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Glucose-1-phosphate uridylyltransferase from *Erwinia amylovora*: Activity, structure and substrate specificity

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ABSTRACT

Erwinia amylovora, a Gram-negative plant pathogen, is the causal agent of Fire Blight, a contagious necrotic disease affecting plants belonging to the *Rosaceae* family, including apple and pear. *E. amylovora* is highly virulent and capable of rapid dissemination in orchards; effective control methods are still lacking. One of its most important pathogenicity factors is the exopolysaccharide amylovoran. Amylovoran is a branched polymer made by the repetition of units mainly composed of galactose, with some residues of glucose, glucuronic acid and pyruvate. *E. amylovora* glucose-1-phosphate uridylyltransferase (UDP-glucose pyrophosphorylase, EC 2.7.7.9) has a key role in amylovoran biosynthesis. This enzyme catalyses the production of UDP-glucose from glucose-1-phosphate and UTP, which the epimerase GalE converts into UDP-galactose, the main building block of amylovoran.

We determined EaGalU kinetic parameters and substrate specificity with a range of sugar 1-phosphates. At time point 120 min the enzyme catalysed conversion of the sugar 1-phosphate into the corresponding UDP-sugar reached 74% for *N*-acetyl-α-D-glucosamine 1-phosphate, 28% for α-D-galactose 1-phosphate, 0% for α-D-galactosamine 1-phosphate, 100% for α-D-xylose 1-phosphate, 100% for α-D-glucosamine 1-phosphate, 70% for α-D-mannose 1-phosphate, and 0% for α-D-galacturonic acid 1-phosphate. To explain our results we obtained the crystal structure of EaGalU and augmented our study by docking the different sugar 1-phosphates into EaGalU active site, providing both reliable models for substrate binding and enzyme specificity, and a rationale that explains the different activity of EaGalU on the sugar 1-phosphates used. These data demonstrate EaGalU potential as a biocatalyst for biotechnological purposes, as an alternative to the enzyme from *Escherichia coli*, besides playing an important role in *E. amylovora* pathogenicity.

1. Introduction

Fire Blight is one of the most costly diseases in pome fruit production worldwide [1]. Its causal agent is *Erwinia amylovora*, a Gram-negative bacterium of the *Enterobacteriaceae* family, closely related to some important mammalian pathogens, such as *Escherichia coli*, *Yersinia pestis* and *Salmonella enterica* [1,2]. *E. amylovora* infection occurs *via* natural openings, such as floral nectaries and wounds on leaves or shoots, spreads in the parenchyma eventually leading to plant death [3]. *E. amylovora* major pathogenicity factors are type III secretion system proteins (T3SS) and the exopolysaccharide (EPS) that contributes to biofilm formation [3,4]. Other factors involved in pathogenicity are the siderophore desferrioxamine [5], secreted metalloproteases [6] and the presence of plasmids involved in modulating bacterial virulence [7].

E. amylovora produces two different types EPS, levan and amylovoran, both of which provide protection to cells under different stress conditions and are involved in biofilm formation. Levan is composed of fructans of variable length, synthesized by the enzyme levansucrase [8–10] that was proved to be involved in pathogenesis [11,12]. Amylovoran is made up of polymerized pentasaccharide units that consist of a backbone of three galactose units and a side chain of one glucuronic

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acid residue and one further galactose bearing a pyruvate and one or two acetyl groups. About 50% of the pentasaccharides also present an accessory side chain of one glucose linked to the branched galactose residue [13–16]. Amylovoran biosynthesis requires the proteins encoded by the twelve genes in the *ams* operon and the proteins involved in the galactose metabolism [14,17–19].

Glucose-1-phosphate uridylyltransferase (GalU; UDP-glucose pyrophosphorylase, EC 2.7.7.9) is the designated enzyme for the synthesis of UDP-glucose from α-D-glucose 1-phosphate (Glc-1P) and UTP, while releasing pyrophosphate. GalU plays a key role in carbohydrate metabolism in every organism, catalysing the production of the main glycosyl donor in oligo- and poly-saccharide biosynthesis [20]. In lower complexity organisms such as bacteria, GalU represents a critical molecular "switch" in sugar metabolism at the crossroads of complex carbohydrate synthesis and breakdown. GalU directs Glc-1P either to become substrates for biosynthetic pathways or to be used as a carbon source. GalU is strictly required for the biosynthesis of lipopolysaccharides and capsular polysaccharides [21–23]. GalU mutants in *E. coli*, *Klebsiella pneumoniae* and *Streptococcus pneumoniae* strains were not able to grow in minimal media with galactose as the unique carbon source and they showed dramatically reduced virulence [21,22,24].

Members of the *Enterobacteriaceae* family share highly conserved genes for galactose metabolism, but their regulation changes among species on the basis of the different galactose utilization purposes [25]. Galactose is not essential for *E. coli* metabolism, hence it is uniquely used as a carbon source *via* the amphibolic Leloir pathway where the galactose mutarotase (GalM), the galactokinase (GalK), the galactose-1-phosphate uridylyltransferase (GalT) and the epimerase (GalE) drive the conversion of galactose into UDP-glucose [20,26]. In the Leloir pathway, GalE is the unique enzyme involved in anabolic processes, while GalK, GalT and GalM are needed exclusively for galactose catabolism. The genes encoding these proteins belong to a unique operon, the *gal* operon, ensuring their common regulation and their simultaneous expression only in the presence of galactose [27]. Worth to note is that *E. amylovora* and *E. coli* harbour the same *gal*-genes for galactose metabolism, but these are differently arranged in the two genomes, indicating different regulation and use. In fact, *E. amylovora galE* does not belong to the *gal* operon, thus providing a separate and autonomous regulation with respect to the other *gal*-genes. In *E. amylovora*, the discoordinated expression of GalE from the other *gal*-genes escapes the catabolic progression of the Leloir pathway and leads to galactose production for biosynthetic processes. GalE catalyses the reversible conversion of UDP-glucose into UDP-galactose, which represents a critical substrate for amylovoran biosynthesis [18].

