction were employed except that crude EryBVII enzyme (2 mg) was added to the incubation mixture.

GC-Mass spectrophotometric analysis Peracetylated alditols were dissolved in 1 ml of chloroform and 10 μl of sample was injected into a GC-Mass Spectrophotometric (Varian GC-MS, Rts-225 capillary column, 0.25 μm × 30 m; injector temperature: 250°C; detector temperature: 260°C; temperature program: 190°C – 240°C at 5°C/min, EI mode, Texas, USA).

Results and Discussion

The putative epimerase gene (*ery*BVII) inserted into the pTrc99A vector successfully expressed a soluble enzyme in *E. coli* as shown in Fig. 1. The substrate of the EryBVII enzyme, TDP-6-deoxy-L-threo-D-glycero-4-hexulose, was prepared by incubating TDP-D-glucose with crude extracts of overexpressed dehydratase enzyme. GC of the dehydratase reaction product which had been derivatized to the peracetylated alditol is shown in Fig. 2. The appearance

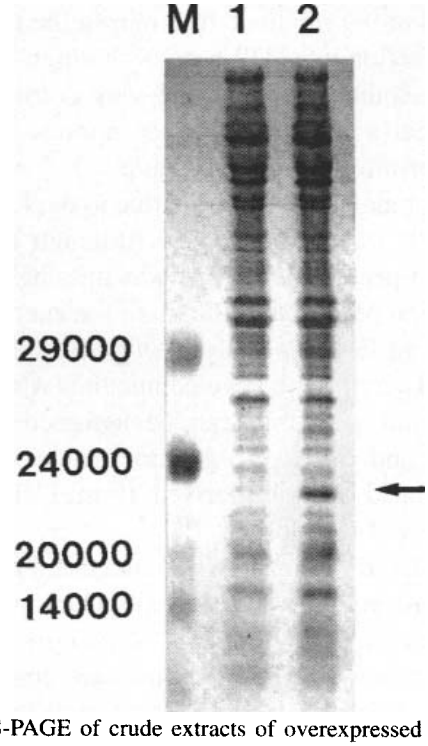

Fig. 1. SDS-PAGE of crude extracts of overexpressed EryBVII enzyme in *E. coli* DH5αMCR. Crude protein extracts (20 μg) were loaded in each lane and electrophoresis was run in 12% SDS-Polyacrylamide gel. An arrow indicates the expected EryBVII enzyme (MW = 21,290). M, molecular weight markers; lane 1, crude extract from *E. coli* DH5αMCR transformed with pTrc99A without the *ery*BVII gene insert; lane 2, crude extract from *E. coli* DH5αMCR transformed with pTrc (*ery*BVII).

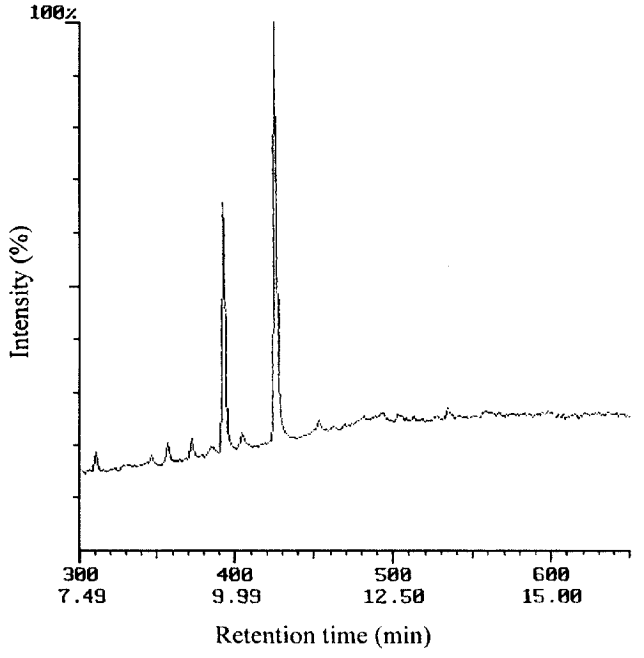

Fig. 2. GC chromatogram of dehydratase reaction product after derivatization. The enzymatic product was immediately reduced with $NaBH_4$ to TDP-D-fucose and TDP-6-deoxy-D-glucose, derivatized to peracetylated alditols, and analyzed by GC as described in Materials and Methods.

