Characterization of the Glycosyltransferase Enzyme from the *Escherichia coli* K5 Capsule Gene Cluster and Identification and Characterization of the Glucuronyl Active Site*

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Bacterial capsular polysaccharides play an important role in virulence and survival. The *Escherichia coli* K5 capsule consists of a repeat structure of -4)GlcA-β(1,4)-GlcNAc α(1-1, identical to N-acetylheparosan. A 60-kDa protein, KfiC, has been identified as a bifunctional glycosyltransferase, responsible for the alternating α and β addition of each UDP-sugar to the nonreducing end of the polysaccharide chain. Using hydrophobic cluster analysis, a conserved secondary structure motif characteristic of β-glycosyltransferases was identified along with two highly conserved aspartic acid residues at positions 301 and 352 within the KfiC protein. Site-directed mutagenesis was used to identify catalytically active amino acids within domain A of the KfiC protein. The conserved aspartic acid residues at 301 and 352 were shown to be critical for the β addition of UDP-GlcA (uridine diphosphoglucuronic acid) to defined nonreducing end oligosaccharide acceptors, suggesting that these conserved aspartic acid residues are catalytically important for β-glycosyltransferase activity. A deleted derivative of the kfiC gene was generated, which encoded for a truncated KfiC (kfiC') protein. This protein lacked 139 amino acids at the C terminus. This enzyme had no UDP-GlcA transferase activity but still retained UDP-GlcNAc transferase activity, indicating that two separate active sites are present within the KfiC protein.

Glycosyltransferases responsible for the addition of UDP-sugars to the growing polysaccharide chain by the formation of an α (16) or β bond (17, 18) have been studied from a wide variety of systems. Hydrophobic cluster analysis (HCA) of processive β-glycosyltransferases that add sugars to the reducing end of the growing polysaccharide chain identified two domains A and B (17). Domain A consists of four alternating β-strands separated by three α helices (Fig. 2). Two conserved aspartic acid residues were identified in the C-terminal loops of the β2- and β4-strands within domain A (Fig. 2, Table I) and it has been suggested that these might be the catalytic amino acids for the nucleophilic substitution reaction involved in β bond formation (17). The secondary structure prediction for domain B is more difficult to interpret, although a conserved aspartic acid residue and the motif QXXRW were identified (Fig. 2/17). Nonprocessive β-glycosyltransferases that add a sugar molecule to the nonreducing end of the polysaccharide chain lack domain B, suggesting that this domain is important for processivity. Similar studies on bacterial α-mannosyltransferases identified a region of approximately 270 amino acids that could be aligned in these enzymes. Two glutamic acid residues were conserved in this region, and it is likely that these are the catalytic amino acids for the two-step process involved in the retaining mechanism of a bond formation (16).

The abbreviations used are: -4)GlcA-β(1,4)-GlcNAc α(1-, a polymer of alternating glucuronic acid and N-acetylglucosamine residues linked by α1,4 and β1,4 glycosidic bonds; UDP-GlcA, uridine diphosphoglucuronic acid; HCA, hydrophobic cluster analysis; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

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To date no biochemical studies have been carried out on a glycosyltransferase like KfiC, which is able to sequentially add UDP-sugars using both an inverting (β-bond) and retaining (α-bond) mechanism. In this study we used HCA (19) to predict regions of secondary structure homology between KfiC and other known β-glycosyltransferases. Based upon this information, potential catalytic aspartic acid residues within the conserved regions were changed by site-directed mutagenesis. The ability of modified KfiC to add each UDP-sugar to defined oligosaccharide substrates was then assessed. These studies confirmed that KfiC is a bifunctional transferase, and that they identified the catalytic aspartic acid residues essential for the UDP-GlcA β-glycosyltransferase activity.

EXPERIMENTAL PROCEDURES

**Bacterial Strains and Plasmids**—JM109(DE3) (Promega) was used for the expression of all constructs. Plasmid pG11 (amp+) contains the kfiC gene encoding the KfiC transferase from the *E. coli* K5 capsule gene cluster amplified by polymerase chain reaction and cloned into EcoRI site of pGEM5zf (+) (Promega). Expression is controlled by T7 promoter. Plasmid pPC6 (K5", Cm") contains the entire K5 capsule gene cluster cloned into pACYC184 (12).

**K5 Bacteriophage Analysis**—Cells were grown to A<sub>600</sub> ~0.6, pelleted by centrifugation, re-suspended in 10 mM MgSO<sub>4</sub>, 100 μl of cells were mixed with 100 μl of the appropriate bacteriophage dilution, incubated for 20 min, mixed with 3 ml of molten soft top agar (~45 °C), and poured onto selective agar plates. Plaque formation was assessed after overnight incubation.

