# Genome-Wide Identification of *Klebsiella pneumoniae* Fitness Genes during Lung Infection

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**ABSTRACT** *Klebsiella pneumoniae* is an urgent public health threat because of resistance to carbapenems, antibiotics of last resort against Gram-negative bacterial infections. Despite the fact that *K. pneumoniae* is a leading cause of pneumonia in hospitalized patients, the bacterial factors required to cause disease are poorly understood. Insertion site sequencing combines transposon mutagenesis with high-throughput sequencing to simultaneously screen thousands of insertion mutants for fitness defects during infection. Using the recently sequenced *K. pneumoniae* strain KPPR1 in a well-established mouse model of pneumonia, insertion site sequencing was performed on a pool of >25,000 transposon mutants. The relative fitness requirement of each gene was ranked based on the ratio of lung to inoculum read counts and concordance between insertions in the same gene. This analysis revealed over 300 mutants with at least a 2-fold fitness defect and 69 with defects ranging from 10- to >2,000-fold. Construction of 6 isogenic mutants for use in competitive infections with the wild type confirmed their requirement for lung fitness. Critical fitness genes included those for the synthesis of branched-chain and aromatic amino acids that are essential in mice and humans, the transcriptional elongation factor RfaH, and the copper efflux pump CopA. The majority of fitness genes were conserved among reference strains representative of diverse pathotypes. These results indicate that regulation of outer membrane components and synthesis of amino acids that are essential to its host are critical for *K. pneumoniae* fitness in the lung.

**IMPORTANCE** *Klebsiella pneumoniae* is a bacterium that commonly causes pneumonia in patients after they are admitted to the hospital. *K. pneumoniae* is becoming resistant to all available antibiotics, and when these infections spread to the bloodstream, over half of patients die. Since currently available antibiotics are failing, we must discover new ways to treat these infections. In this study, we asked what genes the bacterium needs to cause an infection, since the proteins encoded by these genes could be targets for new antibiotics. We identified over 300 genes that *K. pneumoniae* requires to grow in a mouse model of pneumonia. Many of the genes that we identified are found in *K. pneumoniae* isolates from throughout the world, including antibiotic-resistant forms. If new antibiotics could be made against the proteins that these genes encode, they may be broadly effective against *K. pneumoniae*.

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*K*lebsiella pneumoniae is a pathogenic Gram-negative bacterium that is a member of the *Enterobacteriaceae* family. While the bacterium is normally found in the flora of human skin, mouth, and intestinal tract, it is also well characterized as an opportunistic pathogen (1, 2). *K. pneumoniae* infections are associated with hospitalized patients with a weakened immune system. The bacterium causes a wide range of human diseases that include urinary tract infections, pyogenic liver abscess (PLA), and pneumonia (1, 3). Due to the alarming increase in carbapenem-resistant *Enterobacteriaceae* (CRE), of which *K. pneumoniae* comprises the majority of infections, the Centers for Disease Control (CDC) have designated these bacteria as an urgent threat to public health (4).

Because of the decreasing efficacy of antibiotics against *K. pneumoniae*, new treatment modalities must be developed, and a promising approach is through identifying bacterial factors required to cause disease. The few virulence factors identified to date include capsule and lipopolysaccharide (LPS), which vary by structure and serotype, and adhesins and siderophores, which vary in frequency among clinical isolates (1, 5–7). To develop novel therapies that will be broadly effective against *K. pneumoniae*, conserved targets must be identified.

Barriers to identifying new *K. pneumoniae* virulence factors include limitations with genetic sequencing techniques and the difficulties in manipulating the *K. pneumoniae* chromosome. In recent years, newer approaches such as transposon insertion site sequencing (InSeq) have allowed for greatly enhanced genetic screening. This method uses high-throughput sequencing to determine the frequency and chromosomal location of thousands of

transposon mutations from a large pool of mutants in a single experiment (8, 9).

InSeq requires a fully sequenced genome and confirmation of mutant phenotypes by either simultaneously constructing an indexed transposon mutant library or constructing mutants in selected genes that elicit fitness defects. Because of advances in genetic sequencing, a number of high-quality reference sequences of *K. pneumoniae* are now available (10–13). However, many of the standard methods for gene replacement in *Escherichia coli*, such as suicide vector conjugation and linear DNA homologous recombination, are less efficient in *K. pneumoniae* or are complicated by intrinsic and acquired antibiotic resistance (14). The Lambda Red recombinase system, which is incredibly efficient in *E. coli* (15), could greatly increase the efficiency of gene recombination in *K. pneumoniae* (16). These advances now provide the ability to rigorously screen the genome for novel genes that contribute to virulence or *in vivo* fitness. Using this new sequencing approach and the Lambda Red recombinase system, we have identified numerous novel *K. pneumoniae* fitness genes required to infect the lung in a mouse model of pneumonia.

## RESULTS

**Transposon library construction, inoculation, and validation.**
*K. pneumoniae* KPPR1 causes acute pneumonia complicated by bacteremia in a well-established murine model (5, 6). To facilitate InSeq analysis, the KPPR1 genome was sequenced to generate a single, gapless contig and the genes contained in it were annotated (10). The genome contains 5,191 predicted genes, including 25 rRNA, 85 tRNA, and 5,081 protein-coding sequences. To identify fitness factors required during lung infection, a library of ~25,000 transposon mutants was constructed to provide 99% genome coverage. Colony PCR and Southern blotting of representative transconjugants indicated that the library contained single, random transposon insertions at varied locations in the chromosome without integration of the vector (see Fig. S1 in the supplemental material). This library was inoculated into mice using an aspiration model of pneumonia at a dose of $1.4 \times 10^6$ CFU (5, 10, 17). After 24 h, the mean bacterial density increased to $5 \times 10^6$ CFU in the lung (Fig. 1A). To quantify and map each transposon onto the chromosome, genomic DNA was extracted from the inoculum and lung homogenates, and transposon junction fragments were generated. These fragments were sequenced using an Illumina HiSeq2500 instrument, mapped to the chromosome, and enumerated based on read count (18). Each sample had at least 68 million reads corresponding to 25,346 unique transposon insertions within genes (see Table S1), meeting the coverage goal as stated above. Of 5,191 predicted genes, 4,312 had at least one insertion detected, whereas 879 genes did not. Because the transconjugants were required to grow to a colony for inclusion in the library, genes without insertions may be essential. However, a larger mutagenesis library would be required to estimate more precisely the number of essential genes (8).

