Functions of Some Capsular Polysaccharide Biosynthetic Genes in Klebsiella pneumoniae NTUH K-2044

Jin-Yuan Ho1,2,3,4, Tzu-Lung Lin5, Chun-Yen Li2, Arwen Lee1,2, An-Ning Cheng1,6, Ming-Chuan Chen1,2, Shih-Hsiung Wu1, Jin-Town Wang5,7, Tsung-Lin Li2,3,4, Ming-Daw Tsai1,2,3,4.

1 Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan, 2 Genomics Research Center, Academia Sinica, Taipei, Taiwan, 3 Chemical Biology and Molecular Biophysics Program, Taiwan International Graduate Program, Institute of Biochemistry, Academia Sinica, Taipei, Taiwan, 4 Institute of Biochemical Science, National Taiwan University, Taipei, Taiwan, 5 Department of Microbiology, National Taiwan University College of Medicine, Taipei, Taiwan, 6 Institute of Bioinformatics and Structure Biology, National Tsing Hua University, Hsinchu, Taiwan, 7 Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan

Abstract

The growing number of Klebsiella pneumoniae infections, commonly acquired in hospitals, has drawn great concern. It has been shown that the K1 and K2 capsular serotypes are the most detrimental strains, particularly to those with diabetes. The K1 cps (capsular polysaccharide) locus in the NTUH-2044 strain of the pyogenic liver abscess (PLA) K. pneumoniae has been identified recently, but little is known about the functions of the genes therein. Here we report characterization of a group of cps genes and their roles in the pathogenesis of K1 K. pneumoniae. By sequential gene deletion, the cps gene cluster was first re-delimited between genes galF and ugd, which serve as up- and down-stream ends, respectively. Eight genes were identified as virulence factors based on the observation that their suppression for 52 upstream genes. The genes suppressed include those coding for unknown regulatory membrane proteins and six multidrug efflux system proteins, as well as proteins required for the K1 CPS biosynthesis. In support of the suppression of multidrug efflux genes, we showed that these three mutants became more sensitive to antibiotics. Taken together, the results suggest that kp3706, kp3709 or kp3712 genes are strongly related to the pathogenesis of K. pneumoniae K1.

Introduction

Bacterial pathogenicity has been shown to be due to different causes, including the structures of capsular polysaccharides (CPS; the K antigen), lipopolysaccharide (LPS; the O antigen), secreted toxins, drug resistance, and genetics [1,2,3,4,5]. Klebsiella pneumoniae is an opportunistic pathogen of the Enterobacteriaceae and usually causes pneumonia or urinary tract infections [6]. In addition, the hospital outbreak of multidrug resistant Klebsiella spp., especially the so-called extended-spectrum beta lactamase (ESBL) and Klebsiella pneumoniae carbapenemase (KPC) subtypes, has draw much attention in recent years [6,7,8].

The CPS of K. pneumoniae is complex acidic polysaccharide consisting of repeating units of 3-6 sugars. The type of sugars seems to correlate with the virulence, and 78 capsule types have been identified [9]. In the past two decades, a number of K. pneumoniae strains have been found to cause primary pyogenic liver abscess (PLA) [10,11,12,13], with the capsular serotype K1 being the most virulent [14,15]. The K1 structure has been reported previously (lacking the acetyl-decoration on fucose) to possess two unique features - a fucose subunit (also found only in K54 and K63), and a unique cyclic 2,3-(R)-pyruvate appendix differing from a commonly seen 4,6-(R)-pyruvate in CPS repeat units [16,17].

It has been reported previously that magA (mucoviscosity associated gene A) in the cps locus of NTUH-K2044, a PLA-causing serotype K1 strain from National Taiwan University Hospital [18], is associated with the hypermucoviscosity of the strain and considered a virulence factor [18]. A 31-Kb fragment that covers regions from genes galF (kp3726) to ugd (kp3701) was further identified genetically as the K1 cps cluster [15]. Recently, the whole genome for this PLA strain has been sequenced and annotated, where a previously unidentified acetyl transferase gene was shown in the cps cluster [19]. Our collaborators have re-determined the CPS structure [Figure 1B], and demonstrated that the acetylation occurs on either C2 or C3 of the fucose (unpublished results). In this work, we took a systematic approach to study the biosynthetic genes of the CPS of NTUH-K2044. Our goal is to identify the functions of the genes and their relevance to pathogenicity.
Results and Discussion

Identification of specific genes as virulence factors

We examined the functions of individual genes in CPS biosynthesis, aiming to verify the _cps_ locus annotation ([Figure 1A](#fig1)) and to establish the connection between the chemical structure and the biosynthetic genes in their contributions to pathogenicity. First, the genes for NDP-sugar biosyntheses (manB, manC, wcaH, gnd, wcaG, gmd, galF, ugd, and uge) ([Figure 2A–B](#fig2)) were functionally verified by over expressing and purifying recombinant enzymes and identifying their products by high-pressure liquid chromatography (HPLC) and mass spectrometry. The HPLC analyses of the reaction catalyzed by GalF are shown in [Figure 2C](#fig2) as an example. The rest of the HPLC analyses are shown in Figures S1 and S2. The kinetic parameters of Ugd and Gnd are shown in Table S1, and the specificity of ManC and GalF are shown in Tables S2, S3. Detailed procedures are described in Results S1.

