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Characterization and Mutational Analysis of Two UDP-Galactose 4-Epimerases in *Streptococcus pneumoniae* TIGR4

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Abstract—Current clinical treatments for pneumococcal infections have many limitations and are faced with many challenges. New capsular polysaccharide structures must be explored to cope with diseases caused by different serotypes of *Streptococcus pneumoniae*. UDP-galactose 4-epimerase (GalE) is an essential enzyme involved in polysaccharide synthesis. It is an important virulence factor in many bacterial pathogens. In this study, we found that two genes ($galE_{sp1}$ and $galE_{sp2}$) are responsible for galactose metabolism in pathogenic *S. pneumoniae* TIGR4. Both $GalE_{Sp1}$ and $GalE_{Sp2}$ were shown to catalyze the epimerization of UDP-glucose (UDP-Glc)/UDP-galactose (UDP-Gal), but only $GalE_{Sp2}$ was shown to catalyze the epimerization of UDP-N-acetylglucosamine (UDP-GlcNAc)/UDP-N-acetylgalactosamine (UDP-GalNAc). Interestingly, $GalE_{Sp2}$ had 3-fold higher epimerase activity toward UDP-Glc/UDP-Gal than $GalE_{Sp1}$. The biochemical properties of $GalE_{Sp2}$ were studied. $GalE_{Sp2}$ was stable over a wide range of temperatures, between 30 and 70°C, at pH 8.0. The K86G substitution caused $GalE_{Sp2}$ to lose its epimerase activity toward UDP-Glc and UDP-Gal; however, substitution C300Y in $GalE_{Sp2}$ resulted in only decreased activity toward UDP-GlcNAc and UDP-GalNAc. These results indicate that the Lys86 residue plays a critical role in the activity and substrate specificity of $GalE_{Sp2}$.

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Keywords: UDP-galactose 4-epimerase (GalE), *Streptococcus pneumoniae* TIGR4, mutation, substrate specificity

Streptococcus pneumoniae is a notorious human pathogen that can cause various diseases including acute pneumonia, otitis media, and meningitis [1, 2]. Importantly, the 7-valent and 13-valent pneumococcal conjugate vaccines cannot provide protection against all the pathogenic serotypes of *Streptococcus pneumoniae*, such as *S. pneumoniae* TIGR4. Therefore, it is still urgent that new targets for drug development in preventing pneumococcal diseases should be explored.

Capsular polysaccharide (CPS) protects cells against phagocytosis during infection and is an important virulence factor in many pathogenic bacteria [3]. There are at least 90 different types of CPS in *S. pneumoniae* that are differentiated by their polysaccharide structure and immunogenicity [4]. Understanding the process of CPS synthesis is important for its potential value in preventing the various diseases caused by *S. pneumoniae*.

The CPS of *S. pneumoniae* TIGR4 has a linear repeating tetrasaccharide unit that includes an N-acetylgalactosamine (GalNAc), a phosphorylated galactose, an acetylated fucose, and an acetylated mannose [5, 6]. During infection and colonization, abundant carbon sources are needed to support bacterial growth. Biosynthesis of CPS involves a complex pathway that includes the uptake of component monosaccharides and their conversion to nucleotide derivatives. UDP-galactose 4-epimerase (GalE) is always involved in the biosynthesis of capsular or O-antigen polysaccharide units.

GalE is an essential enzyme in the Leloir pathway of galactose metabolism [7, 8]. It catalyzes the interconversion of UDP-galactose (UDP-Gal) and UDP-glucose (UDP-Glc). In many organisms, including *S. pneumoniae*, this enzyme can also catalyze the interconversion of UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-

Abbreviations: CPS, capsular polysaccharide; GalE, UDP-galactose 4-epimerase; UDP-Gal, UDP-galactose; UDP-GalNAc, UDP-N-acetylgalactosamine; UDP-Glc, UDP-glucose; UDP-GlcNAc, UDP-N-acetylglucosamine.

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N-acetylgalactosamine (UDP-GalNAc) [9, 10]. GalE is a key enzyme for the processes of eukaryotic and prokaryotic protein glycosylation and the production or secretion of virulence factors in many bacterial pathogens [11]. Thus, understanding of GalE will be helpful for developing new drugs or vaccine targets and for providing serotype-independent protection. GalE isoenzymes from various organisms, including *Escherichia coli*, *Kluyveromyces fragilis*, *Trypanosoma brucei*, *Drosophila melanogaster*, and humans have been characterized [12-19]. However, little is known about the GalE from *S. pneumoniae*, especially regarding its biochemical properties and substrate specificity.

In this work, the $galE_{sp1}$ and $galE_{sp2}$ genes from *S. pneumoniae* TIGR4 were cloned and heterologously expressed in *E. coli*. The substrate specificity of the two enzymes was characterized. In addition, biochemical characterization of $GalE_{Sp2}$ was further performed, including analysis of amino acid substitution derivatives, to allow for further understanding of *S. pneumoniae* TIGR4 GalE.

