A Computational Model Driven Design of a Novel Therapeutic Strategy for Targeting *Klebsiella pneumoniae*

A Technical Report submitted to the Department of Biomedical Engineering

Presented to the Faculty of the School of Engineering and Applied Science

University of Virginia • Charlottesville, Virginia

In Partial Fulfillment of the Requirements for the Degree

Bachelor of Science, School of Engineering

Mary Elaine Dickenson

Spring, 2022

Technical Project Team Members

Mary E Dickenson

On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

Jason A Papin, Department of Biomedical Engineering

Matthew L Jenior, Department of Biomedical Engineering

A Computational Model Driven Design of a Novel Therapeutic Strategy for Targeting *Klebsiella pneumoniae*

Mary E. Dickenson[a], Matthew L. Jenior[a], Jason A. Papin[a,b,c,1]

[a] Department of Biomedical Engineering, University of Virginia, Charlottesville, VA, USA
[b] Department of Medicine, Division of Infectious Diseases & International Health, University of Virginia, Charlottesville, VA, USA
[c] Department of Biochemistry & Molecular Genetics, University of Virginia, Charlottesville, VA, USA
[1] Correspondence: papin@virginia.edu, MR-5 2041a Box 800759, Health System Charlottesville, VA 22908, 434-924-8195

Abstract

Infections due to carbapenem-resistant Enterobacteriaceae have recently emerged as one of the most urgent threats to hospitalized patients within the United States and Europe. By far the most common etiological agent of these infections is *Klebsiella pneumoniae*, frequently manifesting in hospital-acquired pneumonia with a mortality rate of ~50% even with antimicrobial intervention. We performed transcriptomic analysis of data collected previously from *in vitro* characterization of both laboratory and clinical isolates which revealed shifts in expression of multiple master metabolic regulators across isolate types. Metabolism has been previously shown to be an effective target for antibacterial therapy, and genome-scale metabolic network reconstructions (GENREs) have provided a powerful means to accelerate identification of potential targets *in silico*. Combining these techniques with the transcriptome meta-analysis, we generated context-specific models of metabolism utilizing a well-curated GENRE of *K. pneumoniae* (iYL1228) to identify novel therapeutic targets. Using functional metabolic analyses, we identified increased catabolism of L-valine in clinical isolate-specific growth simulations. We then used genetic engineering techniques to create a single-gene knockout mutant for the identified gene of interest (*ilvE*). Following the successful generation of this mutant, we performed a variety of assays *in vitro* to evaluate the potential efficacy of *K. pneumoniae* valine metabolism as a novel therapeutic target. From these experiments, we were able to identify differences in the overall metabolic abilities when comparing the generated mutant strain to the wild-type strain. These findings warrant future studies to further investigate the potential efficacy of valine transaminase inhibition as a target against *K. pneumoniae* infection.

Keywords: *Klebsiella pneumoniae,* metabolic model, genetic engineering, therapeutics

Introduction

Carbapenem-resistant Enterobacteriaceae (CRE) have emerged as a growing and urgent issue in healthcare facilities around the world, posing a significant threat to public health and resulting in around 3 million infections in the United States each year[1]. Carbapenem antibiotics, currently considered to be the most potent and highly effective class of antimicrobial agents, are often considered a last-resort, reserved specifically for the treatment of severe multidrug-resistant (MDR) bacterial infections[2,3]. This recent surge in CRE-associated infections has been driven primarily by the emergence and dissemination of carbapenemases, a specific type of β-lactamase that has the ability to hydrolyze carbapenems, rendering even carbapenem-class antibiotics ineffective[2]. A large proportion of these CRE-related infections are due to the Gram-negative bacterium *K. pneumoniae*[2,4,5],with over 50% of *K. pneumoniae* infections now being resistant to

carbapenems in parts of the Eastern Mediterranean and Europe[4]. The rapidly increasing prevalence of carbapenem resistant *K. pneumoniae* results in a demand for new treatment options that vastly outweighs the ability of the pharmaceutical industry to research, develop, and get approval for new therapeutics[6].

Since the vast majority of *K. pneumoniae* clinical isolates now demonstrate a wide range of resistance to antibiotics, there are currently almost no effective therapeutic options to combat these infections[7]. Despite the increasing burden these bacteria present to public health, the most optimal treatment for CRE-related infections is largely unknown[8]. The current standard of treatment for *K. pneumoniae* infections begins with a course of antibiotics tailored around the known antibiotic sensitivities of that particular geographic region. If the specific clinical isolate of *K. pneumoniae* causing infection is found to be antibiotic resistant, carbapenem therapy will be initiated[9]. Further, if a CRE-related infection is diagnosed, common treatment options include more antibiotic regimes, high-dose prolonged-infusion of carbapenem, dual therapy carbapenems, or combination antibiotic therapy. Even with optimal therapy from the currently available treatments, *K. pneumoniae*-related infections carries a mortality rate of 30-50% in previously healthy patients, and an even worse prognosis in immunocompromised individuals[6,9]. Due to this increasingly high mortality rate and the overall inability to treat *K. pneumoniae* infections with current therapeutic strategies, it is vital that new treatment options are discovered for these infections.

Throughout the past several years, alarmingly large numbers of bacterial pathogens have been reported as resistant to antibiotics[10], emphasizing the need for identification of novel therapeutic options. Genome-scale metabolic network reconstructions (GENREs) have become powerful tools for elucidating the metabolic mechanisms underlying infectious diseases, allowing for the identification and acceleration of novel metabolism-based strategies for treatment[11]. One strategy that has emerged recently is the targeting of elements of virulence or core metabolism that may be too costly for the organism to accumulate mutations in or diminish the ability to manifest disease[12]. By identifying those characteristics lost during evolution toward sustained laboratory culture, while remaining conserved across infections, it becomes possible to gain insight into important phenotypes that contribute to successful infection. Furthermore, it has been shown that clinical and laboratory isolates of other bacterial pathogens may also be easily differentiated by distinct metabolic capacities[13]. Employing this approach for *K. pneumoniae* within this study, we may highlight "core" metabolic pathways in clinical isolates that may present ideal therapeutic target candidates. Consistent with this strategy, certain elements of metabolism have already been successfully identified as drug targets in bacterial pathogens including other Enterobacteriaceae[14–17].