Then in-frame deletion mutants were constructed using a modified pKO3 system [20] for ugd, gnd, wbAP, wcAL, wcAH, wcAG, gmd, atf, wzy (magA), ptf, wzE, wzB, and wzA in the proposed _cps_ locus.
Tables 1). All mutants were obtained except Δugd and Δgmd. Based on both anti K1 serum test by double immunodiffusion assay (Figure 3A) and string test [15], all mutants obtained (except Δgmd and ΔwcaH) lost the K1 serotype and mucoviscosity while remaining O1 serotype positive (Table 2), suggesting that these mutants produce little or no CPS. Moreover, deletion of glf and uge in the proposed lps locus led to loss of O1 serotype, confirming the proposed boundary between cps and lps loci shown in Figure 1A.

Since the deletion mutants make little or no CPS, they are expected to lose pathogenicity also. Animal inoculation experiments were performed for three mutants as examples. As shown in Table 2, the results indicate that deletion of the acetyltransferase gene (Δatf or Δ3712), the pyruvyltransferase gene (Δptf or Δ3715), or the mucoviscosity associated gene (ΔmagA, Δwzy, or Δ3714) was sufficient to cause a significant decrease in the virulence of the NTUH-K2044 strain (LD₅₀ 10⁷ CFU in intra-peritoneal infection, relative to 10² for WT).

These results taken together have verified the functions of individual genes and proteins. Even though the CPS itself can be considered as a virulence factor, our results suggested that the individual genes or proteins responsible for CPS synthesis can also be considered as virulence factors, and thus are potential targets for designing inhibitors against the pathogen. We further examined the properties of the deletion mutants as described below.

Discovery of a broad gene silencing effect related to fucose biosynthesis

We first examined the deletion mutants at the transcriptional level by Q-PCR. Surprisingly, expressions for genes between wcaI and galF, most of the genes in the cps gene cluster, were found completely silenced in d3706, d3709 and d3712 (Table 3), while they expressed normally (except the deleted gene) in the other deletion mutants (Table S4). To estimate the scopes of the gene silencing effect, genes upstream of the cps locus were also examined and found to be silenced up to kp3767 in these three mutants (Table 4, left 4 lanes). The total region influenced by the effect was about 70 Kb, including 15 of the 20 cps genes and 37 upstream genes (Figure 1A and Table S5). Interestingly, the functions of these three genes are all related to the fucose residue of the trisaccharide repeat unit - WcaI (KP3706) is likely the fucosyl transferase, WcaG (KP3709) is responsible for GDP-fucose synthesis, and Atf (KP3712) is for fucose acetylation.

Further support for the broad gene silencing effect

That the broad gene silencing effect is novel and real is further supported by three experimental approaches: (a) Complementation experiments with plasmids carrying the deleted gene restored only the expression of the deleted gene, not the other silenced genes (Table 4, right 3 lanes). This result suggests that the silencing effect is caused by changes at the level of genomic DNA, not simply due to protein expression. (b) The effect of gene silencing was observed from fucose-related metabolites. Analyses of cell fluids extract showed that GDP-Fuc were hardly detectable in these three mutants, while clearly present in two control mutants d3705 and d3712 (Figure 3B). The results were also verified by MS analyses (Figure S3). This result supports the silencing effect since only KP3709 is involved in GDP-fucose synthesis, and Atf (KP3712) is for fucose acetylation.

Figure 2. Characterization of the biosynthetic pathway of trisaccharide building blocks. A, Biosynthetic pathway of GDP-fucose. B, Biosynthetic pathway of UDP-glucose and UDP-galacturonic acid. C, HPLC traces of GalF reaction, (a) starting substrates, 1 mM glucose-1-phosphate and 1 mM UTP; (b) 1 mM glucose-1-phosphate, in the presence of GalF; (c) 1 mM UTP, in the presence of GalF; (d) 1 mM glucose-1-phosphate and 1 mM UTP, in the presence of GalF; (e) 1 mM commercial UDP-glucose as a product standard. The enzyme reactions were carried out in a buffer solution (50 mM Tris-HCl, pH 8.0, 5 mM MgSO₄) at 37°C for 4 hr.

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formation of GDP-fucose and their deletion should not have affected the production of GDP-fucose if there were no silencing effect.

