A novel method for integrating genomic and Tn-Seq data to identify common *in vivo* fitness mechanisms across multiple bacterial species

Derrick E. Fouts[1*], Thomas H. Clarke[1], Aric N. Brown[2], Elizabeth N. Ottosen[4], Caitlyn L. Holmes[2,3], Bridget S. Moricz[3], Sophia Mason[3], Ritam Sinha[4], Geoffrey B. Severin[2], Mark T. Anderson[2], Victor DiRita[4], Michael A. Bachman[3] and Harry L. T. Mobley[2*]

[1]The J. Craig Venter Institute, Rockville, MD, USA.

[2]Department of Microbiology and Immunology, University of Michigan Medical School

[3]Department of Pathology, University of Michigan Medical School

[4]Department of Microbiology and Molecular Genetics, Michigan State University College of Natural Sciences

*To whom correspondence should be addressed:

dfouts@jcvi.org

hmobley@umich.edu

Abstract

Sepsis is life-threatening organ dysfunction due to an unregulated immune response to infection. Bacteremia is a leading cause of sepsis, and members of the *Enterobacterales* cause nearly half of bacteremia cases annually. While previous Tn-Seq studies to identify novel bacteremia-fitness genes have provided valuable insight into virulence mechanisms, evidence for common pathways across species was lacking. To identify common fitness pathways in five bacteremia-caused *Enterobacterales* species, we utilized the JCVI pan-genome pipeline to integrate Tn-Seq fitness data with multiple available functional data types. Core genes from species pan-genomes were used to construct a multi-species core pan-genome, producing 2,850 core gene clusters found in four out of the five species. Integration of Tn-Seq fitness data enabled identification of 373 protein clusters that were conserved in all five species. A scoring rubric was applied to these clusters, which incorporated Tn-Seq fitness defects, operon localization, and antibiotic susceptibility data to identify seven common bacteremia-fitness pathways. Mutations in *tatC* showed reduced fitness *in vivo* and increased susceptibility to beta-lactams that were restored following *tatC* complementation *in trans*. By integrating known operon structures and antibiotic susceptibility with Tn-Seq fitness data, common genes within the core pan-genome emerged and revealed mechanisms that are essential for colonization of, or survival in, the mammalian bloodstream. Our prediction and validation of *tatC* as a common bacteremia fitness factor and contributor of antibiotic resistance supports the utility of this bioinformatic approach. This study represents a major step forward to identify novel targets of therapy against these deadly widespread sepsis infections.

Author Summary

Bacteremia is a leading cause of sepsis, a life-threatening condition where an unregulated immune response to infection causes systemic organ failure. Nearly half of bacteremia cases are caused by members of the Gram-negative bacterial taxonomic order *Enterobacterales*. Given the public health impact of bacteremia and the reduction of existing antibiotic treatment options, novel strategies are needed to combat these infections. Pan-genome software was used to predict seven shared fitness pathways in these bacteria that may serve as novel targets for treatment of bacteremia. Briefly, a scoring rubric was applied to shared pan-genome clusters, which incorporated multiple data types, including Tn-Seq fitness defects, operon localization, and antibiotic susceptibility data to rank and prioritize fitness genes. To provide an example of our predictions, mutations were constructed in one prioritized gene, *tatC*, which showed both reduced fitness in mice and increased susceptibility to *beta*-lactam antibiotics; complementation restored fitness and antibiotic susceptibility to wild type levels. This study takes a novel bioinformatics approach to build a core pan-genome across multiple distantly related bacteria to integrate computational and experimental data to predict important shared fitness genes and represents a major step forward toward identifying novel targets of therapy against these deadly widespread life-threatening infections.

Introduction

Sepsis is the life-threatening organ dysfunction resulting from a dysregulated and overwhelming immune response to infection. There are an estimated 49 million cases of sepsis that occur annually worldwide [1] and in the United States sepsis accounts for one in every 2-3 hospital deaths [2]. Bacteremia, or the presence of bacteria in the bloodstream, is the leading cause of sepsis with Gram-negative species belonging to the *Enterobacterales* order among the most common causes of bacteremia [3–5]. Drug resistance among this cohort of pathogens is a major limitation to effective treatment [4,6] and contributes to the large burden of antibiotic resistant bacterial threats recognized by the Centers for Disease Control and Prevention [7]. Given the public health impact of *Enterobacterales* bacteremia and the waning efficacy of existing treatments, novel strategies are needed to combat these infections. To develop such approaches, it is critical to first understand the genetic and molecular basis by which these bacteria cause bloodstream infections (BSI).

Bacteremia can be divided into three phases of pathogenesis: initial primary site infection, dissemination to the bloodstream, and growth and survival in blood and filtering organs [8]. The primary site can also serve as a reservoir for the pathogen that intermittently re-seeds the bloodstream and prolongs systemic infection. We have previously determined that *Enterobacterales* species replicate rapidly in the liver and spleen during bacteremia [9]. For some tissue and species combinations, bacterial burdens decrease over time reflecting an ability of the host immune system to overcome this rapid proliferation, while in other instances bacterial growth outpaces clearance and bacterial numbers rapidly expand. This balance between a bacterium's ability to proliferate and withstand immune-mediated clearance can be collectively described as infection fitness.

To define the genetic requirements for "bacteremia-fitness," we previously performed a series of transposon sequencing (Tn-Seq) studies in which bacterial genes disrupted by transposon insertion mutations are assessed for their contribution to bacteremia-fitness [10–13]. These studies have resulted in the identification and characterization of fitness genes ranked by impact for four *Enterobacterales* BSI pathogens; *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Serratia marcescens*. Fitness gene identification has also allowed us to build models of metabolism and characterize bacterial pathways required for replication and survival in the host, enabling opportunities for the development of novel therapeutic strategies against these antibiotic resistant pathogens. While the Tn-Seq studies have provided valuable insight on an organismal level, comprehensive information on shared and unique fitness strategies across Gram-negative species is lacking, due in part to the unique nature and depth of each transposon insertion library. By integrating the biological infection fitness data with comprehensive pan-genomic information on gene content, operon structure, and gene function across species we can gain a better understanding of infection processes for this important group of pathogens.

Our pan-genome pipeline [14] uses PanOCT [15] to cluster orthologous proteins to identify core, variable and singleton gene or protein clusters and provides the data structure to easily link gene functions such as GO terms and antimicrobial resistance (AMR) genes as well as linkage of genes into genomic islands. The core genome of a bacterial pathogen is the collection of genes shared by all or nearly all strains of a species. This core genome is generally enriched for critical functions including energy production, amino acid metabolism, metabolite transport, nucleotide metabolism, and translational machinery [16]. On the other hand, the accessory genome is the set of genes that

vary across strains of a species. These genes are often involved in protein secretion and defense against innate host immunity, as well as many niche-specific functions that include traits such as fimbriae, toxins, or iron acquisition systems, which reside in so-called “flexible genomic islands” or fGIs [17].

In this report, we first constructed individual species pan-genomes to identify genes shared within *E. coli*, *K. pneumoniae*, *C. freundii*, *S. marcescens*, and *Enterobacter hormaechei* genomes. Each species pan-genome represents the sum of the core and accessory genes for all strains of a species under study. To add biological context, known operon structures, virulence gene predictions, AMR genes and Tn-Seq bacteremia-fitness data were overlaid onto each pan-genome, visually depicted by using the program *PanACEA* [18]. Then we took each species' core genes to build a multi–species pan-genome, which identified the core genes shared across these species (*i.e.*, the core of cores). By integrating our multi-species pan-genome and genome-wide Tn-Seq fitness data, we were able to infer fitness genes in *E. hormaechei*, which lacked Tn-Seq data. We identified 373 protein clusters that were conserved in all five species and predicted to contribute to bacteremia in at least one of them. Applying a scoring rubric to these bacteremia protein clusters, which incorporated the magnitude of a cluster’s fitness defect as predicted by Tn-Seq, its operon localization, and published antibiotic susceptibility data, seven common bacteremia-fitness pathways were identified. Finally, to validate our findings within the prioritized list of bacteremia-fitness genes, the twin-arginine translocation (Tat) system was selected for validation of independent mutations for reduced fitness *in vivo* and for increased susceptibility to beta-lactam antibiotics *in vitro*. The TatC transmembrane protein strongly contributes to *C. freundii* bacteremia fitness [11] and was likewise predicted to be a bacteremia-fitness factor in *K. pneumoniae* [13],

but not in the *S. marcescens [10]*, *E. coli* [12] or *E. hormaechei*, which lacked Tn-Seq data. Independent insertional inactivation of *tatC* showed reduced fitness *in vivo*, confirming one of our bioinformatics fitness predictions, even in the three species lacking Tn-Seq evidence. The *tatC* mutants also showed increased susceptibility to beta-lactam and beta-lactam plus inhibitor that was restored following *tatC* complementation *in trans*. This raises the exciting possibility that inhibitors of these gene products could simultaneously decrease *in vivo* fitness and sensitize the bacteria to FDA-approved standard-of-care antibiotics. Combined, this study provides an atlas of gene conservation across the *Enterobacterales* and the fitness contribution of these genes to bacteremia, a powerful resource that can be leveraged to advance our understanding of pathogenesis and explore novel therapeutic targets.

Results

Species Pan-genomes. Protein-level pan-genomes of five common sepsis-causing *Enterobacterales* species were constructed for two key reasons. First, to integrate multiple data types and Tn-Seq datasets to decipher common bacteremia-fitness factors across species. Second, to generate core pan-genomes that can be used downstream to build a "Multi-Species Core" (MSC) pan-genome that represents highly conserved genes that may be targets of interest for future therapies that would treat infections from multiple species (**Fig 1**). Proteins from representative genomes of *E. coli*, *K. pneumoniae*, *S. marcescens*, *C. freundii*, and *E. hormaechei* were used to first build species pan-genomes using PanOCT separately within the JCVI pan-genome pipeline (**S1 Table, S1-5 Datasets**). Centroids (*i.e.*, orthologous protein cluster representatives) of core and accessory pan-genome clusters were searched against curated databases of bacteremia-fitness

genes as defined by transposon insertion-site sequencing (**S2 Table**), classic *E. coli* "essential" genes (**S3 Table**), virulence genes [19] (**S4 Table**), and AMR [20] genes (**Table 1**).