Metabolic model-based growth simulations in other pathogens have successfully highlighted novel enzyme targets which were subsequently validated in the laboratory, effectively accelerating research efforts[16,18,19]. Changes in bacterial transcription have been used to assess differences in active metabolism with higher resolution than metabolomics screens, as shifts can be traced to specific pathways and gene products[20]. Additionally, GENREs can also be utilized to provide improved context for omics data as the network architecture can reveal additive effects of small changes in activity across interconnected pathways[11]. These network-based analyses enable greater insight into metabolic patterns that correspond with growth under specific conditions[18,19].

To identify novel therapeutic targets, a transcriptomic analysis of data collected from *in vitro* characterization of both laboratory and clinical isolates revealed shifts in expression of multiple master metabolic regulators across isolate types. We assembled 56 publicly available transcriptomes of *K. pneumoniae* isolates from both the laboratory and clinical profiles during growth in similar media conditions at multiple institutions[21–24]. Leveraging a GENRE with the transcriptome meta-analysis, we generated context-specific models of metabolism utilizing a well-curated GENRE of *K. pneumoniae* (iYL1228)[25] to identify novel therapeutic targets (Figure 1).

GENREs are computational formalisms of the biochemical reactions encoded for in an organism's genome[18,19]. Functional metabolic analyses of these generated context-specific models revealed that both composition and metabolic activity of clinical isolate-associated context-specific models significantly differs from laboratory isolate-associated models of the bacterium. Most prominent among these predictions was significantly elevated uptake and utilization of environmental L-valine through the increased activity of an Enterobacteriaceae-specific valine transaminase. This elevated uptake of L-valine was observed across >89% of clinical isolate context-specific models, while nearly entirely absent from laboratory strains, supporting the hypothesis of increased importance for survival *in vivo*. These results also agreed with previous findings that macrophages respond to high concentrations of exogenous valine in order to upregulate phagocytosis and the killing of *K. pneumoniae* during infection[26]. This result could be indicative of an immunosuppressive strategy *K. pneumoniae* evolved for survival during infection. Thus, this preliminary work warrants the goals of this study where we will be experimentally evaluating the efficacy of these previously identified novel therapeutic targets for *K. pneumoniae* infection.

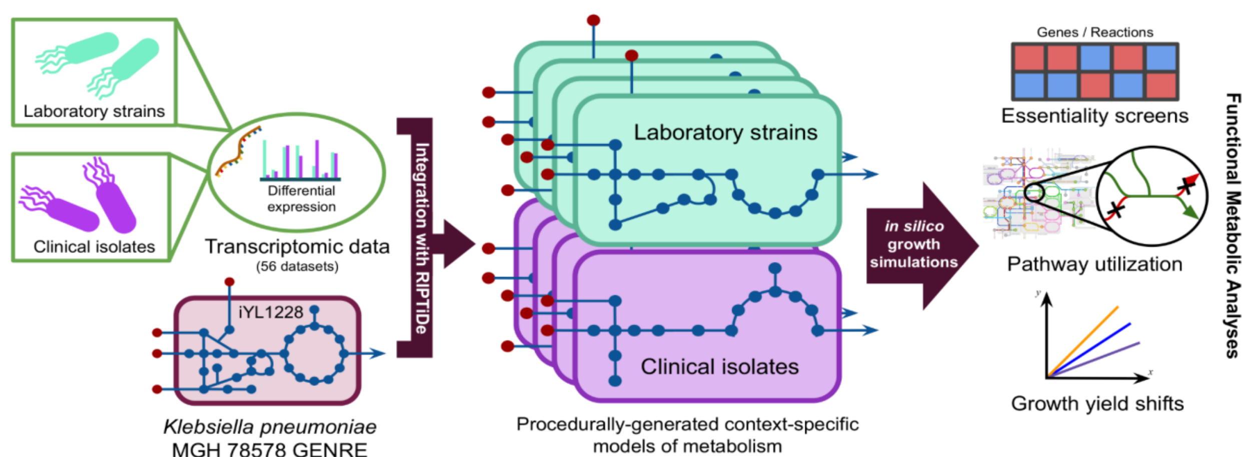


Figure 1. General procedure for generating context-specific models of metabolism from transcriptomic data. All 56 datasets from the transcriptome meta-analysis were used to generate distinct context-specific models of K. pneumoniae metabolism.

Overall, by targeting elements of metabolism specifically related to life in a host, we may be able to interfere with the ability of the organism to colonize or cause disease. Additionally, we have been able to help minimize effects from otherwise potentially problematic inter-study variation through performing a meta-analysis of transcriptomic datasets across multiple studies using a unified curation and analysis pipeline. To further explore possible targets within infection-associated metabolic pathways, we integrated the transcriptomic meta-analysis with a metabolic network-based investigation which allowed us to discern novel conserved components of *K. pneumoniae*'s metabolic strategy specific to active infection. This work highlights the utility of well curated GENREs integrated with transcriptomic data to accelerate molecular target identification. This computational model driven approach to identifying novel therapeutic targets could be a considerable improvement over other existing methods since it vastly expedites the process of identifying plausible targets and mitigates variability otherwise present in studies.

As *K. pneumoniae* has been rapidly acquiring antibiotic resistance and rendering almost all available treatments ineffective, the discovery of new treatment strategies for this bacterial pathogen are critical[3,4]. To address this global health threat, the primary goal of this project is to develop and experimentally validate a computational model driven design of a novel therapeutic strategy for targeting *K. pneumoniae.* To achieve this, we first improved the computational model using previously published data to increase physiological accuracy of computational-model based predications. Using this improved model, we identified novel therapeutic targets for *K.*

pneumoniae infections to subsequently experimentally validate. We then created genetically modified *K. pneumoniae* single-knockout mutants for the identified genes of interest. Finally, we designed and performed assays *in vitro* to test the effects of the generated mutations on the potential ability to cause infections. Positive results gained from this study may lead to the development of novel therapeutic options for *K. pneumoniae* infections which are more effective than current treatment options and harder for the bacteria to evolve resistance to.

