

A Mutagenic Study of β -1,4-Galactosyltransferases from *Neisseria meningitidis*

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N-terminal His-tagged recombinant β -1,4-galactosyltransferase from *Neisseria meningitidis* was expressed and purified to homogeneity by column chromatography using Ni-NTA resin. Mutations were introduced to investigate the roles of, Ser68, His69, Glu88, Asp90, and Tyr156, which are components of a highly conserved region in recombinant β -1,4 galactosyltransferase. Also, the functions of three other cysteine residues, Cys65, Cys139, and Cys205, were investigated using site-directed mutagenesis to determine the location of the disulfide bond and the role of the sulfhydryl groups. Purified mutant galactosyltransferases, His69Phe, Glu88Gln and Asp90Asn completely shut down wild-type galactosyltransferase activity (1-3%). Also, Ser68Ala showed much lower activity than wild-type galactosyltransferase (19%). However, only the substitution of Tyr156Phe resulted in a slight reduction in galactosyltransferase activity (90%). The enzyme was found to remain active when the cysteine residues at positions 139 and 205 were replaced separately with serine. However, enzyme reactivity was found to be markedly reduced when Cys65 was replaced with serine (27%). These results indicate that conserved amino acids such as Cys65, Ser68, His69, Glu88, and Asp90 may be involved in the binding of substrates or in the catalysis of the galactosyltransferase reaction.

Keywords: Galactosyltransferase, Mutagenesis, *Neisseria meningitidis*, PCR

Introduction

β -1,4-galactosyltransferase (EC 2.4.1.22) is involved in the synthesis of many oligosaccharides of biological importance in eucaryotes (*N*-glycan, *O*-glycans, histo-blood group antigens, glycolipids, lactose, etc.), and in the synthesis of cell wall polysaccharides in prokaryotes. This enzyme catalyzes the transfer of galactose from UDP-galactose to glycoproteins or glycolipids containing β -linkage (Ram and Munjal, 1985; Paulson and Colley, 1989). cDNAs encoding galactosyltransferase genes have been isolated from human (Appert *et al.*, 1986; Marsi *et al.*, 1988), bovine (Narimatsu *et al.*, 1986), and murine sources (Shaper *et al.*, 1988). The availability of galactosyltransferase cDNA opens the way to the investigation of the structure-function relationships of galactosyltransferase by recombinant techniques. The functional domains of mammalian galactosyltransferase have been studied by various laboratories by expressing cDNA clones in both bacterial and mammalian cells (Masibay and Qasba, 1989; Aoki *et al.*, 1990; Nakazana *et al.*, 1993). Based on amino acid sequence similarities and conserved structural features, eucaryotic and bacterial galactosyltransferases can be classified into five families; A, B, C, D and E (Breton *et al.*, 1998). Multi-alignment of bacterial galactosyltransferases shows three conserved regions I, II, and III, as shown in Fig. 1. Region II contains an acidic motif, EDD, surrounded by two stretches of hydrophobic residues, which exhibits hydrophobic cluster analysis (Gaboriaud *et al.*, 1987) similarity with the DVD and DXD motifs of families A and B, respectively (Breton *et al.*, 1998). This is located in a region of the catalytic domain that has been implicated in UDP-galactose binding by chemical (Yadav and Brew, 1990, 1991) and mutational studies (Aoki *et al.*, 1990; Zu *et al.*, 1995)

The structural and functional relationships of mammalian galactosyltransferase have been well studied, but bacterial galactosyltransferase is poorly understood. Recently, we cloned and expressed β -1,4-galactosyltransferase from *Neisseria meningitidis* and *Neisseria gonorrhoeae* (Park *et al.*,

