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The C-glycosyltransferase IroB from pathogenic *Escherichia coli*: Identification of residues required for efficient catalysis☆

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ABSTRACT

Escherichia coli C-glycosyltransferase IroB catalyzes the formation of a C–C bond between enterobactin and the glucose moiety of UDP-glucose, resulting in the production of mono-, di- and tri-glucosylated enterobactin (MGE, DGE, TGE). To identify catalytic residues, we generated a homology model of IroB from aligned structures of two similar C-glycosyltransferases as templates. Superposition of our homology model onto the structure of a TDP-bound orthologue revealed residue W264 as a possible stabilizer of UDP-glucose. D304 in our model was located near the predicted site of the glucose moiety of UDP-glucose. A loop containing possible catalytic residues (H65, H66, E67) was found at the predicted enterobactin-binding site. We generated IroB variants at positions 65–67, 264, and 304 and investigated variant protein conformations and enzymatic activities. Variants were found to have T_m values similar to wild-type IroB. Fluorescence emission spectra of H65A/H66A, E67A, and D304N were superimposable with wild-type IroB. However, the emission spectrum of W264L was blue-shifted, suggesting solvent exposure of W264. While H65A/H66A retained activity (92% conversion of enterobactin, with MGE as a major product), all other IroB variants were impaired in their abilities to glucosylate enterobactin: E67A catalyzed partial (29%) conversion of enterobactin to MGE; W264L converted 55% of enterobactin to MGE; D304N was completely inactive. Activity-impaired variants were found to bind enterobactin with affinities within 2.5-fold of wild-type IroB. Given our outcomes, we propose that IroB W264 and D304 are required for binding and orienting UDP-glucose, while E67, possibly supported by H65/H66, participates in enterobactin/MGE/DGE deprotonation.

1. Introduction

Siderophore-mediated iron acquisition is required for virulence by many pathogenic bacteria [1]. The *Escherichia coli* catecholate siderophore enterobactin (Fig. 1a) is not commonly a virulence factor since mammalian hosts can sequester the siderophore *via* the innate immune system protein NGAL (also known as lipocalin 2 or siderocalin). Pathogenic extra-intestinal *E. coli* such as uropathogenic *E. coli* (UPEC) [2], avian pathogenic *E. coli* (APEC) [3], the probiotic *E. coli* strain Nissle 1917 [4], as well as *Salmonella enterica* [5], harbor a five-gene cluster known as *iroA*, which is involved in the glucosylation and linearization of enterobactin prior to its secretion. It has been shown that glucosylated enterobactin (GE) cannot be efficiently bound by NGAL [6]. Pathogens containing the *iroA* cluster can therefore continue to acquire iron from the host while evading innate immune system defenses. The *iroA* cluster is comprised of the genes *iroB*, *iroC*, *iroD*, *iroE*, and *iroN* that encode the proteins IroB, IroC, IroD, IroE, and IroN, respectively. IroB produces GE in the cytoplasm. The inner-membrane transporter IroC facilitates transport of GE to the periplasm, where it is linearized by the esterase IroE and then secreted from the cell by a currently unknown mechanism. Upon acquisition of extracellular Fe^{3+}, Fe-GE is imported into the bacterial cell by the TonB-dependent outer membrane receptor IroN. Finally, the cytoplasmic esterase IroD catalyzes the hydrolysis of the triserine trilactone core of GE, resulting in the formation of glucosylated DHB-serine subunits. This cleavage of GE facilitates the release of iron in the cytoplasm in order to support bacterial growth.

As the first *iroA* gene product required for production of GE, the 42.3 kDa C-glycosyltransferase IroB catalyzes the attachment of one, two, or three glucose moieties to enterobactin, resulting in the formation of mono-glucosylated enterobactin (MGE) (Fig. 1b), di-glucosylated enterobactin (DGE) (Fig. 1c), or tri-glucosylated enterobactin (TGE)

Abbreviations: CD, circular dichroism; DGE, di-glucosylated enterobactin; C-GT, C-glycosyltransferase; DHB, 2,3-dihydroxybenzoic acid; ENT, enterobactin; ESI-MS, electrospray ionization mass spectrometry; FPLC, fast protein liquid chromatography; GE, glucosylated enterobactin; GT, glycosyltransferase; MGE, mono-glucosylated enterobactin; NTA, Ni-nitrilotriacetic acid; OD, optical density; PDB, Protein Data Bank; RMSD, root-mean-square deviation; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TCEP, tris(2-carboxyethyl)phosphine; TGE, tri-glucosylated enterobactin