Materials and Methods

As all hypotheses generated for this project are informed by predictions gathered from the metabolic model of *K. pneumoniae*, it was vital to continue to curate this model throughout the duration of this project. The portion of metabolism of primary interest in this project is valine catabolism, thus we wanted to have the most accurate representation of this metabolism in the metabolic model. An extensive manual evaluation was performed of the valine biosynthesis and valine degradation pathways represented in the model against published genes and reactions associated with these pathways. A variety of databases were used as a basis for this investigation, namely ModelSEED[27], BiGG[28], KEGG[29], and UniProt[30]. The proper stoichiometries were then identified for each of the reactions that needed to be added or altered in the iYL1228 model from appropriate databases. Following this, the metabolic model was curated through the addition and alteration of misrepresented reactions and genes. Specifically, the gene *budB* was added and the gene *ilvC* was altered in the model to complete the valine biosynthesis pathway (Figure 2). Some of the previously conducted metabolic analyses were then rerun using this more curated metabolic model. This curation will allow this study, and future studies, using metabolic model iYL1228 to yield more physiologically relevant results.

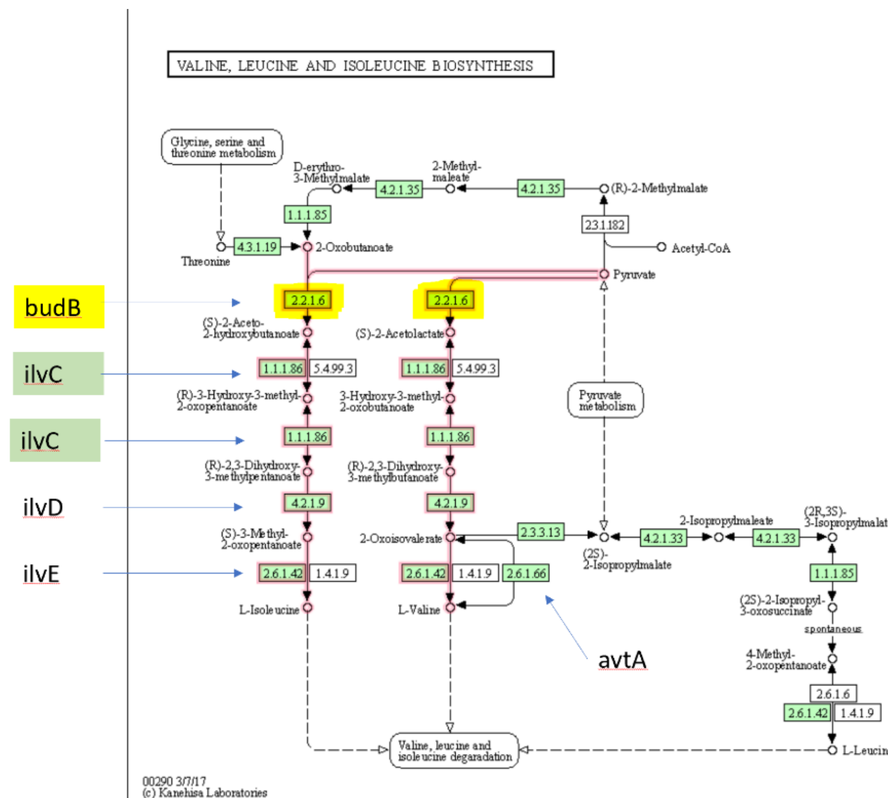


Figure 2. Valine biosynthesis pathway (highlighted in red) with corresponding gene names. Genes highlighted in yellow indicate they were added to the model, genes highlighted in green indicate they were altered in the model, and genes not highlighted indicate they were not altered in the updated model.

To genetically engineer *K. pneumoniae* single gene knockout mutants using site-directed mutagenesis, it is necessary to design appropriate plasmids for each gene of interest. Briefly, primers were selected for the gene of interest using Primer3[31], selecting both the most appropriate forward and reverse primer for the gene of interest, *ilvE* (Figure S1) . This primer set was designed with a restriction site on both the forward and the reverse end, correlating with the available

restriction sites on plasmid pSR47S (Figure S2). Since primer design using Primer3 results in multiple potentially effective primer sets for each gene of interest, it was decided to test multiple primer sets for the gene of interest to improve the chances of being able to successfully create the desired mutant. When performing this step, it was importantly insured that each of the selected restriction sites were not located anywhere within the gene of interest. Each of these designed primer sets was then amplified using polymerase chain reaction (PCR). The volume of each PCR reactant, as well as the cycling temperatures for each PCR reaction, was optimized to maximize the total amount of resulting PCR product. The efficiency of the amplification was then evaluated using gel electrophoresis. A negative control for each set of primers was included to ensure no unexpected amplification occurred. The set of primers for gene *ilvE* that resulted in the highest levels of amplification was selected for use.

Having successfully designed and amplified primers for the gene of interest (*ilvE*), preparation and ligation of this insert into plasmid pSR47S was conducted next. To achieve this, *ilvE* PCR products was isolated using the QIAquick PCR Purification Kit. A restriction enzyme double digestion was then prepared for the designed primers and plasmid pSR47S, using restriction enzymes speI and notI correlating with my *ilvE* primer design. The NEBcloner web application was used to select the proper buffer for this double digestion[32], as different restriction enzymes have different amounts of activity in the various buffer options. Using the NEBcloner, NEBuffer r2.1 was identified as the best option for this particular double digest. The general recommendation for double digestions is that both enzymes need a minimum of 50% activity in the selected buffer, which both speI and notI have in NEBuffer r2.1(Figure S3). Many protocols suggest increasing incubation time if both restriction enzymes do not have 100% activity in the selected buffer[33,34]. Therefore, this double digestion reaction was incubated overnight to ensure maximum digestion. Following this step, the digested PCR products and plasmids were once again isolated using a QIAquick PCR Purification Kit. Finally, the digested insert was ligated into plasmid pSR47S using the standard protocol for T4 DNA Ligase, resulting in the final plasmid used for the gene knockout of *ilvE*.