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2002). In *N. meningitidis*, contains a locus that consisted of three genes (*lgtA*, *lgtB*, and *lgtE*), which encode the glycosyltransferase enzymes that are required for to add of least three sugars in the lacto-*N*-neotetraose chain (Jennings *et al.*, 1995). Of these genes, the *lgtB* gene from the bacterial pathogen *N. meningitidis* is known to encode a β -1,4-galactosyltransferase enzyme (Wakarchuk *et al.*, 1998). The amino acid sequence of the β -1,4-galactosyltransferase of *N. meningitidis* shows homology with those of other bacterial galactosyltransferase from *Haemophilus influenza* (High *et al.*, 1993), *Haemophilus ducreyi* (Sun *et al.*, 2000), *Helicobacter pylori* (Endo *et al.*, 2000), *Pateurella haemolytica* (Potter and Lo, 1995) and *Escherichia coli* (Heinrichs, 1998). These bacterial galactosyltransferases are classified into family C (Breton *et al.*, 1998), one of the five families of galactosyltransferases that include the β -1,3-galactosyltransferase of the rat and the β -1,4-galactosyltransferases *Neisseria* bacteria, which participate in the biosynthesis of the lacto-*N*-neotetraose terminal lipopolysaccharide structure (Gotschlich, 1994; Jennings *et al.*, 1995). In this report, we performed mutational studies upon several conserved amino acids, and we found that the conserved acidic cluster (EDD in Region II of Fig. 1) and amino acids (Fig. 1) are important for catalysis.

Materials and Methods

Construction and cloning of the site-directed galactosyltransferase genes Eight oligonucleotides were designed to construct site-directed mutated galactosyltransferases (Table 1). These were chosen by examining the conserved sequences of other bacterial galactosyltransferases. Mutagenesis was performed by sequential PCR (Higuchi *et al.*, 1988). In first round PCR, the terminal sense sequence 5' CGGGATCCATGCAAACACGTTATCAGC 3' and internal antisense primers (Table 1) were used as a primer set. And for 2nd round PCR, the terminal antisense sequence 5' GCGGTACC GCAAATACGATGTCCATCT 3' and internal sense primers (Table 1) were used as a primer set. The pETgal4M vector containing the galactosyltransferase gene (*lgtB*) of *N. meningitidis* was obtained during a previous study (Park *et al.*, 2002) and used as a template. In 1st round PCR was performed over 30 cycles with an initial denaturation of 10 min at 95°C, and cycles of 60 s at 95°C, 60 s at 55°C and 2 min at 72°C. In the 2nd round, terminal sense and anti-sense primers were used as a primer set to anneal the upstream and downstream flanking sequences just beyond the *lgtB* gene, and which contained the *Bam* HI and *Kpn* I recognition sequences (underlined in the terminal sense and antisense sequences). Two PCR products from the first round reactions were used as templates to produce a single annealed PCR product. The PCR conditions used were as follows: initial denaturation for 2 min at 95°C and then thirty cycles of denaturation for 1 min at 95°C, annealing for 60 s at 55°C, and extension for 2 min at 72°C. The final PCR products were digested with *Bam* HI and *Kpn* I, and ligated to pET28a purchased from Novagen (Darmstadt, Germany). This ligation mixture was used to transform *E. coli* XL1 blue. All mutants were analyzed by DNA sequencing of the plasmid inserts by the

Table 1. Primers used in site-directed mutagenesis

Primer	Sequence
Cys65Ser	GTGGAAAAAGCC <u>AGC</u> TTTATGAGCCAC
Ser68Ala	GCCTGCTTTATGG <u>CCC</u> CACGCCGATTG
His69Phe	TGCTTTATGAGCT <u>TTC</u> GCCCGTATTGTGG
Glu88Gln	ATCGCCGTATTT <u>CAG</u> GACGACGTTTTTA
Asp90Asn	GTATTTGAGGAC <u>AAC</u> GTTTTACTCGGC
Cys139Ser	GTGGCGGACTAC <u>AGC</u> GGGCGTGCCTTT
Tyr156Phe	GGGACGGCGGGC <u>TTT</u> ATCATTTCCAAA
Cys198Ser	GGAATGCCGGTT <u>AGC</u> CAGCTCAATCCC
Cys205Ser	AATCCCGCCTTA <u>AGC</u> GCCCAAGAGCTG

dideoxy termination method. The resulting recombinants are referred to as the pETgal4 series.