**Site-directed Mutagenesis**—Mutagenesis was achieved using the QuikChange site-directed mutagenesis kit from Stratagene. Mutated DNA was fully sequenced using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit and automated sequencer (Applied Biosystems) to confirm that the desired mutation was the only change present.

**Generation of Cell Lysates**—Bacterial cultures were grown to an A<sub>600</sub> 0.6 at 37 °C with shaking at 200 rpm. Protein expression was induced by addition of 0.5 mM IPTG (final) for 90 min. Cells were harvested by centrifugation (10,000 × g, 10 min). The bacterial pellet was washed and resuspended in buffer (50 mM Tris, pH 8.0, 30 mM MgSO<sub>4</sub>), and cells were lysed by sonication (4 × 20-s bursts with cooling) prior to undertaking transferase assays.

**Transferase Assay**—Assays were carried out on cell lysates prepared as described above. The assay measured the incorporation of single radiolabeled UDP-sugars onto defined oligosaccharide substrates (either terminating with glucuronic acid or N-acetylgalactosamine at the nonreducing end) in the presence of 1% Triton X-100. The full methods section is as used by Lind et al. (20).

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis**—Membranes were prepared for Western blot analysis by centrifugation of cell lysates at 10,000 × g for 10 min to remove debris. Subsequently, the supernatant was centrifuged (100,000 × g, 30 min) to separate membranes and cytosolic fractions. The membranes were washed, resuspended in T buffer (50 mM Hepes, pH 7.2, 10 mM MnCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>, 1% Triton X-100), and stored frozen. The membranes were analyzed using standard 12% polyacrylamide electrophoresis gels, and following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes and probed with a rabbit anti-transferase antibody followed by a goat anti-rabbit horseradish peroxidase secondary antibody. Detection was achieved using the ECL kit from Amersham, UK.

**Hydrophobic Cluster Analysis**—HCA was carried out using a computer program obtained from B. Henrissat, Center de Recherches sur les Macromolecules Vegetales, CNRS, BP 53, 38041 Grenoble cedex 9, France. Alignments were made manually.

**RESULTS**

Alignment of the HCA plot of the predicted amino acid sequence of KfiC with plots of other known β-glycosyltransferases revealed a common domain structure of repeating α-helix and β-strand motifs between amino acids 260–360 (Fig. 2). This conserved region corresponded to the previously named domain A of β-glycosyltransferases (17). Within this domain, two aspartic acid residues at 301 and 352 in KfiC were identified as the highly conserved aspartic acid residues in the C-terminal loops of the β2 and β4 strands present in all of the β-glycosyltransferases (Fig. 2, Table I). For reasons of clarity only HCA alignments with hyaluronic synthase and chitin synthase (processive β-glycosyltransferases that utilize alternating UDP-GlcA and UDP-GlcNAc) are shown in Fig. 2, however, most β-glycosyltransferases may be aligned in this way. There is no evidence of domain B in KfiC and no conserved QXXRW amino acid motif (Fig. 2), which is characteristic of this domain (17).

Based upon the identification of the conserved domain A and the presence of the two highly conserved aspartic acid residues, a series of site-directed mutations were made. The aspartic acid (D) residues at 300, 301, 305, 352, and 354 within domain A were replaced with alanine (A) or glutamic acid (E) residues. The nucleotide sequence of the mutated kfiC gene was determined to confirm that no additional mutations had been introduced. The level of expression of each mutated KfiC protein following induction of the T7 promoter using IPTG was established by Western blot analysis using KfiC-specific antisera. All of the mutated KfiC proteins were expressed at similar levels comparable with that of the wild-type KfiC protein (Fig. 3).

The over-expression of the kfiC gene in the absence of other capsule-related proteins results in large amounts of recombinant KfiC protein in inclusion bodies within the cell, but no detectable membrane-associated K5 glycosyltransferase activity. However, overexpression of the kfiC gene in strains harboring the K5 capsule gene cluster results in an increase in membrane-associated K5 glycosyltransferase activity (14). This suggests that other proteins encoded within the K5 capsule gene cluster are essential for the formation of a membrane-associated complex containing the KfiC glycosyltransferase. In order to provide the necessary kfiC minus background, but still retain the other K5 capsule proteins necessary for the formation of the K5 polysaccharide biosynthetic complex, a kfiC mutation was generated in plasmid pPC6 using the streptomycin suicide vector pKNG101 (21). This vector requires the pir gene for replication and will not replicate in laboratory strains of *E. coli* lacking this gene (21). A 0.8-kb DraI-BamHI fragment from within the center of the kfiC gene was cloned into pKNG101 to generate pNJ1. Plasmid pNJ1 was introduced into JM109DE3(pPC6), in which it cannot replicate, and transformants in which pNJ1 had become integrated into pPC6 via homologous recombination within the kfiC gene were selected using chloramphenicol and streptomycin (Fig. 4). The disruption of the kfiC gene in the resulting plasmid pPC6::NJ1 was confirmed by restric-

