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Characterization of D-Glucose α-1-Phosphate Uridylyltransferase (VldB) and Glucokinase (VldC) Involved in Validamycin Biosynthesis of *Streptomyces hygroscopicus* var. *limoneus* KCCM 11405

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Abstract Aminocyclitol antibiotic validamycin A, a prime control agent for sheath blight disease of rice plants, is biosynthesized by *Streptomyces hygroscopicus* var. *limoneus*. Within the validamycin biosynthetic gene cluster, *vldBC* forms an operon of *vldABC* with *vldA*, the gene encoding 2-*epi*-5-*epi*-valiolone synthase. Biochemical studies, employing the recombinant proteins from *Escherichia coli*, established VldB and VldC as D-glucose α-1-phosphate uridylyltransferase and glucokinase, respectively. This finding substantiates that the validamycin biosynthetic gene cluster harbors genes encoding the enzymes for UDP-glucose formation from glucose. Therefore, we propose that validamycin biosynthesis employs its own catalysts to generate UDP-glucose, but not depending on the primary metabolism.

Key words: Glucokinase, D-glucose α-1-phosphate uridylyltransferase, *Streptomyces hygroscopicus*, validamycin biosynthesis

Members of the streptomycetes belong to the most prominently known group, owing their demonstrated importance in the pharmaceutical field. Many useful antibiotics in current use and bioactive metabolites from streptomycetes have been studied by cloning their biosynthetic genes and analyzing the regulatory mechanisms [14]. Recently, the biosynthetic gene clusters for spectinomycin, GERI-155, kanamycin, and kasugamycin were discovered [13, 14, 16, 23]. The validamycin complex, with validamycin A as the main component, is produced by *Streptomyces hygroscopicus* var. *limoneus* [11, 12, 15]. Validamycin A has been widely used as a control agent against *Basidomycetes* that causes sheath blight disease in rice, potatoes, vegetables, and others as well as damping-off disease in cucumber seedlings [18, 19], and the potency is attributed mainly to its trehalase inhibitory effect [2]. Structurally, validamycin A is 4-*O*-β-D-glucopyranosyl-validoxylamine A [21], and validoxylamine A is a pseudodisaccharide of two aliphatic *m*-C_7N molecules linked by the common nitrogen atom. The bioconversion experiment with isotopically labeled precursors established that 2-*epi*-5-*epi*-valiolone, valienone, and validone are intermediates in validamycin biosynthesis [5]. Recently, the validamycin biosynthetic gene cluster was independently isolated from *S. hygroscopicus* subsp. *jinggangensis* [22] and *Streptomyces hygroscopicus* var. *limoneus* KCCM 11405 (GenBank accession no. DQ223652) [20]. Located downstream from *vldA*, the genes encoding 2-*epi*-5-*epi*-valiolone synthase, *vldB* and *vldC*, are predicted to encode glucose-1-phosphate nucleotidyltransferase and glucokinase, respectively.

Computer-Aided Analysis of *vldB* and *vldC* Genes from the Validamycin Biosynthetic Gene Cluster

VldB is composed of 373 amino acids and shows significant homologies with D-glucose α-1-phosphate adenyltransferase (GlgC) orthologs from various microbial sources and bears the highest similarity with GlgC from *B. halodurans* C-125 (BAB04806) (32% identities/49% similarities). VldB also bears putative conserved domains such as nucleotidyl transferase (pfam00483), ADP-glucose pyrophosphorylase

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(GlgC, COG0448), and dTDP-glucose pyrophosphorylase (RfbA, COG1209), and the signatures characteristic to NDP-sugar pyrophosphorylases; 12-GXGXR-16 for the nucleotide-binding and 191-EEKP-194 for the sugar-binding [4].

VldC is composed of 351 amino acids and contains a conserved N-terminal site (14-ADLGGT-19), which is found in the ROK (repressors, ORFs of unknown function, sugar kinase) family [8, 9] and thought to manifest the ATP-binding site [1]. VldC also shows homologies with glucokinases from *B. cereus* G9241 (EAL13845) (30% identities/45% similarities) and 2-*epi*-5-*epi*-valiolone 7-kinase (AcbM) from *Actinoplanes* sp. 50/110 (CAD29482) (32% identities/41% similarities).

The presence of a glucose moiety in the structure of validamycin suggests that VldC catalyzes the formation of D-glucose 6-phosphate, which is then isomerized to D-glucose α-1-phosphate, and then VldB converts D-glucose α-1-phosphate to NDP-glucose for validamycin biosynthesis. To test this idea, we expressed *vldB* and *vldC* in *E. coli* and defined the catalytic roles of their products.

