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Evolution of *Klebsiella pneumoniae* with mucoid and non-mucoid type colonies within a single patient

Haejeong Lee[a,1], Juyoun Shin[b,1], Yeun-Jun Chung[b,c], Jin Yang Baek[d], Doo Ryeon Chung[d,e], Kyong Ran Peck[e], Jae-Hoon Song[d], Kwan Soo Ko[a,d,*]

[a] Department of Molecular Cell Biology and Samsung Medical Center, Sungkyunkwan University School of Medicine, Suwon 16419, Republic of Korea
[b] Department of Microbiology, College of Medicine, The Catholic University of Korea, Seoul 06591, Republic of Korea
[c] Precision Medicine Research Center, Integrated Research Center for Genome Polymorphism, College of Medicine, The Catholic University of Korea, Seoul 06591, Republic of Korea
[d] Asia Pacific Foundation for Infectious Diseases (APFID), Seoul 06351, Republic of Korea
[e] Division of Infectious Diseases, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 06351, Republic of Korea

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ABSTRACT

We obtained nine *Klebsiella pneumoniae* isolates successively isolated from a single patient. Four pairs (M1–M4 and NM1–NM4) obtained simultaneously from the same site showed different colony types, mucoid and non-mucoid, while the final isolate (M5) was isolated alone from the blood and showed a mucoid phenotype. The whole genome of isolate M5 was sequenced *de novo* using the PacBio RSII system, while the others were sequenced with an Illumina Hiseq4000 and mapped to the genome sequences of M5. To identify insertions or deletions in the *cps* locus, we amplified and sequenced *cps* locus genes. We identified insertion sequence (IS) elements in several genes of the *cps* locus or one amino acid substitution in WcaJ in all non-mucoid isolates. Five additional amino acid alterations in RpsJ, LolE, Lon-2, PpsE, and a hypothetical protein were detected in some mucoid and non-mucoid isolates. Based on the genome data and *cps* locus sequences, the mucoid phenotype may have been lost or converted into the non-mucoid phenotype because of the insertion of IS elements or amino acid alterations at this locus. We inferred a within-host evolutionary scenario, in which non-mucoid variants emerged repeatedly from mucoid isolates, but may be short-lived because of their low fitness.

1. Introduction

Klebsiella pneumoniae is one of the common pathogens among gram-negative bacteria (Boucher et al., 2009). This bacterium is the leading cause of hospital infections, including respiratory tract, urinary tract, and bloodstream infections, primarily afflicting young and immunocompromised patients (Paczosa and Mecsas, 2016). One of the most important virulence factors in *K. pneumoniae* is the capsule, which is synthesized by gene products from the capsular polysaccharide synthesis (*cps*) locus and protects the bacteria from opsonophagocytosis thus evading the immune system during infection (Paczosa and Mecsas, 2016). Several studies have shown that acapsular or non-mucoid *K. pneumoniae* strains are markedly less virulent than isogenic encapsulated strains (Yoshida et al., 2000; Lawlor et al., 2005). A recent study showed that modification of the *cps* locus may affect the mucoid feature and thus virulence of *K. pneumoniae* (Lin et al., 2017).

Within-host evolution of bacterial pathogens has been investigated in several bacterial species (Marvig et al., 2014; Golubchik et al., 2013; Kennemann et al., 2011; Witney et al., 2017; Snitkin et al., 2013). The evolution of pathogens through the course of infection was repeatedly observed and has been reported (Workentine et al., 2013; Lieberman et al., 2016). It was also known that high virulence or disease is dead-ends for bacterial evolution. For example, phase shift associated with virulence determinants was observed in the evolution of *Neisseria meningitides* (Meyers et al., 2003). Although the presence of heterogeneous *K. pneumoniae* within a single patient has been reported (Snitkin et al., 2012), within-host evolution, particularly for virulence, has not been widely investigated. Loss of mucoid feature would be a dead-end point in the within-host evolution of *K. pneumoniae*.