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tion mapping and Southern blotting (data not shown). It is predicted that the disruption of the KfiC gene in pPC6::NJC, will result in the expression of a truncated KfiC protein consisting of the first 381 amino acids, KfiC\textsuperscript{9} (Fig. 4). Strains harboring pPC6::NJC were unable to make a K5 capsule and were resistant to the K5-specific bacteriophage. The mutation in the kfiC gene in pPC6::NJC could be complemented by pGG1, which restored K5 capsule production and K5 bacteriophage sensitivity.

The effect of the site-directed mutations in the kfiC gene on UDP-GlcA and UDP-GlcNAc transferase activity was determined by assaying the ability to add radiolabeled GlcA or GlcNAc from the corresponding UDP-sugar to defined oligosaccharides with either a GlcNAc or GlcA at their nonreducing end. The transferase activities were expressed as a percentage of the activity of the wild-type KfiC transferase activity in JM109DE3(pPC6::NJC; pGG1) following induction of kfiC expression by the addition of IPTG. As predicted, strain JM109DE3 had no detectable GlcA or GlcNAc transferase activity (Table II). While strain JM109DE3(pPC6::NJC) lacked any detectable GlcA transferase activity, surprisingly, it still expressed GlcNAc transferase activity at 71.6% of that of strain JM109DE3(pPC6::NJC; pGG1) (Table II). This suggests that the insertion of pNJC into pPC6 had not disrupted GlcNAc transferase activity. All of the site-directed mutations that changed aspartic acid to alanine in domain A, D300A, D301A, D305A, D352A, and D354A, lacked any detectable GlcA transferase activity but retained GlcNAc transferase activity comparable with that of JM109DE3(pPC6::NJC; pGG1) (Table II). The conservative changes D301E, D305E, and D352E also abolished detectable GlcA transferase activity, but had no effect on

| Gene | Organism | Sequence |
|------|----------|----------|
| KfiC | E. coli | 295.EILVCDGCSSDKLEI...NFITFQADDLHPE.360 |
| HasA | Streptococcus pyogenes | 58..EIYIYDGGSSNDAIQ...DVFLTVSDTYIYN.128 |
| Chs1 | Streptococcus cerevisiae | 494.VCVIISDRGSKINERS...NVTLTDAGTMCAP.610 |
| ExoM | Rhizobium meliloti | 39..LRVIVADNDAEFSARA...DFLAFDDDSYSG.106 |
| ExoO | Rhizobium meliloti | 41..EYVYVDSADATPAI...R9IAVLDSDTYPDR.104 |
| ExoU | Rhizobium meliloti | 37...EVVVIDGSDTDDASAV...PLIGVLDADDFPG.102 |
| ExoW | Rhizobium meliloti | 34...HLVVIDDESPYIADI...DFVAFBDDDDVTP.101 |
| NodC | Rhizobium meliloti | 58...RVYVVDGSRNEAIV...DLVNVDSSTIAF.124 |
| RfaA | Salmonella enterica | 35..SELVIDNMGILDRHY...QVIADVDPDDINEP.101 |

![Fig. 2. Hydrophobic cluster analysis alignment of KfiC protein with 2 other known \(\beta\)-glycosyltransferases, hyaluronan synthase (HasA) and chitin synthase (Chs1). Conserved domains A and B are marked. Conserved amino acids are circled. \(\bullet\), glycine; \(\star\), proline; \(\square\), threonine; \(\bigcirc\), serine. The alignment shows that KfiC possesses a conserved domain A only with no domain B or QXXRW amino acid motif. The conserved aspartic acid residues are at positions 301 and 352.](http://www.jbc.org)
DISCUSSION

The induction of expression of the kfiC gene in JM109DE3(pPC6::NJC, pGG1) increases the rate of both GlcNAc and GlcA additions to the defined nonreducing end of K5 oligosaccharides. This confirms earlier studies that KfiC is a bifunctional transferase capable of adding GlcNAc and GlcA from the appropriate UDP-sugar to the nonreducing end of the polysaccharide chain. The same mechanism has been suggested for the chain elongation in proteoglycan biosynthesis (22) where a single protein catalyzes the same transferase reactions (20).