Table 1. Pan-genome Statistics

Pan-genome	#Genomes	#Clusters	#Paralogs (#clusters; fitness genes@)	#Core proteins	#Accessory proteins	#Singleton proteins	# core pan-chrom asmbls (cycle; chain)‡	#fGIs (cycle; chain)	#Bacteremia-fitness@ (core; accessory)	#Essential^ (core; accessory)	#VFDB^ (core; accessory)	#AMR (core; accessory)
*C. freundii**	28	13,825	698 (2,743; 3)	3,997	5,211	4,617	4 (2; 2)	2,336 (7; 2,329)	172 (90; 82)	659 (630; 29)	213 (140; 73)	142 (66; 76)
*E. hormaechei**	58	13,713	770 (2,730; NA)	3,821	5,601	4,291	1 (1; 0)	2,971 (13; 2,958)	NA	605 (583; 22)	239 (130; 109)	140 (62; 78)
E.coli/Shigella spp.*	281	37,889	3,279 (19,551; 57)	3,690	14,899	19,300	3 (1; 2)	13,438 (21; 13,417)	233 (117; 116)	755 (677; 78)	1,575 (158; 1475)	160 (67; 93)
*K. pneumoniae**	205	24,203	1,788 (8,006; 2)	4,453	4,970	14,780	1 (1; 0)	7,232 (20; 7,212)	56 (33; 23)	600 (573; 27)	492 (159; 358)	171 (68; 103)
*S. marcescens**	48	12,874	529 (1,705; 6)	4,077	4,984	3,813	3 (0; 3)	2,922 (6; 2,916)	201 (117; 84)	466 (459; 7)	192 (102; 90)	96 (56; 40)
Multi-species Core (70% cutoff)*	**5**	**7,332**	**155 (35; 20)**	**2,850**	**1,369**	**3,113**	**49 (0; 49)**	**1,649 (0; 1,649)**	**500 (424; 76)**	**571 (502; 69)**	**272 (122; 150)**	**87 (38; 49)**
Multi-species Core (100% cutoff)†	**5**	**7,332**	**ND**	**2,232**	**5,100**	**3,113**	**ND**	**ND**	**373**	**455**	**55**	**27**

***70% core definition; †100% core definition; ‡cycle = a circular pan-chromosome, chain = a linear pan-chromosome; @from Tn-Seq studies; ^Using BLASTP matches with a percent id of >70% and e-value < $1x10^{-5}$; NA = Not Applicable; ND = Not Determined.**

The core genomes of each species varied from 3,690 protein clusters in *E. coli* to 4,453 in *K. pneumoniae*. Based on 281 genomes, *E. coli* had the largest pan-genome, encoding 37,889 protein clusters, which is 8 times the number of proteins encoded by the average *E. coli* genome (*i.e.*, 4,661 proteins) (**Table 1, S1 Table**). *K. pneumoniae* had the second largest pan-genome with 24,203 protein clusters across 205 genomes, but the number of proteins of this pan-genome was 4.5 times that of the average *K. pneumoniae* genome (*i.e.*, 5,322 proteins). The remaining species' pan-genome number of proteins were between 2.7-3.0 times larger than their respective genome averages. The number of strain-specific protein clusters from the species pan-genomes ranged from a minimum of 0 to a maximum of 488 (*Escherichia*: 0 to 488, *Klebsiella*: 1 to 296, *Enterobacter*: 0 to 280, *Citrobacter*: 4 to 449, and *Serratia*: 0 to 449) (**S1 Table**), indicating that each species has a large and variable accessory genome.

Since our pan-genome software, PanOCT, does not collapse or discard paralogs, we were able to determine the extent of paralogy within each species pan-genome. Of the 37,889 total *E. coli* pan-genome protein clusters, 52% were paralogous, reducing the size of the pan-genome to 18,338 protein clusters, which is still considerably larger than the other species pan-genomes (**Table 1**). *Klebsiella* had the second highest number of paralogs (33%) followed by *Enterobacter*/*Citrobacter* (20% each) and *Serratia* (13%).

A total of 569 CARD/RGI-predicted AMR protein clusters were identified across all 4 species with Tn-Seq data (*i.e.*, 160 in *E. coli*, 171 in *K. pneumoniae*, 142 in *C. freundii*, and 96 in *S. marcescens*) (**Table 1**). The distribution of AMR genes across the core and accessory species pan-

genomes was roughly even, but with slightly more in the accessory pan-genome (45% core, 55% accessory) except for *S. marcescens* (58% core, 42% accessory). In stark contrast, genes essential for growth under laboratory conditions were skewed towards core (95% core, 5% accessory). Predicted virulence protein clusters varied across species with *E. coli* and *K. pneumoniae* having more clusters in the accessory pan-genome.

Fitness genes across species.

A total of 695 published bacteremia-fitness genes derived from four Tn-Seq studies [10–13] of *E. coli* (247 genes), *K. pneumoniae* (74 genes), *C. freundii* (172 genes), *S. marcescens* (202 genes) (**S2 Table)** were mapped to 662 species pan-genome clusters (**Table 1**). The distribution of species-specific fitness gene clusters across the core and accessory pan-genomes showed slightly more in the core pan-genome (54% core, 46% accessory), with *K. pneumoniae* (59% core, 41% accessory) and *S. marcescens* (58% core, 42% accessory) each having more bias toward core genes. This indicates that many genes important for causing bacteremia are highly conserved within each species and may be conserved across species as well.

Multi-species Pan-genome.

To identify genes conserved across species and determine if they are multi-species bacteremia-fitness factors, core protein sequences from each of the five species-level pan-genomes were combined and analyzed in a second pan-genome run - the MSC pan-genome. For this MSC pan-genome, clusters were labeled as core if they contained proteins from at least four out of five

species core pan-genomes. There were 2,232 clusters shared across all five species and 2,850 shared in four of five species (**Table 1,** 100% and 70% cutoff respectively**,** and **Fig 2A**). *Serratia* had the largest number of unique core protein clusters within its species core genome at 1,124 with *Klebsiella* having the second largest number of unique core clusters at 892 (**Fig 2A**). Despite *Escherichia* having the most accessory genes (**Table 1**) in the species pan-genome, it only has 385 unique core protein clusters within the MSC pan-genome. All genera but *Serratia* shared 344 clusters, supporting the newly proposed family reclassification of *Serratia* out of the *Enterobacteriaceae* family *[21]*.

Identification of common bacteremia-fitness and virulence genes

Next, we analyzed the MSC pan-genome for bacteremia-fitness genes (**S5 Table, S6 Dataset**). Using a criterion of identification by Tn-Seq in at least one species of the 7,332 proteins used in the MSC pan-genome run, 500 were identified as core bacteremia-fitness factors conserved in 4 of 5 species and 373 were conserved across all species (**Table 1, Fig 2B**). These bacteremia-fitness clusters included proteins from multiple species with published Tn-Seq results implicating them as fitness factors. For example, 11 clusters contained Tn-Seq fitness factors across 3 species: 7 from *C. freundii*, *K. pneumoniae*, and *S. marcescens*, and 4 from *C. freundii*, *E. coli*, and *S. marcescens* (**S6 Table**). Combining these pan-genome clusters with Tn-Seq data can be used to predict potential fitness genes in species and strains that lack experimentally derived data.

MSC pan-genome clusters with homology to known virulence factors were identified by searching against the virulence factor database (VFDB) [19]. Out of 272 total clusters assigned to virulence functions (**Table 1**), 55 (20%) were conserved across all five genera (**Fig 2C**). The maximum

number of shared virulence factors between four, three and two species was 49 (*Citrobacter*:*Enterobacter*:*Escherichia*:*Serratia*), 12 (*Citrobacter*:*Enterobacter*:*Escherichia*) and 7 (*Citrobacter*:*Escherichia*), respectively. *K. pneumoniae* had the highest number of species-specific virulence factors with 45. We then determined how many MSC pan-genome clusters predicted to be bacteremia-fitness factors were also homologous to known virulence factors (**S7 Table**). Of the 500 bacteremia-fitness clusters and 272 virulence clusters conserved in 4 or more species, 34 were predicted bacteremia-fitness factors associated with known virulence mechanisms (*e.g.*, LPS and capsular production for immune modulation, flagella and fimbriae for motility and adherence, and genes for the acquisition of iron and magnesium).

Using an orthologous approach to identifying multi-species fitness factors, we searched for core operons that contained distinct predicted fitness genes across species. We identified 369 operons that contained at least one bacteremia-fitness factor in the MSC pan-genome (**S8 Table**). Of the 67 operons that contained more than one bacteremia-fitness factor in the MSC pan-genome, 14 operons contained bacteremia-fitness genes from 3 species, 29 from 2 species, and 24 from one species (**S8 Table**). One prominent example was the operon encoding the Enterobacterial common antigen (ECA), a 12-gene locus with 9 bacteremia-fitness genes identified in either *S. marcescens*, *C. freundii*, or *K. pneumoniae* (**Fig 3**). The WzxE ECA flippase within this operon is a predicted fitness factor in all 3 species while WecA (UDP-*N*-acetylglucosamine-UP *N*-acetylglucosaminephosphotransferase) and RffH (glucose-1-phosphate thymidylyltransferase) proteins were fitness factors in two species.

To prioritize genes for future investigation and to identify conserved biological processes contributing to bacteremia we devised a scoring rubric using four genotypic and phenotypic characteristics (see Materials and Methods for details). The MSC pan-genome bacteremia-fitness

genes were individually scored based on the magnitude of their fitness defect predicted in any bacteremia Tn-Seq screen, whether the same gene was predicted to be a bacteremia-fitness factor in multiple Tn-Seq screens (*i.e.*, multiple species), if multiple bacteremia-fitness factors were encoded in the same operon, and whether mutation of that gene was previously found to increase antibiotic susceptibility of *E. coli* BW25113 [22,23]. Scores were summed in two ways: first as individual genes to distill the most meaningful bacteremia-fitness factors from the 500 total MSC pan-genome bacteremia-fitness clusters identified, and then as the total score of bacteremia-fitness factors encoded in the same operon to identify biological pathways of interest (**S9 Table**).