We isolated nine *K. pneumoniae* isolates from a single patient. Isolates with different colony types, mucoid and non-mucoid, were isolated simultaneously from a single out-patient, and the pairs were

* Corresponding author at: Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon 16419, Republic of Korea.
E-mail address: ksko@skku.edu (K.S. Ko).
[1] Lee H and Shin J contributed equally to this paper.

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successively isolated four times from the peritoneal fluid or bile of the patient. An additional mucoid isolate was obtained from the blood of the same patient. All isolates co-produced NDM-1 and OXA-232, which are carbapenem-hydrolyzing enzymes, and were resistant to most antibiotics tested except for colistin and fosfomycin. To address how mucoid and non-mucoid phenotypes emerged and were preserved in the host, we performed whole genome sequencing. Based on the results, we identified mutations resulting in the phenotype change from mucoid into non-mucoid colonies, suggesting an in-host bacterial evolution mechanism.

2. Materials and methods

2.1. Bacterial isolates

In November to December 2015, mucoid and non-mucoid phenotype *K. pneumoniae* isolates, which belonged to MLST ST14 and serotype K64, were obtained simultaneously from a 53-year-old male patient with hepatocellular carcinoma at the Samsung Medical Center (Seoul, South Korea). Four pairs of mucoid (M; moisty, clear margin, but not hypermucoviscous phenotype) and non-mucoid (NM; dry and erosive margin colony phenotype) isolates, M1–M4 and NM1–NM4 were obtained. Finally, only one mucoid type colony isolate, M5, was obtained from the patient's bloodstream (Fig. 1).

2.2. Whole genome sequencing

We sequenced the DNA samples of the five mucoid (M1–M5) and four non-mucoid (NM1–NM4) isolates using two different next-generation sequencing techniques. Whole genome sequencing was performed by Macrogen, Inc. (Seoul, Korea) on the PacBio RS II and Illumina-HiSeq 4000 platforms. Only the M5 isolate was sequenced by PacBio platform. Its genome was selected as a reference because it was isolated unilaterally in blood and was not isolated with non-mucoid form. Bioinformatics softwares such as HGAP3, FALCON, and CANU were used for *de novo* assembly, and the assembled sequences were confirmed using Quiver. After the whole genomes were assembled, the locations of the open reading frames were predicted and annotated using Prokka v1.12b (http://www.vicbioinformatics.com/software.prokka.shtml). For the other isolates, Illumina sequencing was performed to determine variants from the genome of isolate M5. The Illumina HiSeq 4000 platform generated 101-base pair (bp) of paied-end reads.

2.3. Variant calling

To map reads in order call variants in the draft genome of M1 to M4 and NM1 to NM4, we used the Burrows-Wheeler aligner MEM algorithm (BWA-MEM) version 0.7.10 and strain M5 as the reference genome. Genetic alterations between the mucoid and non-mucoid isolates, including single-nucleotide polymorphisms and indels (insertions and deletions) were identified using Picard (http://broadinstitute.github.io/picard/) and SAMtools (http://samtools.sourceforge.net/). All non-synonymous mutations were confirmed by Sanger sequencing (primer information is listed in Supplementary Table 1). A phylogenetic tree was drawn based on 11 nucleotide substitutions by the neighbor-joining method. To predict whether the identified amino acid substitutions affect protein function, the PROVEAN scores were calculated (http://provean.jcvi.org/index.php). If the PROVEAN score was equal to or below a predefined threshold, the protein variant was predicted to have a deleterious effect. If the PROVEAN score was above the threshold, the variant was predicted to have a neutral effect (Choi et al., 2012). The annotated sequences of *K. pneumoniae* isolate M5, which were determined by PacBio platform, are deposited in the GenBank nucleotide sequence database under accession numbers CP031734 (chromosome), CP031735 to CP031737 (plasmids). The whole genome sequences of eight *K. pneumoniae* isolates M1 to M4 and NM1 to NM4, which were determined by Illumina platform, are deposited in the NCBI sequence read archive (SRA) under accession number SRP156768.