**InSeq analysis identifies hundreds of potential *K. pneumoniae* lung fitness factors.** To identify *in vivo* fitness genes, the numbers of transposon insertion reads within each gene were compared between the lung and inoculum pools. Of genes with insertions, 3,880 had at least one shared transposon between the inoculum and lung samples (see Data Set S1 in the supplemental material) and were used for subsequent analysis. Since input and output data are "paired" in nature for each insertion, a P value was
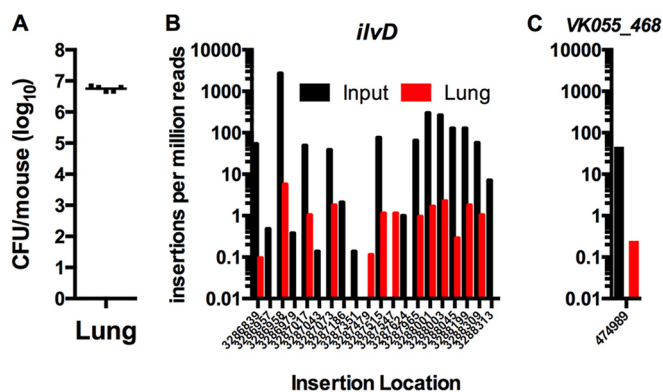


**FIG 1** InSeq analysis indicates that fitness genes are important during pneumonia based on differential transposon read counts between the inoculum and mouse lung. Mice ($n = 5$) inoculated with $1.4 \times 10^6$ CFU of a pool of ~25,000 transposon mutants for 24 h were sacrificed and cultured on LB agar for CFU (A) or for DNA extraction, Illumina sequencing of transposon junctions, and read alignment with the KPPR1 genome to map and count transposon insertions. The locations and frequencies of transposon insertions in *ilvD* (B) and *VK055_468* (C) are shown.

calculated for each insertion using an exact Poisson test and then combined into a single P value for each gene using Fisher's method. This method is similar to an approach called CEDER, which uses information from each exon of a eukaryotic gene to determine if there is consistent differential expression in RNA transcriptome sequencing (RNA-Seq) data for a given gene (19). Therefore, we call this P value a CEDER P value. Genes were sorted based on their fitness factor (total insertion counts in the inoculum/total insertion counts in the lung) and their CEDER P value (see Data Set S3). Inspection of transposon numbers and counts in individual genes suggested that the CEDER P value could be used as a selection criterion for true-positive results of identification of fitness genes by InSeq. For example, *ilvD*, required for branched-chain amino acid (BCAA) synthesis, had a P value of 0 based on 18 insertions in the input and 13 in the lung with 11 shared insertions having an average fitness factor of 192 (Fig. 1B). In contrast, *VK055_468*, a putative transcriptional regulatory gene, had a CEDER P value of 2e−14 based on a 186-fold fitness factor but only one insertion (Fig. 1C).

To confirm fitness defects and test the predictive power of CEDER, seven potential fitness genes identified by InSeq analysis were selected for experimental validation by coinfection. Six genes (*rfaH*, *ilvC*, *ilvD*, *copA*, *aroE*, and *VK055_4417*) with a CEDER P value of 0 and a fitness factor of >10 were tested compared to *VK055_468* ($P = 2e-14$; fitness factor of 168). These genes were mutated using Lambda Red recombinase to replace the entire coding sequence with a kanamycin resistance gene (15). Mice were then infected as described in Materials and Methods, but with a 1:1 ratio of the wild type (WT) and each mutant. Based on the bacterial density of each strain after 24 h, the competitive index was calculated. All six mutants with a CEDER P value of 0 had a fitness defect in the lung compared to the wild type (Fig. 2), ranging from 1.4-fold (*VK055_4417*) to 11,694-fold (*rfaH*). In contrast, *VK055_468* did not have a fitness defect compared to the wild type (Fig. 2). Therefore, genes with a CEDER P value of >0 were not characterized further. In total, 1272 genes had a P value of 0 (top 25 shown in Table 1), including 69 genes with a fitness factor of >10 and 333 genes with a fitness factor of >2. There were
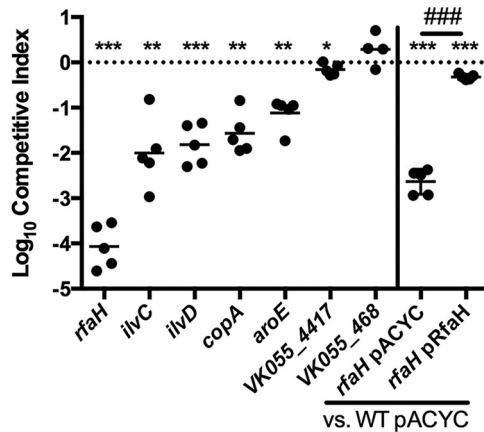
**FIG 2** Competitive infection against the wild-type strain confirms the fitness defect of mutants selected by InSeq. Insertion-deletion mutants of six genes selected and one gene not selected by CEDER analysis (*VK055_468*) of InSeq fitness were constructed using Lambda Red recombinase and used to coinfect mouse lungs with the wild type (WT), or WT carrying pACYC184 where noted, at a 1:1 ratio in a total inoculum of $1 \times 10^6$ CFU. Mean competitive index ($\log_{10}$) for each mutant compared to the wild type at 24 h is shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, by one-sample *t* test on $\log_{10}$-transformed data ($\geq 4$ mice per group); ###, $P < 0.001$ by two-sample *t* test.

also 452 genes with a fitness factor of <0.5 and 97 genes with a fitness factor of <0.1, suggesting that insertion mutants increase lung fitness. Since the goal of the study was to identify mutants with decreased lung fitness, mutants overrepresented in the lung were not pursued further.