We then examined whether the broad silencing effect described above could be due to any known regulatory mechanism. The first to consider was whether the observed silencing effect is a form of well known “polar effect” [21] (suppression of a small number of downstream genes) often observed for insertion mutants. Even though the in-frame deletion mutants in our studies were designed to avoid polar effects [20], some insertion mutants from our previous work [15] were examined to see if they display the broad silencing effect as described above for the deletion mutants. As shown in Table 5, polar effects were observed for i3706, i3709, which are distinctly different from the broad silencing effect in the deletion mutants. Furthermore, the K1 serotype of insertion mutants can be restored by complementation as shown previously [15], but not that of the three deletion mutants (Figure 3A-u~w and Table 4).

Several other known regulatory mechanisms are related to the \(\text{cps}\) gene cluster, but not involved in the expression of upstream genes: (i) regulators of capsule synthesis (\(rcs\)) [22], which sense the extracellular signals then regulate the expression of \(\text{cps}\) gene cluster; (ii) the transcriptional antiterminator \(rfaH\) [1,3], which recognizes Just Upstream of Many Polysaccharide Starts (JUMP-star) to ensure the expression of distal genes; (iii) \(wzb/wzc\) [1], which are believed to be involved in the CPS polymerization; and (iv) \(rmpA2\), which is a transcriptional activator and its absence would only lower the capsule production [15]. The expression level of these regulatory genes shows no significant difference between wild-type and mutants.

Small RNA (sRNA) is specifically used to represent bacterial non-coding RNA. Since it has been suggested that sRNA [23]
effects could be dependent on the temperature of bacterial growth [24] or the state of growth [25], we examined the gene silencing effect at different temperatures (25, 42 and 45°C) (Table S6) and at the stationary phase of the growth (Table S7). The results indicate that these factors did not affect the broad gene silencing effect observed for the three deletion mutants.

Synergy between the fucose-related virulence factors and drug resistance

To test whether the broad gene silencing effect is related to the pathogenicity of the NTUH-K2044 strain, we examined possible functions of the silenced genes. Importantly, the 37 genes upstream of the cps locus affected by the gene silencing effect include many regulators and multidrug efflux genes (from kp3742 to kp3747) (Figure 1A and Table S5). Since multidrug efflux pumps are known to contribute to drug resistance in Gram-negative bacteria [26], this finding led us to predict that the three mutants d3706, d3709, and d3712 would be less drug-resistant to some of the antibiotics. As shown in Figure 4A, in the absence of antibiotics there were no significant differences in growth rates between K2044 and its mutants, confirming that CPS is important for pathogenicity but not for growth (Δugs or d3699, a slow-growing mutant, was used as a positive control as Uge is involved in the biosynthesis of LPS). Then we examined the effects of various antibiotics on the growth rates of these strains. Two of the antibiotics tested, zetocycin, sulfamethoxazole, ciprofloxacin, and geneticin, which are known to bind and cleave DNA (Figure 4B) and erythromycin (a macrolide family of antibiotics known to interfere with protein synthesis) (Figure 4C) showed significant inhibition against ΔwcaI, ΔwcaG, and Δatf (d3706, d3709, and d3712, respectively) but had limited effect against wild-type and the other mutants. In contrast, tetracycline, sulfamethoxazole, ciprofloxacin, and geneticin, which are classified as members of tetracyclines, sulfonamides, quinolones, and aminoglycosides antibiotics, respectively, did not show specific effects on the three mutants (Figure S4A–S4D).

If the effects of zeocin or erythromycin on the three deletion mutants are indeed caused by silencing of multidrug efflux genes, we should be able to identify a single efflux gene, construct the deletion mutant of the single gene, and show that the mutant is sensitive to the antibiotics. To test this possibility, a kp3742 (yegM) deletion mutant was constructed. The inhibition assays showed that the growth of the ΔyegM strain was inhibited by zeocin (Figure 4D) but not erythromycin (Figure 4E) or the other antibiotics (not shown).

There has been growing evidence that fucose [27] and acetylation [28,29,30] are important factors in bacterial pathogenicity and in other diseases. For example, Helicobacter pylori is a well known primary cause of gastritis, duodenal ulcer, and gastric cancer, which infects about one-half of human population. Liu et al. demonstrated the important connection between human L-fucosidase (FUCA2) and H. pylori on the adhesion pathogenesis and escape of host surveillance [31]. In addition, Coyne et al. also demonstrated that bacteriodes in mammalian intestine use a mammalian-like pathway to decorate numerous capsular polysaccharides and glycoproteins with fucose [32]. No mechanism has been suggested about the special roles of fucose. Our results add an example in the potential biological significance of fucose, and we have discovered some link between fucose biosynthetic genes and gene silencing. However the specific functions of fucose or fucose biosynthetic genes remain to be further investigated.

