Genetic analysis of capsular polysaccharide synthesis gene clusters in 79 capsular types of *Klebsiella* spp

Yi-Jiun Pan1,*, Tzu-Lung Lin1,*, Chun-Tang Chen1, Yi-Yin Chen1, Pei-Fang Hsieh1, Chun-Ru Hsu1, Meng-Chuan Wu1 & Jin-Town Wang1,2

A total of 79 capsular types have been reported in *Klebsiella* spp., whereas capsular polysaccharide synthesis (*cps*) regions were available in only 22 types. Due to the limitations of serotyping, complete repertoire of *cps* will be helpful for capsular genotyping. We therefore resolved the rest 57 *cps* and conducted comparative analysis. Clustering results of 1,515 predicted proteins from *cps* loci categorized proteins which share similarity into homology groups (HGs) revealing that 77 Wzy polymerases were classified into 56 HGs, which indicate the high specificity of Wzy between different types. Accordingly, Wzy-based capsular genotyping could differentiate capsule types except for those lacking Wzy (K29 and K50), those sharing identical Wzy (K22 vs. K37); and should be carefully applied in those exhibited high similarity (K12 vs. K42, K2 vs. K13, K74 vs. K80, K79 vs. K22 and K30 vs. K69). Comparison of CPS structures in several capsular types that shared similarity in their gene contents implies possible functions of glycosyltransferases. Therefore, our results provide complete set of *cps* in various types of *Klebsiella* spp., which enable the understandings of relationship between genes and CPS structures and are useful for identification of documented or new capsular types.

The genus *Klebsiella*, especially the species *Klebsiella pneumoniae*, is a common human pathogen that can lead to a wide range of diseases in both hospital and community settings. It causes nosocomial infections, such as septicemia, pneumonia, and urinary tract infections1,2, and is also associated with community-acquired infections, including pneumonia, urinary tract infections and pyogenic liver abscess complicated with meningitis and endophthalmitis3–5. Capsule is a major virulence factor of *K. pneumoniae*, and association between capsular types and particular diseases6,7 or severity of infections was documented8,9. At present, a total of 79 capsular types have been identified and associated with different *Klebsiella* species10, including 77 types from reference strains (recently reclassified into *K. pneumoniae*, *K. variicola*, *K. oxytoca*, *K. michiganensis*, *Raoultella planticola*, *R. ornithinolytica*, and *R. terrigena*) recognized by serological reactivity tests established during the period 1926 to 197711 and 2 new types of *K. pneumoniae* (KN1 and KN2) characterized by molecular genotyping and phage typing in recent years12,13. Serotyping has been used to characterize the K-types of *Klebsiella* spp. since 192614. However, the limitations of serotyping of *Klebsiella* spp. have been reported in several studies, including limitations of sensitivity and specificity15–17. For this reason, capsular genotyping methods have been developed for discriminating the capsular types of *Klebsiella* spp.10,12,18–24. Polymerase chain reaction-based genotyping of the capsular polysaccharide synthesis region, *cps*-PCR genotyping, was first adopted for the detection of specific wzy genes in *Klebsiella* spp. type K118–20 and subsequently applied to other capsular types related to community-acquired pyogenic liver abscess12,21,22. Recently, wzi or wzc sequencing was also used for

1Department of Microbiology, National Taiwan University College of Medicine, Taipei, Taiwan. 2Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to J.-T.W. (email: wangjt@ntu.edu.tw)
Klebsiella spp. capsular typing\textsuperscript{10,25}. However, some types were undistinguished by their sequences and it can be also complicated to determine the capsular types when sequence variation exists in a given type.

Genetic structures of the capsular polysaccharide synthesis (\textit{cps}) gene cluster in Klebsiella spp. have been determined in some types\textsuperscript{12,18,26-31}. A group of six genes (\textit{galf}, \textit{cpsACP}, \textit{wzi}, \textit{wza}, \textit{wzb} and \textit{wzc}) at the 5' end of the \textit{cps} regions that encode proteins involved in CPS translocation and processing at the bacterial surface are highly conserved among different capsulate types and genes encoding glucose-6-phosphate dehydrogenase (\textit{gnd}) and UDP-glucose dehydrogenase (\textit{ugd}) were found at the 3' end. The middle region (variable region) of the \textit{cps} loci which comprises particular genes encoding proteins responsible for polymerization and assembly of specific CPS subunits were therefore considered to be crucial for K-type variation\textsuperscript{15}. Generally, the synthesis of the capsular repeat is initiated by the initial glycosyltransferase (GT)–WbaP and WcaJ\textsuperscript{28,29} and further catalyzed by specific (non-initial) GTs allowing the addition of sugars\textsuperscript{29}. The lipid-linked repeat units are flipped across the plasma membrane by Wzx and then polymerized by Wzy\textsuperscript{29}. Subsequently, the channel Wza, together with regulators Wzb and Wzc, which control the process of polymerization and transportation, exports the polymer to the surface of the bacteria\textsuperscript{29}.

Of the 79 documented capsular types in \textit{Klebsiella} spp., the \textit{cps} gene clusters of 22 types (complete 13 \textit{cps} for K1, K2, K3, K5, K9, K14, K16, K20, K22, K39, K52, K62 and KN2; incomplete 9 \textit{cps} for K15, K23, K37, K45, K50, K54, K57, K79 and KN1) are available\textsuperscript{12,18,25,28}. In order to associate all of 79 \textit{cps} gene clusters with distinct capsular types, we sequenced the \textit{cps} of 57 capsular types of \textit{Klebsiella} spp. and extend the 3' of incomplete 9 \textit{cps}, and conducted comparative analysis of the \textit{cps} gene clusters of various types. Investigation into the relationships between different capsular gene clusters provided further understanding of capsule biosynthesis. Moreover, as we have gained more complete information on the genetic structures of all 79 capsular types, the limitations of current genotyping methods can be more clearly defined and the use of these typing methods can be further improved.