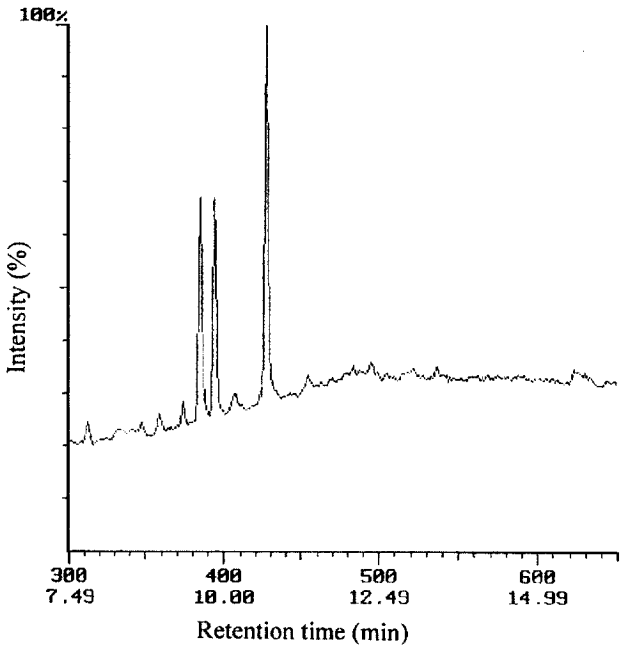

Fig. 3. GC chromatogram of EryBVII reaction product. The enzymatic product was immediately reduced with $NaBH_4$, derivatized to peracetylated alditols, and analyzed by GC as described in Materials and Methods. The compound eluted at 9.7 min is the peracetylated alditol of the EryBVII enzyme product.

of two peaks at 9.9 min and 10.7 min in the GC indicated that the reduction of TDP-6-deoxy-L-threo-D-glycero-4-hexulose by sodium borohydride was nonstereospecific. EI-mass spectra of these two compounds commonly showed a prominent peak at m/e = 317 without any corresponding molecular ion peak due to the loss of acetate ion (m/e = 59) (data not shown). Although the expected molecular ion peak at m/e = 376 was missing, the back of a molecular ion peak due to the loss of acetate is a general phenomenon in EI-mass analysis of peracetylated alditols (Lei, 1995). Therefore, the two compounds which appeared at 9.9 min and 10.7 min can be assigned to D-fucitol pentaacetate and 6-deoxy-D-glucitol pentaacetate, which are peracetylated alditols derived from TDP-6-deoxy-L-threo-D-glycero-4-hexulose.

When the EryBVII enzyme was incubated together with the dehydratase enzyme, a new peak, which was absent in Fig. 2, appeared at 9.7 min as shown in Fig. 3. The EI-Mass spectrum (Fig. 4) of this new compound also showed the characteristic prominent peak at m/e = 317 without the corresponding molecular ion peak at m/e = 376 expected from the epimerization of TDP-6-deoxy-L-threo-D-glycero-4-hexulose due to the loss of acetate ion. Hence, the EI-Mass spectra of all three compounds appearing at 9.7, 9.9, and 10.7 min GC sharing the common prominent peak at m/e = 317 was due to the loss of acetate ion from compounds with the same molecular weight (MW = 376), and the compound that appeared at 9.7 min comes from the