Application of this scoring rubric led to the identification of 32 and 73 individual bacteremia-fitness factors having scores two and one standard deviations above the mean cluster score, respectively (**S1 Fig**). Additionally, summation of the individual scores for bacteremia-fitness factors encoded within the same operon led to the identification of 11 operons with total scores 0.5 standard deviations above the mean operon score. Investigation of the gene functions encoded by these highly-scored individual bacteremia-fitness factors and operons revealed seven biological processes that are predicted to contribute to the full virulence capacity of *Enterobacterales* during bacteremia (**Table 2**). These processes include maintenance of the proton motive force, resistance to antimicrobial peptides and complement, protein and small molecule transport, DNA repair and homologous recombination, shikimate biosynthesis, global gene regulation (pre- and post-transcription), and resistance to oxidative stress. While not an exhaustive accounting of all biological processes that contribute to bacteremia or of the functions encoded within the top scoring genes and operons, these seven biological functions (**Table 2**) include, at a minimum, 19 of the 32 (59%) fitness genes with scores two standard deviations above the mean and 40 of the 73 (55%) fitness genes with scores at least one standard deviation above the mean (**S1 Fig**).

Additionally, these seven biological functions encompass genetic elements encoded by 10 of the 11 highest scoring bacteremia-fitness factor rich operons identified.

Table 2. Core Biological Processes Predicted to be Required for Full Virulence Capacity of *Enterobacterales* during Bacteremia using a Multifactorial Prioritization Scoring Rubric*

Function	Gene(s)
1. Maintenance of Proton-Motive Force across the inner membrane	
ATP Synthase	*atpA*[‡]*B*[†]*D*[†]*EG*[‡]*H*[†]
Electron Transport: Ubiquinone synthesis	*ubiC*[†]*GH*
Iron-Sulfur Cluster biosynthesis for Cytochrome maturation	*iscS*[‡]*U*[†]*A*[†], *hscB*[‡]*A*[†]
2. Resistance to Antimicrobial Peptides and Complement	
Modification of Lipid A of LPS	*arnAD*[‡]*E*[†]*F*[†]*T*
LPS inner core synthesis	*gmhB*[†]
Synthesis of Enterobacterial Common Antigen	*wecA(rfe)*[†], *wzzE*, *wecB*[†], *rffH(rfbA)*[‡], *wecE(rffA)*[†], *wzxE*[‡], *wecF*, *wzyE*, *rffM*
Sensitivity to antimicrobial peptides	*sapA*[‡]*CDF*
Periplasmic protease inactivates complement complex	*prc*[†]
3. Transport	
Phosphate (ABC) transport	*pstSC*[‡]*A*[‡], *phoU*[‡]
Twin arginine transporter	*tatC*[‡]
4. DNA repair and homologous recombination	
DNA recombination	*ruvA*[‡]*BC*[‡]
Tyrosine recombinase	*xerC*[‡]*D*[†]
5. Shikimate Biosynthesis	

Production of siderophores, aromatic amino acids, folates, and quinones (*e.g.*, *ubiGH*)	*aroACB*†*EK*†
6. Global gene regulation	
Aerobic respiration control protein	*arcAB*†
Nucleoid-binding regulator Fis	*fis*†
Nitrogen gene regulation sigma factor	*rpoN*‡
Glutamine synthetase and glutamine sensing	*glnA*†*D*†
mRNA regulation by binding sRNAs to target mRNAs	*proQ*‡
7. Oxidative stress	
Oxidative stress response	*apaGH, pdxA*‡*, sspA*‡

Genes listed here were predicted as a bacteremia-fitness factor in at least one Tn-Seq screen for four species (*E. coli, K. pneumoniae, C. freundii* and *S. marcescens*) in a murine model of bacteremia and are predicted to contribute to these seven biological processes. Genes were scored among all 500 MSC pan-genome bacteremia-fitness clusters in the four species. Genes followed by (‡) or (†) received scores >2 or >1 standard deviation above the mean gene score, respectively.

Validation of a representative core fitness gene function for virulence phenotype in a bacteremia mouse model. In our Tn-Seq screens, numerous genes have been predicted to represent fitness factors during experimental bacteremia. This is a first step in identifying relevant genes that are critical for survival in the bloodstream. To validate our findings within the prioritized MSC genes, the twin-arginine translocation (Tat) system was selected for further study. The Tat system, under Transport functions (**Table 2**), facilitates translocation of folded proteins across the cytoplasmic membrane in bacteria and while the system itself (TatABC) is widely conserved, the translocated proteins that serve as substrates for the system often vary between species [24] and may therefore alter the requirement for the translocation system in different organisms. The *tatC* allele was one of the highest-scoring predicted bacteremia-fitness factors (**Table 2** and **S9 Table**). The TatC transmembrane protein strongly contributes to *C. freundii* bacteremia fitness [11] and

was likewise predicted to be a bacteremia-fitness factor in *K. pneumoniae* [13] (**S2 Table**). However, neither the *S. marcescens [10]* nor *E. coli* [12] Tn-Seq findings identified TatC as a significant fitness factor (**S2 Table**), perhaps due to lack of sufficient representation of *tatC* mutants in the inoculum or the initial transposon library, or genuine species-specific differences in Tat requirements.

To directly test whether TatC was a conserved bacteremia-fitness factor among the remaining four species of interest, *tatC* mutants were constructed in *S. marcescens*, *K. pneumoniae*, *E. hormaechei* and *E. coli* followed by competition infections whereby the wild-type strain and the differentially marked *tatC* mutant strain were co-inoculated in a murine infection model. For all four tested species, the *tatC* mutant was significantly outcompeted by the wild-type strain when bacteria were inoculated into the bloodstream via tail vein injection (TVI), as indicated by competitive indices that were significantly below zero (**Fig 4A-D**). Furthermore, *tatC* was required in every tested organ via this model, demonstrating that folded protein translocation was universally important not only across species but within different infection niches.

Since the TVI route represents direct bacteremia, we additionally sought to determine how TatC contributes to disseminated infection via a bacteremic pneumonia model. Following retropharyngeal inoculation (RI), *S. marcescens* and *K. pneumoniae tatC* mutants were outcompeted in the lungs of infected mice (**Fig 4E-F**). For *S. marcescens*, an even greater competitive disadvantage was observed for bacteria that had escaped the primary infection site and disseminated to the spleen, liver, and kidneys (**Fig 4A and E**). This result was further supported by a trend toward lower recovery of the *tatC* mutant from the bloodstream and suggests that Tat translocation is critically important for escape from the lungs or survival in distal sites for this organism. Similarly, *K. pneumoniae tatC* mutants that disseminated from the primary infection

site were also significantly outcompeted by wild-type bacteria (**Fig 4B and F**). Together, these results establish the importance of the Tat translocation system, and very likely its substrate proteins, in Gram-negative bacteremia and demonstrate the value of comparing Tn-Seq fitness hits between species.

Contribution of conserved fitness genes to intrinsic antibiotic resistance. Previous work in *E. coli* revealed that many core genes had contributions to antibiotic resistance that were not predictable based on their known functions [22,23]. Genes that contribute to both bacteremia fitness and antibiotic resistance could be attractive therapeutic targets as disruption of their function in combination with antibiotic therapy would be expected to have synergistic efficacy. Therefore, we mapped published data on the antibiotic susceptibility of a comprehensive panel of *E. coli* mutants to multiple classes of antibiotics to our MSC pan-genome. *E. coli* mutants in *tatC* were previously shown to have increased susceptibility to beta-lactam antibiotics. Unlike *E. coli*, the species *K. pneumoniae*, *S. marcescens*, *E. hormaechei* and *C. freundii* have intrinsic resistance to ampicillin due to chromosomally-encoded beta-lactamases. To test whether disruption of *tatC* by either a transposon insertion (*tatC::tn*) or deletion (Δ*tatC::nptII*) could counteract this intrinsic resistance, we used Epsilometer tests to determine the minimum inhibitory concentration (MIC) of ampicillin or piperacillin-tazobactam for the wild-type and *tatC* mutants of these three species. The most striking finding was in *K. pneumoniae*, where disruption of *tatC* by either approach lowered the MIC to $\leq$ 8, which is a level that would be considered susceptible to ampicillin in clinical care of an infection, and is similar to disruption of the beta-lactamase itself (*bla::tn*) (**Table 3, Fig 5**). This increased susceptibility to ampicillin in *K. pneumoniae* was counteracted by provision of either *tatC* (pACYC*tatC*) or the *tatABCD* operon (pACYC*tatABCD*) on a multicopy

plasmid, but not the empty vector control (pACYCev). When *tatC* was interrupted in a carbapenem-resistant isolate of *E. hormaechei*, the resistance profile shifted from resistant to sensitive dose dependent (SDD) to piperacillin-tazobactam (**Table 3**). The resistance phenotype of the *tatC* mutant was returned to parental levels when *tatABC* was provided on a plasmid. In the case of *S. marcescens*, which has an extremely high resistance to ampicillin (> 256 µg/ml), mutation of *tatC* caused a substantial reduction in MIC, which could be rescued by provision of the *tatC* in trans from a multicopy plasmid (pBB26) and not the vector control (pBBR1MCS-5) (**Table 3**). In *C. freundii*, we tested piperacillin-tazobactam as a beta-lactam and beta-lactamase inhibitor combination without intrinsic resistance and found that a *tatC* mutation also lowered the MIC and this phenotype was likewise rescued by provision of *tatC* (pBB4 (*tatC*+)) in trans and not the vector control ((pBBR1MCS-5; **Table 3**). Combined with animal models of infection, these data demonstrate that *tatC* represents a conserved bacteremia-fitness factor that also contributes to intrinsic resistance to beta-lactam antibiotics in *Enterobacterales* and has the potential to restore the use of antibiotics to which these species have intrinsic or acquired resistance.