MATERIALS AND METHODS

Bacterial strains and materials. The *E. coli* DH5α used for plasmid maintenance was purchased from Gibco-BRL (USA), and the *E. coli* BL21(DE3) used for protein expression was from Novagen (USA). The genomic DNA of *S. pneumoniae* TIGR4 was kindly provided by Dr. Samantha King (Nationwide Children's Hospital, Columbus, Ohio, USA). The plasmid pMCSG7 was provided by Ohio State University (USA). Restriction enzymes, DNA polymerases, and T4 ligases were purchased from Fermentas (USA). The HisTrap

Table 1. PCR primers, plasmids, and strains used in this study

Name	Sequence of PCR primers and description of plasmids and strains*	Source
PCR primer		
$galE_{sp1}$-F	5'GGGACCTCCATATGGCAATATTGGTAACAGG (NdeI)	
$galE_{sp1}$-R	5'CGGGATCCCAATGCGGATCTCAATTATAGC (BamHI)	
$galE_{sp2}$-F	5'CGCGCCATGGATATGCAAGAAAAGATTTTGGTAA (NcoI)	
$galE_{sp2}$-R	5'CCGCTCGAGTTTATCACTATTTAGTCTTCAAATC (XhoI)	
Plasmid		
pMCSG7	T7 promoters with MCS, PBR322 replicon, copy number >100, IPTG-inducible, Amp^r	Novagen, USA
pMCSG7-$galE_{sp1}$	$galE_{sp1}$ (NdeI/BamHI), Amp^r	this study
pMCSG7-$galE_{sp2}$	$galE_{sp2}$ (NcoI/XhoI), Amp^r	this study
pMCSG7-C300Y	pMCSG7, $galE_{sp2}$ 300 Cys mutant (NcoI/XhoI), Amp^r	this study
pMCSG7-K86G	pMCSG7, $galE_{sp2}$ 86 Lys mutant (NcoI/XhoI), Amp^r	this study
Strain		
E. coli DH5α	F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697*gal*U *gal*K*rps*L (Str^R) *end*A1*nup*G	Gibco-BRL, USA
E. coli BL21(DE3)	F^-, *omp*T, *hsd*SB(rB^-mB^-), *gal*, *dcmrne131* (DE3)	Novagen, USA
Strain A	*E. coli* BL21(DE3) containing pMCSG7-$galE_{sp1}$	this study
Strain B	*E. coli* BL21(DE3) containing pMCSG7-$galE_{sp2}$	this study
Strain C	*E. coli* BL21(DE3) containing pMCSG7-$galE_{sp2}$ C300Y	this study
Strain D	*E. coli* BL21(DE3) containing pMCSG7-$galE_{sp2}$ K86G	this study

* Underlined sequences denote recognition sites of the restriction enzymes.

affinity column used in this research was from Amersham Pharmacia Biotech (USA). The capillary electrophoresis equipment was from Beckman Coulter P/ACE MDQ (USA). All kits and enzymes were used according to the manufacturer's instructions. No endangered or protected species were involved in the field studies.

Construction of recombinant *E. coli* strains. The DNA sequences of $galE_{sp1}$ (NP_346261.1) and $galE_{sp2}$ (NP_346051.1) were extracted from the genome of *S. pneumoniae* TIGR4 (No. AE005672). The primers $galE_{sp1}$-F/R and $galE_{sp2}$-F/R were designed accordingly and are listed in Table 1. DNA fragments were amplified using PCR. The PCR products and plasmid pMCSG7 were digested with the same restriction enzymes (Table 1) and ligated with T4 ligases. Vectors pMCSG7-$galE_{sp1}$ and pMCSG7-$galE_{sp2}$ were constructed and verified by DNA sequencing. The recombinant plasmids pMCSG7-$galE_{sp1}$ and pMCSG7-$galE_{sp2}$ were subsequently transformed into *E. coli* BL21(DE3) cells for protein expression.

Sequence alignment and mutagenesis. The sequences of $GalE_{Sp1}$ and $GalE_{Sp2}$ and those of six other GalE sequences belonging to different groups were aligned using the DNAMAN software. For $GalE_{Sp2}$, four other sequences of GalEs were chosen from group 2 and aligned to identify the essential sites for the isomerase activity. According to differences in their sequences and hypothetical crystal structure, amino acid substitution mutations were produced in $GalE_{Sp2}$ using the Quick-Change site-directed mutagenesis kit from Stratagene (USA). Mutants were constructed in the highly conserved active residues of Cys300 and Lys86 in $GalE_{Sp2}$ using pMCSG7-$galE_{sp2}$ as a template. The mutated plasmids pMCSG7-C300Y and pMCSG7-K86G were transformed into *E. coli* BL21 for protein expression.