Figure 3. Characterization of the in-frame deletion mutants (dxxxx), complementation strains of the deletion mutants (cxxxx), and insertion mutants (ixxxx). A, Double immunodiffusion assay for the K1 serotype. A: wild-type, b: d3695, c: d3699, d: d3704, e: d3713, f: d3714, g: i3705, h: d3705, i: i3706, j: d3706, k: i3709, l: d3709, m: d3712, n: i3715, o: d3715, p: d3718, q: d3719, r: d3720, s: wild-type, t: d3708, u: c3706, v: c3709, w: c3712. Only wild-type, d3695, d3699, d3704, and d3708 remained K1 positive. B, HPLC analysis of NDP-sugars. All peaks were determined by the standard retention time and confirmed by MS (Figure S3). The GDP-fucose peak is indicated by the red arrow. The data for the wild-type was not obtained due to the high mucoviscosity, which prohibited extraction of metabolites.

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Table 2. K1 antigenicity, O antigenicity, and LD50 values of all strains in this study.

| K. pneu. Strains | K1 Antigenicity | O Antigenicity | LD50 (CFU) [NI]* |
|------------------|-----------------|-----------------|------------------|
| NTUH-K2044       | +               | +               | <10⁶ [4]         |
| d3705            | −               | ND              | ND               |
| d3706            | −               | ND              | ND               |
| d3709            | −               | ND              | ND               |
| d3714            | −               | ND              | ND               |
| d3715            | −               | ND              | ND               |
| d3695            | +               | −               | ND               |
| d3699            | +               | −               | ND               |
| d3704            | +               | +               | ND               |
| d3705            | −               | +               | ND               |
| d3706            | −               | +               | ND               |
| d3708            | +               | +               | ND               |
| d3709            | −               | +               | ND               |
| d3712            | −               | +               | >10⁶ [4]         |
| d3713            | −               | +               | ND               |
| d3714          | −               | +               | >10⁶ [4]         |
| d3715            | −               | +               | >10⁶ [4]         |
| d3718            | −               | +               | ND               |
| d3719            | −               | +               | ND               |
| d3720            | −               | +               | ND               |
| d3706            | −               | ND              | ND               |
| d3709            | −               | ND              | ND               |
| d3712            | −               | ND              | ND               |

ND: not determined,
*mice numbers for inoculation,
†(Ref. 15).
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In summary, our results on the CPS of the NTUH-K2044 strain of the PLA K. pneumoniae add to the emerging evidence that fucose [32] and O-acetylation [28] are important factors in bacterial pathogenicity, and our genetic analyses of deletion mutants uncover a possible link between three fucose-related genes and the multidrug efflux genes via an unprecedented broad gene networking. This phenomenon potentially represents a newly uncovered pathogenesis mechanism. These results led us to hypothesize that the broad silencing effects observed for the three in-frame deletion mutants are caused by DNA structure alteration in the deletion mutants of the three fucose-related genes, and that the silencing effects may be relevant to the specific roles of the fucose and its acetylation in the pathogenicity of the strain. The validation of the hypothesis, and the mechanism of the broad silencing effect, are important subjects for future studies.

Materials and Methods

Bacterial strains and plasmid vectors

Bacterial strains and plasmids used in this study are listed in Table 1. Clinically isolated K. pneumoniae strains were collected at National Taiwan University Hospital (NTUH) [18].

Serum resistance assay and animal inoculation

The serum resistances of K. pneumoniae strains were determined as previously described [33]. Female BALB/cByl 5-week-old mice were used for inoculation. BALB/cByl mice were bred and housed in specific pathogen-free rooms within animal care facilities of the Laboratory Animal Center at the National Taiwan University College of Medicine (NTUCM) with free access to food and water. All procedures were approved by the National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee (IACUC), following with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and Taiwanese Animal protection act. IACUC approval number: 20060139. K. pneumoniae inoculation consisting of 10⁵–10⁶ mid-logarithmic growth phase CFUs were diluted in 100 µl normal saline and injected intraperitoneally [34,35]. Four mice were used to test the effects of each inoculum. After inoculation, the mice were observed for 30 days. The LD50 was calculated using the method established by Reed and Muench [35].

Construction of K. pneumoniae deletion mutant strains

The deletion mutants were generated using a modified pKO3-Km vector that contained a temperature-sensitive origin of replication and markers for positive and negative selection for chromosomal integration and excision [36,37]. The genes and its flanking regions were amplified by PCR using primers listed in Table S8 and cloned into a pGEM-T easy vector. The deletion fragment was generated by inverse PCR using primers listed in Table S8. The deletion fragments described above were cloned into a NotI site of a pKO3-Km plasmid separately [36]. The resulting constructs were then electroporated into wild-type strain. The transformants were cultured at 43°C. Five colonies were picked in 1 ml LB broth followed by serial dilution and

Table 3. Gene expression of mutant strains.