Results 
\textbf{\textit{cps} gene clusters of 79 \textit{Klebsiella} spp. capsular types.} We obtained all the 79 \textit{cps} gene clusters which extend from \textit{galf} to \textit{ugd} (except for K4 and K50) by retrieving sequences from Genbank database (13 complete \textit{cps} and 9 incomplete \textit{cps}), extending the 3' \textit{cps} sequences in the 9 types and resolving 57 \textit{cps} of \textit{Klebsiella} spp. (Supplementary Table S1). In K4, we failed to extend the downstream sequences of \textit{gnd}; in K50, conserved \textit{gnd} or \textit{ugd} genes were not found in this locus although a ~21 kb region from \textit{galf} to the downstream genes \textit{HisA} and \textit{HisF} (which encode enzymes associated with histidine biosynthesis and are generally located downstream of \textit{cps} gene clusters in \textit{Klebsiella} spp.) was resolved. Moreover, we identified \textit{K. pneumoniae} strain BIDMC 47 as K13 by wzc genotyping\textsuperscript{26} (100% DNA sequence identity). Thus, the full \textit{cps} sequences of BIDMC47 (accession number AB924555) was included to represent K13 type. For these 78 \textit{cps}, a total of 1515 coding sequences were annotated, including \textit{galf} (n = 79), \textit{cpsACP} (n = 80), \textit{wzi} (n = 78), \textit{wza} (n = 81), \textit{wzb} (n = 78), \textit{wzc} (n = 78), \textit{gnd} (n = 79), \textit{manB} (n = 43), \textit{manC} (n = 44), \textit{rmLA} (n = 30), \textit{rmLB} (n = 29), \textit{rmLC} (n = 30), \textit{rmLD} (n = 30), \textit{wcaJ} (n = 40), \textit{wbaP} (n = 39), \textit{gnd} (n = 6), \textit{wcaG} (n = 6), \textit{gld} (n = 5), \textit{wxx} (n = 77), \textit{wxv} (n = 77) and genes encoding non-initial GTs (n = 318), glycosyl hydrolase (n = 33), acetyltransferases (n = 35), pyruvyltransferases (n = 35), transposases (n = 21), nitroreductase (n = 2), potassium/proton antiporter (n = 2), tail fiber (n = 6), acetylneuraminic acid synthetase (n = 1), UDP galacturonate 4-epimerase (n = 1), carbohydrate lyase (n = 1), CMP-N-acetylneuraminic acid synthetase (n = 1), coenzyme F420 hydrogenase (n = 1) and hypothetical proteins (n = 49) (Supplementary Table S2).

By NCBI blast, the \textit{cps} gene clusters of \textit{Klebsiella} spp. K31, K47, K61 and K63 were almost identical (>96% DNA identity) to those of \textit{Escherichia coli} 5-172-05_S1_C3 (JQ0501000075.1), \textit{Escherichia coli} HS (CP000802), \textit{Escherichia coli} MS 85-1 (ADWQ01000010.1) and \textit{Escherichia coli} KTE222 (ASUP01000016.1), respectively. Similarly, previous studies reported that \textit{E. coli} and \textit{Klebsiella} spp. indicated highly similar \textit{cps} sequences\textsuperscript{23,31}. The \textit{cps} sequences of \textit{Klebsiella} spp. K4 also share high similarity (99% DNA identity) with those of \textit{Serratia} spp. (AEQT01000901).

General and atypical features of the \textit{cps} locus in 79 capsular types of \textit{Klebsiella} spp. The commonality of genetic features of the \textit{cps} locus has been revealed in previous studies\textsuperscript{18,28}. Conserved genetic organization at the 5' end of the \textit{cps} locus extends from \textit{galf} through \textit{cpsACP}, \textit{wzi}, \textit{wza}, \textit{wxb} and \textit{wzc} and at the 3' end of \textit{cps} locus from \textit{gnd} to \textit{ugd}. The \textit{wzc-gnd} region which usually contains genes encoding GTs, flipase (\textit{wzx}), polymerase (\textit{wzy}) and modifying enzymes (acytetylttransferase, pyruvyl transferase, etc.) varies among different capsular types\textsuperscript{15}. The \textit{gnd-ugd} region possibly is composed of genes involved in GDP-D-mannose synthesis (\textit{manB} and \textit{manC}) or dTDP-L-rhamnose synthesis (\textit{rmLA}, \textit{rmLB}, \textit{rmLC} and \textit{rmLD})\textsuperscript{29}. Analysis of the 79 \textit{cps} gene clusters from \textit{Klebsiella} spp. indicated that these general features were observed in most capsular types, meanwhile, some notably uncommon features were characterized as well.

In K4, \textit{cpsACP} was not followed by a \textit{wzi} gene; instead, one acetyltransferase gene, two potassium/proton antiporter genes and a transposase gene were located between \textit{cpsACP} and \textit{wza} (Fig. 1). Similarly, the \textit{wzi} gene was absent in K33 and K40 \textit{cps} loci; instead, three hypothetical proteins were identified in the region of K33 (Fig. 1), and three GT genes, three genes encoding hypothetical proteins and three transposase genes were located in the region of K40 (Fig. 1). Besides, two \textit{gnd} genes were found in the K41 \textit{cps} region and most interestingly, the K4 \textit{cps} was composed of a \textit{wza-wzb-wzc} region and an additional
wzi-wza-wzb-wzc region (wza was interrupted by a transposase gene). The additional three genes and the upstream wzi gene showed high DNA sequence identity with those of K1 (99% for each gene), indicating that K4 cps included several K1 cps genes (Fig. 1). Another atypical feature is that no wzx-like gene was found in capsular types K11 and K34 and no wzy-like gene was found in capsular types K29 and K50.