2.4. cps locus sequencing

Because insertion sequence (IS) is hard to determine the number and location in next-generation sequencing techniques, we identified insertions or deletions in the *cps* locus by polymerase chain reaction (PCR) and sequencing. The obtained *cps* locus sequences were compared with that of *K. pneumoniae* K64 isolate NCTC 8172 (GenBank accession no., AB924600.1) as a reference. Also, we compared the sequence using EMBOSS needle pairwise sequence alignment program (https://www.ebi.ac.uk/Tools/psa/emboss_needle/).

3. Results

3.1. Non-synonymous mutations in mucoid and non-mucoid isolates

The chromosome of *K. pneumoniae* M5 is composed of 5,374,875 bp, which contains 4,905 coding sequences, 85 tRNA genes, and 7 rRNA gene clusters. The overall G + C content was 57.4%. Additionally, we identified three plasmids of 253 kb (pKPM501), 250 kb (pKPM502) and 6 kb (pKPM503). Genes encoding carbapenemases NDM-1 and OXA-232 were found in the plasmids pKPM502 and pKPM503, respectively. After the whole genome of M5 was assembled, we mapped the HiSeq reads of the remaining mucoid isolates (M1 to M4) and non-mucoid isolates (NM1 to NM4) and compared the results to identify genetic variations. The average alignment depth of Illumina sequencing was 301.8 (ranging from 233.32 to 509.05).

Genome sequence analysis revealed 11 genetic variations in the genomes of the eight *K. pneumoniae* isolates compared to in isolate M5. A phylogenetic tree deduced from the 11 variable sites were shown in Supplementary Fig. 1. The tree showed that the mucoid isolates M3 to M5 and the non-mucoid isolates NM1 to NM4 originated from M1 and M2. In addition, the mucoid isolate M3 did not evolve into M4 and M5, and non-mucoid isolates NM3 and NM4 did not come from the other non-mucoid isolates NM1 and NM2.

Among the 11 nucleotide variations (Table 1), six variations were non-synonymous, which result in amino acid substitution was confirmed by Sanger sequencing. All substitutions resulting in no amino acid alteration were identified in non-coding regions in M3 to M5, and NM3. No substitutions were found in promoters, which identified by a promoter finding program, Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/). Because they did not affect

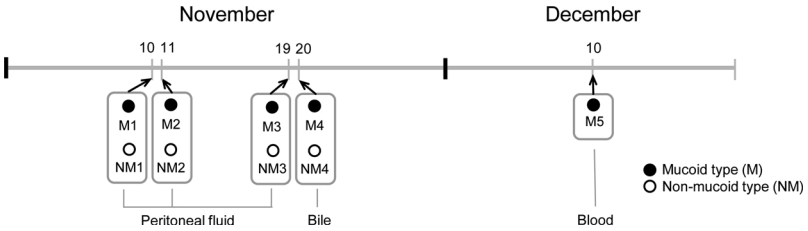

Fig. 1. Timeline of isolation of mucoid and non-mucoid type *K. pneumoniae* isolates. The four pairs with mucoid (M) and non-mucoid (NM) colony types were isolated simultaneously from the same specimen (peritoneal fluid or bile) of a single patient, while the other mucoid isolate (M5) was isolated from the blood.

Table 1
Substitutions and IS insertions between non-mucoid and mucoid *K. pneumoniae* isolates.