Work on transforming the generated plasmid into *E. coli* began following the generation of the desired plasmid. To accomplish this, the strain of *E. coli* being used for this project had to be made electrocompetent. Briefly, this involved growing one *E. coli* colony in Super Optimal Broth (SOB) media overnight, adding a few drops of this overnight culture to two different flasks of SOB media, and growing each of these cultures until they reached an OD600 of 0.5-0.7 (actual OD600 value measured to be 0.5). Each of these cultures was then subjected to multiple rounds of centrifugation at 4 degrees Celsius, removal of the supernatant, and subsequent resuspension in a 10% glycerol mixture. Long term freezer stocks were then created from the electrocompetent cells for future use. Having both the constructed plasmid and electrocompetent cells, work began to electroporate the constructed plasmids into the now electrocompetent *E. coli* cells. To do this, a standard electroporation protocol was followed[35]. Briefly, a mixture of the electrocompetent cells and the isolated plasmids was electroporated at 1.8 kV, the mixture was resuspended in SOC media, the cells were incubated for 1 hour at 37 degrees Celsius, and cells were then plated at 100-fold and 1000-fold dilutions on both Luria-Bertani (LB) media and LB-kanamycin plates prior to incubating overnight. The resulting plates indicate that the *E. coli* was successfully transformed, as the plasmid contains a kanamycin-resistant gene.

Liquid cultures were then created of both the transformed *E. coli* and *K. pneumoniae* to be used for co-culture, and each of these cultures was incubated overnight. 100 microliters of transformed *E. coli* and 100 microliters of wild-type *K. pneumoniae* were then co-cultured overnight on LB plates. To select for only transformed colonies, colonies from the overnight LB plates were streaked onto LB + kanamycin plates and grown them overnight. Finally, *K. pneumoniae* colonies were selected for by streaking colonies selected from the LB + kanamycin plates onto MacConkey media, since *E. coli* and *K. pneumoniae* colonies turn different colors on this type of media. Individual *K. pneumoniae* colonies were randomly selected to use for verification of the mutant genotype. PCR was run to amplify the *ilvE* gene on both the mutant and the wild-type *K. pneumoniae* to verify the desired gene was knocked out.

Two assays were performed *in vitro* to assess the effects of the generated mutations on *K. pneumoniae's* ability to successfully infect, and these assays were designed based off of previously published assays[26]. For the first assay, both the mutant strain and the wild-type strain were grown *in vitro* on LB plates as well as in liquid LB media at 37 degrees Celsius for 24 hours, with 2 replicates of each condition. The optical density (OD600) was measured at six different time points (0, 2, 4, 6, 8, and 24 hours) to determine growth in each condition. Pictures of the plates were also taken at each time point. For the second assay, both the mutant strain and the

wild-type strain were grown in both liquid M9 minimal media and liquid M9 minimal media supplemented with valine[36]. They were grown at 37 degrees Celsius for 24 hours and shaken at 150 rpm, with 2 replicates of each condition. The OD600 was measured at six different time points (0, 2, 4, 6, 8, and 24 hours) as a measurement of growth for each condition. Following the collection of data from each of these assays, the average OD600 measurements for each condition at each time point were used as relative representations of growth.

Results

Network topological analysis and essentiality screens highlight valine catabolism as differentially critical in clinical isolates

To identify novel therapeutic targets using the created contextualized models, we performed an analysis of unique subnetwork topology to each isolate type-specific group of models. After subtracting "core" reactions that were present in all 56 models, we were left with a median of 52 and 72 reactions that were unique to either laboratory or clinical-specific models, respectively. Finally, to focus the analysis on those reactions most shared within each group we further limited the scope of reactions to only those shared by at least 55% of models within each group respectively, revealing 15 differentially active reactions (Figure 3A). Among the most prominent patterns from this analysis were reactions for the import of environmental L-valine in clinical isolate models that were not present in their laboratory counterparts. This finding was interesting as it has been recently discovered that exogenous L-valine promotes increased macrophage phagocytosis *in vivo*, thereby pressuring a lung pathogen to evolve to remove excess valine from the environment[26].