In addition, we further examined the correlation between the sugar composition and presence/absence of related cps genes. Of the 79 documented capsular types, to our best knowledge, 74 capsule structures are publicly available (the chemical structure are unavailable for the five types, K29, K42, K65, KN1 and KN2) (references were provided in Supplementary Table S3). Sugars found in different K-types are mannose for 37 types, fucose for 6 types, rhamnose for 28 types, and galactofuranose for 3 types. Among the 37 types with mannose as a structural unit, genes for GDP-D-mannose synthesis (manB and manC) were found in their cps regions, except in K4 (with manC only) and in K50 (both absent). Moreover, even there is no mannose incorporated into their capsule structures, capsular types K1, K6, K16, K54, K58 and K63 harbored manCB genes. As the five types were known to use fucose as one of the components of their capsules and GDP-L-fucose is converted from GDP-D-mannose, mannose would be an intermediate rather than the final product incorporated into capsule. The six capsular types (K1, K6, K16, K54, K58, K63) that contain fucose as a structural unit possessed both gmd (gene encodes GDP-D-mannose 4, 6-dehydratase) and wcaG (a nucleotide sugar epimerase/dehydratase with bifunctional activity: GDP-4-dehydro-6-deoxy-D-mannose epimerase and GDP-4-dehydro-6-L-deoxygalactose reductase) genes, which are responsible for conversion from GDP-D-mannose to GDP-L fucose. Conversely, the types with capsules that do not contain fucose lacked both gmd and wcaG in their cps regions.

The rmlA, rmlB, rmlC and rmlD genes are known to be responsible for dTDP-L-rhamnose synthesis, and the four genes were usually found together, with the exception of the K65 cps region, which contained only the rmlA, rmlC and rmlD genes and not rmlB. From the resolved CPS structure, we found that the presence/absence of rhamnose in repeat units was perfectly correlated with presence/absence of rmlBADC genes. Galactofuranose was found in K12, K14 and K41, consistent with the presence of glf genes (encoding UDP-galactopyranose mutase, which catalyzes the conversion of UDP-galactopyranose into UDP-galactofuranose).

With respect to the correlation between capsule modifications and modifying enzymes (acyetyltransferases and pyruvyltransferases), 12 types exhibited acetylated capsules and 10 of them carried genes encoding acetyltransferases (the two exceptions are K33 and K59) (Supplementary Table S3). Sixty-two types express capsule without acetylation, but genes encoding acetyltransferases were found in 19 types.
Twenty-eight types have pyruvylated capsules and contained genes that encoded pyruvyltransferases in their \( cps \) regions except for K11 (Supplementary Table S3). Forty-six types express capsule without pyruvylation, but gene for adding pyruyl groups were found in 4 types (K8, K22, K37 and K66).

**Homology group (HG) assignment of \( cps \) genes.** We used the TribeMCL program to assemble 1,515 predicted proteins into 361 HGs. The clustering result showed that 143 of the 361 HGs (40\%) contained 2 to 81 members each, and the remainders formed 218 single-member HGs (Supplementary Table S2).

The products of galF, wzi, wza, wzb, wzc, gnd, wcaJ, wbaP, manC, manB, rmlA, rmlB, rmlC and \( rmlD \) fell into a single HG, suggesting these proteins were conserved among different capsular types. In contrast, non-initial GTs, Wzy polymerases and Wzx flippases were clustered into 142, 56 and 28 different HGs, respectively, indicating they were diverse in various types. Intriguingly, proteins for capsule modification (acetyltransferases and pyruvyltransferases) also classified into multiple groups (26 and 16 HGs, respectively), suggesting different modifying enzymes were needed for distinct capsule structures.

**Applications of \( cps\)-PCR genotyping.** Due to the limitations of capsular serotyping, polymerase chain reaction-based genotyping of the capsular polysaccharide synthesis region, \( cps\)-PCR genotyping, was developed based on available sequences to detect specific \( cps \) genes in some capsular types of *Klebsiella* spp.

Because \( cps\)-PCR genotyping is a rapid and accurate method for detecting the \( cps \) genotype, the availability of \( cps \) sequences in all 79 types will be very useful for discriminating capsular types based on capsular type-specific genes. According to the results of the protein clustering, non-initial GTs, Wzx and Wzy were specific to distinct capsular types, indicating these genes could be selected for genotyping. Because more than one non-initial GT gene was present in a given type, it would be easier to choose wzy or wzy typing genes. In addition, since the 77 Wzy were classified into 56 HGs compared to the 77 Wzx categorized into 28 HGs, wzy exhibits more diversity than wzx in different types.

There were 78 Wzi clustered into 4 HGs, suggesting that the wzi was less discriminatory than wzx or wzy. Therefore, wzy would be most specific for capsular \( cps\)-PCR genotyping. We further analyzed the amino acid and DNA sequence identity of the wzy genes that were grouped into the same HG groups. Most of the Wzy proteins shared < 60\% amino acid sequences identity even within a single HG group and shared DNA sequence similarity with < 600 bp matching sequences over ~ 1.2 kb gene length except K22 vs. K37, K12 vs. K41, K2 vs. K13, K74 vs. K80, K79 vs. K91 and K30 vs. K69 (Table 1). Previous studies have documented that K22 and K37 possess identical wzy genes and are only distinguishable by the acetyltransferase encoding genes \( ^{23} \). For the types exhibited high similarity (> 600 bp matching sequences over ~ 1.2 kb gene length), primers should be designed according to the variable region of their wzy genes; alternatively, other \( cps \) genes can be used for differentiating these types. Another limitation is the inapplicability in the capsular types lacking wzy-like genes (K29 and K50).