Heterologous expression and enzymatic purification of $GalE_{Sp1}$ and $GalE_{Sp2}$. The *E. coli* BL21(DE3) strain harboring the recombinant plasmids was grown in 1 liter of Luria–Bertani (LB) broth with shaking at 220 rpm and 37°C. When the OD_{600} reached 0.6-0.8, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to final concentration 0.2 mM for induction. Expression proceeded overnight at 16°C, and then cells were harvested by centrifugation at 4°C. The cells were washed and resuspended in 40 ml of start buffer (500 mM NaCl, 20 mM Tris-HCl buffer, pH 8.0) and lysed on ice by sonication for 20 min at 400 W. The lysate was centrifuged at 12,000*g* at 4°C for 30 min, and the supernatant was loaded onto a HisTrap affinity column (5 ml) preequilibrated with a binding buffer. After washing with 10 column volumes of washing buffer (500 mM NaCl, 20 mM Tris-HCl buffer, 20 mM imidazole, pH 8.0), the target His-tagged proteins were eluted with elution buffer (500 mM NaCl, 20 mM Tris-HCl buffer, 500 mM imidazole, pH 8.0). Fractions containing the purified enzymes were harvested and desalted with 50 mM Tris-HCl buffer (pH 7.5) at 4°C. The purity and molecular mass were analyzed using 12% SDS-PAGE. Protein concentrations were determined using the Bradford method [20].

Enzymatic assay. The activities of proteins $GalE_{Sp1}$ and $GalE_{Sp2}$ toward UDP-Glc, UDP-Gal, UDP-GlcNAc, and UDP-GalNAc were assayed as described before with a minor modification [21]. Briefly, the reaction mixture consisted of 1 mM substrate (UDP-Glc, UDP-Gal, UDP-GlcNAc, or UDP-GalNAc) and varying amounts of freshly prepared enzyme in 20 mM Tris-HCl, pH 8.0, in a total volume of 50 μl. The reactions were performed at 37°C for 120 min and then quenched by boiling for 5 min before centrifugation. The supernatant was analyzed using capillary electrophoresis (see Supplement to this paper on the site of the journal http://protein.bio.msu.ru/biokhimiya and Springer site Link.springer.com). The conditions used were as follows: 75 μm, 25 kV/80 μA, 5-s vacuum injection, monitoring at 262 nm, a 25-mM sodium tetraborate running buffer, pH 9.4. The activities of enzymes $GalE_{Sp1}$ and $GalE_{Sp2}$ toward UDP-Gal, UDP-GlcNAc, and UDP-GalNAc were assessed using the same method.

RESULTS

Sequence analysis of $GalE_{Sp1}$ and $GalE_{Sp2}$ from *S. pneumoniae* TIGR4. The genes of $GalE_{Sp1}$ and $GalE_{Sp2}$ cloned from the genome of *S. pneumoniae* TIGR4 have sizes of 1011 and 1020 bp, respectively. Sequence analysis showed high similarity (52.22%) between eight UDP-hexose 4-epimerases' amino acid sequences (Fig. 1).

UDP-hexose 4-epimerases belong to the superfamily of short-chain dehydrogenase/reductase (SDR) having two-domain structure. The N-terminal domain with conserved sequence GxxGxxG forms a modified Rossmann fold and is involved in binding of the cofactor NAD^+, whereas a smaller domain with conserved sequence YxxxK is involved in substrate binding [8]. Both functional motifs conserved in the SDR superfamily members were identified in $GalE_{Sp1}$ and $GalE_{Sp2}$ (Fig. 1).

Based on its substrate specificity, GalEs can be divided into three groups. Group 1 epimerases strongly prefer non-acetylated substrates (UDP-Glc/Gal), such as the *E. coli* GalE, with a corresponding "Y300" residue. Group 2 epimerases can epimerize both acetylated (UDP-GlcNAc/GalNAc) and non-acetylated substrates, such as the GalE from *Drosophila melanogaster*. Group 3 epimerases include WbgU from *Pl. shigelloides* [22] and WbpP from *Ps. aeruginosa* [23] and show a strong preference for acetylated substrates with a corresponding "G86" residue.

According to Ishiyama et al. [23], there are six key residues (marked in Fig. 1) related to the group identification. GalE enzymes belonging to group 1 contain "L-S-Y-N-H-L or K-S-Y-N-N-Y" in the amino acid sequences, enzymes belonging to group 2 contain "K-S-