**rfaH is required for serum resistance.** RfaH is an antiterminator that specifically promotes the transcription of long virulence operons, including capsule and lipopolysaccharide (LPS)

synthesis genes, in *E. coli* and *Salmonella enterica* serotype Typhimurium (20–22). In KPPR1, an *rfaH* mutant is severely attenuated in the lung (>10,000-fold compared to wild type), and the fitness of the mutant is significantly restored by complementation with the *rfaH* gene ligated into pACYC184 (Fig. 2). This mutant forms a small colony compared to the wild type, suggesting a defect in capsule biosynthesis. Indeed, India ink staining of bacteria delineated copious capsule in KPPR1 but no visible capsule in the *rfaH* mutant (Fig. 3A and B). To assess mucoviscosity, a phenotype associated with hypervirulent capsular serotypes in *K. pneumoniae* clinical isolates, low-speed centrifugation was performed and the optical density of the supernatant was measured (23, 24). Whereas the wild-type suspension remained turbid, the *rfaH* mutant pelleted readily, and complementation restored mucoviscosity significantly (Fig. 3D). Both capsule and O antigen of LPS can contribute to resistance to complement-mediated killing (25), which has been correlated with the ability to cause patient infections (1). Incubation of the *rfaH* mutant in serum demonstrated a >1,000-fold loss in viability, greater than that for the susceptible control strain KP297, whereas there was no loss of viability in the wild type (Fig. 4). Complementation restored serum resistance. In RPMI medium with 10% heat-inactivated serum, complement is disabled and *K. pneumoniae* grows robustly but requires the siderophore enterobactin or its glycosylated derivative salmochelin to acquire iron; yersiniabactin does not support growth under this condition (5, 26). Whereas the wild type replicates robustly, the *rfaH* mutant had a moderate growth defect comparable to the loss of salmochelin expression (*iroA ybtS* mutant) but not as severe as the siderophore null *entB ybtS* mutant (Fig. 5). In contrast, the *rfaH* mutant grew well in RPMI medium without added serum (see Fig. S2A in the supplemental material). Together, these data indicate that *rfaH* is required for wild-type

**TABLE 1** Top 25 *K. pneumoniae* KPPR1 lung fitness genes[a]

| Locus ID (*VK055_x*) | Gene | Product | Ratio (input/lung) |
|---|---|---|---|
| 5014 | | Capsule assembly Wzi family protein | 2820.95 |
| **3141** | ***rfaH*** | **Transcriptional activator** | **2696.77** |
| 5096 | | Hypothetical protein | 2486.08 |
| **3202** | ***ilvC*** | **Ketol-acid reductoisomerase** | **2248.61** |
| 3832 | *argR* | Arginine repressor | 1140.74 |
| 5025 | | Undecaprenyl-phosphate glucose phosphotransferase | 840.55 |
| 5012 | *galF* | Regulatory protein | 704.99 |
| 3206 | *ilvE* | Branched-chain amino acid aminotransferase | 581.10 |
| 3515 | | Rhodanese-like domain protein | 508.09 |
| **4417** | | **MarR family protein** | **442.14** |
| 4811 | *purF* | Amidophosphoribosyltransferase | 378.16 |
| 4619 | *purL* | Phosphoribosylformylglycinamidine synthase | 312.03 |
| 1194 | *trpD* | Anthranilate synthase component II | 301.31 |
| 4135 | *serA* | D-3-Phosphoglycerate dehydrogenase; provisional | 248.61 |
| 2495 | *leuC* | 3-Isopropylmalate isomerase subunit, dehydratase component | 246.77 |
| 3142 | *tatC* | Twin arginine-targeting protein translocase | 219.96 |
| **3205** | ***ilvD*** | **Dihydroxy acid dehydratase** | **203.61** |
| 5023 | | Hypothetical protein | 172.11 |
| 4883 | *rcsB* | Transcriptional regulator | 126.39 |
| 2215 | *phoR* | Phosphate regulon sensor kinase | 110.92 |
| 4579 | *pheA* | Bifunctional chorismate mutase/prephenate dehydratase | 94.55 |
| 3368 | | 2-Oxo-3-deoxygalactonate 6-phosphate aldolase | 90.01 |
| **2084** | ***copA*** | **Copper-translocating P-type ATPase** | **69.70** |
| 3086 | *purH* | Phosphoribosylaminoimidazole carboxamide formyltransferase | 66.84 |
| **3791** | ***aroE*** | **Dehydroshikimate reductase** | **61.93** |

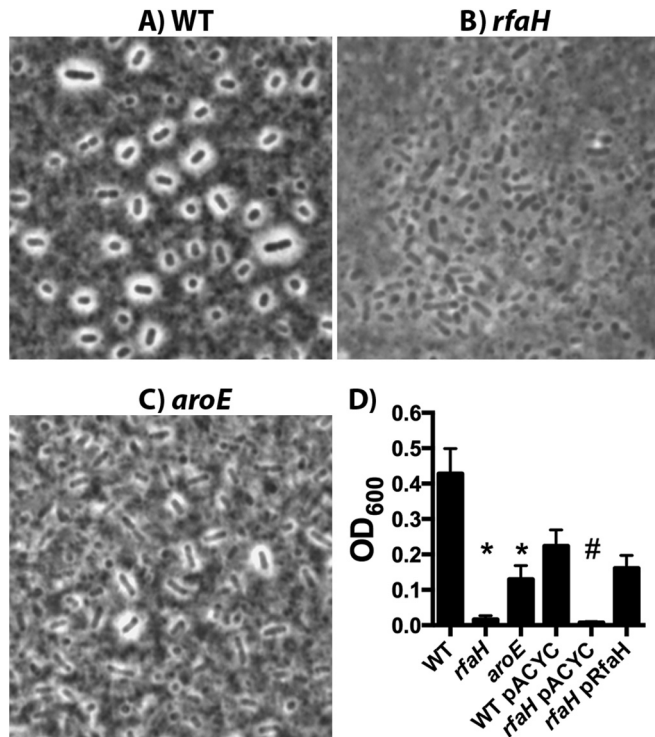[a] Experimentally validated genes are shown in boldface.