**Enzymes for synthesis of capsular repeat unit.** WbaP and WcaJ regarded as initial GT for capsule synthesis are UDP-hexose transferase enzymes that transfer galactose-1-phosphate and glucose-1-phosphate, respectively, to undecaprenol phosphate \(^{28,37} \). Additional transferases (non-initial GT) further add sugars to form repeat units \(^{29,38} \) and polymerase enzyme, Wzy, subsequently assemble the lipid-linked repeat units \(^{29} \). We found that either wbaP or wcaJ were present in the 79 \( cps \) loci, and the clustering results showed that the initial GTs (WbaP and WcaJ) were assembled into a single group each, implying they were conserved among different types. Furthermore, a perfect correlation was observed in the 74 types with available capsule structures, that is, WbaP genes co-exist with the presence of galactose in the repeat unit, and WcaJ co-exist with the presence of glucose. Moreover, possible polymerization linkage of the repeat unit can be predicted based on which type of initial GT they possess. For example, the presence of WcaJ indicates that glucose is the initial sugar of K1 capsular repeat units, therefore, the polymerization linkage of K1 capsular repeat units could be \( 3\text{-D-Glcp}(1 \rightarrow 2)\text{-D-Glcp} \) according to reported chemical structure of its capsule \(^{39} \) and K1 Wzy (MagA) is supposed to be responsible for the linkage formation. In addition, K12 and K41 which share 82% amino acid sequences identity in Wzy seem to have the same predicted polymerization linkage for their capsular repeat units, i.e., \( \alpha\text{-D-Galp}(1 \rightarrow 2)\text{-D-Galp} \).

The clustering results showed that 318 non-initial GTs were clustered into 142 different HGs, which provide some information on the possible functions of the GTs. For example, one HG (HG20) contains 14 GTs from K3, K7, K21, K24, K26, K28, K29, K39, K40, K43, K53, K65, K74 and K80 (the GTs show 37-64\% amino acid identity to their members). Based on the available CPS structures (except for K29 and K65), 10 of the 12 types (K3, K7, K21, K24, K26, K28, K43, K53, K74 and K80) share the same linkage \( \alpha\text{-D-Manp}(1 \rightarrow 2)\text{-D-Manp} \). Therefore, we suggested that these GTs grouped into the same HG (named as WcuE) probably has catalytic activity for the specific sugar linkage. Accordingly, the relationship of GTs and CPS structures lays the foundations for understanding the putative functions of different GTs.

**Capsular types with related \( cps \) genes and similar capsule structure.** According to the protein clustering results, 9 pairs of capsular types (K1 and K58, K2 and K13, K12 and K41, K14 and K64, K10 and K61, K30 and K69, K33 and K35, K74 and K80, and K57 and K68) have 5 or more genes that located within the variable region (wzz-ugd, excluding man and rml genes) shared similarity (clustered into the
same HG). Therefore, we compared the CPS structures of these capsular types and indicated correlations between genes and products. Below are 6 examples with clearer implications (others are described in Supplementary Fig. S1a–S1c):

**K1 and K58.** The same linkage of $\beta\text{-D-GlcpA}(1\rightarrow4)\alpha\text{-L-Fucp}$ was found in the capsular repeat units of K1 and K58, thus, we suggest that the fucosyl transferase WcaI present in both types is responsible for the synthesis of this linkage. Moreover, The specific GT in K1, wcsS, most likely accounts for the linkage $\alpha\text{-L-Fucp}(1\rightarrow3)\beta\text{-D-Glcp}$, whereas the two GTs in K58, WcqS and WcqT, likely accounts for the $\alpha\text{-L-Fucp}(1\rightarrow3)\alpha\text{-D-Glcp}$ linkage or the side chain synthesis (Fig. 2a and Supplementary Table S4).

**K2 and K13.** K2 and K13, which are known to cross-react by serotyping, share similar capsule structures that only differ in the side chain, i.e., $\alpha\text{-D-GlcpA}(1\rightarrow3)\beta\text{-D-Manp}$ in K2 and $3\text{-Pyr}\cdot\beta\text{-D-Galp}(1\rightarrow4)\alpha\text{-D-GlcpA}(1\rightarrow3)\beta\text{-D-Manp}$ in K13. The pyruvyl transferase (WcuL) and the GT (WcoW) present only in K13 but not in K2 may contribute to the addition of the pyruvyl group and the synthesis of the linkage $\beta\text{-D-Galp}(1\rightarrow4)\alpha\text{-D-GlcpA}$, respectively (Fig. 2a and Supplementary Table S4). The function of WcoW was also evidenced by the co-existence of WcoW and the linkage in K74. In addition, we also found that K2 has an acetyltransferase-encoding gene, however, the previously reported K2 capsule structure is not acetylated.

### Table 1. Amino acid and DNA sequences identity of the members in Wzy HG groups.

| Wzy group | Capsular types of the members | Types for comparison | Sequences identity (%) |
|-----------|------------------------------|---------------------|------------------------|
|           |                              |                     | Amino acid           | DNA                   |
| 1(HG46)   | K52, K53, K81                | K52 vs. K81         | 144/376(38%)          | 193/292(66%)          |
|           |                              | K52 vs. K53         | 133/377(35%)          | —                     |
|           |                              | K81 vs. K53         | 134/377(36%)          | —                     |
| 2(HG49)   | K42, K59, K65                | K59 vs. K65         | 167/415(40%)          | —                     |
|           |                              | K59 vs. K42         | 152/388(39%)          | —                     |
|           |                              | K65 vs. K42         | 156/388(40%)          | —                     |
| 3(HG50)   | K36, K48, K67                | K48 vs. K36         | 127/303(42%)          | 146/214(68%)          |
|           |                              | K48 vs. K67         | 110/296(37%)          | —                     |
|           |                              | K36 vs. K67         | 118/295(38%)          | —                     |
| 4(HG51)   | K27, K38, K2              | K27 vs. K38         | 128/409(31%)          | —                     |
|           |                              | K27 vs. K2             | 145/403(36%)          | —                     |
|           |                              | K38 vs. K2             | 143/423(34%)          | —                     |
| 5(HG56)   | K22, K25, K37                | K22 vs. K25         | 194/380(51%)          | 540/842(64%)          |
|           |                              | K22 vs. K25         | 381/381(100%)         | 1143/1143(100%)       |
| 6(HG58)   | K3, K24, K28                 | K3 vs. K24           | 179/373(48%)          | 173/269(64%)          |
|           |                              | K3 vs. K28           | 167/394(42%)          | —                     |
|           |                              | K24 vs. K28           | 145/394(37%)          | —                     |
| 7(HG80)   | K30, K69                     | K30 vs. K69          | 269/412(65%)          | 819/1192(69%)         |
| 8(HG88)   | K79, K11                     | K79 vs. K11          | 239/364(66%)          | 803/1167(69%)         |
| 9(HG91)   | K74, K80                     | K74 vs. K80          | 308/407(76%)          | 831/1141(73%)         |
| 10(HG101) | K45, K72                     | K45 vs. K72          | 149/389(38%)          | —                     |
| 11(HG105) | K35, K46                     | K35 vs. K46          | 158/387(41%)          | 380/602(63%)          |
| 12(HG110) | K11, K82                     | K11 vs. K82          | 135/349(39%)          | —                     |
| 13(HG121) | K2, K13                      | K2 vs. K13           | 228/415(55%)          | 747/1180(63%)         |
| 14(HG140) | K14, K64                     | K14 vs. K64          | 201/397(51%)          | 241/357(68%)          |
| 15(HG125) | K12, K41                     | K12 vs. K41          | 324/393(82%)          | 894/1174(76%)         |