| Strains | d3706 | d3709 | d3712 | d3713 | d3715 |
|---------|-------|-------|-------|-------|-------|
| Genes   |       |       |       |       |       |
| kp3589  | 0.13  | 0.54  | 0.99  | 0.53  | 0.27  |
| kp3593  | 0.11  | 0.47  | 0.73  | 0.78  | 0.28  |
| kp3694  | 0.12  | 0.38  | 0.69  | 0.52  | 0.27  |
| kp3695  | 0.23  | 0.99  | 0.88  | 0.82  | 0.44  |
| kp3696  | 0.15  | 0.64  | 0.59  | 0.98  | 0.34  |
| kp3699  | 0.22  | 1.02  | 0.76  | 1.14  | 0.48  |
| kp3701  | 0.10  | 0.32  | 0.10  | 0.86  | 0.54  |
| kp3702  | 0.10  | 0.44  | 0.18  | 1.06  | 0.62  |
| kp3703  | 0.11  | 0.35  | 0.09  | 0.60  | 0.61  |
| kp3704  | 0.17  | 0.56  | 0.50  | 0.70  | 0.61  |
| kp3705  | 0.00  | 0.00  | 0.00  | 0.62  | 0.53  |
| kp3706  | 0.00  | 0.00  | 0.00  | 1.12  | 0.50  |
| kp3708  | 0.00  | 0.00  | 0.00  | 0.80  | 0.56  |
| kp3709  | 0.00  | 0.00  | 0.00  | 0.65  | 0.78  |
| kp3711  | 0.00  | 0.00  | 0.00  | 0.40  | 0.75  |
| kp3712  | 0.00  | 0.00  | 0.00  | 0.36  | 0.63  |
| kp3713  | 0.00  | 0.00  | 0.00  | 0.00  | 0.66  |
| kp3714  | 0.00  | 0.00  | 0.00  | 0.45  | 0.59  |
| kp3715  | 0.00  | 0.00  | 0.00  | 0.52  | 0.00  |
| kp3716  | 0.00  | 0.00  | 0.00  | 0.86  | 0.81  |
| kp3718  | 0.00  | 0.00  | 0.00  | 0.37  | 0.81  |
| kp3719  | 0.00  | 0.00  | 0.00  | 0.56  | 0.89  |

Genes that are no expression are highlighted in bold black.
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Table 4. Gene expression of deletion mutants and complementation strains.

| Strains | d3706 | d3708 | d3709 | d3712 | c3706 | c3709 | c3712 |
|---------|-------|-------|-------|-------|-------|-------|-------|
| Genes   |       |       |       |       |       |       |       |
| kp3699  | 0.22  | 1.04  | 0.99  | 0.59  | 0.27  | 0.46  | 0.32  |
| kp3701  | 0.10  | 0.43  | 0.34  | 0.25  | 0.24  | 0.43  | 0.16  |
| kp3702  | 0.10  | 0.32  | 0.40  | 0.18  | 0.55  | 0.29  | 0.11  |
| kp3703  | 0.11  | 0.26  | 0.41  | 0.18  | 0.07  | 0.05  | 0.12  |
| kp3704  | 0.17  | 0.74  | 0.59  | 0.30  | 0.23  | 1.10  | 0.23  |
| kp3705  | 0.00  | 0.26  | 0.01  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3706  | 0.00  | 0.17  | 0.00  | 0.00  | 0.93  | 0.00  | 0.00  |
| kp3708  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3709  | 0.00  | 0.23  | 0.00  | 0.00  | 0.00  | 2.58  | 0.01  |
| kp3711  | 0.00  | 0.26  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3712  | 0.00  | 0.27  | 0.00  | 0.00  | 0.00  | 4.92  | 0.00  |
| kp3713  | 0.00  | 0.26  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3714  | 0.00  | 0.18  | 0.01  | 0.01  | 0.00  | 0.03  | 0.01  |
| kp3715  | 0.00  | 0.28  | 0.00  | 0.01  | 0.00  | 0.00  | 0.00  |
| kp3716  | 0.00  | 0.20  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3718  | 0.00  | 0.31  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3719  | 0.00  | 0.32  | 0.00  | 0.01  | 0.00  | 0.00  | 0.00  |
| kp3720  | 0.00  | 0.24  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3721  | 0.00  | 0.33  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3725  | 0.00  | 0.19  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3726  | 0.01  | 0.22  | 0.01  | 0.00  | 0.01  | 0.01  | 0.01  |
| kp3731  | 0.00  | 0.42  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3736  | 0.01  | 0.68  | 0.01  | 0.01  | 0.00  | 0.00  | 0.00  |
| kp3744  | 0.00  | 0.57  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3751  | 0.03  | 0.69  | 0.01  | 0.01  | 0.00  | 0.00  | 0.00  |
| kp3759  | 0.03  | 0.43  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3767  | 0.00  | 1.26  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  |
| kp3768  | 2.31  | 0.44  | 0.48  | 1.17  | 2.67  | 0.62  | 0.00  |
| kp3770  | 0.03  | 0.18  | 0.13  | 0.08  | 0.10  | 0.35  | 0.73  |