**FIG 3** The *rfaH* mutant is deficient in extracellular capsule. (A to C) India ink staining of *K. pneumoniae* KPPR1 (WT) (A) and isogenic *rfaH* (B) and *aroE* (C) mutants after overnight culture in LB broth is shown by phase-contrast microscopy, in which the presence of capsule is indicated by an area of negative staining around the bacteria. Magnification, ×1,000. (D) Mucoviscosity, as measured by optical density (OD$_{600}$) after centrifugation for 5 min at 1,000 × *g* with a starting turbidity of 1.0, is shown as the mean and standard deviation from 6 biological replicates. *, $P < 0.0001$ by ANOVA with Fisher's LSD test compared to the wild type; #, $P < 0.0001$ compared to both WT carrying pACYC and the *rfaH* mutant carrying pRfaH.



**FIG 4** The *K. pneumoniae* rfaH and *aroE* mutants are susceptible to serum killing. Viability of *K. pneumoniae* KPPR1 (WT) and isogenic mutants, without (A) or containing pACYC184 with or without *rfaH* (B), after a 3-h incubation in human serum is shown as mean log$_{10}$ CFU/ml ± standard deviation from at least two replicates per group. *, $P < 0.0001$ by ANOVA with Fisher's LSD test compared to the wild type.

despite production of capsule as measured by India ink (Fig. 3C). These data indicate that *aroE* is dispensable for bacterial growth *in vitro* but is required to evade complement-mediated serum killing.

**copA is required to prevent copper toxicity.** Mutation of the *copA* gene, encoding a P-type ATPase copper efflux pump (29), causes a 37-fold fitness defect in competitive infection with the wild type (Fig. 2). To determine whether deletion of *copA* increases susceptibility to copper toxicity, as seen in *E. coli*, the wild type and *copA* mutant were incubated in M9 minimal medium with increasing amounts of copper (5 to 25 μM). We found that CFU are significantly lower in *copA* mutants on average ($P = 0.02$) and the rate of decreasing CFU with increasing Cu concentration is significantly higher in a *copA* mutant ($P = 0.0003$) than in the wild type (WT) (Fig. 7A). Complementation with a plasmid-expressed copy of *copA* restored copper resistance to the mutant (Fig. 7B). In *E. coli*, copper toxicity occurs through inactivation of

capsule production, to resist complement-mediated serum killing, and for maximal growth in iron-limited serum.

***K. pneumoniae* requires branched-chain amino acid synthesis for full virulence.** The *ilv* locus encodes enzymes for synthesis of the branched-chain amino acids isoleucine and valine and the precursors for leucine (27). *ilvC* and *ilvD* mutants had 100- and 66-fold defects in competition with the wild type (Fig. 2), respectively, indicating that branched-chain amino acid synthesis is required for fitness in the lung. To confirm that the *ilvC* and *ilvD* mutants are auxotrophs for branched-chain amino acids, their growth in minimal medium was compared to that of the wild type (Fig. 6). Neither mutant grew significantly, but growth of both was restored by supplementation of the medium with the branched-chain amino acids isoleucine and valine at 10 mM concentrations. These data indicate that synthesis of branched-chain amino acids is required for lung fitness.

***aroE* is required for serum resistance.** Disruption of the *aroE* gene, which encodes shikimate dehydrogenase required for aromatic amino acid synthesis, causes a 13-fold decrease in fitness in competitive infection (Fig. 2). Unlike for *ilvC* and *ilvD* mutants, the *aroE* mutant is not defective for growth in minimal medium (see Fig. S2B in the supplemental material), perhaps due to compensation by the *ydiB*-encoded quinate/shikimate dehydrogenase (10, 28). However, the *aroE* mutant does have a moderate defect in serum survival (Fig. 4) and a defect in mucoviscosity (Fig. 3D),
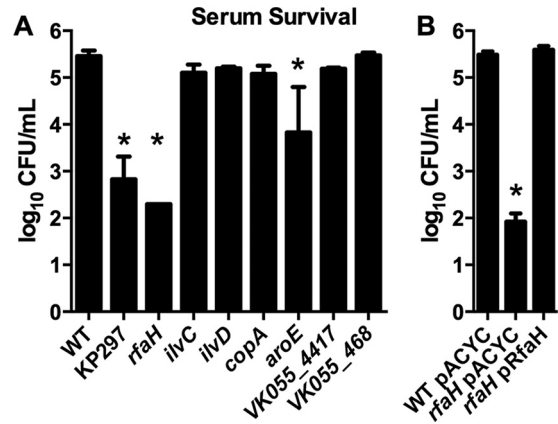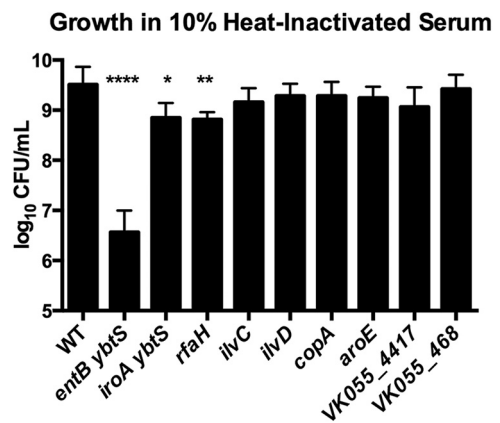