335 **Table 3. Susceptibility of *tatC* mutants to Beta-lactam antibiotics**

Species (strain)	Genotype	Antibiotic	Mean MIC (µg/ml)	CLSI Interpretation
K. pneumoniae (KPPR1)	wild-type	ampicillin	24.0	I
	wild-type + pACYCev		18.7	I
	***tatC*::tn**		8.0	S
	tatC*::*nptII		6.7	S
	ftn-e		3.0	S
	***tatC*::tn + pACYCev**		6.7	S
	tatC*::tn + pACYC*tatC		14.7	I
	tatC*::tn + pACYC*tatABCD		18.7	I
S. marcescens (UMH9)	wild-type	ampicillin	>256	R
	***tatC*::*nptII* + pBBR1MCS-5**		85.3	R
	***tatC*::*nptII* + pBB26 (*tatC*+)**		>256	R
C. freundii (UMH14)	wild-type	piperacillin-tazobactam	6	S
	***tatC*::*nptII* + pBBR1MCS-5**		0.75	S
	***tatC*::*nptII* + pBB4 (*tatC*+)**		5.3	S
E. hormaechei (CRE14)	wild-type	piperacillin-tazobactam	96	R
	***tatC*::*acc(3)IV*+ pBBR1Hyg**		16	SDD
	tatC*::*acc(3)IV*+ pBBR1Hyg_*tatABC		85.3	R

336 **S=sensitive, SDD=sensitive dose dependent, I=intermediate, R=resistant**

Discussion

In this report, we leveraged the JCVI pan-genome pipeline as a tool to integrate multiple disparate data types to identify key pathways and mechanisms used by *Enterobacterales* to survive in the bloodstream. Within the core genes (2,850/2,232, 70%/100% core, respectively) shared by *E. coli*/*Shigella spp.*, *K. pneumoniae*, *S. marcescens*, *C. freundii*, and *E. hormaechei*, statistically significant bacteremia-fitness factors were identified in core clusters (424/373, 70%/100% core, respectively) containing at least one bacteremia-fitness factor identified in any of the four Tn-Seq studies performed in our TVI model of bacteremia. Using a multifactorial scoring rubric, we propose prioritization of 73 of the 500 total conserved bacteremia-fitness factors found in the MSC pan-genome, that have scores at least one standard deviation above the mean gene score, for future investigation. Of these 73 prioritized fitness factor genes, 36 were predicted by Tn-Seq to contribute to bacteremia in at least two species and 41 were found to increase antibiotic susceptibility in *E. coli* BW25113 when mutated [22,23]. Summation of individual gene scores encoded in the same operon revealed 11 operons enriched for bacteremia-fitness factors with scores 0.5 standard deviations above the mean. Investigation of the biological functions encoded by these operons and high scoring bacteremia fitness factors revealed a minimum of seven common biological functions that significantly contribute to *Enterobacterales* fitness during bacteremia. These data support a model in which full virulence of *Enterobacterales* during bacteremia in-part requires the seven biological functions outlined in **Table 2** and discussed below.

To aid in the discovery of potential novel antibiotic targets to clear bacteremia, we conducted species-level and family-level pan-genomic analyses that identified the core pathogenic genome

of these *Enterobacterales* species. The identification of unique and common virulence determinants among these Gram-negative bacterial pathogens also provided substantial advances toward determining mechanisms of pathogenesis for these pathogens that disseminate to and survive in the bloodstream. By integrating the pan-*Enterobacterales* genome, genome-wide Tn-Seq fitness data, and published lists of essential genes [25–29], antibiotic mutational susceptibility [22], virulence [19] and operon data [30,31], we have identified 373 conserved fitness genes across all five species that were shown to be important in at least one species during experimental bacteremia. Inhibition of these bacteremia-fitness factors *in vivo* could allow the immune system to clear infecting bacteria more rapidly or even kill them directly. Furthermore, 95 mutants in these bacteremia-fitness genes also confer increased susceptibility to common antibiotics in a laboratory strain of *E. coli [22,23]*. This raises the exciting possibility that inhibitors of these gene products can simultaneously decrease *in vivo* fitness and sensitize the bacteria to FDA-approved standard-of-care antibiotics.

The most serious consequence of bacteremia, sepsis, is characterized by an overwhelming innate immune response to pathogen-associated molecular patterns (PAMPs) such as LPS, peptidoglycan, flagella, and other macromolecules produced by pathogenic bacteria that translocate into the bloodstream from the urinary tract, lung, wounds, intraperitoneal cavity, and the gut. Interaction of PAMPs with Toll-like receptors triggers a well-understood cascade that can lead to catastrophic events including coagulation, vascular permeability, and death [32]. In contrast, bacterial factors that allow survival of bacteria in the bloodstream are poorly understood. The literature has primarily focused on *E. coli* for Gram-negative pathogens and includes surveys of isolates that identify their phylogenetic group and their “traditional” virulence factors assayed

by PCR of relevant genes. These studies implicate ExPEC strains of the B2 and D phylogroups (although other groups are also found in lower proportions) [33,34]. Virulence factors associated with bacteremic isolates include *papC, afa, sfa* (adhesins), *fyuA, iutA, iroN* (iron acquisition), microcins (small bacteriocins), *cnf1* (cytotoxic necrotizing factor), and *hlyA* (hemolysin) [34–39]. Isolates are more often serum-resistant than control strains and represented by certain O-antigen types [40,41].

Mechanistic studies for evaluation of specific genes in animal models of bacteremia have been conducted only sparingly [42]. Investigations of fitness genes for other Gram-negative species in bacteremia are rare [43,44]. In practice, characteristics of Gram-negative bacterial species causing bacteremia have been hard to identify. However, Tn-Seq has become a powerful tool to simultaneously assess the contribution of all non-essential genes in animal models of infection. The technique, employed prior to conducting this project, provided a standard approach that we have exploited. While there have been more than 200 published Tn-Seq studies dealing with bacterial pathogens *in vitro* or *in vivo* (*e.g.*, [12,39,45–81]), only a fraction of these have addressed Gram-negative pathogens in animal models of infection; for example: [12,39,45,52,56,58,61,68,70,71,75,79,81–94]. Indeed, only a few studies [68,95,96], beyond our work [10,12,13,83,97–99], have sought fitness and virulence factors in models of bacteremia.

Several conserved and shared *Enterobacterales* genes were identified, following integration of Tn-Seq with operon structure and AMR, that fall into common bacterial cell functions of which we discuss seven (**Table 2**). These included 1) maintenance of proton-motive force across the inner membrane, 2) resistance to antimicrobial peptides and complement, 3) substrate transport, 4) genome maintenance, 5) shikimate biosynthesis, 6) global gene regulation, and 7) oxidative stress.

While it is beyond the scope of this study to discuss all these findings, it is instructive to summarize notable predicted bacteremia-fitness factors below in the context of the seven highlighted common bacterial cell processes that support survival of *Enterobacterales* species in the mammalian bloodstream.

Maintenance of proton-motive force. Tn-Seq studies of *Enterobacterales* species in the murine model of bacteremia clearly implicate the importance of genes that support aerobic respiration and maintenance of a proton-motive force. First, multiple hits in the operon encoding ATP Synthase underscore the importance of this enzyme, central to the chemiosmotic theory, as critical for infection. This function is clearly supported by electron transport which depends on synthesis of electron transport chain cofactors such as ubiquinone that are embedded in the cytochromes. In addition, this system is dependent on the biosynthesis of Fe-S clusters that carry electrons within cytochromes as critical to maintenance of proton motive force. The ISC system is encoded by the *iscRSUA-hscBA-fdx-iscX* operon and predominantly carries out the Fe-S cluster biosynthesis under normal conditions used for cytochrome maturation and proton motive force. Indeed, iron-sulfur (Fe-S) proteins contribute to several cellular processes that include respiration, nitrogen fixation, and gene regulation. *E. coli* and closely related enterobacteria possess two pathways [100]. While oxygen is tightly bound by hemoglobin within erythrocytes circulating in the blood, we nevertheless presume that this milieu represents an aerobic environment, and that the bacterial species undergo aerobic respiration and thus would require the activities of ATP synthase coupled with the electron transport chain.

Resistance to antimicrobial peptides, complement, and phagocytosis. Enterobacterial Common Antigen is a critical outer membrane component in *Enterobacterales* that may affect resistance to antimicrobial peptides and complement [101]. Interestingly, while identified by Tn-Seq in *K. pneumoniae*, *C. freundii*, and *S. marcescens* (**Fig 3**), none of the ECA genes were identified by Tn-Seq as fitness factors in *E. coli* suggesting that either the biological role of ECA during infection differs in this species or that ECA genes were not adequately targeted by transposon mutagenesis in *E. coli*. While this discrepancy is currently under investigation, the clear benefit of such a comparison is in forming testable hypotheses regarding the conserved function of ECA or other shared operons across Gram-negative bacteremia species. Combined, our findings provide strong evidence that production of ECA is an important fitness determinant since mutation of even a single gene in the processive ECA biosynthesis pathway may result in a lack of ECA production or its proper localization in the bacterium. Clearly, surface-exposed macromolecules on the Gram-negative outer membrane, when present or absent or biochemically modified, may modulate surface charge. These changes in charge can confer, in some cases, resistance to binding by complement components and host-derived antimicrobial peptides, thus conferring protection from these host innate immune responses.

Likewise, GmhB (YaeD) is a multi-species fitness factor involved in biosynthesis of the LPS inner core. Specifically, GmhB is a D,D-heptose 1,7-bisphosphate phosphatase that is a member of the ADP-heptose enzymatic cascade [102]. While ADP-heptose is a part of the LPS inner core, it is also a soluble inflammatory mediator recognized by host cytosolic receptors [103,104]. GmhB has been validated as a bacteremia fitness factor in *K. pneumoniae*, *E. coli*, and *C. freundii [13,105]*. Interestingly, GmhB is dispensable for fitness in the lung but is required for spleen and liver fitness

during bacteremia. Thus, GmhB is likely linked to bacteremia fitness across species in a manner unique to bloodstream infection. Indeed, exponential replication in the spleen at 24 h during murine bacteremia [9] may require substantial LPS export. In the absence of GmhB, lower abundance of normal LPS may be produced, leaving Gram-negative species more susceptible to killing by phagocytosis by immune cells such as splenic macrophages.

Transport. Twin-arginine translocation system [106] represents perhaps the most critical transport system and fitness factor overall required for bacteremia. Specifically, *tatC* allele was demonstrated to make a significant contribution to the fitness of all four species tested in this study, as well as *C. freundii* [11], in a murine model of bacteremia (**Fig 4**). While it is possible that disruption of the TatC transmembrane component may have unintended consequences, such as altering bacterial envelope integrity, the most likely explanation for this shared phenotype is a requirement for appropriate localization of Tat-secreted proteins for infection fitness. Indeed, we have previously attributed at least some of the fitness defect associated with *C. freundii tatC* mutation to SufI loss of function [11]. Whether SufI is required for infection of these additional species remains to be determined. However, it may also be informative in future work to compare the repertoire of Tat-secreted proteins in each species using the conserved N-terminal twin-arginine motif, since it is additionally possible that the requirement for TatC is due to species-specific translocation of different proteins.