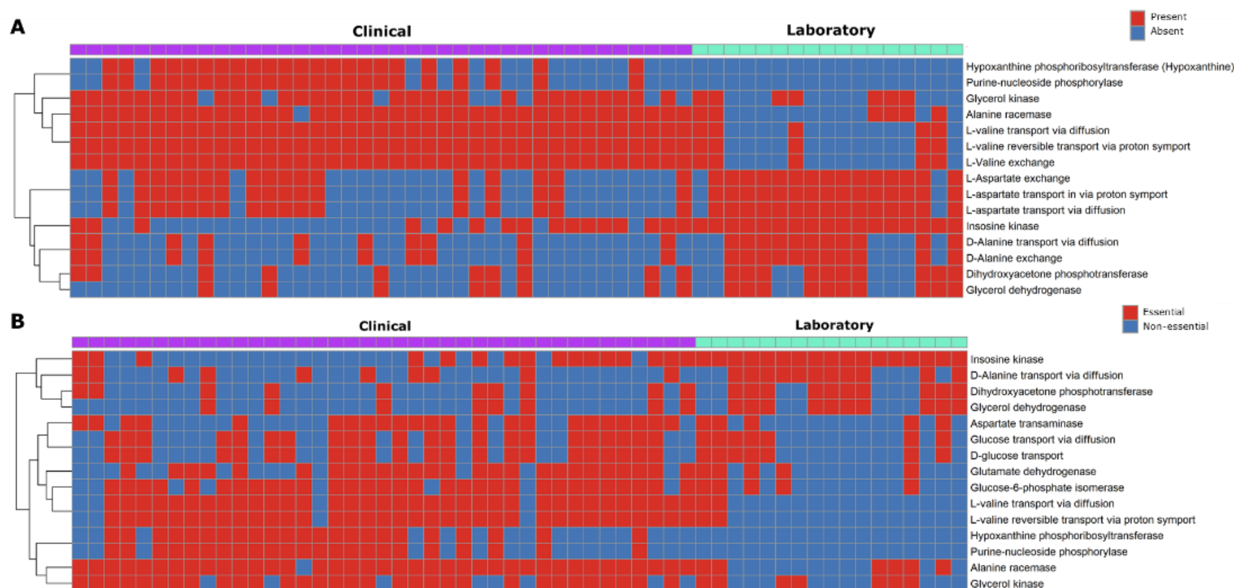


Figure 3. Environmental valine is differentially essential in clinical-isolate context-specific models of *K. pneumoniae.* (A) Reaction topology differentially present between the context-specific model groups. **(B)** Differential reaction essentiality between isolate model groups, essentiality was determined through single reaction knockout screen with a cutoff of 1% of the biomass flux. Inclusion in final analysis was determined by cross reference against uncontextualized GENRE and a within-group shared threshold of >55% of models possessing a given feature. Color within the figure area indicates essentiality/presence (red) and non-essentiality/absence (blue), and color on the top margin denotes strain-type of origin for the associated transcriptome with clinical isolate (purple) or laboratory strain (teal).

Next, we sought to identify differentially essential metabolic pathway elements between clinical and laboratory isolates in an effort to ultimately provide a basis for future drug discovery efforts. To accomplish this goal, we performed both single gene and reaction knockout simulations

across context-specific models using a threshold of a minimum of 1% of optimal biomass for a gene or reaction to be deemed essential[37,38]. This functional analysis resulted in a median of 262 and 282 essential reactions in laboratory and clinical associated models, respectively. We then cross referenced these results against the uncontextualized iYL1228 to limit potential targets to only those components of metabolism that were environment-specific, and likely not due to strict user-applied constraints, as well as subtracting the "core" essential reactions, resulting in a reduction to a median 52 (laboratory) and 72 (clinical) essential reactions. Then, using a similar 55% threshold of shared elements to the previous topology analysis, our combined essentiality screen reported a total of 15 reactions as differentially essential between isolate types (Figure 3B). This analysis indicated that bioconversion of environmental valine is essential in clinical isolates, but not for laboratory strains of *K. pneumoniae*. Of the 15 differentially essential reactions parsed, three reactions were directly related to valine metabolism (Figure 3B). These three reactions had the highest levels of consistent essentiality among analyzed reactions, being essential for growth in approximately 90% of clinical isolate context-specific models. These results seem to agree with the prior topology-based findings, indicating that valine catabolism may play an important role in the metabolism of *K. pneumoniae* during infection. Due to these GENRE-based analyses indicating valine metabolism to be a potential therapeutic target, we aimed to validate these results experimentally, specifically interrogating the valine transaminase *ilvE*.

Site-directed plasmid mutagenesis successfully yields K. pneumoniae single-gene (ilvE) knockout mutant

To be able to evaluate the potential efficacy of valine metabolism as a novel therapeutic target, we aimed to genetically engineer a *K. pneumoniae* single gene knockout mutant. Specifically, we used site-direct plasmid mutagenesis to create this mutant. After optimizing and conducting the necessary protocol for creating a genetically engineered *K. pneumoniae* *ilvE* gene knockout mutant, we were able to confirm the desired genotype of this mutant. To do this, we selected specifically for the transformed *K. pneumoniae* colonies by plating them on LB + kanamycin media (Figure 4A). The wild-type strains are unable to grow on this media, as they are susceptible to kanamycin. However, the plasmid that was transformed into the mutant strains has a kanamycin resistant coding region, making these mutants resistant to kanamycin. Following this successful selection via plating, we performed PCR to amplify the *ilvE* gene on DNA from both wild-type *K. pneumoniae* and from the mutant *K. pneumoniae*. The successful knockout of the *ilvE* gene was confirmed by there being no amplification of the *ilvE* gene for the mutant strain (Figure 4B). This successful generation and confirmation of a genetically engineered mutant without the *ilvE* gene allowed for tests to be performed *in vitro* to assess the potential efficacy of this metabolic pathway as a therapeutic target.

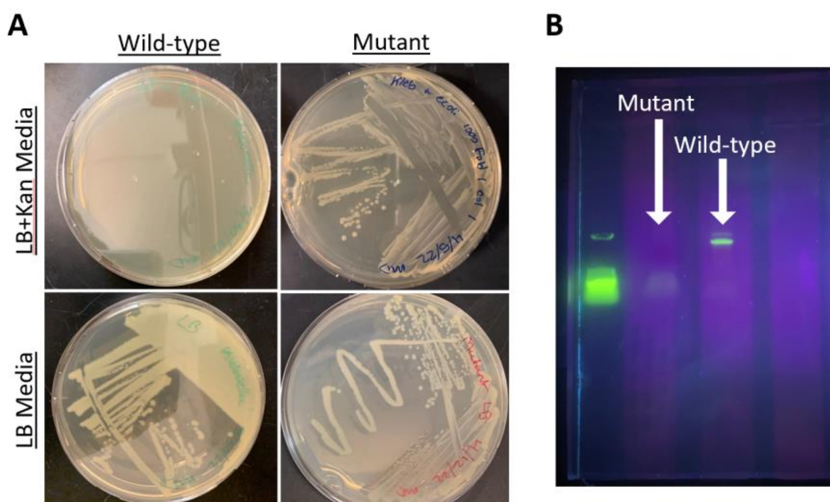


Figure 4. Validation of successful *ilvE* gene knockout mutation. (A) Overnight growth of wild-type strain and mutant strain in both LB media and selective LB + kanamycin media. **(B)** Gel electrophoresis results of *ilvE* amplification via PCR for both wild-type strain and mutant strain.

Growth assays in different media types indicate differences in metabolic abilities between wild-type and mutant strains

Having successfully created a *K. pneumoniae* mutant, we sought to design and perform assays to evaluate the effects of the *ilvE* gene knockout on the bacteria's potential ability to cause infections. Each of the assays performed were designed to help assess the potential efficacy of the therapeutic target identified via GENRE-based approaches. The two primary factors we aimed to evaluate was the overall ability to grow in a rich media environment, and the ability to utilize valine in a minimal media environment.