**FIG 5** The *K. pneumoniae* rfaH mutant has a mild growth defect in heat-inactivated serum. *K. pneumoniae* KPPR1 (WT) and isogenic mutant growth (log$_{10}$ CFU/ml) after overnight incubation of a 1 × 10$^3$-CFU/ml inoculum in RPMI medium supplemented with 10% heat-inactivated serum is shown as the mean ± standard deviation from 4 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$, by ANOVA with Fisher's LSD test.
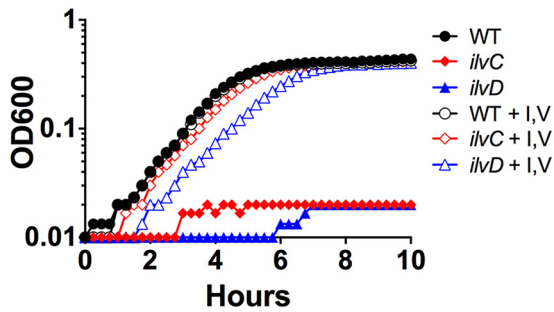
FIG 6  *K. pneumoniae ilvC* and *ilvD* mutants are unable to replicate in minimal medium without branched-chain amino acids. Optical density (OD$_{600}$) of *K. pneumoniae* KPPR1 (WT) and *ilvC* and *ilvD* mutants in M9 minimal medium with or without 10 mM isoleucine (I) and valine (V) is shown as the mean from triplicate samples representative of 3 independent experiments.

dehydratases such as IlvD in the branched-chain synthesis pathway (30). The *ilvD* mutant was more resistant to copper than the *copA* mutant, suggesting additional cellular targets of copper toxicity (Fig. 7C). However, the addition of branched-chain amino acids significantly protected the *copA* mutant (Fig. 7C). These data indicate that copper resistance and branched-chain amino acid metabolism pathways may overlap in their contribution to *K. pneumoniae* lung fitness.

**InSeq identifies conserved genes as critical fitness factors for *K. pneumoniae* lung infection.** To define broad pathways that may contribute to *K. pneumoniae* fitness in the lung, gene enrichment analysis was performed. The KEGG classification of the 333 genes in which mutants were >2-fold underrepresented in the lung was determined, and the representation of each class was compared to the total gene set in the KPPR1 genome as annotated based on KEGG assignment. The pathways for 2-oxocarboxylic acid metabolism; valine, leucine, and isoleucine biosynthesis; and biosynthesis of amino acids were significantly overrepresented among genes identified as fitness factors by InSeq (corrected *P* values of <0.01), indicating the critical importance of branched-chain amino acid synthesis during lung infection.

The products of conserved fitness genes could serve as targets for novel therapeutics against *K. pneumoniae*. To identify conserved genes in other clinically significant isolates, the KPPR1 genome was compared to the following reference sequences: MGH78578, an isolate from a patient with pneumonia; NTUH-K2044, an isolate that caused a PLA; NJST258_1, a carbapenem-resistant (KPC) ST258 isolate; and Kp342, a plant endophyte. All combinations of shared, full-length chromosomal genes were analyzed, and overlapping gene sets were tabulated (Fig. 8A; see also Data Set S3 in the supplemental material). KPPR1 shares a conserved set of 3,865 genes (quadrant X; 76% of predicted protein-encoding genes) with all strains; 121 genes with human isolates MGH78578, NTUH-K2044, and NJST258_1 but not the plant endophyte Kp342 (quadrant XIV); and 20, 89, 40, and 27 genes exclusively with MGH78578 (quadrant II), NTUH-K2044 (quadrant XV), NJST258_1 (quadrant IV), and Kp342 (quadrant VIII), respectively. To assess the conservation of the top fitness genes identified by InSeq analysis, the 69 genes with a fitness factor of >10 were mapped to the regions of overlap between KPPR1 and 4 reference genomes. In fact, 60 of 68 mapped genes were conserved between all *K. pneumoniae* isolates (Fig. 8B), and all 6 fitness genes verified by targeted mutation are conserved. These data indicate
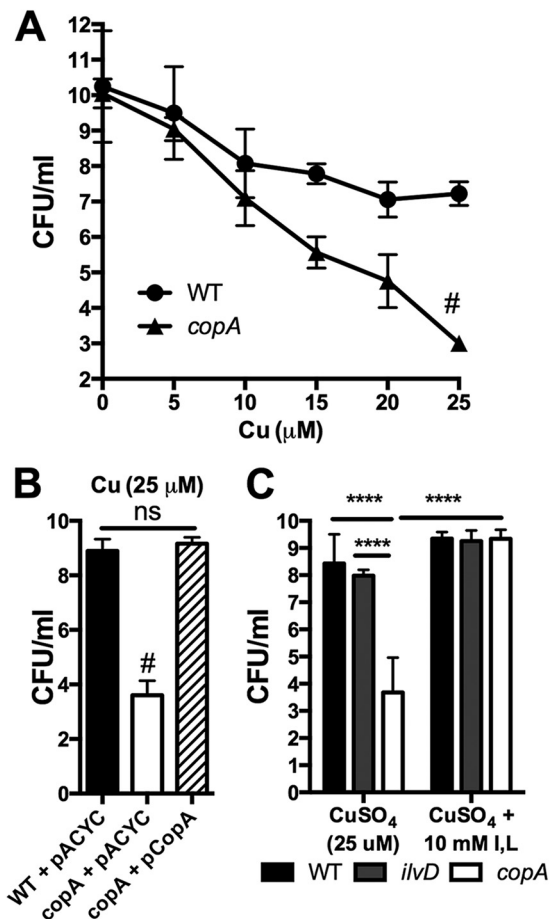


FIG 7  The *K. pneumoniae copA* mutant has increased sensitivity to copper. (A) Viability of a $1 \times 10^8$-CFU inoculum of *K. pneumoniae* WT and *copA* mutant after an 18-h incubation in M9 broth with concentrations of cupric sulfate indicated is shown as mean ± standard deviation of results from at least 3 independent experiments. Based on a linear regression model (modeling log-transformed CFU as a function of the Cu concentration, group [WT or *copA*], and interaction between concentration and group), the *copA* mutant has a significantly faster-decreasing rate over concentrations than the wild type (#). (B) Viability of *K. pneumoniae* harboring pACYC184 (pACYC) or pACYC184 with *copA* (pCopA) after incubation in 25 μM CuSO$_4$ is shown as the mean ± standard deviation from three independent experiments. #, $P <$ 0.0001 compared to both strains by ANOVA with Fisher's LSD test; ns, not significant. (C) Viability of *K. pneumoniae* mutants as indicated in 25 μM CuSO$_4$ with or without 10 mM isoleucine and valine is shown as the mean ± standard deviation from at least three independent experiments. ****, $P <$ 0.0001 by ANOVA with Fisher's LSD.

that InSeq analysis detects lung fitness genes conserved in reference strains causing nosocomial pneumonia, pyogenic liver abscess, and carbapenem-resistant infections.