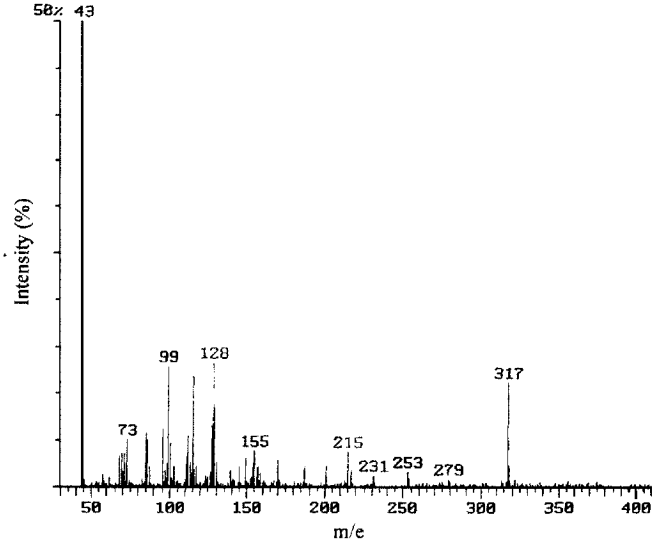

Fig. 4. EI-Mass spectrum of the compound eluted at 9.7 min in Fig. 3. Analytical conditions are described in Materials and Methods.

epimerization of TDP-6-deoxy-L-threo-D-glycero-4-hexulose. In addition, the appearance of only one new peak at 9.7 min in the the EryBVII enzymatic reaction indicated that the reduction at C-4 of the epimerized product by sodium borohydride was stereospecific. In conclusion, TDP-6-deoxy-L-threo-D-glycero-4-hexulose is indeed the substrate of the EryBVII enzyme and the function of the

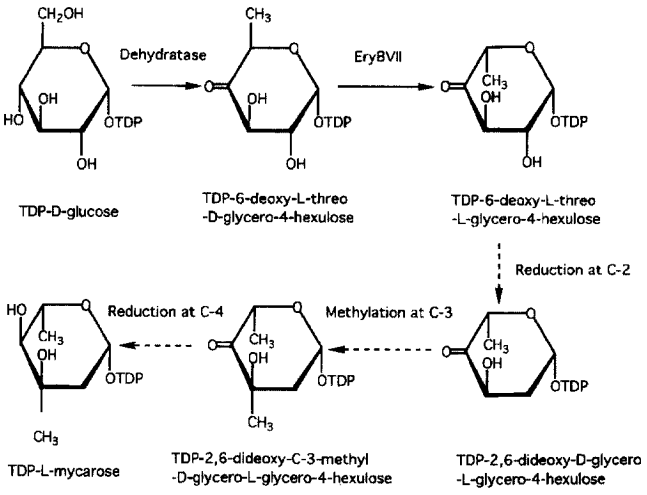

Fig. 5. Possible biosynthetic pathway of TDP-L-mycarose in *Saccharopolyspora erythrea*. The epimerization step was confirmed in this study and dotted arrows represent the deduced biosynthetic sequences (Summers *et al.*, 1997; Gaisser *et al.*, 1998).

*ery*BVII gene is to convert TDP-6-deoxy-L-threo-D-glycero-4-hexulose into its epimer. It is not clear at this point whether the epimerization occurs at C-5 and/or C-3. However, the configuration of the hydroxyl group at C-3 in TDP-6-deoxy-L-threo-D-glycero-4-hexulose is not changed in TDP-L-mycarose, and thus the EryBVII enzyme may catalyze the epimerization at C-5 only.

Biosynthesis of TDP-L-mycarose from TDP-6-deoxy-L-threo-D-glycero-4-hexulose requires at least four chemical transformations. They are an epimerization at C-5, a deoxygenation at C-2, a reduction at C-4, and a methylation at C-3. The reduction at C-4 is likely to be the final step since the 4-keto functionality is needed for the rest of the transformations. The deoxygenation at C-2 would proceed the methylation at C-3 since a deprotonation at C-3 is required for the deoxygenation at C-2 (Wegel *et al.*, 1992). Because the EryBVII enzyme could catalyze the epimerization of TDP-6-deoxy-L-threo-D-glycero-4-hexulose, the epimerization would be the first step. Therefore, the order of biosynthetic steps of TDP-L-mycarose from TDP-6-deoxy-L-threo-D-glycero-4-hexulose would be the epimerization at C-5, the deoxygenation at C-2, the methylation at C-3, and the reduction at C-4. Based on the function of the EryBVII enzyme and chemical intuitions discussed above, the minimal biosynthetic scheme of TDP-L-mycarose from TDP-glucose can be drawn as Fig. 5.