GalU isoforms are present in all organisms, both eukaryotes and prokaryotes. Bacterial GalUs share high amino acid sequence identity but they diverge significantly from their eukaryotic counterparts, with an estimated sequence identity as low as 8% [21,28]. This very limited similarity reveals substantial evolutionary divergence for this enzyme between the two domains [21,29]. Since in *E. amylovora* GalU is involved in the production of the main pathogenicity factor upstream of the *ams* gene cluster and the galactose metabolism protein-encoding genes, it may represent a good target to block amylovoran biosynthesis and thereby compromise *E. amylovora* infection. The clear distinction from eukaryotic counterparts suggests GalU as a promising target to develop new selective bioactive compounds against *E. amylovora* and perhaps also for other pathogens of the *Enterobacteriaceae* family [21,22,30,31].

Besides its biological significance, GalU also has potential importance in biotechnology as an alternative to the enzyme from *Escherichia coli*. In a previous study, we found that *Escherichia coli* UDP-glucose pyrophosphorylase (glucose-1-phosphate uridylyltransferase) in conjunction with *Saccharomyces cerevisiae* inorganic pyrophosphatase was a very active combination for converting 5-substituted UTP derivatives into a range of *gluco*-configured 5-substituted UDP-sugar derivatives in good yield [32].

For a better understanding of the structure and function relationship of *E. amylovora* GalU (EaGalU) we focused on the recombinant expression of GalU from *E. amylovora* strain CFBP1430 in *E. coli*. We determined EaGalU kinetic parameters and substrate specificity on a range of sugar 1-phosphates. At time point 120 min the enzyme catalysed conversion of the sugar 1-phosphate into the corresponding UDP-sugar reached 74% for *N*-acetyl-α-D-glucosamine 1-phosphate (GlcNAc-1P), 28% for α-D-galactose 1-phosphate (Gal-1P), 0% for α-D-galactosamine 1-phosphate (GalN-1P), 100% for α-D-xylose 1-phosphate (Xyl-1P), 100% for α-D-glucosamine 1-phosphate (GlcN-1P), 70% for α-D-mannose 1-phosphate (Man-1P), and 0% for α-D-galacturonic acid 1-phosphate (GalA-1P). We compared these results with the corresponding enzyme from *Escherichia coli*, which we previously used to develop chemo-enzymatic approaches for the synthesis of nucleobase-modified UDP-sugars [32]. We determined the crystal structure of the protein and from the molecular docking of the substrates, we were able to identify the determinants of substrate specificity making it possible to decide putative sites for mutagenesis in order to modify the catalytic properties of the enzyme or choose the best strategy for chemo-enzymatic reactions. The substrate promiscuity of EaGalU as well as the wealth of information provided in this study make it a good alternative to other GalUs as a biocatalyst for biotechnological applications.

2. Materials and methods

2.1. Protein expression and purification

The cloning of the Ea*galU* gene and the procedure from protein expression to protein crystallization and data collection are briefly summarised here as they were previously reported [33]. The *galU* gene from *E. amylovora* CFBP 1430 was cloned into the expression vector pETM-11 [34] which was then transformed into competent *E. coli* BL21(DE3). The transformed cells were grown at 310 K under shaking at 180 rpm until an OD600 of 0.6 ± 0.1, the temperature was decreased to 293 K, left to equilibrate for 1 h and inducted with 0.5 mM IPTG. After 16 h, cells were harvested by centrifugation at 4500*g* for 20 min at 277 K and resuspended in cold PBS. After disruption by sonication, cell debris were removed by centrifugation at 18000*g* for 20 min at 277 K. Protein purification was carried out by IMAC using Ni loaded His-Trap HP 5 ml column (GE Healthcare, Sweden), followed by cleavage of the N-terminal His-tag by rTEV protease [35]. After a second His-Trap HP column, the fractions containing pure and cleaved EaGalU were loaded onto a Sephadex S200 16/60 column (GE Healthcare, Sweden) and eluted at 1 ml min^{-1}. All purification steps were carried out at 293 K. The purity of recombinant EaGalU was confirmed by SDS-PAGE electrophoresis and the protein molecular mass confirmed by MALDI-TOF mass spectrometry [33].

Cloning of the Ec*galU* gene, protein expression and purification were carried out as described earlier [36].

2.2. Enzymatic activity

The activity of EaGalU in the synthetic direction was determined using a method described in Mok and Edwards [37] and used in Fang et al. [38] for UDP-*N*-acetylglucosamine pyrophosphorylase. Steady-state kinetics of EaGalU were studied under linear, initial rate conditions, with no more than 15% substrate conversion. The assay was performed in triplicate in a 96-well plate at 37 °C with each well containing 50 mM HEPES, pH 8.0, 5 mM KCl, 10 mM $MgCl_2$, inorganic pyrophosphatase (5 U/ml, final concentration), EaGalU (1.9–2.4 nM) and varying concentrations of Glc-1P and UTP in a final volume of 100 µl. In the K_m of Glc-1P assay, the concentrations were 100 µM UTP and 100 µM, 50 µM, 12.5 µM, 6.25 µM and 3.13 µM glucose 1-phosphate. In the K_m of UTP assay, the concentrations were 100 µM Glc-1P and 100 µM, 50 µM, 25 µM, 12.5 µM and 6.25 µM UTP. The reaction was incubated for 5 min and terminated by the addition of 100 µl