Table 5. Gene expression of insertion mutant strains.

| Strains | i3705 | i3706 | i3709 |
|---------|-------|-------|-------|
| Genes   |       |       |       |
| kp3705  | 0.325 | 0.061 | 0.129 |
| kp3706  | 2.313 | 0.062 | 0.160 |
| kp3708  | 2.567 | 1.064 | 0.071 |
| kp3709  | 2.567 | 1.007 | 0.022 |
| kp3711  | 2.378 | 0.683 | 0.953 |
| kp3712  | 2.479 | 1.094 | 1.050 |
| kp3713  | 2.990 | 0.722 | 0.889 |
| kp3714  | 3.117 | 0.908 | 1.094 |
| kp3715  | 4.170 | 1.141 | 1.580 |
| kp3716  | 5.389 | 1.636 | 2.144 |
| kp3718  | 2.514 | 0.559 | 0.774 |
| kp3719  | 5.657 | 2.028 | 1.892 |

If the colony could be stretched to a string longer than 0.5 cm then it was defined as positive string test; otherwise as negative.

K1 and O serotyping
Capsule and lipopolysaccharide were purified as previously described [15]. The K1 serotyping was performed by the double immunodiffusion assay using a serotype K1-specific antisera (Statens Serum Institute) as previously described [15]. After separation in 12% SDS-PAGE gel, the O antigen was detected by sera from mice infected with a capsule-deficient magF deletion mutant.

Quantitative PCR
1 μg of extracted RNA was reverse transcribed into cDNA in 20 μl reaction as the manufacturer protocol (Roche, US). 20 μl of cDNAs were then diluted into 1 ml. 5 μl of diluted cDNA samples were added into the Q-PCR reaction plate, and also the 10 μl of reaction solution. The analysis of Q-PCR result is that each gene was first normalized with 23S RNA. Each gene in each strain was then normalized with wild-type to obtain the relative gene expression pattern. The primers used for Q-PCR are listed in Table S9.

Gene cloning, protein purification, and functional assay
Genes (upa, ugd, manB, manG, gmd, wcaH, wcaG, gmd, and gaf) were cloned from NTUH K-2044 genomic DNA into pET28a (his-tagged). Proteins (Uga, Ugd, ManB, ManG, Gmd, WcaH, WcaG, Gmd, and Gaf) were over-expressed in E. coli BL21 star (DE3). His-tagged protein purification was followed by PRO-BOND Invitrogen, US) manufacturer’s protocol. Purified proteins were concentrated to 1 mg/ml then 10 μl of protein together with 10 μl of 10 mM substrates (in the figure legend) were added in reaction buffer, 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂ in final 100 μl solution. The reaction solution was extracted by chloroform to remove the proteins, then analyzed by HPLC (Angilent, HP1100, US) with ammonium formate gradient.

Extraction and separation of nucleotides and sugar nucleotides
For isolation of soluble fractions containing sugar nucleotide/nucleotides from K. pneumoniae strains, cells were collected when...
OD\textsubscript{600} was 1.0. The cells were harvested by centrifugation at 5000 rpm for 30 min at 4°C. Cell pellets were re-suspended in 1 ml phosphate-buffered saline (PBS, pH 7.4), followed by addition of a mixture of chloroform/methanol (1:2) and vortexing for 10–15 min. The mixture was then centrifuged at 4000 rpm for 15 min, the upper phase containing soluble components was collected and dried under air. It was redissolved in distilled water and further purified by Amicon filter (YM-10 cut-off), and the filtrate was collected and monitored by anion exchange HPLC using ammonium formate. The peaks were identified by comparing the retention times and MS with known standards.

Growth curves of WT and mutants and effects of antibiotics
NTUH-K2044 and knock-out strains were grown in LB broth at 37°C. For growth curves, log phase cultures were diluted to OD\textsubscript{600} = 0.005 in LB broth with desired antibiotics. The growth curve was plotted by measuring OD\textsubscript{600} periodically.

Supporting Information

Results S1 Characterization of enzymes for the synthesis of UDP-glucose (UDP-Glc), UDP-glucuronic acid (UDP-GlcA), UDP-galacturonic acid (UDP-GalA), GDP-mannose (GDP-Man) and GDP-fucose (GDP-Fuc).

Figure S1 HPLC traces. A, Ugd (KP3701) reaction buffers containing: (a) UDP-glucose+NAD\textsuperscript{+}, (b) UDP-glucose+KP3701, (c) NAD\textsuperscript{+}+KP3701 (d) UDP-glucose+NAD\textsuperscript{+}+KP3701. B, Uge (KP3699) reaction buffers containing: (a) UDP-gluconate+Mg\textsuperscript{2+}, (b) UDP-gluconate+Mg\textsuperscript{2+}+KP3699.