To achieve this, we first performed a growth assay comparing both the mutant and wild-type strains' ability to grow in a rich LB media over 24 hours. While there was no significant difference between the two strains growth over the first 8 hours, the wild-type strain was observed to reach a significantly higher OD600 over the 24-hour period than the mutant strain (Figure 5). The *ilvE* knockout was not predicted to have a detrimental effect on the ability of the organism to grow, as *K. pneumoniae* is not known to be auxotrophic for valine. Yet, it was interesting to see that the removal of this portion of the valine metabolism pathway did result in a lower maximum potential growth rate. These results potentially indicate a slight inhibition in the organism's ability to grow, and hence successfully infect a host, due to the interruption of the valine metabolism pathway.

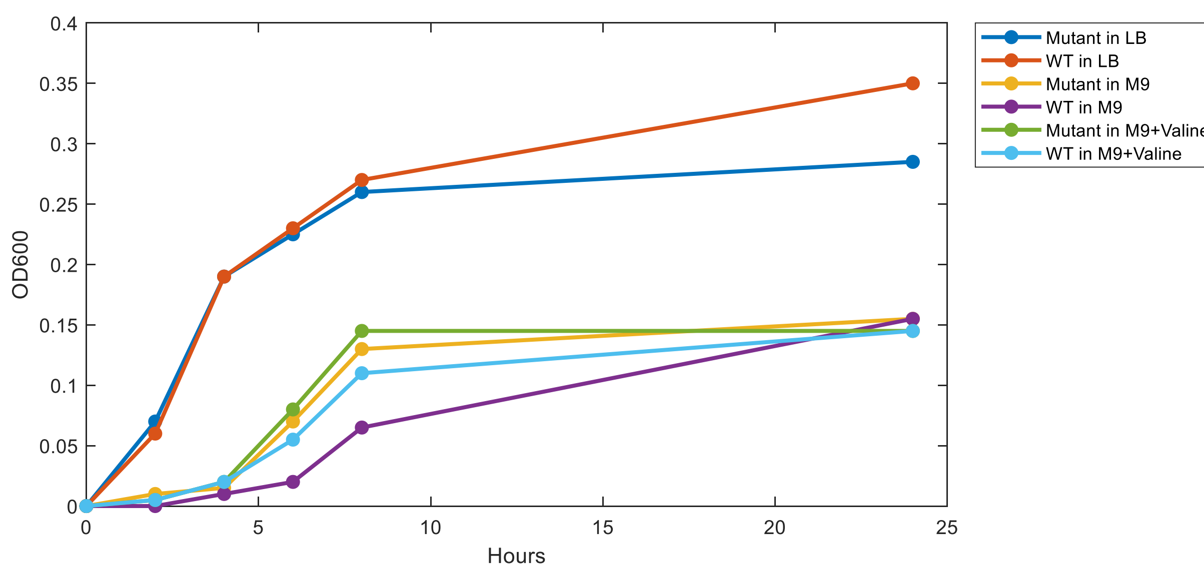


Figure 5. Growth curves taken over a range of 24 hours in a variety of media conditions. Both mutant and wild-type strains were grown in LB media, M9 minimal media, and M9 minimal media supplemented with valine overnight at 37 degrees Celsius shaking at 150 rpm. Each point on the plot represents the average of OD600 measurements taken from 2 replicates for each given condition.

We subsequently performed an assay to evaluate the effect of the gene knockout on the organism's overall need to consume valine to successfully grow. Both the mutant and wild-type strains were grown in M9 minimal media and M9 minimal media supplemented with valine over 24 hours. While there was some variability in how quickly the different strains reached stationary phase in the two different media types, all four of the tested conditions notably resulted in very similar maximum OD600 values. Interestingly, the mutant strain reached the stationary phase for both the M9 minimal media and the M9 minimal media supplemented with valine faster than the wild-type strain did in either of these conditions (Figure 5). One possible explanation for this result is that the site-directed plasmid mutagenesis protocol resulted in unintended mutations in addition to the desired knockout[39]. An additional plausible explanation is that a slightly larger colony was

inoculated into the mutant medias than the wild-type medias at the first, resulting in there being relatively more cells to proliferate from the beginning. Alternatively, since both strains in both conditions ended at roughly the same OD600 when reaching stationary phase, presumably neither strain of bacteria's metabolic machinery required valine to achieve a state of maximum growth in this particular media condition. Future work should be done to further interrogate *K. pneumoniae's* ability to sequester exogenous valine, and the potential impact that interrupting valine metabolism may have on the organism's ability to infect.

Discussion

To address the urgent healthcare threat attributed to the bacterial pathogen *K. pneumoniae* which has been rapidly gaining antibiotic resistance in recent years, the primary goal of this project was to develop and experimentally validate a computational model driven design of a novel therapeutic strategy for targeting these infections. Through this study, we successfully identified and subsequently began to test the potential efficacy of valine transaminase inhibition as a future therapeutic target against *K. pneumoniae* infections. Furthermore, this study helped to provide a better understanding of the role of L-valine in *K. pneumoniae* colonization and virulence, which may be further researched in future studies. Results gleaned from this study regarding the potential of valine transaminase inhibition as a therapeutic strategy could help lead to novel clinical treatment regimens for *K. pneumoniae* infections. Finally, the methods described here may be applied to other recalcitrant bacterial pathogens in the future as a platform for accelerated drug target discovery and validation.

While this study was able to present several novel insights into potential new therapeutic options for *K. pneumoniae* infections, some limitations are present. Sequencing the final created *K. pneumoniae* mutant was not within the overall scope of this project. Therefore, while there were multitudes of verification steps throughout the mutagenesis process to minimize any potential errors, the exact final sequence has not yet been confirmed. Additionally, the mutants used in this study were made from a base laboratory strain, rather than from clinical strains. Since we know that there are metabolic differences between laboratory and clinical strains of *K. pneumoniae*, a clinical strain with the same gene inhibited may act significantly differently than what we have seen here. However, since we focused on core metabolism throughout this study, it is less likely for there to be any large differences between the machinery of laboratory and clinical strains in this particular context. Additionally, all evaluation experiments have only been tested *in vitro*, which does not take into account the many other factors which may play a role in infections, including the immune system and other germs that may be present in the environment. I additionally was unable to evaluate the entire pathway of interest due to time constraints for this project. Some genes may be more impactful than the one selected for this particular project, which would be interesting other future directions of study.