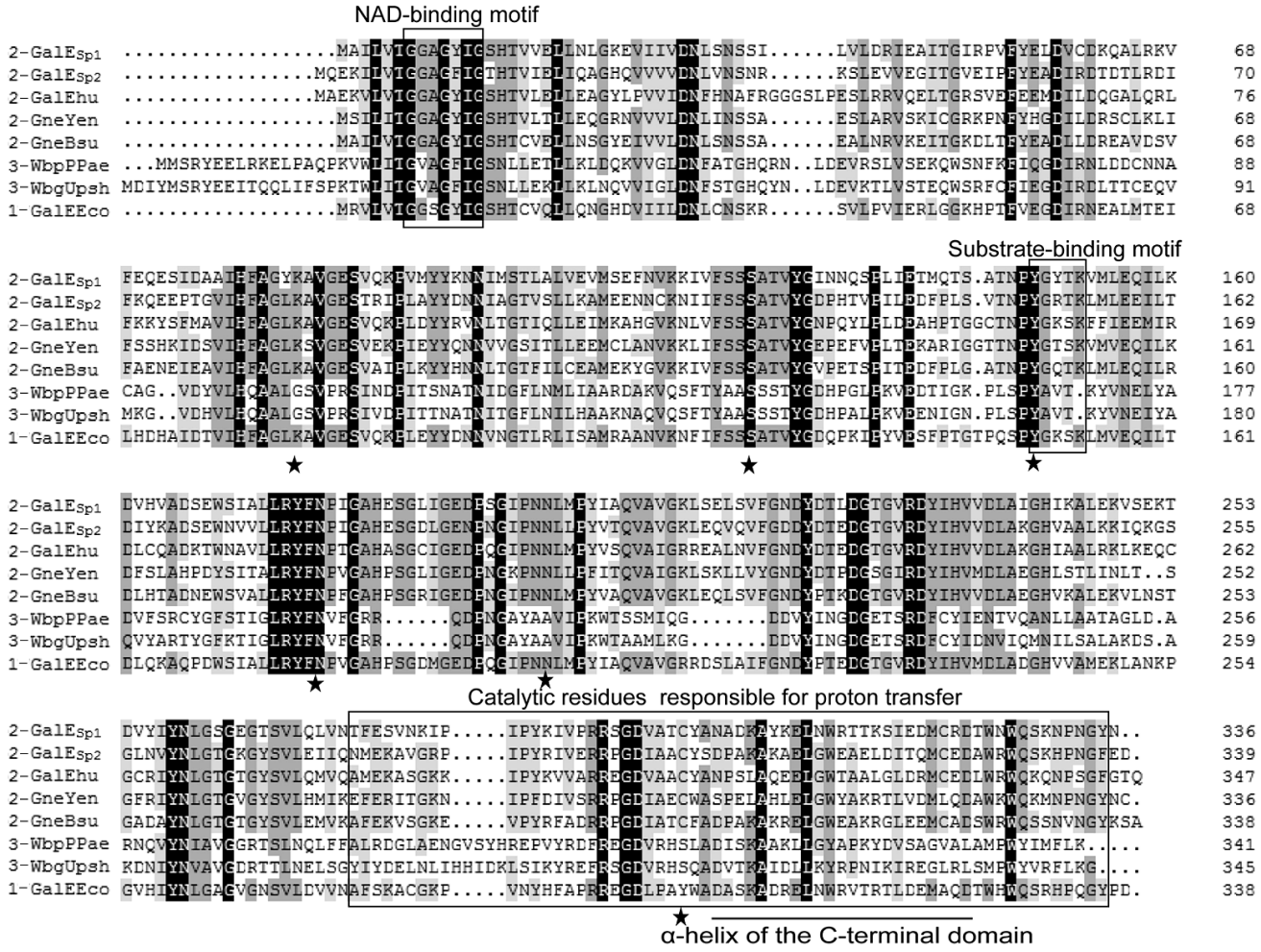

Fig. 1. Sequence alignments of GalE from different organisms. The protein sequences are as follows: $GalE_{Sp1}$ (WP_000996560.1, group 2), $GalE_{Sp2}$ (WP_001156518.1, group 2), GalEhu from *Homo sapiens* (NP_001008217.1, group 2), GneYen from *Yersinia enterocolitica* (WP_005167749.1, group 2), GneBsu from *Bacillus* (WP_003244356.1, group 2), WbpPPae from *Pseudomonas aeruginosa* (AAF23998.1, group 3), WbgUPsh from *Plesiomonas shigelloides* (AAG17409.1, group 3), and GalEEco from *Escherichia coli* (EFJ65355.1, group 1). Identical and similar amino acids are shaded in black and gray, respectively. Sequence numbering corresponding to each protein is shown on the right. The key six residues related to the group identification including the mutation sites K86 and C300 are indicated by stars. The conserved NAD-binding motif (GxxGxxG), the substrate-binding motif (YxxxK), and catalytic amino acid residues responsible for proton transfer are boxed, respectively.

Y-N-N-C", and enzymes belonging to group 3 contain "G-S-Y-N-A-S". Based on the rules, both the $GalE_{sp1}$ and $GalE_{sp2}$ sequences from *S. pneumoniae* TIGR4 were more consistent with those of the group 2 epimerases. Moreover, it was expected that changing C (group 2) to Y (group 1) or C (group 2) to S (group 3), as well as K (group 2) to G (group 3), might change the substrate specificity of the epimerases.

Heterologous expression and purification of enzymes $GalE_{Sp1}$ and $GalE_{Sp2}$. To identify the enzymes $GalE_{Sp1}$ and $GalE_{Sp2}$, their encoding genes were cloned into plasmid pMCSG7. The recombinant enzymes were expressed in *E. coli* and purified by nickel-affinity chromatography (Fig. 2). Fractions containing the purified enzymes were collected and dialyzed against 20 mM Tris-HCl buffer (pH 8.0). $GalE_{Sp1}$ and $GalE_{Sp2}$ were shown to be purified to homogeneity with apparent molecular mass of about 38 kDa, which is consistent with the molecular masses calculated from their amino acid sequences.