Characterization of VldB as D-Glucose α-1-Phosphate Uridylyltransferase

In order to obtain VldB, *vldB* was subcloned into pQE30 to generate pMJS2; the primer pair used was 5'-CGAGCTC-ATGGACGGAGTG-3' (sense; SacI site is boxed)/5'-AAAACTGCAGTCACAGCGCCAC-3' (antisense; PstI site is boxed). The IPTG induction resulted in an accumulation of product of 50 kDa in *E. coli* M15/pMJS2 (Fig. 1A, lane 3); however, the product was not seen with the pQE30 control (Fig. 1A, lane 2). A nickel-affinity chromatography resulted in a protein mixture containing His_6-tagged VldB as a major component (Fig. 1A, lane 5). Attempt to optimize the nickel-affinity chromatographic procedure failed to give a homogenous His_6-tagged VldB, which led us to employ the cell extract for characterization. The cell extract with His_6-tagged VldB was incubated with D-glucose α-1-phosphate and either ATP, CTP, GTP, dTTP, or UTP for 30 min, and the mixture was analyzed with HPLC: The reaction mixture, containing 20 mM Tris-HCl buffer (pH 8.0), 5 mM $MgCl_2$, 5 mM glucose-1-phosphate, 2 mM NTP, and 100 μl of the cell extract with or without His_6-tagged VldB in a total volume of 1 ml, was incubated at 37°C for 30 min. The resulting assay solution was boiled for 2 min and filtered after centrifugation to remove precipitate. HPLC analysis was done by following the method previously described [3]. One unit of the enzyme activity was defined as the amount of enzyme to catalyze the synthesis of 1.0 μmol of UDP-glucose per minute.

The chromatogram of a reaction mixture with UTP showed the formation of UDP-glucose, as judged by comparison with the authentic UDP-glucose (from SIGMA) (Fig. 1B, panel 4), whereas there was no NDP-glucose formation with either of the other nucleotides (data not shown). When the incubation was conducted with the cell extract from *E. coli* M15/pQE30, no UDP-glucose was found. It should be noted that UTP was efficiently hydrolyzed to