Figure S2 HPLC traces. A, ManC (KP3703) reaction buffers containing: (a) mannose-1-phosphate+GTP+Mg\textsuperscript{2+}, (b) mannose-1-phosphate+GTP+Mg\textsuperscript{2+}+KP3703, (c) GTP+Mg\textsuperscript{2+}+KP3703, (d) mannose-1-phosphate+GTP+Mg\textsuperscript{2+}+KP3703, (e) GDP-mannose standard. B, ManB (KP3702) and ManC (KP3703) reaction buffers containing: (a) mannose-6-phosphate+GTP+Mg\textsuperscript{2+}+KP3702, (b) mannose-1-phosphate+GTP+Mg\textsuperscript{2+}+KP3702, (c) mannose-6-phosphate+GTP+Mg\textsuperscript{2+}+KP3703, (d) mannose-6-phosphate+GTP+Mg\textsuperscript{2+}+KP3702+KP3703. C, Gnd (KP3711) reaction buffers containing: (a) GDP+Mg\textsuperscript{2+}, (b) GDP-mannose+Mg\textsuperscript{2+}, (c) GDP-mannose+GTP+Mg\textsuperscript{2+}+KP3711. D, WcaG and Gnd reaction buffers containing: (a) GDP-mannose+NADPH+KP3711+KP3709, (b) GDP-mannose and NADPH standard, (c) GDP-fucose standard. E, WcaH (KP3708) reaction buffers containing: (a) GDP+Mg\textsuperscript{2+}, (b) GDP-mannose+Mg\textsuperscript{2+}, (c) GDP-mannose+GTP+Mg\textsuperscript{2+}+KP3708. F, Gnd (KP3704) reaction buffers containing: (a) NADPH\textsuperscript{+}, (b) glucuronate-6-phosphate+NADPH\textsuperscript{+}, (c) glucuronate-6-P+NADPH\textsuperscript{+}+KP3704.

Figure S3 Verification of GDP-fucose in Fig. 3B by mass spectrometry obtained on a LTQ mass spectrometer. The peak at 588.1 is GDP-fucose, which is clearly present in Δ3715, minimally detectable in Δ3712, and absent in Δ3709.

Figure S4 Growth curves of wild type and mutants in the presence of antibiotics. A, tetracycline (0.5 μg/ml). B, sulfamethoxazole (500 μg/ml). C, ciprofloxacin (0.025 μg/ml). D, gentamicin (12.5 μg/ml).

Table S1 Kinetic parameters for KP3701 (UgD) and KP3704 (Gnd).

Table S2 The enzyme specificity test for ManC (KP3703).

Table S3 The enzyme specificity test for GalF (KP3726).
Table S4  Q-PCR results of non-silencing effect mutants. (EPS)
Table S5  Gene annotation of NTUH K2044 from kp3689 to kp3769. (EPS)
Table S6  Gene expression results of mutants strains at different temperature growth condition. (EPS)
Table S7  Gene expression results of mutants strains at stationary phase (OD600: 2.0). (EPS)