Table 2. Activity of $GalE_{Sp1}$ and $GalE_{Sp2}$ enzymes

Enzyme	Substrate	Product	Activity, mU/mg
$GalE_{Sp1}$	UDP-Glc	UDP-Gal	5.4
	UDP-GlcNAc	UDP-GalNAc	0
$GalE_{Sp2}$	UDP-Glc	UDP-Gal	18.3
	UDP-GlcNAc	UDP-GalNAc	18.5

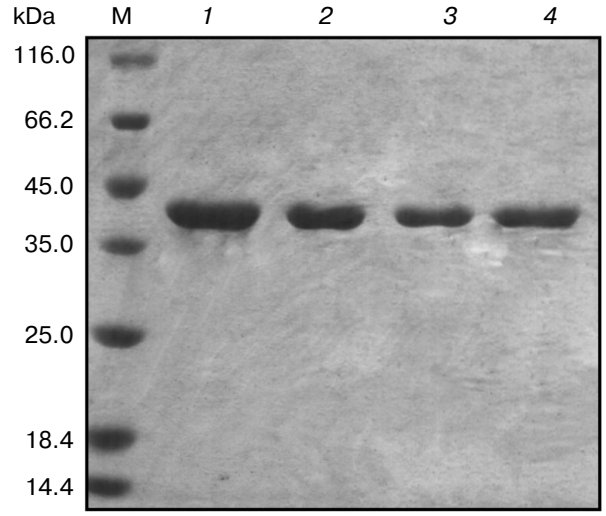

Fig. 2. SDS-PAGE of $GalE_{Sp1}$ and $GalE_{Sp2}$. Lanes: *M*) protein standards; *1*, *2*) $GalE_{Sp1}$ samples eluted by the elution buffer; *3*, *4*) $GalE_{Sp2}$ samples eluted by the elution buffer.

Substrate specificity of $GalE_{Sp1}$ and $GalE_{Sp2}$. The reaction supernatant was analyzed by capillary electrophoresis after the enzymatic assay. Both enzymes were used in an activity assay with UDP-Glc and UDP-GlcNAc using the methods described above. $GalE_{Sp1}$ could use only UDP-Glc and UDP-Gal as its substrate, and its conversion ratios were 30 and 10%, respectively. $GalE_{Sp2}$ could convert both UDP-Glc/UDP-Gal and UDP-GlcNAc/UDP-GalNAc (Fig. 3) with conversion ratios of 29 and 28% for the UDP-Glc and UDP-GlcNAc substrates. The experimental results suggest that $GalE_{Sp2}$ belongs to group 2 as predicted; however, they also suggest that $GalE_{Sp1}$ belongs to group 1 according to its substrate specificity.

The activity of $GalE_{Sp2}$ (18.3 mU/mg) was approximately 3-fold higher than that of $GalE_{Sp1}$ (5.4 mU/mg) when UDP-Glc/UDP-Gal was used as the substrate (Table 2).

Optimum temperature assay of $GalE_{Sp2}$. To further characterize $GalE_{Sp2}$, the optimum temperature of