Note: the two sequences were aligned by NCBI blastp for amino acids analysis and NCBI blastn for nucleotide analysis; the ratio of identity indicates no. of matching nucleotides or amino acids/total no. of nucleotides or amino acids; — indicates < 100 bp nucleotides were matched.
Figure 2. Comparison of cps gene clusters and capsule structures in capsular types with similar cps gene content. Open reading frames (ORFs) are shown as arrows. Conserved genes, man genes, rml genes or transposases are shown in black. Other gene products that were clustered into the same HGs are shown in same colors, and the amino acid similarities (%) are indicated below the ORFs. Genes only present in either of the two types are shown in white. GT, glycosyltransferase. Enzymes most likely involved in linkage formation are indicated along with their capsule structures. The differences of capsule structures from two types are shown in red. a, K1 and K58; b, K2 and K13; c, K12 and K41; d, K30 and K69; e, K74 and K80; f, K57 and K68.
**K12 and K41.** Serological cross-reactions between K12 and K41 are known to occur\(^\text{47,48}.\) The two capsular types exhibit the same repeat unit but distinct side branches\(^\text{49,51}.\) The side chain of K12 was determined to be 5, 6-Pyr-\(β\)-D-Galp\((1→4)\)\(β\)-D-GlcpA\((1→3)\)\(β\)-D-Galf and that of K41 is \(β\)-D-Glcp\((1→4)\)\(α\)-D-Manp\((1→3)\)\(β\)-D-Galf. A GT (wckG) and a pyruvyl transferase (wckH) were found only in K12, suggesting that these are the key enzymes involved in the synthesis of \(β\)-D-Galp\((1→4)\)\(β\)-D-GlcpA and pyruvlation, respectively (Fig. 2c and Supplementary Table S4). And The two GTs (WcpT and WcpU) in K41 are likely involved in the synthesis of \(β\)-D-Glcp\((1→6)\)\(α\)-D-GlcpA\((1→4)\)\(β\)-D-GlcpA.

**K30 and K69.** Even no cross-reaction has been reported between K30 and K69, the capsule structures of the two types are almost identical with the exception of the linkage between \(β\)-D-Galp and the pyruvyl group\(^\text{49,50}.\) The cps regions of the two types were also highly similar (Fig. 2d and Supplementary Table S4). With the major difference between these two strains being pyruvlation, the pyruvyltransferases from K30 and K69 which shared 73% amino acid identity (named as WcuL) could catalyze both pyruvlation linkages or the dissimilarity of the two proteins is critical for their specificity.

**K74 and K80.** K74 and K80 exhibit similar capsule structures\(^\text{46,51}.\) and cps genes but do not show serological cross-reactivity. The differences between these two types reside within the side chains: 4, 6-Pyr-\(β\)-D-Galp\((1→4)\)\(α\)-D-GlcpA\((1→3)\)\(α\)-D-Manp and 3, 4-Pyr-\(β\)-L-Rhap\((1→4)\)\(α\)-D-GlcpA\((1→3)\)\(α\)-D-Manp in K74 and K80, respectively. Comparing the gene content of the K74 and K80 cps loci, genes for rhamnose synthesis (rmlABCD) were found in K80 but not in K74, which is consistent with the use of rhamnose in the side chain of K80 (Fig. 2e and Supplementary Table S4). Moreover, K74 and K80 each possess a unique pyruvyl transferase and a GT, suggesting that WcuL is involved in the synthesis of 4, 6-Pyr-\(β\)-D-Galp (the predicted function of WcuL is the same as what we proposed for the K69 structure); WcoW is involved in the synthesis of \(β\)-D-Galp\((1→4)\)\(α\)-D-GlcpA (the predicted function of WcoW is the same as what we proposed for the K13 structure); WcuN likely accounts for the synthesis of 3, 4-Pyr-\(β\)-L-Rhap; and WcuS likely accounts for the synthesis of \(β\)-L-Rhap\((1→4)\)\(α\)-D-GlcpA. Moreover, because WbaZ is known to catalyze the \(α\)-D-Manp\((1→3)\)\(β\)-D-Galp glycosidic linkage\(^\text{38}.\) WcuD and WcuE were presumably responsible for the synthesis of the rest of linkages, i.e., \(α\)-D-Manp\((1→2)\)\(β\)-D-Manp or \(α\)-D-GlcpA\((1→3)\)\(α\)-D-Manp.