In contrast, the Pst phosphate transport system imports one critical nutrient and is also predicted to contribute to bacterial survival in the bloodstream. The *pst* operon encodes a high-affinity inner membrane ABC transport system of inorganic phosphate [107]. The system includes the permeases

PstA (C) and PstC (C, K), the regulator PhoU (C), the periplasmic phosphate binding protein PstS (C), and the ATP-binding protein PstB (not a predicted bacteremia-fitness factor). Phosphate concentrations are sensed by PhoBR, with PhoB as the histidine kinase and PhoR as the response regulator. The Pst system is required for signal transduction in response to inorganic phosphate, and a mutant in any of the genes causes constitutive expression of the Pho operon encoding alkaline phosphatase. This leads to pleiotropic effects in the cell that attenuate virulence and lower the MIC to polymyxin [108]. This includes reduction in the amount of capsule, decreased resistance to serum, antimicrobial peptides, acid and oxidative stress, and decreased production of type 1 fimbriae. Under phosphate starvation, bacteria modify their phospholipids, including lipid A. Pst mutants also lead to imbalance of cyclopropane and unsaturated fatty acids and an overall increase in outer membrane permeability. Some components of the Pst system (*e.g.*, PhoA, PhoE) have detectable antiserum in previously infected patients with hemolytic uremic syndrome. The Pst system, clearly implicated as a requisite mechanism for phosphate import and predicted as a fitness operon in two of the four species, could also represent an attractive target for novel therapeutics, since blocking of any of the proteins could cause pleiotropic attenuation of virulence and fitness.

DNA repair and recombination. DNA recombinases XerC and RuvABC were predicted to be critical fitness factors for *Enterobacterales* species. RuvABC enzyme complexes are involved in DNA recombination and repair and facilitate Holliday junction branch migration and resolution [109,110]. RuvABC repairs damage by catalyzing homologous exchanges between damaged and undamaged DNA [111]. XerC responds to DNA damage mediated by reactive oxygen species produced by neutrophils during infection and thus defends against the innate immune system in the bloodstream. The recombinase is also required for induction of the SOS response, with the

result that a mutant defective in *xerC* is more susceptible to a range of DNA-damaging antibiotics including ciprofloxacin and immune-mediated killing [112].

Shikimate biosynthesis. Iron acquisition is required by Gram-negative bacilli and all bacterial species to survive in a host where free iron is found in negligible concentrations. While some iron salt import systems contribute (*e.g.*, ferric citrate uptake [113]) to iron acquisition, the highest affinity and effective iron acquisition systems include siderophores that bind iron with extraordinarily high affinity. Genes that contribute to the synthesis of specific siderophores including enterobactin and salmochelin provide the ability to survive in the bloodstream. Shikimate biosynthesis [114], a seven-step enzymatic process that terminates with the production of chorismate, is one such pathway that contributes to the biosynthesis of some siderophores, like enterobactin [115]. Chorismate originating from the shikimate biosynthesis pathway is also a fundamental precursor for the biosynthesis of folates, aromatic amino acids, and the quinones; ubiquinone (mentioned above) and menaquinone, which contribute to aerobic- and anaerobic respiration, respectively. Alleles encoding five of the seven enzymatic steps of the shikimate biosynthesis pathway were predicted to contribute to bacterial fitness during bacteremia in at least one Tn-Seq study, with two alleles, *aroB* and *aroK*, being scored in the top 73 prioritized bacteremia-fitness factors (**S2 Table, S9 Table**). Strains carrying mutations within the shikimate biosynthesis pathway likely experience a fitness defect in the host resulting from the cumulative pleiotropic effects of folate deprivation, aromatic amino acid auxotrophy, loss of energy production through respiration, and iron starvation. In agreement with its prediction as a bacteremia-fitness pathway in our studies, the shikimate biosynthesis pathway has been shown to

play a fundamental role during infection for many bacterial pathogens in diverse disease models [83,116–118]**.**

Global Regulation. ArcA is a global gene regulator of aerobic respiration that contributes to the maintenance of a proton motive force when oxygen is available. In a recent study [119], we constructed deletion mutants in the aerobic respiration control sensor *arcA* across four species and confirmed that ArcA is a bacteremia-fitness factor in *K. pneumoniae*, *C. freundii*, and *S. marcescens* but not *E. coli*. ArcA is conserved across the *Enterobacterales* and clearly contributes to fitness in a murine bacteremia model [119]. Because this regulator, along with ArcB, mediates transition from aerobic to anaerobic growth, mutants in *arcA* do not grow well under anaerobic conditions. In addition, *arcA* mutants grew more slowly than isogenic wild-type strains when cultured under iron-limited conditions because of their inability to easily shift to anaerobic growth. Clearly ArcA, in conjunction with ArcB, promotes aerobic growth during bloodstream infections but also provides flexibility to shift to microaerobic or anaerobic growth when oxygen is more limited in the portions of blood-filtering organs distal from the artery [120].

Oxidative stress. The *pdxA* operon contributes to the alleviation of oxidative stress. This multi-functional operon contains six genes, *lptD-surA-pdxA-rsmA-apaGH*, five of which are predicted bacteremia-fitness genes. The operon begins with *lptD*, an essential gene encoding a protein for lipopolysaccharide assembly [121]. *surA* encodes a periplasmic ATP-independent chaperone protein involved in the proper folding of outer membrane proteins, including LptD [122]. *pdxA* encodes 4-Hydroxythreonine-4-phosphate dehydrogenase (UniProt P19624) as part of the pyridoxal -5’-phosphate (Vitamin B6) synthesis pathway that is required for many steps in

biosynthesis and transformations of amino acids [121]. Whereas most prokaryotes have a simple pathway encoded by *pxtST*, gammaproteobacteria have a complex pathway spread across multiple operons. In contrast, Vitamin B6 is an essential nutrient for humans. The *rsmA* (a.k.a. *ksgA*) locus encodes a (adenine-N6, N6-)-dimethyltransferase that methylates two adenines in the 16S ribosomal subunit as part of ribosomal biogenesis [123]. While the *apaG* gene product has an unknown function, the downstream *apaH* locus encodes a diadenosine tetraphosphatase (UniProt P05637) that degrades diadenosine tetraphosphate (Ap4A). Under disulfide stress, Ap4A accumulates as a by-product of amino-acyl tRNA synthetases and ApaH is deactivated. This leads to capping of RNA transcripts with Ap4A and other dinucleotides with adenosine (Np4A) that ApaH removes and targets the RNA for degradation. The overall function of this operon and the effect of these genes on bacteremia fitness is not entirely clear. It may be that this operon is required for fitness under stresses encountered *in vivo*, including oxidative/disulfide stress that would affect periplasmic protein folding (*lptD*, *surA*) and induce the *apaH* system to regulate RNA stability and amino acid usage (*pdxA*) in protein synthesis (*rsmA*). Whereas 5 of 6 genes of this operon are bacteremia-fitness factors, only 3 of 7 genes involved in Vitamin B6 synthesis are fitness factors, one of which (*serC* in *C. freundii*), is also needed for serine biosynthesis.

Conclusions. The use of our pan-genome pipeline as a powerful tool for integrating multiple data types and sources to infer biological functions was demonstrated in this study. Comparison of the four *Enterobacterales* Tn-Seq datasets revealed, at first inspection, limited common mutations and functional insight. It wasn't until those results were mapped to orthologous protein clusters and overlaid upon operon structure that common pathways emerged (**Table 2**). This enabled the prediction of common genes within this core pan-genome and brought to light mechanisms that

are essential for colonization of, or survival in, the mammalian bloodstream. This represents a step forward in the quest to identify novel targets of therapy against these deadly widespread infections often accompanied by development of life-threatening sepsis. Our prediction and subsequent validation of *tatC* as a common bacteremia fitness factor (**Fig 4**) and further demonstration of increased antibiotic susceptibility of strains carrying mutations in this allele (**Fig 5**, **Table 3**) supports the utility of our bioinformatic analyses. Having predicted the importance of many more genes (**S9 Table**) and pathways, highlighted in **Table 2**, it is now critical to follow up this study by investigating the importance of these bacteremia-fitness factor genes across representative species of the *Enterobacterales* and validate their attenuation in the murine model of bacteremia.

Materials and Methods

Species-level pan-genome analysis. The pan-genome for the five *Enterobacteriaceae* species were constructed using all the proteins from a curated list of publicly available genomes for each species using the JCVI pan-genome pipeline [14]. Specifically, we used the "user_core_cutoff" branch of the JCVI pan-genome pipeline found on GitHub [124]. These included the reference genomes used for Tn-Seq as well as genome quality, UMH and common laboratory strains, and epidemiological importance. The number of genomes in each species-level pan-genome run ranged from a minimum of 28 (*Citrobacter*) to a maximum of 281 (*Escherichia*) and consisted mainly of a single species per genus (*e.g.*, *C. freundii*, *E. hormaechei*, *K. pneumoniae*, and *Serratia marcescens*) where only the *Escherichia* pan-genome also contained *Shigella* spp. The default parameters for the pan-genome pipeline were used, except for a core cutoff (i.e., run_pangenome.pl --core_cutoff) of 70% of genomes as opposed to the 95% default.

***Enterobacteriaceae* Multi-Species Core (MSC) Pan-genome analysis.** The pan-genome pipeline results were used to create a core "pseudo-genome" for each species from the sequences of the core centroids and their location on the species-level consensus pan-chromosome as shown in the Core.att attribute output file [125]. These core pseudo-genomes from the five species were integrated in a MSC pan-genome using the same parameters including the 70% core cutoff. A consensus MSC pan-chromosome was constructed as described for the species-level pan-chromosomes. For all species pan-genomes and the MSC pan-genome, the TIGR roles and AMR were assigned to centroids using the best match for the HMMer3 [126] alignment of the TIGRFAMs [127] and the CARD [20], respectively as part of the pan-genome pipeline [14]. Other high-level gene features were integrated into the pan-genomes as described below.