Overall, this project has identified a potential novel therapeutic strategy for treating *K. pneumoniae* infections, thus motivating future work in this field as well. Specifically, continuing *in vitro* assays to further evaluate the potential efficacy of this treatment option would be highly useful. For example, an assay could be performed to measure exogenous valine levels at different time points when growing either the mutant or wild-type strain in a rich media environment. Additionally, since exogenous valine is known to upregulate macrophage phagocytosis in infection environments, macrophage assays could be performed to determine if the mutant strains could be more easily cleared than wild-type strains by macrophages. If further positive results are gleaned from *in vitro* studies to demonstrate viability of this metabolic pathway as a potential target, this therapeutic option should be moved into *in vivo* studies. Namely, it would be interesting to further investigate if animal immune systems are able to more easily clear mutant strains than wild-type strains. The differences in macrophage activity and exogenous valine levels between mutant and wild-type strain infections could be further investigated during these studies. Finally, if proven to be an effective therapeutic target through both *in vitro* and *in vivo* studies, researchers should begin evaluating the drug development phase to repurpose a currently available drug or design a new drug in order to target this portion of metabolism.

Our results indicate that targeting valine metabolism in *K. pneumoniae* may be an effective new treatment option against these infections in the future. Future studies may build on the targets that have been identified and validated within this study to further interrogate the role of valine metabolism in *K. pneumoniae* colonization and virulence. Finally, the pipeline outlined within this

study may be applied for the accelerated identification and validation of novel therapeutic targets for other bacterial pathogens in the future.

End Matter

Author Contributions and Notes

M.E.D., M.L.J., and J.A.P. conceptualized the study. M.E.D., performed research, analyzed data, and wrote the paper.
The authors declare no conflict of interest.

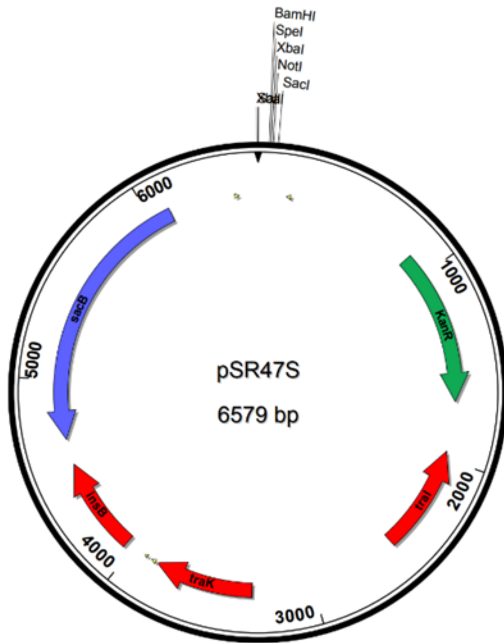
Acknowledgments

The authors would like to thank the members of the Papin Lab, namely Dr. Glynis Kolling, for many helpful conversations and suggestions throughout the duration of this project. This work was supported by funding from the US National Institutes of Health award 5R01AI154242-02 to JP.

Supplementary Figures

	sequence	restriction enzyme	sequence + restriction enzyme	tm (deg C)	temp PCR ran at
Forward:		SpeI	ACT AGT + TAT CTT TCC TCG CTG CTG GT	59.4	55
Reverse:		NotI	TTA ACA TCG AGG GCG ATA CCC GCC GGC	68.7	55

Supplementary Figure 1. Table showing selected primers for *ilvE* gene knockout.



Supplementary Figure 2. Visual map of restriction sites and correlating locations on plasmid pSR47S.

Name	Cat #	Temp °C	Supplied Buffer	Add SAM	% Activity in NEBuffer™			
					r1.1	r2.1	r3.1	rCutSmart
SpeI	R0133	37	rCutSmart Buffer	No	75	100	25	100
NotI	R0189	37	NEBuffer r3.1	No	10	50	100	25

Supplementary Figure 3. Table showing *ilvE*-associated restriction enzyme efficiencies in variety of buffer options for single or double digestion.

References

1. CDC. Antibiotic-resistant Germs: New Threats. *Centers for Disease Control and Prevention* https://www.cdc.gov/drugresistance/biggest-threats.html (2021).
2. Iovleva, A. & Doi, Y. Carbapenem-Resistant Enterobacteriaceae. *Clinics in Laboratory Medicine* **37**, 303–315 (2017).
3. Papp-Wallace, K. M., Endimiani, A., Taracila, M. A. & Bonomo, R. A. Carbapenems: Past, Present, and Future. *Antimicrob. Agents Chemother.* **55**, 4943–4960 (2011).
4. Zhu, W., Yuan, Z. & Zhou, H. Risk factors for carbapenem-resistant Klebsiella pneumoniae infection relative to two types of control patients: a systematic review and meta-analysis. *Antimicrob Resist Infect Control* **9**, 23 (2020).
5. Kotb, S. *et al.* Epidemiology of Carbapenem-resistant Enterobacteriaceae in Egyptian intensive care units using National Healthcare-associated Infections Surveillance Data, 2011-2017. *Antimicrob Resist Infect Control* **9**, 2 (2020).
6. Xu, L., Sun, X. & Ma, X. Systematic review and meta-analysis of mortality of patients infected with carbapenem-resistant Klebsiella pneumoniae. *Ann Clin Microbiol Antimicrob* **16**, 18 (2017).
7. Nirwati, H. *et al.* Biofilm formation and antibiotic resistance of Klebsiella pneumoniae isolated from clinical samples in a tertiary care hospital, Klaten, Indonesia. *BMC Proc* **13**, 20 (2019).
8. Morrill, H. J., Pogue, J. M., Kaye, K. S. & LaPlante, K. L. Treatment Options for Carbapenem-Resistant Enterobacteriaceae Infections. *Open Forum Infectious Diseases* **2**, (2015).
9. Ashurst, J. V. & Dawson, A. Klebsiella Pneumonia. in *StatPearls* (StatPearls Publishing, 2021).
10. Banin, E., Hughes, D. & Kuipers, O. P. Editorial: Bacterial pathogens, antibiotics and antibiotic resistance. *FEMS Microbiology Reviews* **41**, 450–452 (2017).
11. Blazier, A. S. & Papin, J. A. Integration of expression data in genome-scale metabolic network reconstructions. *Front. Physio.* **3**, (2012).