β-Glycosyltransferases can be divided into two families on the basis of HCA of their predicted amino acid sequence (17). β-Glycosyltransferases that add sugars to the reducing end of the polysaccharide chain, such as HasA or ChsI, have two conserved domains A and B (17), while those that add sugar residues to the nonreducing end of the polysaccharide chain have only domain A (17). HCA of the predicted amino acid sequence of KfiC demonstrated the presence of a domain A, but there was no evidence for domain B (Fig. 2). This is in agreement with the current knowledge on K5 biosynthesis, which is believed to occur by the sequential addition of GlcA and GlcNAc residues to the nonreducing end of the growing K5 polysaccharide (23, 24). In contrast to the membrane-associated hyaluronate synthase (HasA) enzyme of group A streptococci which, when expressed alone in E. coli, is capable of synthesising hyaluronic acid (25, 26), KfiC is unable to synthesize K5 polysaccharide in the absence of other K5 proteins (14). The formation of a membrane-bound biosynthetic complex consisting of up to 10 proteins would appear necessary for the functioning of the KfiC enzyme and the biosynthesis of K5 polysaccharide.3 It is likely that KfiC acts to extend a membrane-bound oligosaccharide acceptor synthesized by either the KfiA

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**TABLE II**

The effect of each single amino acid change on the two glycosyltransferase activities of the KfiC enzyme

The ability to add UDP-glucuronic acid and UDP-N-acetylglucosamine was measured separately using defined nonreducing end oligosaccharide acceptors. The specific activity for KfiC in JM109DE3(pPC6::NJC, pGG1) was taken as 100% activity, and all other specific activities were expressed as a percentage of that figure. The figures in parentheses are the standard deviations.

| Construct          | GlcNAc activity (percentage of wild type) | GlcA activity (percentage of wild type) |
|--------------------|------------------------------------------|----------------------------------------|
| JM109DE3           | 0.1 (0.04)                               | 0.1 (0.06)                             |
| pPC6::NJC          | 71.6 (0.6)                               | 0.1 (0.02)                             |
| D300A              | 89.6 (24.8)                              | 0.3 (0.10)                             |
| D300E              | 112.8 (24.8)                             | 2.4 (1.10)                             |
| D301A              | 100.9 (11.3)                             | 0.2 (0.17)                             |
| D301E              | 131.2 (50.8)                             | 0.2 (0.20)                             |
| D305A              | 94.9 (25.6)                              | 0.3 (0.20)                             |
| D305E              | 112.9 (29.7)                             | 0.1 (0.02)                             |
| D352A              | 103.9 (25.2)                             | 0.2 (0.20)                             |
| D352E              | 71.7 (31.1)                              | 0.1 (0.06)                             |
| D354A              | 132.8 (50.1)                             | 0.3 (0.04)                             |
| D354E              | 152.7 (30.0)                             | 75.7 (21.0)                            |

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or KfiB proteins. The nature of this starting oligosaccharide and its linkage to the membrane is unknown, but presumably it terminates with either GlcA or GlcNAc at its nonreducing end.

The catalytic mechanism of polysaccharide growth involves the formation of a glycosidic bond via the addition of sugars from UDP-sugar precursors to the growing polysaccharide chain. Depending on whether an \( \alpha \) or \( \beta \) linkage is being formed, catalysis can take place either by an inversion mechanism with the \( \alpha \)-linked UDP-sugar forming a \( \beta \)-linked product or by a retaining mechanism where an \( \alpha \)-linked product is formed (27). In the case of the inversion reaction, a single nucleophilic substitution at the anomeric carbon atom of the UDP-sugar will be sufficient to generate the \( \beta \) configuration. This will involve two acidic active site amino acids that act as acid-base catalysts. In polysaccharide hydrolases, which are believed to function by a similar reaction mechanism, the catalytic amino acids have been shown to be either aspartic or glutamic acid (27). It is hypothesized that one of the two acidic residues acts as an acid catalyst to protonate the substrate, while the second residue acts as a base catalyst by deprotonating a water molecule (27). Inspection of the HCA of KfiC identified two highly conserved aspartic acid residues within domain A at positions 301 and 352. It is likely that these aspartic acid residues that are also conserved in the family of \( \beta \)-glycosyltransferases are likely to be the catalytically active amino acids for \( \beta \)-transferase activity (Table I). The occurrence of these conserved residues in close proximity to other aspartic acids D300, D305, D354, and D355 in KfiC may allow effective ionization of the residues (28).