Mutation type	Location (function)	Non-mucoid				Mucoid				
		NM1	NM2	NM3	NM4	M1	M2	M3	M4	M5
Amino acid substitution [a]	*rpsJ* (30S ribosomal protein S10)			V57L	V57L					
	wcaJ (undecaprenyl-phosphate glycosyltransferase)	V260D								
	lolE (lipoprotein-releasing system transmembrane protein)		V154G							
	lon_2 (Lon protease)								K417N	K417N
	TRKP067_0849[c] (Hypothetical protein)							M1I		
	ppsE (phthiocerol synthesis polyketide synthase)				N1506T					
Substitution in non-coding region [b]	Upstream of *mltB* (Membrane-bound lytic murein transglycosylase B)							A > G		
	Upstream of hypothetical gene 1562									G > C
	Upstream of *hpxO* (FAD-dependent urate hydroxylase)			A > C						
	Upstream of hypothetical gene 4320			G > T						
	Upstream of *murI* (Glutamate racemase)								G > A	
IS insertion	*wzc* (tyrosine-protein kinase)	IS5								
	wzy (O-antigen and lipid-linked capsular repeat unit polymerase)		IS5							
	wcaJ (undecaprenyl-phosphate glycosyltransferase)		IS5/IS1182	IS5/IS1182						
	wzb (protein tyrosine phosphatase)			IS5/IS1182	IS5/IS1182					

[a] The substitutions were shown in amino acids.
[b] The substitutions were shown in nucleotides.
[c] In *K. pneumoniae* strain KP67 (GenBank accession number, AP018753.1).

function of proteins, they might not be significant in the within-host evolution of bacterial pathogen. While four amino acid changes were identified in non-mucoid isolates, two non-synonymous substitutions including that in a hypothetical protein (TRKP067_0849 in *K. pneumoniae* KP67, GenBank accession number AP018753.1) were found in mucoid isolates. Of these, *wcaJ* encoding undecaprenyl-phosphate glycosyltransferase is a gene consisting of a *cps* locus. The six amino acid changes occurred in only one or two isolates, and there were no common amino acid changes between the mucoid and non-mucoid isolates. According to the PROVEAN scores, three mutations, V260D in WcaJ, V154 G in LolE, and K417 N in Lon_2, were predicted to be deleterious. The other amino acid substitutions were predicted to be neutral.

3.2. Insertion sequence (IS) in cps locus

To identify the difference in the *cps* locus between the mucoid and non-mucoid type isolates in addition to mutations in *wcaJ*, we compared the PCR product size of nine isolates and sequenced the larger PCR products. As a result, we identified IS elements in several genes of the *cps* locus of four non-mucoid isolates (Fig. 2).

The IS5 family of NM1 and NM2 were highly similar to each other (98.0% sequence similarity), and high sequence similarities of > 99% were observed among four IS5/IS1182 family. We found 44.8% sequence similarity between the IS5 and IS5/IS1182. In the first non-mucoid isolate NM1, the IS5 of 1,200 bp was identified within *wzc*, in addition to a V260D mutation in *wcaJ* of the NM1 isolate. The IS5 of 1,215 bp was also found within *wzy* of the second non-mucoid isolate NM2. In NM2, IS5/IS1182 of 1,210 bp was also found within *wcaJ*. In addition to the *cps* locus of NM2, IS5/IS1182 was found in the other non-mucoid isolates, NM3 and NM4. IS5/IS1182 was identified in two genes of the *cps* locus of NM3, *wzb* and *wcaJ*. While IS5/IS1182 of *wzb* was incorporated at the same site between NM3 and NM4, that of *wcaJ* was inserted at different sites between NM2 and NM3, the 689 nucleotide (nt) position and 1,145 nt position of *wcaJ*, respectively.