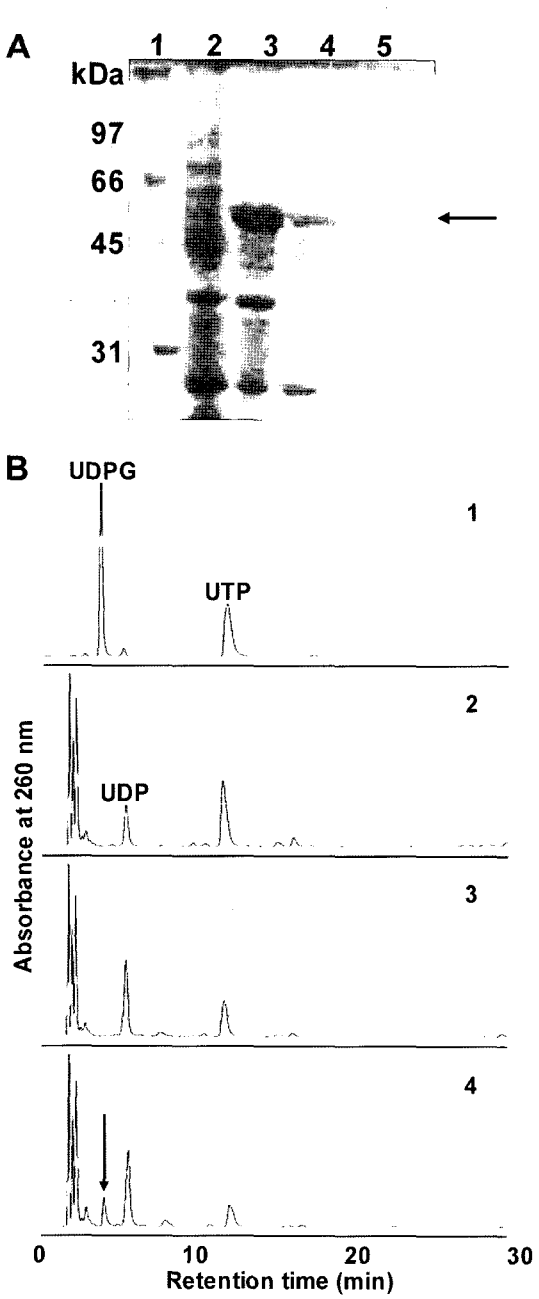

Fig. 1. A. SDS-PAGE analysis of the expressed VldB protein in *E. coli* M15. Lane 1, molecular weight marker; lane 2, cell-free extract of *E. coli* M15/pQE30; lane 3, total protein of *E. coli* M15/pMJS2; lane 4, cell-free extract of *E. coli* M15/pMJS2; lane 5, cell-free extract of *E. coli* M15/pMJS2 after a nickel-affinity chromatography. **B.** HPLC analysis for the formation of UDP-glucose by His_6-tagged VldB. 1, Authentic samples of UTP and UDP-glucose; 2, assay with the cell extract of *E. coli* M15/pMJS2 denaturated by boiling; 3, assay with the cell extract of *E. coli* M15/pQE30; 4, assay with the cell extract of *E. coli* M15/pMJS2.

UDP in the samples of both *E. coli* M15/pQE30 (Fig. 1B, panel 3) and *E. coli* M15/pMJS2; the lesser UDP in *E. coli* M15/pMJS2 with concomitant formation of UDP-glucose (Fig. 1B, panel 4). The UTP hydrolytic activity was also seen in the boiled control (Fig. 1B, panel 2). Similarly, other nucleotides such as ATP and dTTP were also readily hydrolyzed by the cell extract of *E. coli* M15/pQE30 (data not shown). It was assumed that the UTP hydrolysis is mediated by the host's alkaline phosphatase(s), which has broad substrate specificity toward diverse phosphorylated molecules such as ribonucleoside polyphosphates, deoxyribonucleoside polyphosphates, and inorganic pyrophosphate [10]. It was also demonstrated that the phosphatase activity of *E. coli* cell could survive boiling [10].

The activity of UDP-glucose formation in the cell extract of *E. coli* M15/pMJS2 was measured as 0.35 U/mg, and about 50% of UTP was converted to UDP-glucose in 30 min, when compared with the UTP level left in the incubation mixture with the cell extract of *E. coli* M15/pQE30. This result indicates that *vldB* encodes D-glucose α-1-phosphate uridylyltransferase, substantiating that the validamycin biosynthetic pathway does not recruit UDP-glucose from the primary metabolism, but employs the UDP-glucose synthase dedicated to the validamycin pathway. However, we could not presently answer whether other UDP-glucose synthase isozyme(s) played a pivotal role in supporting the metabolic integrity of *S. hygroscopicus*.

Characterization of VldC as Glucokinase

In order to obtain VldC, *vldC* was subcloned into pET32a to generate pMJS4, which was expressed in *E. coli* BL21 (DE3); the primer pair used was 5'-CGCGGATCCATG-GTGACGGCACTTACC-3' (sense; BamHI site is boxed)/ 5'-CCCAAGCTTCGCCGTCACCTCGAC-3' (antisense; HindIII site is boxed). After a nickel-affinity chromatography, His_6-tagged VldC was purified to homogeneity, and the molecular mass of the purified protein was estimated to be 50 kDa (Fig. 2A). Based on the predicted role of VldC as glucokinase, the formation of glucose-6-phosphate in the reaction mixture was monitored by using the enzyme assay coupled with glucose-6-phosphate dehydrogenase [6], whose activity was determined by photometrically measuring NADPH formation at 340 nm [7]. The result showed that the glucokinase activity from the cell extract of *E. coli* BL21 (DE3)/pMJS4 was 6.67 U/mg, whereas the activity from that of BL21 (DE3)/pET32a was 2.12 U/mg. The glucokinase activity from the purified His_6-tagged VldC was 8.71 U/mg, and there was no significant activity found without ATP. The glucokinase activity of VldC was further tested by a thin-layer chromatography (TLC) analysis by following the method previously described [17]. The TLC analysis further verified the formation of glucose-6-phosphate by His_6-tagged VldC, further confirming VldC as the glucokinase (Fig. 2B). This result