Site-directed mutagenesis of the aspartic acid residues within domain A combined with in vitro assays using oligosaccharides with defined nonreducing ends confirmed that this region is important for GlcA transferase activity. Mutations D300A, D301A, D305A, D352A, and D354A within domain A abolished GlcA transferase activity but left GlcNAc transferase activity unaffected. Conservative changes in which a glutamic acid residue was inserted in place of the aspartic acid, allowed the the catalytic aspartic acid residues to be further refined. The aspartic acid residues at positions 301 and 352 that are conserved in domain A of all of the \( \beta \)-glycosyltransferases (Table I) were essential for GlcA transferase activity, and changes to glutamic acid abolished any detectable activity. This is the first biochemical evidence that these conserved aspartic acid residues are important for \( \beta \)-glycosyltransferase activity. The inability to replace aspartic acid with a glutamic acid suggests that the size of the side chain as well as the charge is important for catalysis and \( \beta \)-glycosyltransferase activity. Likewise, the aspartic acid residue at position 305 was also essential for GlcA transferase activity in KfiC and could not be substituted by a glutamic acid residue. This aspartic acid residue is not conserved in other \( \beta \)-glycosyltransferases with no strong preference for an acidic amino acid at this position (Table I). This suggests that the absolute requirement for an aspartic acid residue at this position for GlcA transferase activity in KfiC may reflect structural considerations and the correct folding to generate the active site rather than playing a direct role in acid-base catalysis. The aspartic acid residue at position 354 in KfiC is highly conserved among the other \( \beta \)-glycosyltransferases, with only the chitin synthase enzyme of \textit{Saccharomyces cerevisiae} lacking an aspartic acid residue at this position (Table I). The observation that the D354A mutation abolished GlcA transferase activity while the conservative D354E change only reduced GlcA transferase activity to 75% of that of the wild type (Table II) suggests that there is a requirement for an acidic amino acid at this position but that the size of the side chain is less critical.

None of the mutations within domain A affected GlcNAc transferase activity, indicating that a separate distinct active site is responsible for UDP-GlcNAc transferase activity. The insertion of the suicide vector pNJC1 (containing part of the KfiC sequence) into the kfiC gene on pPC6 resulted in the expression of a truncated KfiC protein consisting of the first 381 amino acids of the 520-amino acid full-length protein. The in vitro transferase assays using defined oligosaccharide acceptors clearly show that the truncated KfiC' protein has lost the ability to add GlcA from UDP-GlcA to its acceptor but still retains the ability to add GlcNAc. Domain A is still present on the C terminus of the truncated KfiC' protein, but the lack of GlcA transferase activity may indicate that sequences C-terminal to this region are also important for this activity and the correct folding of the protein to generate the GlcA transferase active site. The presence of GlcNAc transferase activity on KfiC' indicates that the active site for the addition of GlcNAc is located in the first 381 amino acids of KfiC (Fig. 4). The observation that none of the mutations in domain A abolished GlcNAc transferase activity (Table II) would indicate that domain A is not involved in GlcNAc transferase activity and suggest that the GlcNAc transferase activity is located in the first 260 amino acids of KfiC prior to domain A (Fig. 4). HCA of the first 260 amino acids of KfiC did not show any strong similarity to known \( \alpha \)-mannosyltransferases. This may be a consequence that these \( \alpha \)-glycosyltransferases are transfering mannose from GDP-mannose rather than GlcNAc from UDP-GlcNAc as well as the fact that KfiC is a bifunctional enzyme having both \( \alpha \) and \( \beta \)-transferase activities. The association of the truncated KfiC' protein with the K5 polysaccharide biosynthetic complex would indicate that the interaction between KfiC and other members of the K5 polysaccharide biosynthetic complex is mediated by structures present in the first 381 amino acids of KfiC.

In summary, these results demonstrate that KfiC is a bifunctional transferase with both \( \alpha \) and \( \beta \)-glycosyltransferase activities. The site-directed mutagenesis and in vitro transferase assays using defined oligosaccharide acceptors demonstrated the role of conserved aspartic acid residues within domain A for \( \beta \)-glycosyltransferase activity. The two transferase activities could be separated with the UDP-GlcNAc \( \alpha \)-glycosyltransferase activity being located in the first 300 amino acids of KfiC. Experiments are under way in order to determine the exact location and nature of the second active site responsible for the \( \alpha \) addition of UDP-GlcNAc to the nonreducing end of the K5 polysaccharide.

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