Integration of Tn-Seq bacteremia-fitness genes with pan-genomes. As the original Tn-Seq fitness experiments were performed on single bacterial strains [10–13] whose genomes were included in the individual species pan-genomes (*e.g.*, *C. freundii* UMH14 biosample SAMN07729546, *E. coli* CFT073 biosample SAMN02604094, *K. pneumoniae* ATCC 43816 KPPR1 biosample SAMN02982872, and *S. marcescens* UMH9 biosample SAMN06164063), we were able to annotate the fitness gene clusters using their unique gene identifiers (**S2 Table**). If the species pan-genome bacteremia-fitness clusters were core (*i.e.*, orthologous sequences present in greater than 70% of the genomes), they were used in the MSC pan-genome and the MSC pan-genome clusters were labeled a fitness centroid.

Integration of operon structures, essential and virulence factors, and KEGG ontology.

Experimentally-determined operon structures for *E. coli* and *Klebsiella[30,31,128]* were obtained and mapped to the MSC pan-genome centroids with BLASTP (reciprocal best matches with e-value < 1×10^{-5}). For the *E. coli* operons, additional information, such as the Gene Symbol and the Blattner identifier, were also included (**S5 Table**). To include as many Blattner identifiers as possible for the MSC pan-genome clusters, in instances where the MSC clusters were not in operons, we used the Blattner identifier and the gene symbol of any gene from the genome of *E. coli* str. K-12 substr. MG1655 (BioSample SAMN02604091) with a good BLASTP match (same cutoff as above) to the MSC pan-genome clusters.

Genes that were found to be essential for growth of *E. coli* K-12 and derivatives under laboratory growth conditions were obtained from five different manuscripts/databases[25–29]. A unique set of "essential" genes was created (**S3 Table**) from these combined data sets and assigned the unique *E. coli* K-12 composite (ECK*) identifier. BLASTP was used to map the translated essential proteins to the *Escherichia* pan-genome centroids.

Similarly, we used the full set of protein sequences from the Virulence Factor Database (VFDB) (**S4 Table**) [19] downloaded on August 31, 2021, to do a similar BLASTP mapping to species-level pan-genome centroids. If the reciprocal best matches had an e-value < 1×10^{-5} and an additional requirement that the BLASTP match had to have more than 70% identity, then the centroid was annotated as "Essential" or a "Virulence Factor." The cluster annotation for the species-level pan-genome was passed to the MSC pan-genomes as described for bacteremia-fitness proteins above.

Additionally, KEGG annotations were mapped to the MSC centroids using hmmsearch v 3.3.2 [129] against the KEGG ontology dataset collected in KOfam [130] with an e-value < 1×10^{-5} (**S5 Table**).

Venn Diagrams. Venn Diagrams were created with R with the Venn Package [131], using data from **S5 Table.** For each of the Venn diagrams comprising **Fig 2,** a different list of MSC cluster IDs was used as input for each species in the MSC pan-genome to count the numbers of shared orthologs in: A) all MSC clusters; B) all MSC clusters with at least one Tn-Seq bacteremia-fitness protein; and C) all MSC clusters with at least one virulence factor protein.

Pan-chromosome visualizations. Browsable pan-chromosome visualizations, including the fitness, antimicrobial resistance, and TIGRFam annotations were constructed for each species pan-genome and the MSC pan-genome with PanACEA using default parameters (**S1-5 Dataset, S5 Table**) [18]. The species pan-genome comparison was constructed with data from **S5 Table**, using a polar graph from *ggplot2* [132]. Orthologs for each MSC cluster were identified in each species as well as the relative position in each species pan-genome relative to *dnaA*. MSC clusters that were not present in a species were noted with a gap. The radial position of orthologs on the figure was the relative location of the *Escherichia* ortholog, while the color was based on the location of ortholog in each species. The fitness clusters with an *Escherichia* ortholog are shown at the *Escherichia* ortholog position.

Prioritization of fitness genes and operons. We ranked MSC pan-genome bacteremia-fitness clusters and operons identified among the four species Tn-Seq data using a scoring rubric. The

rubric was based on four additive criteria: 1) The fold-change of the fitness defect associated with transposon interruption of a gene in any one species, 2) a single gene being identified as a fitness factor in multiple species, 3) whether multiple fitness factors were encoded in the same operon, and 4) a fitness factor single-gene-knockout was previously found to confer increased antibiotic susceptibility in *E. coli* BW25113 [22,23].

The top 60 bacteremia-fitness factors in any one species Tn-Seq data set (*i.e.*, the top 60 least fit transposon mutants) were awarded points based on the magnitude of the fitness defect determined in any one species. The top 1 - 20 least fit bacteremia-fitness factors were awarded 3 points, 21 - 40 were awarded 2 points, and 41 - 60 were awarded 1 point. All scores for each MSC bacteremia-fitness factor were then summed across species. (*e.g.*, *wzxE* received 6 total points in this category by accumulating 3 points each for providing the 6th and 15th greatest fitness defects when mutated in *S. marcescen*s UMH9 and *K. pneumoniae* KPPR1, respectively.) One item of note; *K. pneumoniae* KPPR1 bacteremia Tn-Seq [13] only identified 55 fitness factors, therefore this species was slightly underrepresented in this scoring category.

The MSC pan-genome bacteremia-fitness factor clusters were also awarded points based on their identification in multiple species Tn-Seq datasets, regardless of the magnitude of their defect. If a cluster member was identified as a bacteremia-fitness factor in three species or two species it was awarded 3 points or 2 points, respectively. (*e.g.*, *wzxE* received 3 points for being identified as a bacteremia-fitness factor in Tn-Seq datasets from *C. freundii* UMH14, S. *marcescen*s UMH9, and *K. pneumoniae* KPPR1.)

The MSC pan-genome bacteremia-fitness factor clusters were awarded points based on the identification of at least one other bacteremia-fitness factor encoded in the same operon, regardless of which species any one fitness factor was identified in. Fitness factors found encoded in an operon with 5 to 9 other fitness factors were awarded 3 points, those encoded with 3 to 4 other fitness factors were awarded 2 points, and those encoded in an operon with 1 to 2 other fitness factors were awarded 1 point. An additional point was awarded to fitness factors found in an operon where at least 50% of the genes in that operon were found to be fitness factors. (*e.g.*, *wzxE* is encoded in a 12 gene operon with 9 other fitness factors being identified in at least one species' Tn-Seq dataset. Therefore, *wzxE* received a total of 4 points; 3 points for being encoded in an operon with 5 to 9 other fitness factors and 1 additional point for greater than 50% of the genes in that operon being identified as fitness factors.)

Finally, MSC pan-genome bacteremia-fitness factor clusters were given additional points if mutation of that gene in *E. coli* BW25113 conferred increased susceptibility to an antibiotic [22,23]. Fitness factor clusters received another 2 points if mutation of the orthologous gene in the Keio collection was found to have increased sensitivity to any antibiotics, regardless of the mode of action.

Generation of *tatC* mutant derivatives and complementation constructs. Mutations in *tatC* were generated by lambda red recombineering using established protocols [10,11,83,133]. For *C. freundii*, *E. coli*, *K. pneumoniae,* and *S. marcescens*, the *nptII* kanamycin resistance cassette was PCR-amplified from pKD4[133,134] and targeted to an in-frame deletion of the *tatC* ORF via 5'-end homologous sequences (**S10 Table**). For *E. hormaechei*, the *acc(3)IV* apramycin resistance

cassette was amplified from pUC18-miniTn7T-Apr [135]. Recombination was facilitated by Red functions encoded on pKD46, pSIM19, or pSIM18 depending on the species mutated[133,134]. All mutations were confirmed by sequencing of PCR-amplified alleles or by PCR amplicon size and recombineering plasmids were cured prior to phenotypic analysis.

Genetic complementation of the *S. marcescens tatC* mutation was achieved by insertion of the UMH9 *tatC* ORF into plasmid vector pBBR1MCS-5 [136] via isothermal assembly, followed by sequencing verification. The resulting plasmid, pBB26, was transformed into *S. marcescens* via electroporation. Genetic complementation of the *K. pneumoniae tatC* mutation with the $pAYCYC_{tatC}$ plasmid was achieved by using SOE PCR [137] to connect the promoter of the *tat* operon to the *tatC* ORF. The $pACYC_{tatABCD}$ plasmid insert was made by amplifying *tatABCD* including its native promoter. The amplicons were inserted into pACYC184 at the *Hin*dIII and *Sal*I sites and the resulting plasmids were sequenced for verification. Correct plasmids were transformed into *K. pneumoniae* via electroporation. The *E. hormaechei tatC* mutant was complemented by insertion of wild-type *tatABC* into pBBR1Hyg, a hygromycin resistant derivative of pBBR1MCS-5 (this study), by isothermal assembly. The resulting plasmid, pBBR1Hyg_*tatABC* was transformed into *E. hormaechei* by electroporation.

Murine bacteremia models. All experiments involving the use of laboratory animals were conducted with protocols approved by the University of Michigan or Michigan State University Institutional Animal Care and Use Committee and were performed in accordance with the Office of Laboratory Animal Welfare guidelines. Wild-type and *tatC* mutant derivatives were cultured separately in LB medium, then washed with PBS and normalized to the final density prior to

mixing 1:1. Tail vein injections were performed on 7-8 week old C57BL/6J mice as previously described for all tested species [9,42] and organs were collected for homogenization and CFU determination at 24 h post-injection. For the bacteremic pneumonia model, 1×10^7 CFU *S. marcescens* or $1x10^6$ CFU *K. pneumoniae* was administered retropharyngeally into lightly anesthetized mice, as previously described [83]. Mice were sacrificed at 24 h post-inoculation for CFU determinations. Competitive indices for both infection models were determined as the ratio of mutant/wild-type bacteria after 24 h compared to that same ratio in the inoculum.

Determination of minimum inhibitory concentration. The minimum inhibitory concentration (MIC) of ampicillin and piperacillin-tazobactam was determined using Epsilometer (E-test) strips. This experiment was carried out following the guidelines of the Clinical Microbiology Procedure Handbook [138]. Bacteria were cultured on Lysogeny Broth (LB) agar, then inoculated in 3 mL of LB broth the following day. After reaching OD_{600} of 0.1, cultures were spread onto Mueller-Hinton agar using a sterile cotton-swab and allowed to dry. Then E-test strips were placed on the dried plates and incubated at 37°C overnight. The MIC was determined by observing the point at which the zone of clearing touches the test strip. The Clinical and Laboratory Standards Institute (CLSI) interpretations were obtained from *Performance Standards for Antimicrobial Susceptibility Testing* [139].