Acknowledgments This research was supported by the Korean Ministry of Education through a Genetic Engineering Research Fund grant to O. Han and in part by HRC 97-K3-0401-03-02-2. The authors would like to personally thank Drs. J. Yoo and K. Sohng for providing pRSJ462-B.

References

Blakeney, A. B., Harris, P. J., Henry, R. J. and Stone, B. A. (1983) A single and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohyd. Res.* **113**, 291–299.

Cortes, J., Haydock, S. F., Roberts, G. A., Bevitt, D. J. and Leadlay, P. F. (1990). An unusually large multifunctional polypeptide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythrea*. *Nature* **348**, 176–178.

Cupp-Vickery, J. R., Han, O., Hutchinson, C. R. and Poulos, T. L. (1996) Substrate assisted catalysis in cytochrome P450eryF. *Nature: Struct. Biol.* **3**, 632–637.

Dhilson, N., Hale, R. S., Cortes, J. and Leadlay, P. F. (1989) Molecular characterization of a gene from *Saccharopolyspora erythrea* which is involved in erythromycin biosynthesis. *Mol. Microbiol.* **3**, 1405–1414.

Gaisser, S., Bohm, G. A., Doumith, M., Raynal, M.-C, Dhilson, N., Cortes, J. and Leadlay, P. F. (1998) Analysis of *ery*BI, *ery*BIII and *ery*BVII from the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythrea*. *Mol. Gen. Genet.* **258**, 78–88.

Haydock, S. F., Dowson, J. A., Dhilson, N., Roberts, G. A., Cortes, J. and Leadlay, P. F. (1991) Cloning and sequence analysis of genes involved in erythromycin biosynthesis in *Saccharopolyspora erythrea*: Sequence similarities between EryG and a family of S-adenosylmethionine-dependent methyltransferases. *Mol. Gen. Genet.* **230**, 120–128.

Jacobson, J. R., Hutchinson, C. R., Cane, D. E. and Khosla, C. (1997) Precursor-directed biosynthesis of erythromycin analogs by an engineered polyketide synthase. *Science* **277**, 367–369.

Lei, Y. (1995) *Mechanistic Studies of Enzymes Involved in Biosynthesis of CDP-Ascarylose and TDP-Rhamnose*, PhD Thesis, University of Minnesota, pp. 239–240.

Sohng, J. K. and Yoo, J. (1996) Cloning, sequencing and expression of dTDP-D-glucose-4,6-dehydratase gene from *Streptpmyces antibioticus* Tu99, a producer of chlorothricin. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **29**, 183–191.

Sohng, J. K., Oh, T. J. and Kim, C. G. (1998) Method for cloning and biosynthetic genes of secondary metabolites including deoxysugar from *Actinomycetes*. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **31**, 475–483.

Summers, R. G., Donadio, S., Staver, M. J., Wendt-Pienkowski, E., Hutchinson, C. R. and Katz, L. (1997) Characterization of ten genes from *Saccharopolyspora erythrea* that are involved in L-mycarose and D-desosamine production. *Microbiology-UK*, **143**, 3251–3262.

Weigel, T. M., Miller, V. P. and Liu, H.-W. (1992) Mechanistic and stereochemical studies of a unique dehydration catalyzed by CDP-4-keto-6-deoxy-D-glucose-3-dehydrase: A pyridoxamine dependent enzyme isolated from *Yersinia pseudotuberculosis*. *Biochemistry* **31**, 2140–2147.