**K57 and K68.** K57 and K68 do not exhibit serological cross-reactivity but showed similarity in CPS structures\(^\text{45,52}.\) and cps genes. The GT WbaZ which is known to form the \(α\)-D-Manp\((1→3)\)\(β\)-D-Galp disaccharide backbone is present in both strains. The pyruvlation of the capsule in K68 is also indicated by the presence of a pyruvyl transferase gene within its cps region instead of disrupting it. For example, it has been documented that the wzi and wzy genes were highly variable while wzi genes were relatively conserved. wzy PCR genotyping needed specific primers from each already resolved sequences, however, it was more specific and no sequencing was necessary. In contrast, wzi genotyping could use relatively conserved sequences as primers, but it needed PCR and sequencing of PCR products to obtain final results. Both methods would encounter difficulties in some capsular types unless full cps sequences available. wzc genotyping\(^\text{25}.\)
was similar to wzi PCR and sequencing. However, it can differentiate much more reported genotypes than wzi. Therefore, wzy PCR would be preferable to rapid identify a specific genotype while wzc PCR with sequencing would be best to test isolates with unknown type prevalence.

Comparative analysis of different capsular types showed their relatedness, and the genetic differences (presence or absence of genes, sequence changes and gene truncation etc.) can be linked to the various structures of the expressed capsules. Our results indicated that some types exhibit similar capsule structures because of the high similarity in their cps regions. In terms of serological reactions, some of the capsular types that share related cps genes are known to cross-react by serotyping (K1 vs. K58; K2 vs. K13; K12 vs. K41), indicating that anti-sera recognize their common structures; other strains do not exhibit cross-reactivity despite sharing very similar structures (K30 vs. K69; K74 vs. K80; K37 vs. K68), suggesting that distinct epitopes are crucial for serological differentiation. In addition, putative functions of cps genes were also indicated according to the presence of specific genes and unique linkages. The existence of genes for capsule modifications in cps region also revealed the possibility of presence of undefined capsule modifications in certain types. Besides, we also provide some evidence of sugar composition in types with unknown CPS structure. Therefore, as all cps gene clusters from different capsular types of Klebsiella spp. have been resolved, the functions of genes involved in capsule synthesis will be much clear.

In conclusion, the available cps sequences and comparative analysis of various capsular types has an impact on understanding of the functions of cps genes and provides complete information on the relatedness of different capsular types through evolutionary history. Furthermore, these data are an important basis for the application of capsular genotyping as well as new type identification in Klebsiella spp.

Methods

Bacterial strains. A total of 77 K-serotype Klebsiella spp. reference strains were purchased from Statens Serum Institute, Copenhagen, Denmark. Two additional strains with novel type KN1 and KN2 capsules identified in our laboratory were also included 12,13.

Sequencing of cps loci. We amplified the cps loci from Klebsiella spp. strains using multiple pairs of conserved primers as previously described 12,56 (Supplementary Table S5 and Supplementary Fig. S2). PCR amplifications were performed with the Long and Accurate PCR system, and the cycling programs were used in accordance with previously described procedures 52. The PCR amplicons were subjected to sequencing by high-throughput sequencing (Yang-Ming Genome Research Center) using the Illumina/ Solexa GAII sequencing platform. When PCR amplifications failed, cps sequences were obtained by previously described inverse PCR and DNA sequencing methods 56 based on the available wzc sequences of these types 53. The cps sequences (approximately 20–30 kb) were deposited in Genbank (Accession Numbers are shown in Supplementary Table S1).

Gene annotation and homology group (HG) assignment. Coding sequences were predicted by vector NTI and annotated by NCBI-protein blast. Predicted proteins were classified into HGS using the TribeMCL algorithm (Centre for Mathematics and Computer Science and EMBL-EBI) with a cut-off of 1e−50. Gene names were assigned for cps genes encoding GTs, acetyltransferases and pyruvyltransferases in accordance with the Bacterial Polysaccharide Gene Database 58 if they had not been given names previously. Proteins within the same HGS were given the same name, and hypothetical proteins with uncertain roles in capsule synthesis were given names according to the number of HGS. The polymerases (Wzy) that fell into multiple HGS were each assigned a number to indicate the different groups.

References

1. Abbott, S. L. Klebsiella, Enterobacter, Citrobacter, Serratia, Plesiomonas, and other Enterobacteriaceae. In: Murray P. R., Baron E. J., Jorgensen J. H., Pfaffer M. A., Yolken R. H., eds. Manual of clinical microbiology. 8th ed. Washington DC, American Society for Microbiology Press, 684–700 (2003).
2. Podschun, R. & Ullmann, U. Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clinical Microbiology Reviews 11, 589–603 (1998).
3. Lin, W. H. et al. Clinical and microbiological characteristics of Klebsiella pneumoniae isolates causing community-acquired urinary tract infections. Infection 38, 459–64 (2010).
4. Tsai, F. C., Huang, Y. T., Chang, L. Y. & Wang, J. T. Pyogenic liver abscess as endemic disease, Taiwan. Emerg Infect Dis 14, 1592–600 (2008).
5. Wang, J. L. et al. Changing bacteriology of adult community-acquired lung abscess in Taiwan: Klebsiella pneumoniae versus anaerobes. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 40, 915–22 (2005).
6. Fang, C. T. et al. Klebsiella pneumoniae genotype K1: an emerging pathogen that causes septic ocular or central nervous system complications from pyogenic liver abscess. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 45, 284–93 (2007).
7. Fung, C. P. et al. A global emerging disease of Klebsiella pneumoniae liver abscess: is serotype K1 an important factor for complicated endophthalmitis? Gut 50, 420–4 (2002).
8. Cortes, G. et al. Molecular analysis of the contribution of the capsular polysaccharide and the lipopolysaccharide O side chain to the virulence of Klebsiella pneumoniae in a murine model of pneumonia. Infect Immun 70, 2583–90 (2002).
9. Mizuta, K. et al. Virulence for mice of Klebsiella strains belonging to the O1 group: relationship to their capsular (K) types. Infect Immun 40, 56–61 (1983).
10. Brisse, S. et al. wzi Gene sequencing, a rapid method for determination of capsular type for Klebsiella strains. J Clin Microbiol 51, 4073–8 (2013).
11. Orskov, I. & Fife-Asbury, M. A. New Klebsiella capsular antigen K82 and the deletion of five of those previously assigned. International Journal of Systematic Bacteriology 27, 366–7 (1977).
12. Pan, Y. J. et al. Capsular polysaccharide synthesis regions in Klebsiella pneumoniae serotype K57 and a new capsular serotype. *Journal of Clinical Microbiology* **46**, 2231–40 (2008).