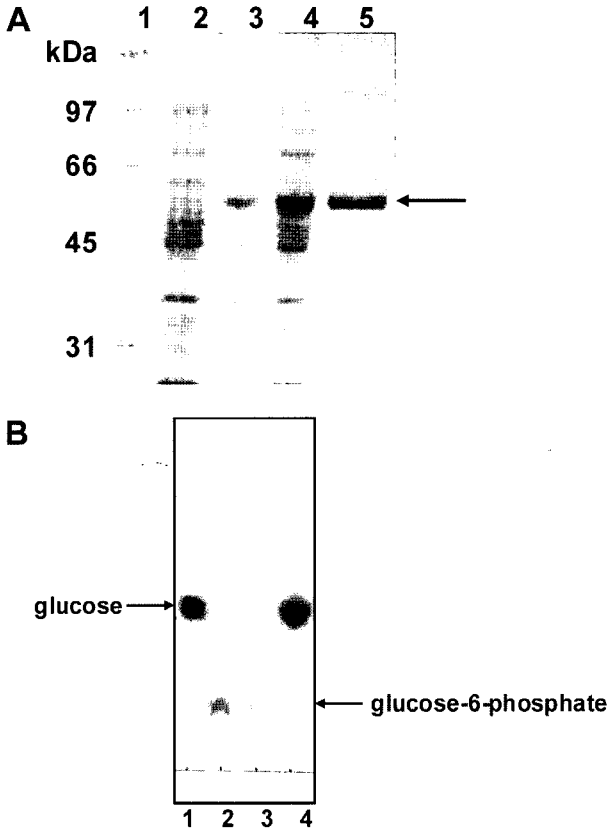

Fig. 2. A. SDS-PAGE analysis of the expressed VldC protein in *E. coli* BL21 (DE3). Lane 1, molecular weight marker; lane 2, cell-free extract of *E. coli* BL21 (DE3)/pET32a; lane 3, cell-free extract of *E. coli* BL21 (DE3)/pMJS4; lane 4, total protein of *E. coli* BL21 (DE3)/pMJS4; lane 5, the purified His_6-tagged VldC. **B.** Phosphorylation of glucokinase VldC. Lane 1, glucose; lane 2, glucose-6-phosphate; lane 3, glucose, ATP, and VldC; lane 4, glucose and VldC.

substantiates that, similar with the case of VldB, the validamycin biosynthetic pathway has its own way to make glucose-6-phosphate, being independent of the primary metabolism.

Biosynthetic Pathway of Validamycin A

Definitions of VldB and VldC led us to propose the validamycin biosynthetic pathway, in which VldI hydrolyzes starch to D-glucose [20], which is shuttled to VldC to be converted to glucose-6-phosphate (Fig. 3B). VldH, a phosphomutase candidate, is proposed to catalyze the conversion of glucose-6-phosphate to D-glucose-α-1-phosphate, which is then converted to UDP-glucose by VldB (Figs. 3A, 3B). The information presented in this study substantiated that validamycin biosynthesis employs starch as the ultimate intermediate to generate a glucose moiety, making the validamycin biosynthetic pathway as a unique example; a secondary metabolic pathway harboring a full set of genes for glucose assimilation.

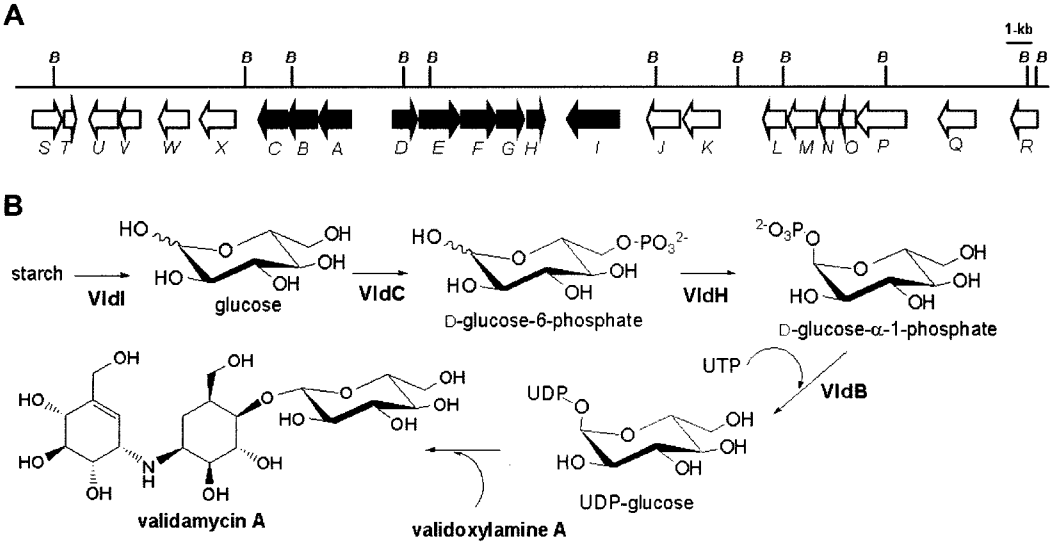

Fig. 3. A. Validamycin biosynthetic gene cluster. **B.** The proposed biosynthetic pathway of validamycin A.

Acknowledgments

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