Expression and purification of the recombinant enzyme After inductions (4 h at 37°C and 8 h at 25°C) for several hours with isopropyl-D-thiogalactopyranoside (IPTG), *E. coli* BL21 (DE3) cells harboring pETgal4 series were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM PMSF). Cells were then sonicated using a microtip at 50% power for five 20-s intervals on ice, and soluble and insoluble fractions were separated by centrifugation, when needed. Whole cell lysates and soluble and insoluble fractions from 0.1 ml cultures were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and bands were visualized by Coomassie blue staining using previously described standard procedures (Maniatis *et al.*, 1982).

The recombinant proteins were purified by column chromatography using Ni-NTA resin (Koh *et al.*, 2001). BL21 (DE3)/pET-gal4 series were grown in 100 ml of LB medium containing 50 μ g/ml kanamycin at 25°C in a shaking flask. When the culture reached $A_{600} = 0.5$, the T7 promoter was induced with 0.5 mM IPTG for 8 h. Cells were then harvested by centrifugation, resuspended in 30 ml of lysis buffer, and sonicated using a multitip at 50% power for ten 1-min intervals on ice. The lysates were then centrifuged at 75,000 *g* for 90 min, and pellets were extracted with 10 ml of the same buffer. The combined supernatants were then loaded on a 5-ml Ni-NTA column pre-washed with lysis buffer, proteins were eluted with elution buffer (50 mM Tris pH 8.0, 300 mM NaCl, 250 mM imidazole, 1 mM PMSF), and eluted fractions were dialyzed against a storage buffer (50 mM Tris pH 8.0, 20 mM NaCl, 20% glycerol). The column fractions were identified at each purification step by 12% SDS-PAGE.

Galactosyltransferase assay with purified β -1,4-galactosyltransferase

The galactosyltransferase assay used was a modification of a previously described method (Kim *et al.*, 1997; Park *et al.*, 2002). The enzyme activities of the purified proteins were measured *in vitro* in 30-ml reaction mixes containing 20 mM MOPS (pH 7.85), 10 mM $MnCl_2$, 10 mM ATP, 20 mM N-acetylglucosamine (GlcNAc), and 0.8 pmol (5×10^4 cpm) of dried [³H] UDP- α -D-galactose (60 Ci/mmol) in an Eppendorf tube. After incubation at 37°C for 1 h, the reaction was terminated by adding 1 ml of distilled water. Reaction mixtures were loaded onto a 1-ml Dowex (AG1-X8) pipette column pre-equilibrated with 5% sodium borate. After

the column had been washed with 1 ml of 5% borate solution 5 times, the amount of tritium labeled galactose β -1,4 N-acetylglucosamine (Gal β 1-4GlcNAc) in each fraction was quantified using a liquid scintillation counter. In order to identify the disaccharide (Gal β 1-4GlcNAc) reaction product, the reaction mixture was loaded on a Bio-Gel P-4 column (1.5 \times 100 cm) preequilibrated with 5% sodium borate solution. The tritium labeled galactose incorporated in disaccharide was counted using a liquid scintillation counter.

Results and Discussion

The galactosyltransferases constitute a large and heterogeneous class of enzymes, and the amino acid sequences of galactosyltransferases from different sources show a high degree of conservation. On the basis of the type of reaction catalyzed and protein sequences, these enzymes can be classified into seven α -galactosyltransferase and five β -galactosyltransferase groups, respectively (Breton *et al.* 1998). This classification also reflects the natures of the glycosidic linkages formed.