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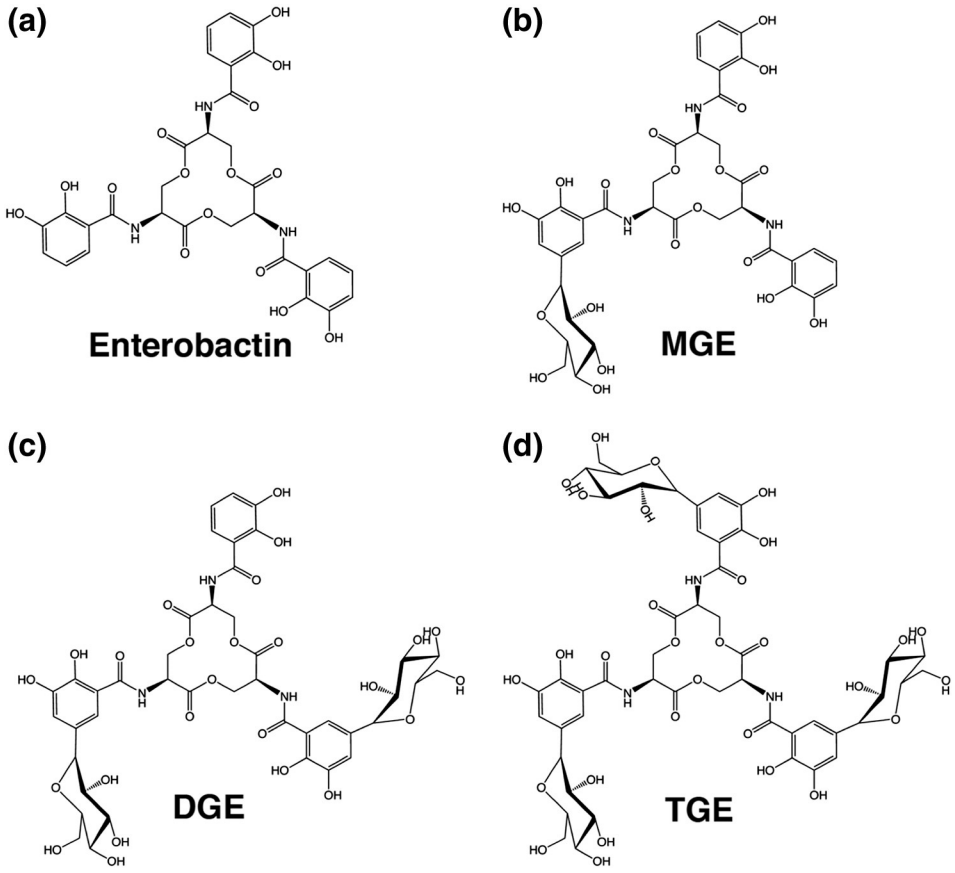

Fig. 1. Chemical structures of enterobactin and IroB enzymatic products. (a) Enterobactin; (b) MGE: mono-glucosylated enterobactin; (c) DGE: di-glucosylated enterobactin; (d) TGE: tri-glucosylated enterobactin.

(Fig. 1d). In each round of glucosylation, IroB transfers the glucose moiety from a bound UDP-glucose donor co-substrate to one DHB subunit of the enterobactin acceptor co-substrate. The glucose moiety is attached to a DHB subunit *via* C–C bond formation between the anomeric C1′ atom of the glucose portion of UDP-glucose and the C5 atom of DHB. It has been shown that DGE, also known as salmochelin S4, is the principal product of IroB catalysis *in vivo* [5]. The mechanistic aspects of C-glycosyltransferases are now beginning to be understood. Recent studies on glycosyltransferases that catalyze O- and C-glycosylation of hedamycin [7] and urdamycin [8] have explored two possible mechanisms for glucose attachment to aryl groups on acceptor substrates: in the first mechanism, glucose is first directly attached to a deprotonated phenolic group on the acceptor, followed by rearrangement of the O-glycoside; in the second mechanism, deprotonation of a phenolic group either *ortho* or *para* to the site of glucose attachment results in aromatic delocalization conferring a nucleophilic character to the carbon atom at which direct glucose attachment occurs. Experimental work on urdamycin glycosylation by UrdGT2 [8] supported the latter mechanism, in which direct glycosylation of urdamycin occurred following deprotonation of a phenolic group *ortho* to the attachment site. This direct glucosylation mechanism was supported by subsequent research on IroB by the Walsh group in which nucleophilicity of the C5 atom of DHB was suggested to be caused by deprotonation of the phenolic group on the DHB C2 carbon *para* to the C5 position of glucose attachment [9].

Glycosyltransferase enzymes are widely found in nature, and are grouped into two families of folds: GT-A and GT-B (for a recent review, see Chang et al. [10]). Proteins of the GT-A family are single-domain folds and generally have a requirement for metals such as Mg^{2+} or Mn^{2+}. Proteins from the GT-B family possess two major Rossmann-like domains: a C-terminal donor-binding domain and an N-terminal acceptor-binding domain [11]. The products of glycosyltransferase enzymes can either retain anomeric configuration of the sugar or invert it; however, there is no correlation between whether a GT is retaining or inverting, and whether its fold is GT-A or GT-B [10]. The three-dimensional structure of IroB has not yet been reported, but the enzyme is thought to belong to the inverting GT-B family of glycosyltransferases, consistent with other C-glycosyltransferase structures [9]. Although the nature of acceptor molecules across the GT-B family is diverse, most donor molecules are nucleotide cofactors such as TDP-glucose or UDP-glucose. In the case of IroB, the acceptor molecule can be enterobactin, MGE, or DGE. UDP-glucose has been shown *in vitro* to be a donor molecule for IroB [9].

Here we have used homology modeling to gain structural insights into IroB substrate binding and catalysis. Our model revealed five residues (H65, H66, E67, W264, and D304) as being potentially involved in IroB catalysis. The roles of these residues were experimentally verified by enzymological assays and biophysical approaches. Taken together, our results demonstrate that IroB residues E67, W264, and D304 are necessary for efficient IroB catalysis, likely *via* their roles in UDP-glucose binding or deprotonation of DHB moieties of bound acceptor molecules. Residues H65 and H66 also appear to play a role in IroB catalysis, perhaps in the recognition of GE substrates.

2 . Materials and methods

2.1 . Reagents

All chemicals were purchased from Bioshop Canada, Inc. (Burlington, Ontario) unless otherwise indicated. Enterobactin was purchased from Sigma-Aldrich (St. Louis, Missouri). Genomic DNA from *E. coli* CFT073