13. Hsu, C. R., Lin, T. L., Pan, Y. J., Hsieh, P. F. & Wang, J. T. Isolation of a bacteriophage specific for a new capsular type of Klebsiella pneumoniae and characterization of its polysaccharide depolymerase. *PLoS One* **8**, e70092 (2013).

14. Julianelle, L. A. A Biological Classification of Encapsulatus Pneumoniae (Friedlander's Bacillus). *J Exp Med* **44**, 113–28 (1926).

15. Fung, C. P. et al. A 5-year study of the seroepidemiology of Klebsiella pneumoniae: high prevalence of capsular serotype K1 in Taiwan and implication for vaccine efficacy. *The Journal of infectious diseases* **181**, 2073–9 (2000).

16. Tsay, R. W., Liu, L. K., Fung, C. P. & Chang, E. Y. Characteristics of bacteria between community-acquired and nosocomial Klebsiella pneumoniae infection: risk factor for mortality and the impact of capsular serotypes as a herald for community-acquired infection. *Arch Intern Med* **162**, 2021–7 (2002).

17. Jenney, A. W. et al. Seroepidemiology of Klebsiella pneumoniae in an Australian Tertiary Hospital and its implications for vaccine development. *Journal of Clinical Microbiology* **44**, 102–7 (2006).

18. Chuang, Y. P., Fang, C. T., Lai, S. Y., Chang, S. C. & Wang, J. T. Genetic determinants of capsular serotype K1 of Klebsiella pneumoniae causing primary pyogenic liver abscess. *Journal of Infectious Diseases* **193**, 645–54 (2006).

19. Fang, F. C., Sandler, N. & Libby, S. J. Investigation of the putative virulence gene magA in a worldwide collection of 495 Klebsiella isolates: magA is restricted to the gene cluster of Klebsiella pneumoniae capsular serotype K1. *J Med Microbiol* **54**, 1111–3 (2005).

20. Arakawa, Y. et al. Genomic organization of the Klebsiella pneumoniae cps region responsible for serotype K2 capsular polysaccharide synthesis in the virulent strain Chedid. *Journal of Bacteriology* **177**, 1788–96 (1995).

21. Rahn, A., Drummlsmith, J. & Whitfield, C. Conserved organization in the cps gene clusters for expression of Escherichia coli group 1 K antigens: relationship to the colanic acid biosynthesis locus and the cps genes from Klebsiella pneumoniae. *J Bacteriol* **181**, 2307–13 (1999).

22. Yu, W. L. et al. Polymerase chain reaction analysis for detecting capsule serotypes K1 and K2 of Klebsiella pneumoniae causing abscesses of the liver and other sites. *J Infect Dis* **195**, 1235–6; author reply 1236 (2007).

23. Pan, Y. J. et al. Capsular Types of Klebsiella pneumoniae Revisited by wzc Sequencing. *PLoS ONE* **8**, e80670 (2013).

24. Brisse, S., Issenhuth-Jeanjean, S. & Grimont, P. A. Molecular serotyping of Klebsiella species isolates by restriction of the amplified capsular antigen gene cluster. *J Clin Microbiol* **42**, 3388–94 (2004).

25. Pan, Y. J. et al. Capsular Types of Klebsiella pneumoniae revisited by wzc sequencing. *PLoS One* **8**, e80670 (2013).

26. Arakawa, Y. et al. Genomic organization of the Klebsiella pneumoniae cps region responsible for serotype K2 capsular polysaccharide synthesis in the virulent strain Chedid. *Journal of Bacteriology* **177**, 1788–96 (1995).

27. Rahn, A., Drummlsmith, J. & Whitfield, C. Conserved organization in the cps gene cluster expression of Escherichia coli group 1 K antigens relationship to the colanic acid biosynthesis locus and the cps genes from Klebsiella pneumoniae. *J Bacteriol* **175**, 7778–92 (1993).

28. Shu, H. Y. et al. Genetic diversity of capsular polysaccharide biosynthesis in Klebsiella pneumoniae clinical isolates. *Microbiology* **155**, 4170–83 (2009).

29. Whitfield, C. Biosynthesis and assembly of capsular polysaccharides in Escherichia coli. *Annu Rev Biochem* **75**, 39–68 (2006).

30. Whitfield, C. & Roberts, I. S. Structure, assembly and regulation of expression of capsules in Escherichia coli. *Molecular microbiology* **31**, 1307–19 (1999).

31. Rahn, A., Drummlsmith, J. & Whitfield, C. Conserved organization in the cps gene cluster expression of Escherichia coli group 1 K antigens: relationship to the colanic acid biosynthesis locus and the cps genes from Klebsiella pneumoniae. *Journal of Bacteriology* **181**, 2307–13 (1999).

32. Albermann, C., Distler, J. & Piepersberg, W. Preparative synthesis of GDP-beta-L-fucose by recombinant enzymes from E. coli: Biological sources. *Glycobiology* **10**, 875–81 (2000).

33. Giraud, M. F. & Naimis, J. H. The rhhamnose pathway. *Curr Opin Struct Biol* **10**, 687–98 (2000).

34. Koplin, R., Wang, G., Hotte, B., Prierer, U. B. & Pulher, A. A 3.9-kb DNA region of Xanthomonas campestris pv. campestris that is necessary for lipopolysaccharide production encodes a set of enzymes involved in the synthesis of GDP-rhamnose. *J Bacteriol* **175**, 7786–92 (1993).