4. Discussion

The mucoid and non-mucoid *K. pneumoniae* variants explored in this study are phenotypic variants of the same isolate. In addition to the

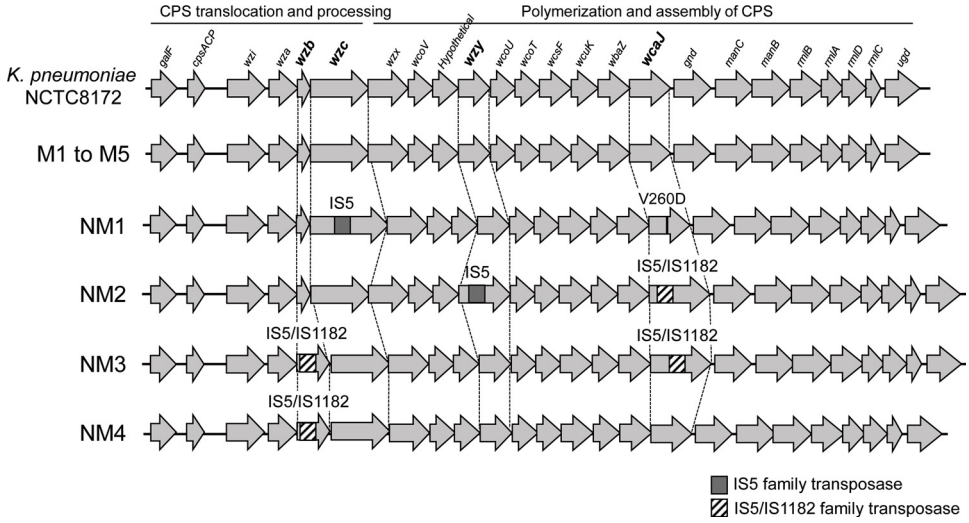

Fig. 2. Comparison of *cps* locus of *K. pneumoniae* isolates investigated in this study. Compared with a reference isolate, NCTC8172, the mucoid isolates (M1–M5) showed no variations in genes of the *cps* locus. However, four non-mucoid isolates (NM1–NM4) showed insertion of IS elements (IS5 or IS5/IS1182 family) or an amino acid alteration (V260D in WcaJ of NM1).

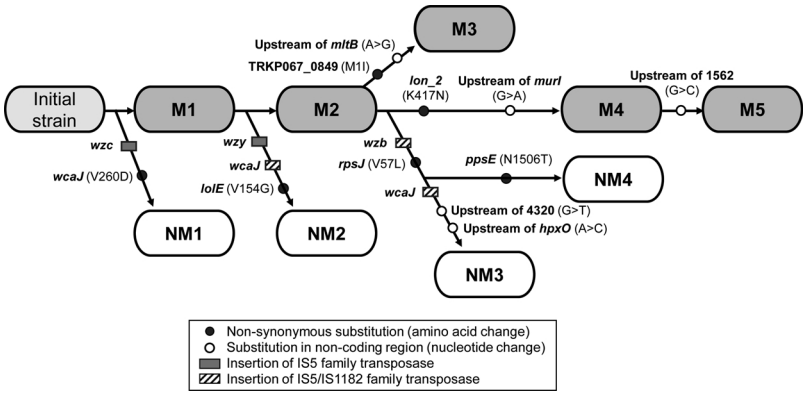

Fig. 3. Inferred evolutionary scenario of mucoid and non-mucoid *K. pneumoniae* isolates mainly based on phylogenetic tree in Supplementary Fig. 1. In this scenario, few amino acid mutations occurred in the lineage of the mucoid isolate. Non-mucoid isolates were formed by insertion of IS elements or amino acid alterations in the *cps* gene locus, and additional amino acid substitutions were identified. However, the non-mucoid isolates may not persist for a long time because of their low fitness relative to mucoid isolates.

genotype (ST14) and serotype (K64), limited genome variations were observed among the isolates. The phenotypic variation such as mucoid vs. non-mucoid may be the result of insertion of an IS or amino acid substitution in genes of the *cps* locus. Although it remains unclear whether insertion of the IS element alters gene expression and further affects function (Siguier et al., 2014), several IS elements were detected within the *cps* locus of *K. pneumoniae* clonal complex 258 which reduced the expression of capsular biosynthesis (Wyres et al., 2015). It was shown that the *wcaJ* mutant isolate synthesized less capsular polysaccharides compared to the wild-type isolate of *K. pneumoniae* NTUH-K2044 (Lin et al., 2009). Additionally, several previous studies reported that mutations in *cps* loci lead to an acapsular phenotype (Rahn et al., 2003; Lin et al., 2009).