12. Panayidou, S. *et al.* Pseudomonas aeruginosa core metabolism exerts a widespread growth-independent control on virulence. *Sci Rep* **10**, 9505 (2020).

13. Bundy, J. G., Willey, T. L., Castell, R. S., Ellar, D. J. & Brindle, K. M. Discrimination of pathogenic clinical isolates and laboratory strains of *Bacillus cereus* by NMR-based metabolomic profiling. *FEMS Microbiology Letters* **242**, 127–136 (2005).

14. Hasan, S., Daugelat, S., Rao, P. S. S. & Schreiber, M. Prioritizing Genomic Drug Targets in Pathogens: Application to Mycobacterium tuberculosis. *PLoS Comput Biol* **2**, e61 (2006).

15. Song, J.-H. & Ko, K. S. Detection of Essential Genes in Streptococcus pneumoniae Using Bioinformatics and Allelic Replacement Mutagenesis. in *Microbial Gene Essentiality: Protocols and Bioinformatics* (eds. Osterman, A. L. & Gerdes, S. Y.) vol. 416 401–408 (Humana Press, 2008).

16. Ramos, P. I. P. *et al.* An integrative, multi-omics approach towards the prioritization of Klebsiella pneumoniae drug targets. *Sci Rep* **8**, 10755 (2018).

17. Hadizadeh, M. *et al.* Genome-Wide Identification of Potential Drug Target in *Enterobacteriaceae* Family: A Homology-Based Method. *Microbial Drug Resistance* **24**, 8–17 (2018).

18. Gu, C., Kim, G. B., Kim, W. J., Kim, H. U. & Lee, S. Y. Current status and applications of genome-scale metabolic models. *Genome Biol* **20**, 121 (2019).

19. Ruppin, E., Papin, J. A., de Figueiredo, L. F. & Schuster, S. Metabolic reconstruction, constraint-based analysis and game theory to probe genome-scale metabolic networks. *Current Opinion in Biotechnology* **21**, 502–510 (2010).

20. Ma, C., Yang, X. & Lewis, P. J. Bacterial Transcription as a Target for Antibacterial Drug Development. *Microbiol Mol Biol Rev* **80**, 139–160 (2016).

21. Long, D. *et al.* Phenotypical profile and global transcriptomic profile of Hypervirulent Klebsiella pneumoniae due to carbapenemase-encoding plasmid acquisition. *BMC Genomics* **20**, 480 (2019).

22. Guilhen, C. *et al.* Transcriptional profiling of Klebsiella pneumoniae defines signatures for planktonic, sessile and biofilm-dispersed cells. *BMC Genomics* **17**, 237 (2016).
23. Lee, M. *et al.* Network Integrative Genomic and Transcriptomic Analysis of Carbapenem-Resistant *Klebsiella pneumoniae* Strains Identifies Genes for Antibiotic Resistance and Virulence. *mSystems* **4**, e00202-19, /msystems/4/4/msys.00202-19.atom (2019).
24. Bruchmann, S. *et al.* Deep transcriptome profiling of clinical *K lebsiella pneumoniae* isolates reveals strain and sequence type-specific adaptation: Comparative transcriptomics of *Klebsiella pneumoniae. Environ Microbiol* **17**, 4690–4710 (2015).
25. *Model: iYL1228 Klebsiella pneumoniae subsp. pneumoniae MGH 78578*. (BiGG Model Database, 2019).
26. Chen, X. *et al.* Exogenous l-Valine Promotes Phagocytosis to Kill Multidrug-Resistant Bacterial Pathogens. *Front. Immunol.* **8**, (2017).
27. ModelSEED. https://modelseed.org/.
28. King, Z. A. *et al.* BiGG Models: A platform for integrating, standardizing and sharing genome-scale models. *Nucleic Acids Res* **44**, D515–D522 (2016).
29. KEGG Pathway Maps. https://www.genome.jp/kegg/kegg3a.html.
30. UniProt. https://www.uniprot.org/.
31. Primer3 Input (version 0.4.0). https://bioinfo.ut.ee/primer3-0.4.0/.
32. NEBcloner. https://nebcloner.neb.com/#!/redigest.
33. Addgene: Plasmid Cloning by PCR (with Protocols). https://www.addgene.org/protocols/pcr-cloning/.
34. Optimizing Restriction Endonuclease Reactions | NEB. https://www.neb.com/tools-and-resources/usage-guidelines/optimizing-restriction-endonuclease-reactions.
35. Making your own electrocompetent cells | NEB. https://www.neb.com/protocols/2012/06/21/making-your-own-electrocompetent-cells.
36. M9 Minimal Media Recipe (1000 ml). http://www.thelabrat.com/protocols/m9minimal.shtml.

37. O'Brien, E. J., Monk, J. M. & Palsson, B. O. Using Genome-scale Models to Predict Biological Capabilities. *Cell* **161**, 971–987 (2015).

38. Opdam, S. *et al.* A Systematic Evaluation of Methods for Tailoring Genome-Scale Metabolic Models. *Cell Systems* **4**, 318-329.e6 (2017).

39. DeCero, S. A., Winslow, C. H. & Coburn, J. Method to Overcome Inefficiencies in Site-Directed Mutagenesis of A/T-Rich DNA. *J Biomol Tech* **31**, 94–99 (2020).