References
1. Whitfield C (2006) Biosynthesis and assembly of capsular polysaccharides in Escherichia coli. Annu Rev Biochem 75: 39–60.
2. West NP, Samomoebi F, Mounier J, Eday RM, Pansot C, et al. (2005) Optimization of virulence functions through glycosylation of Shigella LPS. Science 307: 1313–1317.
3. Rahn A, Whitfield C (2003) Transcriptional organization and regulation of the Escherichia coli K10 group 1 capsule biosynthesis (cps) gene cluster. Mol Microbiol 47: 1045–1060.
4. Brisse S, Fevre C, Passet V, Isenhuth-Jeanjean S, Tourelbiez R, et al. (2009) Virulent clones of Klebsiella pneumoniae: identification and evolutionary scenario based on genomic and phenotypic characterization. PLoS ONE 4: e4862.
5. Bina XR, Provenzano D, Nguyen N, Bina JE. (2008) Vibrio cholerae RND family eflux systems are required for antimicrobial resistance, optimal virulence factor production, and colonization of the infant mouse small intestine. Infect Immun 76: 3595–3605.
6. Podschun R, Ullmann U (1998) Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 11: 589–603.
7. Woodford N, Tierno PM, Jr., Young K, Tysall L, Palepou MF, et al. (2004) Class A beta-lactamase, KPC-3, in a New York Medical Center. Antimicrob Agents Chemother 48: 4795–4799.
8. Nordmann P, Cuzon G, Naas T (2009) The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria. Lancet Infect Dis 9: 228–236.
9. Pan YJ, Fang HC, Yang HC, Lin TL, Hsieh PF, et al. (2008) Capsular polysaccharide synthesis regions in Klebsiella pneumoniae serotype K57 and a new capsular serotype. J Clin Microbiol 46: 2231–2240.
10. Ko WC, Paterson DL, Nagatomi A, Hansen DS, Von Gottberg A, et al. (2002) Community-acquired Klebsiella pneumoniae bacteremia: global differences in clinical patterns. Emerg Infect Dis 8: 160–166.
11. Yang CC, Yen CH, Ho MW, Wang JH (2004) Comparison of pyogenic liver abscess caused by non-Klebsiella pneumoniae and Klebsiella pneumoniae. J Microbiol Immunol Infect 37: 176–184.
12. Lederman ER, Crum NF (2005) Pyogenic liver abscess with a focus on Klebsiella pneumoniae serogroup C evolved apart from other bacterial sialate O-acetyltransferases. J Clin Microbiol 47: 1045–1060.
13. Liu TW, Ho CW, Huang HH, Chang SM, Popat SD, et al. (2009) Role for class A β-lactamase and insertions in the genome of wild-type Escherichia coli: application to open reading frame characterization. J Bacteriol 191: 4492–4501.
14. Link AJ, Phillips D, Church GM (1997) Methods for generating precise deletions and insertions in the genome of wild-type Escherichia coli: application to open reading frame characterization. J Bacteriol 179: 6228–6237.
15. Franklin NC, Luria SE (1961) Transduction by bacteriophage P1 and the properties of the lac gene region in E. coli and S. dysenteriae. Virology 15: 299–311.
16. Majdalani N, Gottsmann S (2005) The Rcs phosphorylase: a complex signal transduction module. Annu Rev Microbiol 59: 379–405.
17. Dambach MD, Wacker WC (2009) Expanding roles for metabolite-sensing regulatory RNAs. Curr Opin Microbiol 12: 161–169.
18. Narberhaus F, Waldminghaus T, Chowdhury S (2006) RNA thermometers. FEBS Microbiol Rev 30: 3–16.
19. Vogel J, Barrels V, Tang TH, Charakov G, Slatter-Jager JG, et al. (2003) Rck NARs in Escherichia coli detect new sRNA species and indicate parallel transcriptional output in bacteria.核酸 Nucleic Acids 48: 653–6443.
20. Nikaido H (1996) Multidrug efflux pumps of gram-negative bacteria. J Bacteriol 178: 5053–5059.
21. Wu JH, Wu AM, Tsai CG, Chang YX, Tsai SF, et al. (2008) Contribution of fucose-containing capsules in Klebsiella pneumoniae to bacterial virulence in mice. Exp Biol Med (Maywood) 233: 64–70.
22. Lewis AL, Nazer V, Varki A (2004) Discovery and characterization of sialic acid O-acetylation in group B Streptococcus. Proc Natl Acad Sci U S A 101: 11123–11128.
23. Lewis AL, Cao H, Patel SK, Diaz S, Ryan W, et al. (2007) NeuA sialic acid O-acetylersterase activity modulates O-acetylation of capsular polysaccharide in group B Streptococcus. J Biol Chem 282: 27562–27571.
24. Bergfeld AK, Claus H, Lorenzen NK, Spieelmans F, Vogel U, et al. (2007) The polysialic acid-specific O-acetylersterase OatC from Neisseria meningitidis serogroup C evolved apart from other bacterial sialate O-acetylersterases. J Biol Chem 282: 6–16.
25. Liu TW, Ho CW, Huang HH, Chang SM, Popat SD, et al. (2009) Role for α-L-fucosidase in the control of Helicobacter pylori-infected gastric cancer cells. Proc Natl Acad Sci U S A.
26. Coyne MJ, Reinaip B, Lee MM, Comstock LE (2003) Human symbions use a host-like pathway for surface fucosylation. Science 307: 1778–1781.
27. Yamamoto M, Sato S, Henni H, Sanjo H, Uematsu S, et al. (2002) Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature 420: 324–329.
28. Mizuta K, Okita M, Mori M, Haegawa T, Nakashima I, et al. (1983) Virulence for mice of Klebsiella strains belonging to the O1 group: relationship to their capsular (K) types. Infect Immun 40: 56–61.
29. Reed L, Munrich H (1938) A simple method of estimating fifty percent endpoints. J Hyp 27: 493–497.
30. Hsieh PF, Lin TL, Lee CZ, Tsai SF, Wang JT (2008) Serum-induced iron-regulated outer membrane protein expression of Klebsiella pneumoniae carbapenemase-producing bacteria. Lancet Infect Dis 9: 228–236.
31. Wu K, Li L, Yen J, Tsao N, Liao T-L, et al. (2009) Genome Sequencing and Comparative Analysis of Klebsiella pneumoniae NTUH-K2044, a Strain Causing Liver Abscess and Meningitis. J Bacteriol 191: 4492–4501.