Within individual groups, proteins are expected to be evolutionally related and to have similar overall 3D structures. By combined hydrophobic cluster analysis (Gaboriaud *et al.*, 1987) and BLAST analysis, Breton *et al.* (1998) searched for local homologies in the different groups of galactosyltransferases and in other glycosyltransferases. This study resulted in the definition of five families (A, B, C, D, and E) which included protein sequences of glycosyltransferases from various sources and classes. The lengths and locations of the conserved regions in galactosyltransferase vary from one family to another (Breton *et al.*, 1998). The present study identified conserved amino acids in family C in galactosyltransferase of bacterial species that infect mammals (Fig. 1). These galactosyltransferases exhibit partial homology with other protein sequences of β -1,3-galactosyltransferase from mammals (Breton *et al.*, 1998). Yuan *et al.* (1997) reported that a class of signaling molecules involved in developmental processes, such as Fringe and Brainiac, may be glycosyltransferases, and found that they show local homology with bacterial galactosyltransferases. A multialignment of the three most conserved regions found in proteins of family C is shown in Fig. 1. Region II, which contains an acidic motif (EDD) present immediately after a vertical hydrophobic cluster, indicative of a β -strand, was found in almost all families, except family D, which contained a consensus sequence instead, i.e., ExxxxxxxE. As this motif is widespread in different classes of glycosyltransferases, it is likely to be involved in enzyme function. The use of a UDP-sugar was found to be a common feature of enzymes belonging to families A, B, C, and E. Therefore, the acidic motif could be involved in either UDP binding and/or the catalytic process.

In this study the use of PCR to introduce mutations into the DNA sequence required either two or three steps. PCR was

performed using mismatched primers as shown in Table 1 to introduce mutations into the galactosyltransferase DNA sequence. Two primary PCR reactions produced two overlapping DNA fragments, both bearing the same mutations, which were introduced into the region of overlap *via* primer mismatch.

As discussed previously, mutations were introduced into recombinant β -1,4-galactosyltransferases to investigate the possible roles of a conserved sequence region, which is a plausible location for a binding site for the catalytically essential cation. The entire sequences of mutants were checked by DNA sequencing to confirm the presence of the desired mutation in each case, and to ensure that no unwanted mutations were introduced by the PCR mutagenesis procedure.

E. coli BL21 (DE3) cells harboring pET-gal4 series containing the mutant galactosyl transferase gene were induced by IPTG at 25°C and 37°C. Whole cell lysates were then analyzed by 12% SDS-PAGE. One major band appeared approximately at the 33-kDa position in the case of IPTG induction (data not shown), as was previously reported by Park *et al.* (2002). The mutant recombinant galactosyltransferases were purified to homogeneity by Ni-NTA affinity chromatography from overexpressed cells that were grown at 25°C (data not shown).

In order to further investigate, the structurally related functions of galactosyl transferase, highly conserved amino acids, such as Ser68, His69, Glu88, Asp90, and Tyr156 were substituted with structurally analogous amino acids. The enzyme activities of wild type and mutant galactosyltransferases were then measured. The mutants His69Ala, Glu88Gln, and Asp90Asn showed dramatic reductions in enzyme activities as shown in Fig. 2. Substitutions of Asp90 and Glu88 into Asn90 and Gln88 severely shut down galactosyltransferase activity (1% and 2.4%), indicating that the acidic group formed by these amino acids may have an essential role in enzymatic activity. A recent study showed that the acidic motif (DVD) of bovine β -1,4-galactosyltransferase is involved in metal coordination (Boeggeman and Qusba, 2002). Thus it seems that the substitutions of Glu88 and Asp90 into Gln88 and Asn90 might interrupt the coordination of Mn⁺² to galactosyltransferase; moreover, the substitution of His69 into Ala69 almost abolished the enzyme activity (3.0%), and replacing Ser68 with Ala68 reduced the activity (by 19%). These results indicate that His69 and Ser68 in galactosyltransferase are probably involved in galactosyltransferase catalysis or are closely located to the binding sites of UDP galactose or other substrates. On the other hand, the substitution of Tyr156 with Phe156 had little affect on enzyme activity (90%) indicating that the hydrophobic properties of tyrosine and phenylalanine may be important for the catalytic reaction. These results suggest that the hydrophobic group of tyrosine is important for the catalytic activity of galactosyltransferase.