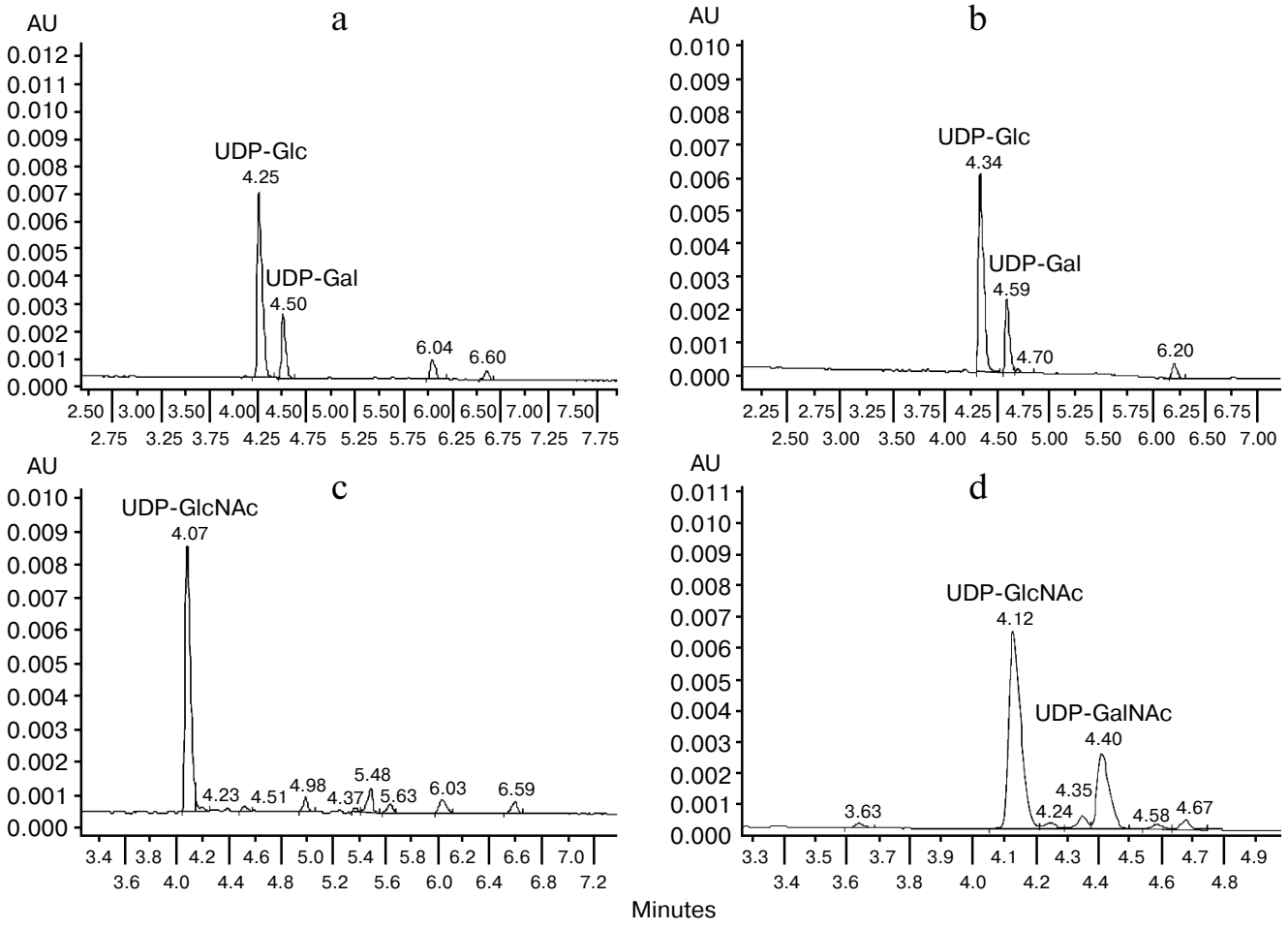

Fig. 3. Activities of $GalE_{Sp1}$ and $GalE_{Sp2}$ toward UDP-Glc and UDP-GlcNAc. a) Activity of $GalE_{Sp1}$ toward UDP-Glc; b) activity of $GalE_{Sp2}$ toward UDP-Glc; c) activity of $GalE_{Sp1}$ toward UDP-GlcNAc; d) activity of $GalE_{Sp2}$ toward UDP-GlcNAc. The activity of $GalE_{Sp1}$/$GalE_{Sp2}$ toward Gal is similar to the activity toward Glc. Similarly, the activity of $GalE_{Sp1}$/$GalE_{Sp2}$ toward UDP-GalNAc is similar to their activity toward GlcNAc.

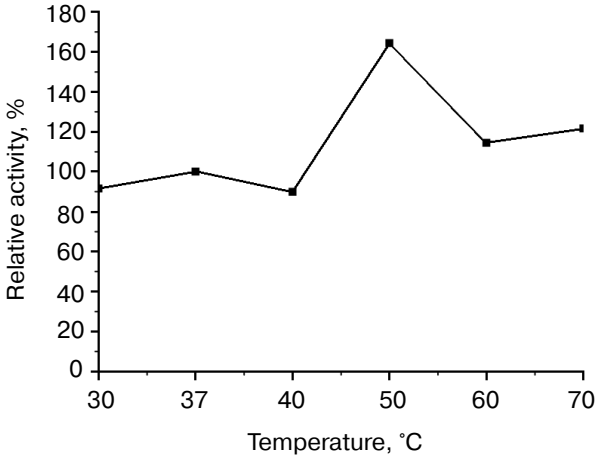

Fig. 4. Effects of temperature on the activity of $GalE_{Sp2}$. The activity of $GalE_{Sp2}$ was determined by measuring the formation of UDP-Gal from UDP-Glc at 37°C at pH 8.0, which was defined as 100%.

$GalE_{Sp2}$ was explored in 20 mM Tris-HCl, pH 8.0, with a total volume of 50 µl (with 2 µg/ml enzyme and 0.5 mM UDP-Glc). Activities were checked between 30 and 70°C in 10°C increments as well as at 37°C for 5 min. We found that $GalE_{Sp2}$ still had strong activity even at 70°C (Fig. 4).

Mutation construction and enzymatic assay of $GalE_{Sp2}$. To find the functional amino acids of $GalE_{Sp2}$, mutational studies were completed on the conserved catalytic motif.