## DISCUSSION

By performing InSeq analysis of *K. pneumoniae* KPPR1, leveraging high-quality genome sequence and a well-established animal model, this study identified over 300 fitness genes important in lung infection. Detection of known virulence genes such as the *wzi* capsule gene validated this approach. Adaptation of CEDER analysis for ranking of fitness genes, based on the concordance of read count ratios from multiple transposon insertions within genes, provided a reliable list of fitness genes that were experimentally
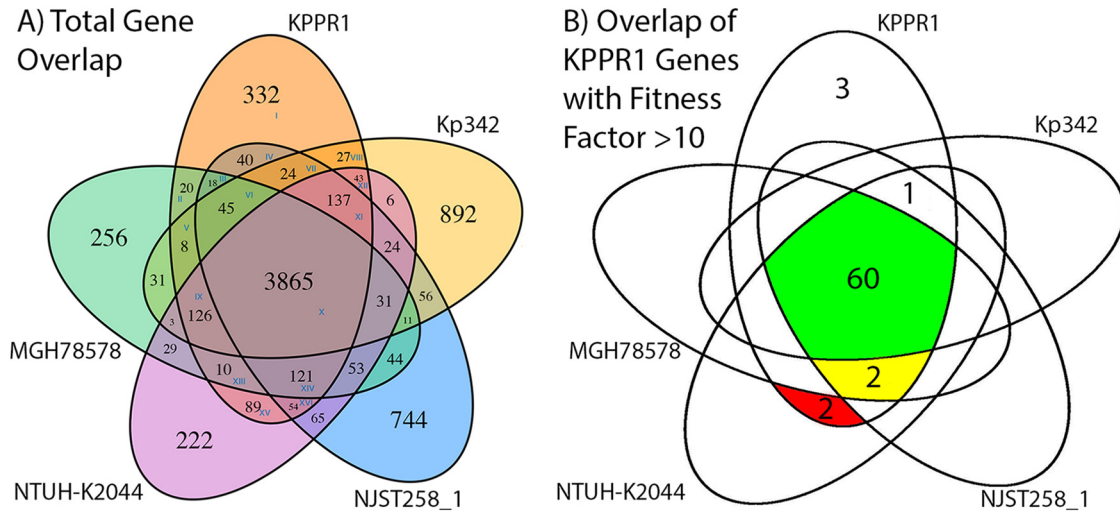
**FIG 8** *K. pneumoniae* conserved genes are fitness factors during pneumonia. (A) The number of genes shared between KPPR1 (orange), pneumonia isolate MGH78578 (green), pyogenic liver abscess isolate NTUH-K2044 (pink), carbapenem-resistant isolate NJST258_1 (blue), and plant endophyte Kp342 (yellow) is shown with the number of genes in each quadrant (labeled by Roman numerals) indicated as calculated by CloVR comparative software. (B) Based on InSeq analysis, 60 of 69 genes with a fitness factor of >10 were conserved in all five isolates (green), 2 were present in human isolates but not Kp342 (yellow), and 2 were shared with hypervirulent NTUH-K2044 (red).

validated. These validation experiments demonstrated that aromatic and branched-chain amino acid synthesis pathways, copper efflux, and global virulence gene regulators such as RfaH are critical for *K. pneumoniae* to cause pneumonia. The identification of genes such as *rfaH*, with a profound contribution to lung fitness, indicates the benefit of applying InSeq even when prior forward genetic screens have been performed (6, 7). Enrichment analysis further substantiated amino acid synthesis as central to lung fitness, and comparative genomics indicated that many critical fitness genes are highly conserved in *K. pneumoniae*. The large set of fitness genes in KPPR1 newly identified by InSeq should accelerate progress in defining the virulence strategies of *K. pneumoniae*.

Enrichment analysis and experimental validation of InSeq-selected genes indicate that *K. pneumoniae* must synthesize amino acids that the host cannot. Since essential amino acids are acquired through diet, it does not necessarily follow that these amino acids would be limiting in the lung. However, based on measurements in human subjects, the concentrations of these amino acids are significantly lower in pulmonary epithelial lining fluid than in plasma (31). InSeq identified mutants with decreased fitness in pathways that synthesize the host's essential amino acids histidine (*hisG*); isoleucine, valine, and leucine (*ilvABCDEN* and *leuABC*); methionine/cysteine (*metACBFL*); phenylalanine and tryptophan (*aroE*, *pheA*, and *trpD*); and threonine (*thrBC*) (see Data Set S2 in the supplemental material) (32). This indicates that synthesis of amino acids that are essential to its host is also critical for *K. pneumoniae* to infect the lung.

Branched-chain amino acid metabolism may be critical for *K. pneumoniae* lung fitness for two reasons: nutrient limitation and copper toxicity. In contrast to reduced branched-chain amino acid levels, copper is increased in human lungs compared to serum (33). Our data show that *K. pneumoniae* must synthesize branched-chain amino acids in the lung, but in *E. coli*, this pathway is blocked by copper through its inhibition of dehydratases such as IlvD (30). In *K. pneumoniae*, the *copA* mutant is exquisitely sensitive to copper but is protected by exogenous isoleucine and

valine (Fig. 7C), consistent with this mechanism of copper toxicity. Sulfonylurea herbicides that inhibit acetohydroxy acid synthase in the branched-chain amino acid synthesis pathway can protect mice from *Pseudomonas aeruginosa* and *Burkholderia pseudomallei* pneumonia, but *K. pneumoniae* is predicted to contain a resistant isozyme (34). The nine *K. pneumoniae* synthesis genes identified by InSeq (*ilvABCDEN* and *leuABC*) could provide novel targets to block branched-chain amino acid (BCAA) synthesis.