It is well known that the internal disulfide bond of human

		Region I	
<i>N.meningitidis</i>	(2)	NHVISLASAA	ERRAHIADTF GRHGIPFQFF DALMPSERLE QAMAEVLPGL
<i>N.gonorrhoeae</i>	(2)	NHVISLASAA	ERRAHIAATF GSRGIPFQFF DALMPSERLE RAMAEVLPGL
<i>H.ducreyi</i>	(7)	NYVISLKTAD	ARRQHIIQEF AKHNIPFQFF DACSIENPLY MDIEKHLPLM
<i>P.hemolytica</i>	(2)	NYVISLTSAQ	ERRKHIEAEF GKQNIPFQFF DAITPD..LI KEKAKAFNID
<i>E.coli</i>	(4)	IYIVSLKRDI	ERRNKINDVF HRLNINFDFF DAIDAKDPQN KEIIDKMRLS
<i>H.pylori</i>	(40)	DAIYSKTFEG	GLHPLVKKHL HPYFITQNIK DMGITTNLIS EVSKFYALK
<i>H.influenzae</i>	(22)	QAQFELLFSN	NLIQEIHFFD AIYGKSNPNH PLFQRYNENK RLNAKGYPL.
Consensus	sl....	.rr..i...fi.f.ff da....n... ..l.
		Region II	
<i>N.meningitidis</i>	(10)	KACFMSHAVL	WKQALDEGLP YITVFEDDVL LGEGBEKFLA EDAWLQERFD
<i>N.gonorrhoeae</i>	(10)	KACFMSHAVL	WEQALDEGVP YIAVFEDDVL LGEGBAQFLA EDTWLQERFD
<i>H.ducreyi</i>	(9)	KGCLMSHFL	WKKCVLDDIP YMTIFEDDII LSDESNEFIS DYSWVNNRFY
<i>P.hemolytica</i>	(10)	IACALSHIAL	WHLAKQQNLD YICIFEDDIY LGNNAFELL.KTYNI
<i>E.coli</i>	(10)	IACTLSHQLI	YKDMIDKNIE WAVILEDDVI VNEKFKKFLQ YFNLPKDKL
<i>H.pylori</i>	(10)	LGCYASHYSL	WEKCIELNEA .ICILEDDIT LKEDFKEGL. ..DFLEKHIQ
<i>H.influenzae</i>	(4)	LGCYASHYSM	WEKCVELDYP .IIVLEDDAK FKNNFLEVL. ..DFINSDKN
Consensus		.gC.. <u>SH</u> ..l	w.kc...n.. .i.il <u>EDD</u> .. l.n.f.e.l.
		Region III	
<i>N.meningitidis</i>	(33)	LESEHWGTAG	YIISRKAMRF FLDRFAALPP EGLHPVDLMM(93)
<i>N.gonorrhoeae</i>	(33)	LESEHCGTAG	YIISRKAMRF FLDRFAVLPP ERLHPVDLMM(97)
<i>H.ducreyi</i>	(33)	LISRHFGTAS	YLISKEAAKY LILLYEKLPA DELIAVDESI(94)
<i>P.hemolytica</i>	(34)	LNSRHVGTAG	YILTNGAELF LINILKTLN. ...IPIDDLI(92)
<i>E.coli</i>	(40)	NKNKIRRTCS	YLMNKDMAQK LLKLTQDYGT YRADSWKLMH(44)
<i>H.pylori</i>	(36)	AYSHGVGTQG	YVITPKIAKV FKKHSRKW.. ..VVPVDTIM(38)
<i>H.influenzae</i>	(32)	GFA...GATG	YYLTPQAARK FLTQSKEW.. ..YLTVDVTM(45)
Consensus		..s...gt.g <u>Y</u> ..t...a..	fl...k....vd..m