In this study, specific residues Cys300 and Lys86, which are highly conserved and supposed to be determined based on the substrate specificity of $GalE_{Sp2}$, were mutated. Substitution C300Y was expected shift the epimerase from a group 2 to group 1 sequence signature, and substitution K86G was expected shift the enzyme from a group 2 to group 3 sequence signature. Therefore, substitutions C300Y and K86G were constructed in $GalE_{Sp2}$.

The activities of the mutants were analyzed in the same way as for the native $GalE_{Sp2}$ using capillary elec-

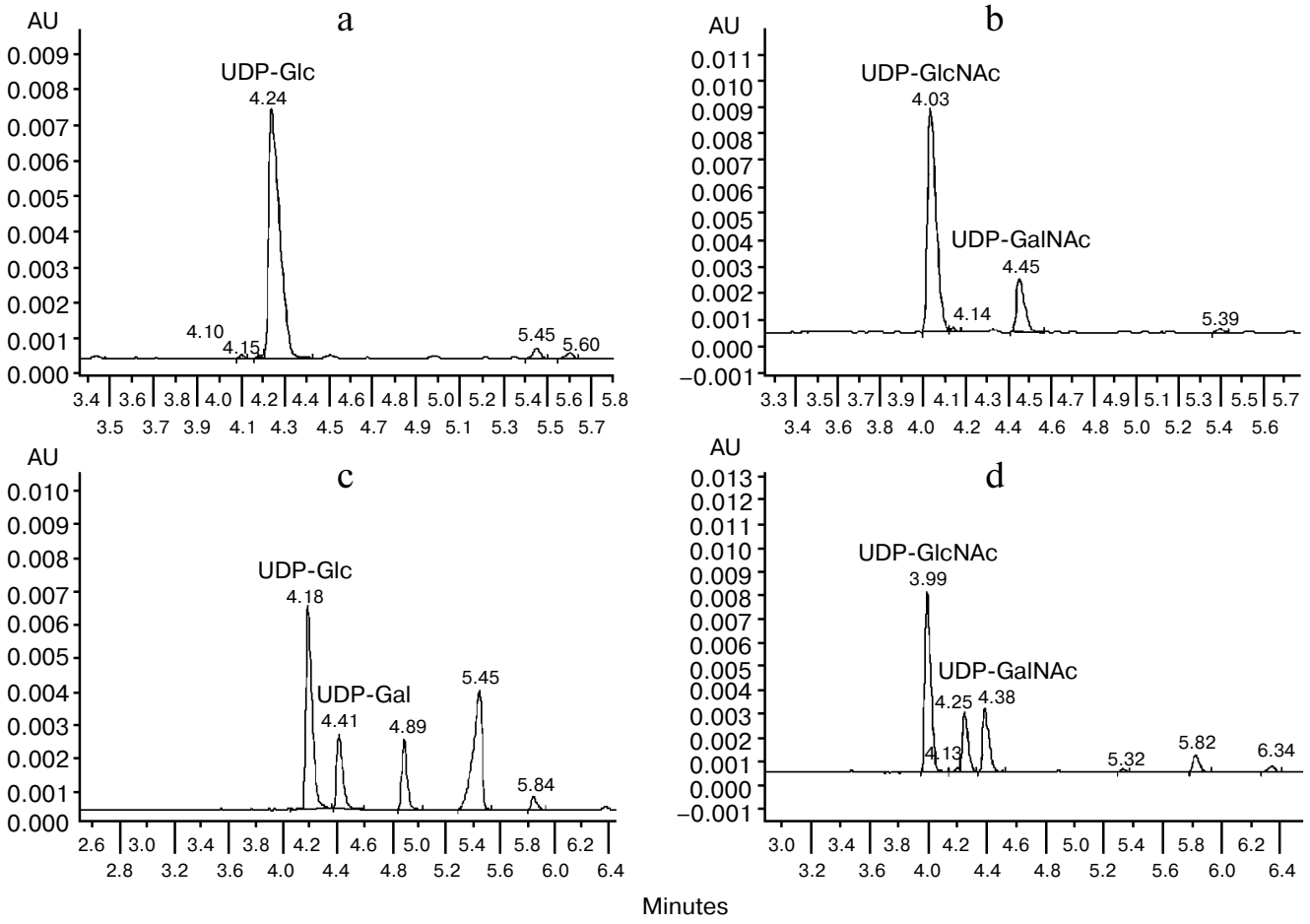

Fig. 5. Activity of $GalE_{Sp2}$ with substitutions K86G and C300Y toward UDP-Glc and UDP-GlcNAc. a) Activity of $GalE_{Sp2}$ with substitution K86G toward UDP-Glc; b) activity of $GalE_{Sp2}$ with substitution K86G toward UDP-GlcNAc; c) activity of $GalE_{Sp2}$ with substitution C300Y toward UDP-Glc; d) activity of $GalE_{Sp2}$ with substitution C300Y toward UDP-GlcNAc. The activity of the enzymes with substitutions K86G and C300Y toward Gal is similar to the activity toward UDP-Glc, and the activity of the enzymes with substitutions K86G and C300Y toward UDP-GalNAc is similar to the activity toward UDP-GlcNAc.

trophoresis. Substitution C300Y did not change the $GalE_{Sp2}$ substrate character specificity, but it greatly reduced its activity toward UDP-GlcNAc/UDP-GalNAc (Fig. 5d). Substitution K86G in $GalE_{Sp2}$ cancelled the ability of the enzyme to transform UDP-Glc/UDP-Gal completely (Fig. 5a). This result indicates that Lys86 is vital to $GalE_{Sp2}$ when functioning with non-acetylated substrates.