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Figure Captions

Fig 1. Funnel Chart Detailing the Flow of Bioinformatics Processes used in this Study. The chart is read from top to bottom beginning with the numbers of genomes from sepsis-causing *Enterobacterales* and ending with the number of predicted conserved fitness pathways. Pan-genomes were constructed using the JCVI pan-genome pipeline. Tn-Seq data was from previously published studies. Orthologous protein clusters were considered to be core if an ortholog was present in at least 70% of the input genomes.

Fig 2. Proteins Shared Across Five bacteremia-causing *Enterobacterales species* in the MSC Pan-genome. All orthologous protein clusters (**A**), the clusters labeled as "bacteremia-fitness" from Tn-Seq data (**B**), and the clusters labeled as "virulence factor" with matches to the VFDB (**C**) from the MSC pan-genome were binned, counted, and placed into a Venn diagram by whether clusters contained proteins from *C. freundii*, *E. hormaechei*, *E. coli/Shigella* spp., *K. pneumoniae*, and *S. marcescens*. Singleton clusters, representing species-specific core genes, are noted in the outermost ellipses of the Venn diagram. The Venn diagram is not to scale.

Fig 3. The ECA operon is a shared bacteremia-fitness locus between species. Genes within the conserved ECA operon are represented by arrows and colored lines indicate genes that were identified as significant bacteremia-fitness genes in previously published Tn-Seq studies.

Fig 4. Validation of *tatC* contributions to bacteremia fitness. Mice were inoculated via tail vein injection (TVI) or retropharyngeal inoculation (RI) with a mixture of wild-type and *tatC* mutant

derivatives of *S. marcescens* (A and E), *K. pneumoniae* (B and F), *E. coli* (C), or *E. hormaechei* (D). Viable bacteria were enumerated in the spleen (S), liver, (L), kidneys (K), lungs (Lg) or blood (B) at 24 h post-inoculation and the relative fitness of each strain was determined as the log competitive index. Only *S. marcescens* was assayed in the kidneys based on previously determined infection profiles [8]. Mean indices (solid line) that deviated significantly from the hypothetical value of zero (dotted line), which represents neutral fitness, were determined by one-sample t-test: * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), **** ($P<0.0001$). Red symbols designate samples from which *tatC* mutant bacteria were at or below the limit of detection.

Fig 5. Disruption of *K. pneumoniae tatC* confers susceptibility to ampicillin. Epsilometer (E-test) results for ampicillin are shown with minimum inhibitory concentration (MIC) at the point the zone of clearing touches the test strip for A) WT KPPR1 + pACYCev (pACYC184), B) KPPR1 *bla* transposon mutant VK055_0891_bla, C) KPPR1 Δ*tatC* + pACYCev and D) KPPR1 Δ*tatC* + pACYC*tatC*.

Supporting information

S1 Table. Genomes Used in this Study.

S1 Dataset. *E. coli* PanACEA Output.

S2 Dataset. *K. pneumoniae* PanACEA Output.

S3 Dataset. *E. hormaechei* PanACEA Output.

S4 Dataset. *C. freundii* PanACEA Output.

S5 Dataset. *S. marcescens* PanACEA Output.

S2 Table. Tn-Seq Fitness Genes used in this Study.

S3 Table. *E. coli* Essential Genes used in this Study.

S4 Table. Virulence Genes used in this Study.

S5 Table. Multi-species Core Pan-genome Clusters.

S6 Dataset. MSC Pan-genome PanACEA Output.

S6 Table. Tn-Seq Fitness Factors Shared Across 3 Species.

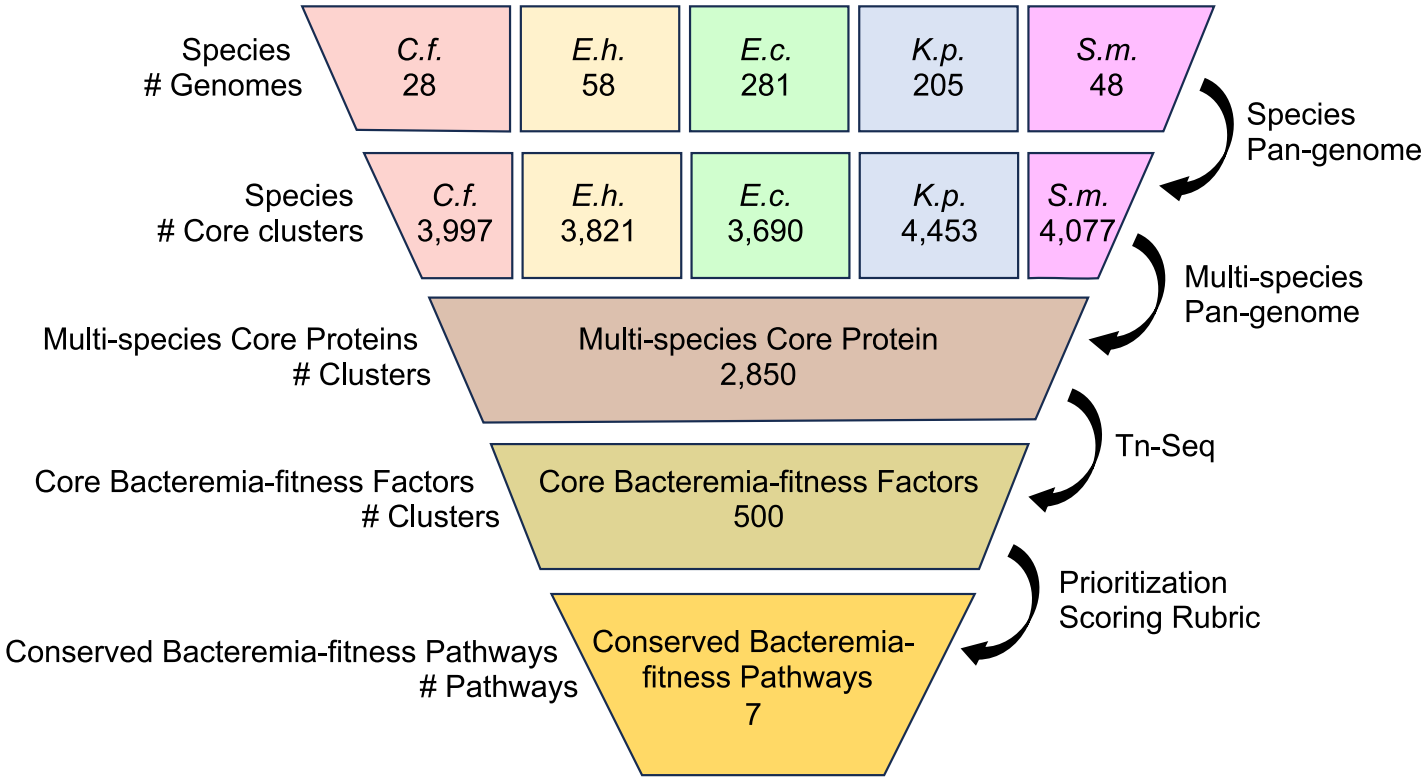
S7 Table. Bacteremia-fitness and Virulence MSC clusters.

S8 Table. Fitness Genes Mapped to Known Operons.

S9 Table. Ranking of Fitness Genes.

S1 Fig. Distribution of Bacteremia-fitness factor centroid scores. Summary of total centroid scores for all 500 bacteremia-fitness factors scored in S9 Table. The vertical dotted line depicts the mean total centroid score (2.52 points). Light gray box indicates the 73 bacteremia-fitness factors with a total centroid score 1 standard deviation (SD) above the mean (i.e., genes receiving at least 6 total points, and includes both † and ‡ genes highlighted in Table 2). The dark gray box indicates the 32 bacteremia-fitness factors with a total centroid score 2 SDs above the mean (i.e., genes receiving at least 8 total points, including only ‡ genes highlighted in Table 2).

S10 Table. Oligonucleotide Primers Used in this Study.


Species
Genomes
C.f.
28
E.h.
58
E.c.
281
K.p.
205
S.m.
48
Species
Pan-genome
Species
Core clusters
C.f.
3,997
E.h.
3,821
E.c.
3,690
K.p.
4,453
S.m.
4,077
Multi-species
Pan-genome
Multi-species Core Proteins
Clusters
Multi-species Core Protein
2,850
Tn-Seq
Core Bacteremia-fitness Factors
Clusters
Core Bacteremia-fitness Factors
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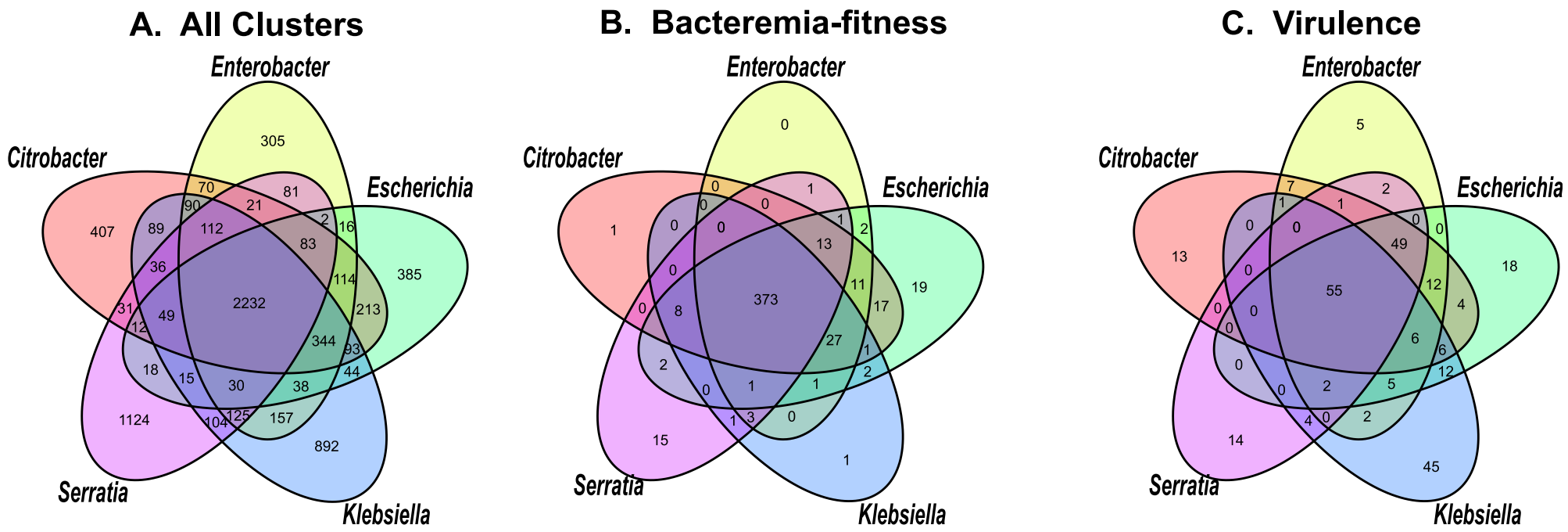
Prioritization
Scoring Rubric
Conserved Bacteremia-fitness Pathways
Pathways
Conserved Bacteremia-
fitness Pathways
7