35. Assael, P. M. et al. Galactofuranosyl biosynthesis in Escherichia coli K-12: identification and cloning of UDP-galactopyranose mutase. *J Bacteriol* **178**, 1047–52 (1996).

36. Lee, R. et al. Enzymatic synthesis of UDP-galactofuranose and an assay for UDP-galactopyranose mutase based on high-performance liquid chromatography. *Anal Biochem* **242**, 1–7 (1996).

37. Liu, D., Haase, A. M., Lindqvist, L., Lindberg, A. A. & Reeves, P. R. Glycosyl transfersase of O-antigen biosynthesis in Salmonella enterica: identification and characterization of transferase genes of groups B, C2, and E1. *J Bacteriol* **175**, 4308–13 (1993).

38. Drummlsmith, J. & Whitfield, C. Gene products required for surface expression of the capsular form of the group 1 K antigen in Escherichia coli (O9aX30). *Mol Microbiol* **31**, 1321–32 (1999).

39. Ho, I. J. Y. et al. Functions of some capsular polysaccharide biosynthetic genes in Klebsiella pneumoniae NTUH K-2044. *PLoS One* **6**, e21664 (2011).

40. Beurret, M., Joseleau, J. P., Vignon, M., Dutton, G. G. & Savage, A. V. Proof of the occurrence of 5,6-O-(1-carboxyethylidene) D-galactofuranose units in the capsular polysaccharide of Klebsiella K12. *Carbohydr Res* **189**, 247–60 (1989).

41. Beurret, M., Joseleau, J. P., Dutton, G. G. & Savage, A. V. Homologous and heterologous reactions of bacteriophages phi 41 and phi 12 on the capsular polysaccharides from Klebsiella K41 and K12. *Carbohydr Res* **189**, 237–46 (1989).

42. Dutton, G. S. & Savage, A. V. Structural investigation of the capsular polysaccharide of Klebsiella serotype K58. *Carbohydr Res* **84**, 297–305 (1980).

43. Pieron, P., Rennie, R. P., Ziola, B. & Deneer, H. G. The use of bacteriophages to differentiate serologically cross-reactive isolates of Klebsiella pneumoniae. *J Med Microbiol* **41**, 423–9 (1994).

44. Orskov, I. & Orskov, E. Serotyping of Klebsiella. *Methods Microbiol.* **14**, 143–64 (1984).

45. Geyer, H., Himmelspach, K., Kwiatkowski, B., Schlecht, S. & Stirn, S. Degradation of bacterial surface carbohydrates by virus-associated enzymes. *Pure & Appl. Chem.* **55**, 637–53 (1983).

46. Dutton, G. S. & Paulin, M. Structure of the capsular polysaccharide of Klebsiella serotype K74. *Carbohydr Res* **87**, 119–27 (1980).

47. Riser, E., Noone, P. & Poulton, T. A. A new serotyping method for Klebsiella species: development of the technique. *J Clin Pathol* **29**, 296–304 (1976).

48. Murcia, A. & Rubin, S. J. Reproducibility of an indirect immunofluorescent-antibody technique for capsular serotyping of Klebsiella pneumoniae. *J Clin Microbiol* **9**, 208–13 (1979).

49. Lindberg, B., Lindh, F., Lonngren, J. & Sutherland, I. W. Structural studies of the capsular polysaccharide of Klebsiella type 30. *Carbohydr Res* **76**, 281–4 (1979).

50. Hackland, P. L., Parolis, H. & Parolis, L. A. A structural investigation of the capsular polysaccharide of Klebsiella K69. *Carbohydr Res* **172**, 209–16 (1988).

51. Dutton, G. S. & Karunarathne, D. Structural investigation of the capsular polysaccharide of Klebsiella serotype K80. *Carbohydr Res* **134**, 103–14 (1984).
52. Dutton, G. G., Parolis, H. & Parolis, L. A. The structural elucidation of the capsular polysaccharide of Klebsiella K68. *Carbohydr Res* **152**, 249–59 (1986).
53. Wyres, K. L. et al. Extensive capsule locus variation and large-scale genomic recombination within the Klebsiella pneumoniae clonal group 258. *Genome Biol Evol* (2015).
54. Bentley, S. D. et al. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. *PLoS Genet* **2**, e31 (2006).
55. Brisse, S., Issenhuth-Jeanjean, S. & Grimont, P. A. Molecular serotyping of Klebsiella species isolates by restriction of the amplified capsular antigen gene cluster. *Journal of Clinical Microbiology* **42**, 3388–98 (2004).
56. Chang, K. C., Yeh, Y. C., Lin, T. L. & Wang, J. T. Identification of genes associated with natural competence in Helicobacter pylori by transposon shuttle random mutagenesis. *Biochem Biophys Res Commun* **288**, 961–8 (2001).
57. Enright, A. J., Van Dongen, S. & Ouzounis, C. A. An efficient algorithm for large-scale detection of protein families. *Nucleic Acids Res* **30**, 1575–84 (2002).
58. Reeves, P. R. et al. Bacterial polysaccharide synthesis and gene nomenclature. *Trends Microbiol* **4**, 495–503 (1996).

**Acknowledgements**

This study was supported by grants from the National Science Council, National Taiwan University, National Taiwan University Hospital, and the Liver Disease Prevention and Treatment Research Foundation in Taiwan.

**Author Contributions**

J.T.W. designed the study, analyzed data and wrote the article. Y.J.P. and T.L.L. carried out experiments; collected, analyzed, and interpreted data; and wrote the Article. C.T.C., Y.Y.C., P.F.H., C.R.H. and M.C.W. carried out experiments, collected and analyzed data.

**Additional Information**

**Supplementary information** accompanies this paper at http://www.nature.com/srep

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Pan, Y.-J. et al. Genetic analysis of capsular polysaccharide synthesis gene clusters in 79 capsular types of *Klebsiella* spp. *Sci. Rep.* **5**, 15573; doi: 10.1038/srep15573 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/