DISCUSSION

Sugars in capsular tetrasaccharide units of *S. pneumoniae* TIGR4 can be biosynthesized via the Leloir or other pathways. Many genes can affect the biosynthesis of capsular tetrasaccharide units, such as glucokinase (GluK), phosphoglucomutase (Pgm), UTP-glucose-1P uridylyltransferase (GalU), galactokinase (GalK), UDP-galactose 4-epimerase (GalE), etc. [24].

UDP-galactose 4-epimerase (GalE) is involved in the biosynthesis of CPS of *S. pneumoniae* and plays an essential role in the virulence of some bacteria. A deficiency of the GalE enzyme is associated with many diseases, including an embryonic-lethal phenotype in fruit flies and type III galactosemia in humans. Considering the essential role of GalE in CPS synthesis and the *S. pneumoniae* virulence, the characterization of this enzyme may provide more information for controlling diseases caused by pneumococcal infection.

GalE from *Pyrococcus horikoshii* has been used to develop a new process for enzymatic production of UDP-Gal from UDP and is an inexpensive starting material [25]. This work has shown the great potential of UDP-galactose 4-epimerase in synthesis of compounds containing unnatural galactose derivatives, in drug target design, and in vaccine development. Until now, GalE from *S. pneumoniae* TIGR4 had not been studied.

The amino acid sequence alignment showed that both $GalE_{Sp1}$ and $GalE_{Sp2}$ from *S. pneumococcus* TIGR4 were more consistent with group 2 epimerases. Both were predicted to epimerize acetylated and non-acetylated substrates. However, our experimental results suggested that only $GalE_{Sp2}$ has both activities, while $GalE_{Sp1}$ could not epimerize the acetylated substrates UDP-GalNAc and UDP-GlcNAc.

Most studies of C4 epimerases have focused on refining the catalytic mechanism involved, but few have examined the molecular basis for their substrate specificity. GalE variants from different organisms differ significantly in their DNA sequences yet are relatively homologous in their quaternary structure and belong to the same enzyme superfamily of short-chain dehydrogenases (SDR) [26, 27]. The SDR enzyme family has two characteristic signature sequences. One is GxxGxxG, which is contained within an alternating α and β structure popularly known as the Rossman fold, located near the amino-terminal end of the enzyme and the cofactor-binding pocket. The other is YxxxK, which is involved in catalysis, in which Y and K function to abstract the 4′-hydroxyl hydrogen from the sugar substrate. Based on sequence analysis, group 1 epimerases, such as the *E. coli* GalE, have a corresponding "Y300" residue. Group 3 epimerases, including WbgU from *P. shigelloides* [22] and WbpP from *P. aeruginosa* [23], have a corresponding G86 residue.

Our results clearly indicate that the determinants of substrate specificity are not exclusively located directly on the surface of the substrate-binding pocket. The site-directed mutagenesis data suggest that the Lys86 residue, which is not located in the GxxGxxG or YxxxK motif, is necessary for $GalE_{Sp2}$ to have its full activity. $GalE_{Sp2}$ belonging to group 2 can transform to group 3 with the amino acid substitution K86G. Since both $GalE_{Sp1}$ (group 1) and $GalE_{Sp2}$ (group 2) from TIGR4 have related Lys86 residues, these results indicate that Lys86 residues in $GalE_{Sp1}$ (group 1) and $GalE_{Sp2}$ (group 2) play a critical role in the activity of UDP-Glc/UDP-Gal. Substitution $GalE_{Sp2}$ C300Y greatly decreased its activity toward acetylated substrates, which suggests that $GalE_{Sp2}$ belonging to group 2 can be transformed to group 1 with amino acid substitution C300Y. Compared to the amino acid sequence of other GalEs, the Lys86 is conserved in group 2 but not in groups 1 and 3. Interestingly, when the Gly102 (corresponding to Lys86 in $GalE_{Sp2}$) in WbpP (GalE of *P. aeruginosa*) was changed to Lys, the WbpP was transformed to group 2 from group 3 [23]. This illustrates that having the amino acids Gly or Lys in these sites may have decisive influences on the substrate specificity of the enzymes.

A single point mutation of a residue can change the activity of GalEs, such as L320C in *A. nidulans*, Y299C in *E. coli*, and A209H in *P. aeruginosa* [28, 29]. This residue is thus called a gatekeeper, and there can be more than one gatekeeper in a given GalE. The Lys86 residue could be a new potential gatekeeper in $GalE_{Sp2}$.

Acknowledgments

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