Fig. 1. Multiple sequence alignment of bacterial galactosyltransferase protein sequences. The aligned protein sequences and their GenBank accession numbers are: *Neisseria meningitidis* β -1,4-galactosyltransferase (U25839), *Neisseria gonorrhoeae* β -1,4-galactosyltransferase (U14544), *Haemophilus ducreyi* galactosyltransferase II (AAF32397), *Pasteurella hemolytica* LpsA gene (U15958), *Escherichia coli* putative β -1,4-galactosyltransferase (AAC69683), *Helicobacter pylori* β -1,4-galactosyltransferase (BAA88524) and *Haemophilus influenzae* Lex1 gene (Q03974). The invariant residues in consensus are given in bold type and underlined. Dots indicate gaps. Numbers in brackets indicate the numbers of amino acids between two conserved regions. Numbers indicate amino acid residues.

galactosyltransferase has an important function in terms of its secondary structure (Wang *et al.*, 1994). The substitution of cysteine residues with another amino acid like serine, would be expected to prevent the formation of an internal disulfide bond, and alter the structure if it is required for disulfide bond formation. Serine appears to be a reasonable substitution for cysteine because this change would be expected to minimally affect the conformation.

The functions of the three cysteine residues in galactosyltransferase were investigated using site-directed mutagenesis to determine the locations of the disulfide bond and of the sulfhydryl groups. The enzyme remains active

when two of its cysteine residues at positions 139 and 205 were mutated separately to serine (92% and 102%). However, its enzyme activity was markedly reduced when Cys65 was replaced with serine (27%). Further substitution of Cys139 or Cys205 with serine did not affect the Cys65Ser mutant galactosyltransferase, as shown in Fig. 3. Our experiments shows that the sulfhydryl group of Cys65 is important required for the catalytic activity of galactosyltransferase.

The above results suggest that the highly conserved amino acids common to bacterial galactosyltransferase are required for proper enzymatic activity. Further studies are under way to characterize the mutant galactosyltransferases.

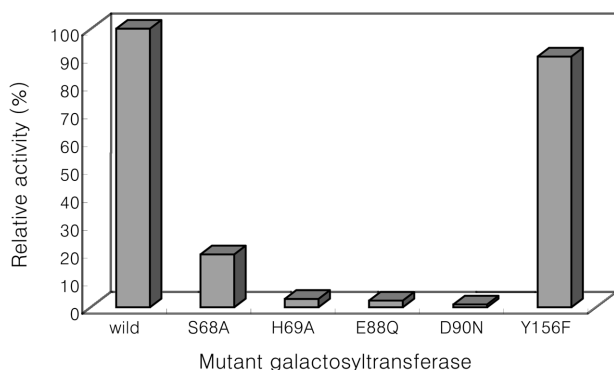


Fig. 2. The galactosyltransferase activity of *Neisseria meningitidis* β -1,4-galactosyltransferase and its site directed mutants. The activity detected for the mutant enzyme is indicated as a percentage of the activity of wild-type galactosyltransferase (0.24 pmoles of galactose transferred per min per mg protein).

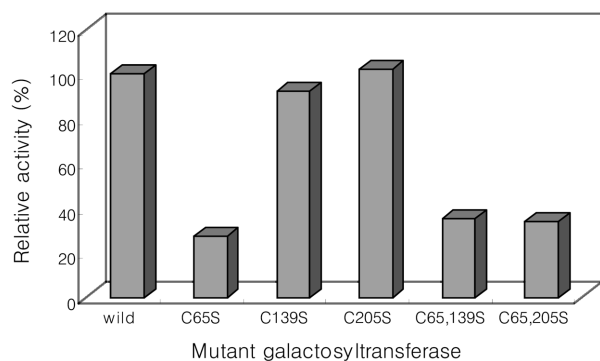


Fig. 3. The galactosyltransferase activity of *Neisseria meningitidis* β -1,4-galactosyltransferase and its cysteine mutants. The activity detected for the mutant enzyme is indicated as a percentage of the activity of wild-type galactosyltransferase (0.24 pmoles of galactose transferred per min per mg protein).

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