In contrast to *ilv* mutants, the *aroE* mutant is not auxotrophic in minimal medium but has reduced fitness in the lung. In *E. coli*, the YdiB quinate/shikimate dehydrogenase can perform the same function as AroE, albeit with lower efficiency (28). The fact that the *aroE* mutant is susceptible to serum killing suggests a specific defect that affects complement resistance. *Salmonella enterica aro* mutants are also attenuated *in vivo* and are susceptible to serum killing in addition to their auxotrophy (35). The mechanism for the increased serum sensitivity of the *K. pneumoniae* aroE mutant is unclear.

In addition to synthesizing building blocks for replication despite nutrient limitations, *K. pneumoniae* must also evade host defenses to cause disease. Polysaccharide capsule is a critical virulence factor of *K. pneumoniae* that evades complement-mediated killing and opsonophagocytosis. Detection of the capsular assembly gene *wzi* as the most significant fitness gene by InSeq analysis reinforces the critical role of capsule. Phenotypic assays indicate that *rfaH* is important in capsular synthesis and is required for survival *in vivo* and in serum. *S.* Typhimurium and uropathogenic *E. coli rfaH* mutants are also severely attenuated in animal models, corresponding to defects in *E. coli* capsular synthesis and LPS synthesis in both (20, 36). RfaH appears to be a critical virulence factor that is conserved among *K. pneumoniae* strains and with other pathogenic *Enterobacteriaceae*.

Treatment of *K. pneumoniae* infections has become increasingly challenging due to carbapenem and extended-spectrum β-lactamase resistance (4). Therefore, novel therapeutic ap-

proaches are needed to fight these common and life-threatening nosocomial infections. Targets for antimicrobials should be conserved within a species or a group of closely related species at a minimum, have a low potential for toxicity or disruption of human metabolism, and ideally not have a preexisting resistance mechanism. This study identifies 60 genes that are conserved among diverse representatives of *K. pneumoniae* and have a 10- to 10,000-fold defect when disrupted, and many are in prokaryote-specific functions such as synthesis of amino acids that humans do not make or transcription of polycistronic RNAs (*rfaH*). Through careful study of their function during infection, some of these gene products could be pursued as targets for novel antimicrobials.

## MATERIALS AND METHODS

**Bacterial strains and media.** *K. pneumoniae* KPPR1 (10) and isogenic mutants were cultured in Luria-Bertani (LB) broth at 37°C with shaking or 30°C on agar (Becton, Dickinson and Company, Sparks, MD) supplemented with kanamycin (25 $\mu$g/ml), spectinomycin (50 $\mu$g/ml), chloramphenicol (20 $\mu$g/ml), and rifampin (30 $\mu$g/ml) as indicated.

**Murine pneumonia model.** Six- to 8-week-old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with isoflurane and inoculated retropharyngeally with $1 \times 10^6$ CFU of pooled *K. pneumoniae* transposon mutants. LB broth-grown cultures were centrifuged, resuspended, and diluted in phosphate-buffered saline (PBS) to a concentration of $2 \times 10^7$ CFU, and 50 $\mu$l of the suspension was administered. After 24 h, mice were euthanized by $CO_2$ asphyxiation. To determine bacterial density, lungs and spleens were removed, homogenized in 1 ml PBS, and cultured on LB agar with appropriate antibiotics. To determine competitive indices, mice (at least 4 per group) were infected as described above with 50 $\mu$l of a 1:1 mixture of wild-type and mutant *K. pneumoniae* strains in a total inoculum of $1 \times 10^6$ CFU. After 24 h, mice were euthanized, lungs were homogenized in 1 ml PBS, wild-type CFU were measured by culture on LB plates supplemented with rifampin, and mutant CFU were measured on LB plates with rifampin and kanamycin. The competitive index was calculated as (mutant lung CFU/wild-type lung CFU)/(mutant inoculum CFU/wild-type inoculum CFU).

**Transposon library construction and sequencing.** The transposon library was constructed by conjugation of the pSAM_Cam plasmid, a modified version of pSAM_Ec (37) containing the chloramphenicol acetyltransferase gene from pKD3 (15), into KPPR1 (see Text S1 in the supplemental material for detailed methods). Insertion sequencing (InSeq; Sequence Read Archive ID SRP051394) was performed as previously described (18).

**Construction and complementation of mutants.** Lambda Red mutagenesis was performed as previously described (15). To make electrocompetent cells, *K. pneumoniae* containing the pKD46 plasmid (15), modified to encode spectinomycin resistance (a gift from Chris Alteri), was cultured overnight in LB broth containing spectinomycin at 30°C with shaking. The following day, cultures were diluted 1:100 in LB broth with spectinomycin and 50 mM L-arabinose and cultured at 30°C until reaching a reading of optical density at 600 nm ($OD_{600}$) of 0.5 to 0.6 (approximately 4 h). Cultures were placed on ice for at least 30 min and centrifuged in sterile cold bottles at $8,000 \times g$ for 15 min at 4°C. The supernatant was decanted, and bacteria were serially washed and centrifuged in ice-cold sterile volumes of 50 ml 1 mM HEPES at pH 7.4 (Invitrogen, Carlsbad, CA), 50 ml distilled water ($dH_2O$), and 20 ml 10% glycerol. Pellets were resuspended in ice-cold sterile 10% glycerol at a final density of $2 \times 10^{10}$ to $3 \times 10^{10}$ CFU/ml and stored at $-80°C$ in 50-$\mu$l aliquots.

To generate null mutants, *E. coli* BW25141 containing the pKD4 plasmid was cultured overnight in LB broth containing 50 $\mu$g/ml kanamycin (MP Biomedicals, Santa Ana, CA) at 30°C. The pKD4 plasmid was isolated using a Spin Miniprep kit (Qiagen, Valencia, CA). Oligonucleotide primers were then designed with 60-bp homology flanking the region

targeted for deletion added to 5′ ends of P1 and P2 sites of the pKD4 kanamycin resistance cassette (see Table S2 in the supplemental material). The targeting fragment was generated by PCR consisting of 95°C for 5 min; 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and 72°C for 5 min. PCR products were pooled and purified using a Qiagen PCR purification kit. PCR products were digested overnight at 37°C with DpnI, and 10 $\mu$l was added to the electrocompetent pKD46 cells, gently mixed, and incubated on ice for 10 min. The mixture was electroporated using a 0.1-cm-gap cuvette (Fisher Scientific; catalog no. FB101) at 1.8 kV, 400 $\Omega$, 25 $\mu$F, with a Bio-Rad Micropulser (Bio-Rad, Hercules, CA), and cells were recovered with SOC medium and incubated overnight at 30°C with shaking in sterile culture tubes. Next, cells were spun down, resuspended in LB broth, plated onto LB broth plates containing kanamycin, and incubated at 37°C overnight. Transformants were restreaked on LB agar and confirmed by colony PCR using flanking primers (see Table S2).