Fig 2


A. All Clusters
Enterobacter
Citrobacter
Escherichia
Serratia
Klebsiella
305
407
385
1124
892
70
81
90
21
89
112
2
16
83
36
114
2232
31
49
213
12
344
93
18
15
30
38
44
104
125
157
B. Bacteremia-fitness
Enterobacter
Citrobacter
Escherichia
Serratia
Klebsiella
0
1
19
15
1
0
1
0
0
0
1
2
13
0
11
373
17
0
8
27
1
2
2
0
1
1
1
3
0
C. Virulence
Enterobacter
Citrobacter
Escherichia
Serratia
Klebsiella
5
13
18
14
45
7
2
1
1
0
0
0
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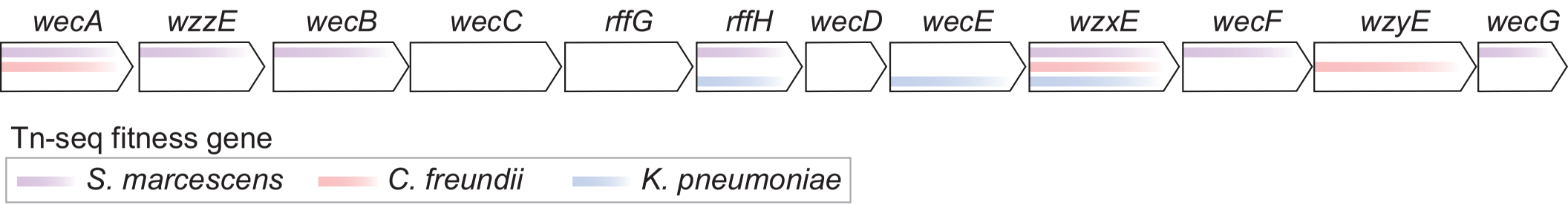
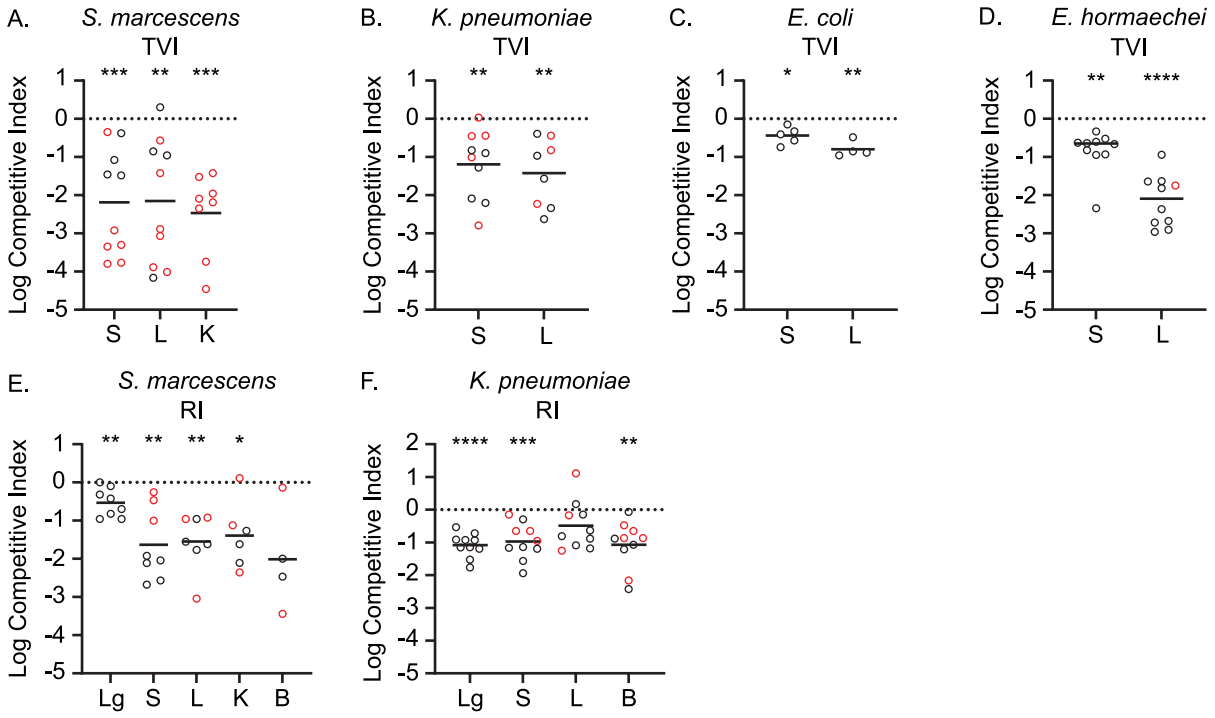

wecA
wzzE
wecB
wecC
rffG
rffH
wecD
wecE
wzxE
wecF
wzyE
wecG
Tn-seq fitness gene
S. marcescens
C. freundii
K. pneumoniae

Fig 4


A.
S. marcescens
TVI
Log Competitive Index
***
**
***
S
L
K
B.
K. pneumoniae
TVI
**
**
S
L
C.
E. coli
TVI
*
**
S
L
D.
E. hormaechei
TVI
**
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S
L
E.
S. marcescens
RI
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**
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*
Lg
S
L
K
B
F.
K. pneumoniae
RI
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***
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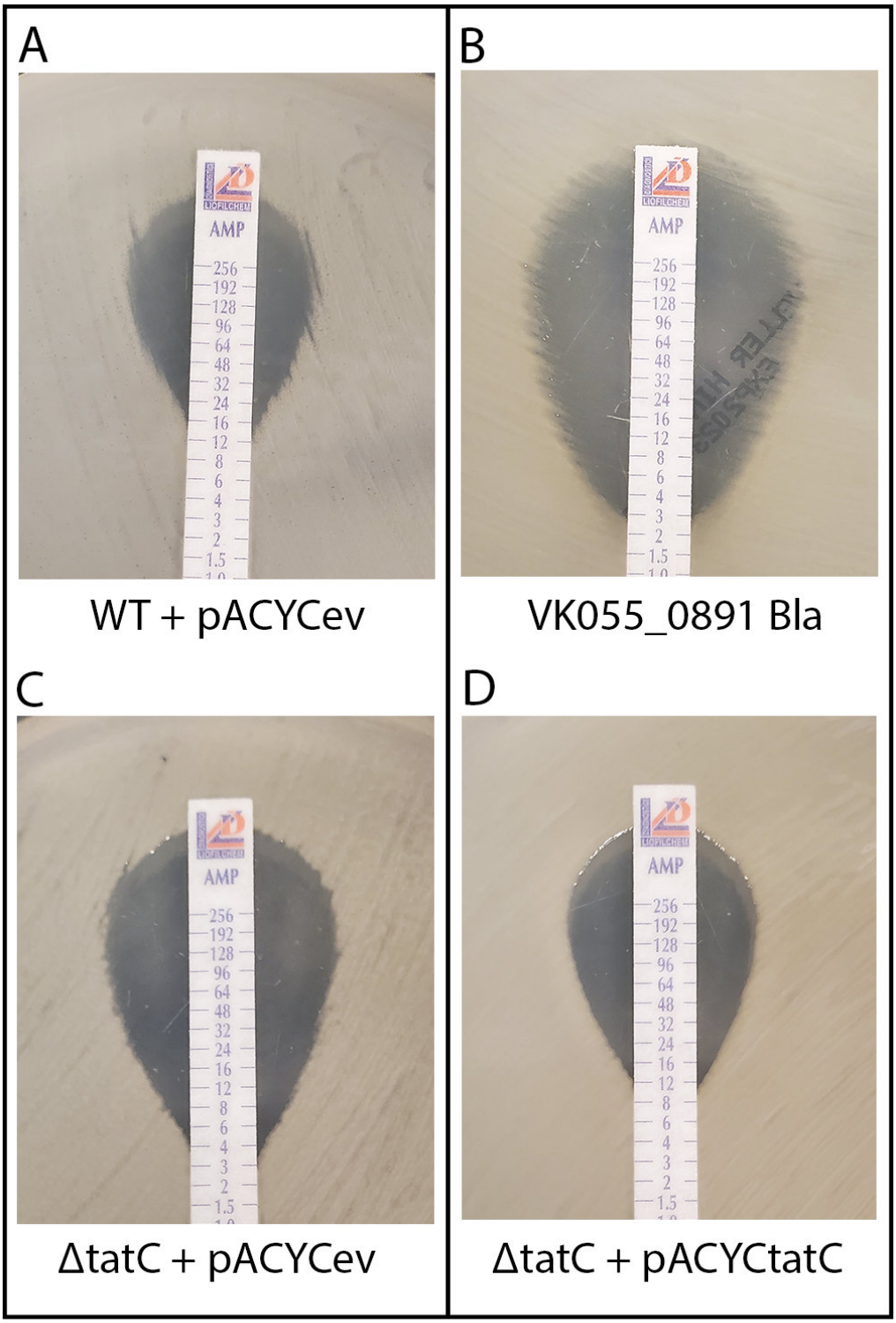
Lg
S
L
B

Fig 5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AMP
256
192
128
96
64
48
32
24
16
12
8
6
4
3
2
1.5
WT + pACYCev
B
AMP
VK055_0891 Bla
C
AMP
ΔtatC + pACYCev
D
AMP
ΔtatC + pACYCtatC