Based on the genome data, we constructed evolutionary scenario of mucoid and non-mucoid *K. pneumoniae* isolates (Fig. 3). In the evolutionary scenario, only mucoid isolates persisted and evolved subsequently, but each non-mucoid variant emerged from its mucoid isolate and then disappeared. Phylogenetic tree based on 11 nucleotide substitutions supports the evolutionary scenario (Supplementary Fig. 1). All isolates The mucoid and non-mucoid variants may have originated from a common isolate, as they shared very similar genome sequences only differing in one or two amino acid sequences and IS insertion. Particularly, the mucoid variants appear to have evolved continuously from the previous isolates, except for M3. There were no amino acid alterations between M1 and M2 and between M4 and M5. Only one amino acid substitution, K417 N in Lon_2, was identified between M2 and M4. M3 contained one amino acid substitution in a hypothetical gene. Unlike mucoid isolates, non-mucoid variants may not have evolved subsequently. The second and third non-mucoid variants (NM2 and NM3) may not have originated from the first and second non-mucoid variants (NM1 and NM2), respectively. Although IS5 and IS5/IS1182 were identified commonly between NM1 and NM2 and between NM2 and NM3, respectively, the insertion sites differed. Additionally, it is unlikely that amino acid mutation in WcaJ in NM1 and LolE in NM2 were reversed in NM2 and NM3, respectively. However, the last non-mucoid isolate, NM4, may have originated from NM3 because an amino acid substitution in RpsJ was preserved. Movement or disappearance of IS5/IS1182 in *wcaJ* and occurrence of a single mutation in *ppsE* in NM4 likely occurred.

Thus, non-mucoid variants likely emerged subsequently from their mucoid isolates by insertion of IS5 or IS5/IS1182 or amino acid alterations in genes of the *cps* locus. A single event is at the origin of IS insertion in NM1 and NM4, whereas two independent events affected NM2 and NM3 in *wcaJ*. The ISs might originate from other locations of the genome of *K. pneumoniae* isolate, because the same IS are present in the genome. However, the IS may came from other bacteria populations of the microbiota. Non-mucoid variants may have disappeared or survived only to a very small extent. Non-mucoid isolates show lower fitness compared to mucoid isolates (Lee et al., 2018). This partly supports the dominant-lineage model of within-host evolution, in which lineages with beneficial traits become fixed, eliminating isolates with lower fitness (Lieberman et al., 2016). Bacterial within-host evolution may vary with the host condition, bacterial species, and feature under selection pressure, among other factors. Particularly, the dominant-lineage model is observed if there is a clear difference in fitness, such as vaccine escape and antibiotic resistance (Croucher et al., 2014). Mucoid features related to capsule formation are important for bacterial survival against the host's immune system, and thus within-host bacterial evolution associated with the feature may follow the dominant-lineage model.

In this study, we sequenced the whole genome of nine *K. pneumoniae* isolates isolated successively from a single patient. Four pairs obtained simultaneously from the same site showed different colony phenotypes, mucoid and non-mucoid. The mucoid phenotype may have been lost or converted into the non-mucoid by insertion of IS elements or amino acid alterations in genes of the *cps* locus. However, the type of IS element, insertion site, and amino acid alteration differed among non-mucoid variants. Based on genomic data, we established a within-host evolutionary scenario in which the mucoid isolates may have evolved continuously from previous isolates and non-mucoid variants emerged repeatedly from mucoid isolates, but may be short-lived because of their low fitness.

Conflicts of interest statement

None to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2019.03.003.

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