To complement the *rfaH* and *copA* mutants, PCR products containing the open reading frame and upstream sequence (see Table S2 in the supplemental material) were inserted into pCR 2.1 by TOPO TA cloning (Life Technologies, Carlsbad, CA) and transferred to pACYC184 by ligation after digestion with XbaI and HindIII. The resulting complementation plasmid or pACYC184 alone was introduced into WT or mutant *K. pneumoniae* by electroporation.

**Serum growth assay.** RPMI medium (Invitrogen, Grand Island, NY), supplemented with 10% (vol/vol) heat-inactivated pooled human serum, was inoculated with $1 \times 10^3$ CFU/ml of an overnight culture of *K. pneumoniae* and incubated overnight in a final volume of 100 $\mu$l in 96-well plates at 37°C with 5% $CO_2$. To determine bacterial density, samples were serially diluted and plated on LB agar (Thermo Fisher Scientific, Waltham, MA) with appropriate antibiotics.

**Growth curves.** *K. pneumoniae* strains were cultured overnight in LB broth. On the following day, cultures were incubated in LB or M9 (Invitrogen) medium with or without isoleucine and valine supplementation (Sigma-Aldrich, St. Louis, MO) at a starting density of $2.6 \times 10^7$ CFU/ml and cultured for 8 h at 37°C. Absorbance readings at 600 nm were taken every 15 min using an Eon microplate spectrophotometer with Gen5 software (BioTek, Winooski, VT).

**Capsule staining.** *K. pneumoniae* strains were cultured overnight in LB broth. On the following day, 10 $\mu$l of culture was added to slides and allowed to air dry. The dried bacteria were then heat fixed to the slide (3 min at 56°C); 10 $\mu$l of India ink (Becton, Dickinson and Company, Sparks, MD) diluted 1:3 in PBS was added and wet mounted with a coverslip. Superfrost slides (Thermo Fisher Scientific, Waltham, MA) were viewed and imaged using an Axioplan2 phase-contrast microscope (Zeiss, Irvine, CA) with a SPOT diagnostic camera and software (SPOT Imagine Solutions, Sterling Heights, MI).

**Mucoviscosity assay.** Overnight cultures were pelleted by centrifugation at $9,400 \times g$ and resuspended in PBS to an $OD_{600}$ of ~1. The suspensions were centrifuged for 5 min at $1,000 \times g$, and the $OD_{600}$ of the supernatants was measured. Final readings were normalized to the $OD_{600}$ of the wild type before centrifugation.

**Serum killing assay.** *K. pneumoniae* strains were cultured overnight in LB broth. On the second day, cultures were diluted to $2.5 \times 10^5$ CFU/ml in non-heat-inactivated human serum and incubated for 3 h at 37°C. Aliquots at $t = 0$ and $t = 3$ h were plated for CFU on LB agar with appropriate antibiotics.

**Copper sensitivity assay.** Sensitivity to copper was measured by washing 3 ml of stationary-phase cultures twice with PBS and then centrifuging them at $10,000 \times g$ and resuspending them in M9 minimal medium. Next, 2-ml reaction mixtures were prepared with defined concentrations of copper sulfate (98% pure; Sigma, St. Louis, MO) in M9 medium with bacterial cultures normalized to an $OD_{600}$ of 0.2. Reaction mixtures were incubated for 18 h at 37°C in six-well plates. Following incubation, CFU were enumerated by quantitative culture.

**Genome comparison and gene enrichment analysis.** To identify genes shared between KPPR1 (GenBank identifier [ID] CP009208) and

reference *K. pneumoniae* isolates MGH78578 (GenBank ID CP000647), NTUH-K2044 (GenBank ID AP006725), NJST258_1 (GenBank ID CP006923.1), and Kp342 (GenBank ID CP000964), reference genomes were compared by CloVR comparative software (on the DIAG computing platform), generating clusters of genes shared between genomes. The number of genes shared between each genome was tabulated, and a Venn diagram was generated with free R (v.3.1.1) software and the Venn diagram package. To identify biological pathways associated with lung fitness, enrichment analysis was performed using KOBAS 2.0 (38).

**Statistical analysis.** To quantify the significance of each gene as a potential fitness factor, we first tested the difference in counts between inoculum and lung at each transposon insertion using an exact Poisson test and then combined the insertion-level *P* values into a *P* value for each gene using Fisher's method (using R 3.1.1 software) in a modification of CEDER analysis (19). A small *P* value indicates an important fitness factor. To test whether the *copA* mutant is more sensitive to copper than the wild type, a linear regression model was used (the relation between log-transformed CFU and Cu concentration is approximately linear). Statistical analysis of CFU data was performed on log-transformed data by a one-sample *t* test for competitive index and a two-sample *t* test or analysis of variance (ANOVA) with Fisher's least significant difference (LSD) test as indicated using Prism 6 (GraphPad Software, La Jolla, CA).

**Ethics statement.** This study was performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (39). The University of Michigan Institutional Animal Care and Use Committee (IACUC) approved this research (PRO00005795).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00775-15/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.
Data Set S1, XLSX file, 2.2 MB.
Data Set S2, XLS file, 0.8 MB.
Data Set S3, XLSX file, 0.8 MB.
Figure S1, TIF file, 0.2 MB.
Figure S2, TIF file, 0.1 MB.
Table S1, PDF file, 0.02 MB.
Table S2, PDF file, 0.1 MB.

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