Genome-Wide Identification by Transposon Insertion Sequencing of *Escherichia coli* K1 Genes Essential for *In Vitro* Growth, Gastrointestinal Colonizing Capacity, and Survival in Serum

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ABSTRACT *Escherichia coli* K1 strains are major causative agents of invasive disease of newborn infants. The age dependency of infection can be reproduced in neonatal rats. Colonization of the small intestine following oral administration of K1 bacteria leads rapidly to invasion of the blood circulation; bacteria that avoid capture by the mesenteric lymphatic system and evade antibacterial mechanisms in the blood may disseminate to cause organ-specific infections such as meningitis. Some *E. coli* K1 surface constituents, in particular the polysialic acid capsule, are known to contribute to invasive potential, but a comprehensive picture of the factors that determine the fully virulent phenotype has not emerged so far. We constructed a library and constituent sublibraries of ~775,000 Tn*5* transposon mutants of *E. coli* K1 strain A192PP and employed transposon-directed insertion site sequencing (TraDIS) to identify genes required for fitness for infection of 2-day-old rats. Transposon insertions were lacking in 357 genes following recovery on selective agar; these genes were considered essential for growth in nutrient-replete medium. Colonization of the midsection of the small intestine was facilitated by 167 *E. coli* K1 gene products. Restricted bacterial translocation across epithelial barriers precluded TraDIS analysis of gut-to-blood and blood-to-brain transits; 97 genes were required for survival in human serum. This study revealed that a large number of bacterial genes, many of which were not previously associated with systemic *E. coli* K1 infection, are required to realize full invasive potential.

IMPORTANCE *Escherichia coli* K1 strains cause life-threatening infections in newborn infants. They are acquired from the mother at birth and colonize the small intestine, from where they invade the blood and central nervous system. It is difficult to obtain information from acutely ill patients that sheds light on physiological and bacterial factors determining invasive disease. Key aspects of naturally occurring age-dependent human infection can be reproduced in neonatal rats. Here, we employ transposon-directed insertion site sequencing to identify genes essential for the *in vitro* growth of *E. coli* K1 and genes that contribute to the colonization of susceptible rats. The presence of bottlenecks to invasion of the blood and cerebrospinal compartments precluded insertion site sequencing analysis, but we identified genes for survival in serum.

KEYWORDS *Escherichia coli*, essential genes, neonates, rat infection model, transposon sequencing

Early-onset sepsis and associated septicemia and meningitis are major causes of morbidity and mortality in the first weeks of life. In the developed world, encapsulated *Escherichia coli* and group B streptococci are responsible for the large majority

of these infections (1–3). Over 80% of *E. coli* blood and cerebrospinal fluid isolates from infected neonates express the α,2-8-linked polysialic acid (polySia) capsular K1 polysaccharide (4, 5), a polymer facilitating the evasion of neonatal immune defenses due to its structural similarity to the polySia modulator of neuronal plasticity in the developing human embryo (6). Infections arise due to colonization of the neonatal gastrointestinal (GI) tract by maternally derived *E. coli* K1 at or soon after birth, from where the bacteria invade the systemic circulation to gain entry to the central nervous system (CNS) (7, 8).

Essential features of human infection can be reproduced in the neonatal rat, enabling investigation of the pathogenesis of invasive neonatal infections by *E. coli* K1 (9–11). In susceptible 2-day-old (P2) rat pups, the protective mucus layer in the small intestine (SI) is poorly developed but matures to full thickness over the period from P2 to P9, coincident with the development of resistance to invasive infection from GI tract-colonizing *E. coli* K1 (12). Thus, oral administration of *E. coli* K1 initiates stable colonization of the small intestine in both P2 and P9 pups but elicits lethal systemic infection only in younger animals (13). In the absence of an effective mucus barrier at P2, the colonizing bacteria make contact with the apical surface of enterocytes in the midregion of the small intestine before translocation to the submucosa by an incompletely defined transcellular pathway (12). The bacteria subsequently gain access to the blood compartment by evading mesenteric lymphatic capture (10, 14). *E. coli* K1 cells strongly express polySia in blood (15), and the capsule may protect the bacteria from complement attack during this phase of infection by facilitating the binding of complement regulatory factor H to surface-bound C3b to prevent the activation of the alternative pathway (16, 17). Following hematogenous spread, the bacteria enter the CNS via the blood-cerebrospinal barrier at the choroid plexus epithelium to colonize the meninges (15). Some microorganisms that invade the CNS enter across the cerebral microvascular endothelium of the arachnoid membrane (18), although the restricted distribution of *E. coli* K1 within the CNS suggests that this is not a primary route of entry for this pathogen.

Only a limited number of pathogenic bacteria have the capacity to invade the CNS from a remote colonizing site, and the large majority elaborate a protective capsule that facilitates the avoidance of host defenses during transit to the site of infection (19). Although the polySia capsule is clearly necessary for the neonatal pathogenesis of *E. coli* K1 (11), the large majority of bacterial virulence factors that facilitate transit from the GI tract to the brain are unknown. A number of potential virulence factors associated with neonatal bacterial meningitis have been defined by phylogenetic analysis (20), and there is good evidence that the genotoxin colibactin and the siderophore yersiniabactin contribute to the pathogenesis of *E. coli* K1 in experimental rats (21–23); however, a more detailed understanding of virulence mechanisms of *E. coli* K1 invasive disease will present opportunities for new modes of therapy for these devastating infections.

Transposon insertion sequencing (24, 25), a combination of traditional transposon mutagenesis and massively parallel DNA sequencing, is a powerful tool for the genome-wide enhanced genetic screening of large pools of mutants in a single experiment. This method was recently used to determine the full complement of genes required for the expression of the K1 capsule by a uropathogenic *E. coli* isolate (26). This technique can be used to detect variations in the genetic fitness of individual mutants undergoing selection in colonized and infected hosts. There are a number of variations of this procedure, but they all rely on the creation of a pool of insertion mutants in which every locus has been disrupted at multiple sites; determination of the site of transposon insertion by sequencing of transposon junctions within chromosomal DNA before and after the application of selective pressure will identify mutants attenuated under selective conditions (27). Thus, genes that confer fitness during *Klebsiella pneumoniae* (28) and *Acinetobacter baumannii* (29) lung persistence, systemic and mucosal survival of *Pseudomonas aeruginosa* (30), and spleen colonization in the mouse by uropathogenic *E. coli* (31) have been identified by this approach. In this study, we

employ transposon-directed insertion site sequencing (TraDIS) (24) to interrogate a library of ~775,000 Tn*5* mutants or constituent sublibraries of *E. coli* K1 strain A192PP for genes essential for growth *in vitro* and for GI colonization, invasion, and systemic survival in susceptible P2 rat pups. In addition, we identified "bottlenecks" (32) to systemic invasion that restrict population diversity and limit the potential for transposon insertion site analysis of infection in neonatal rats with GI colonization.

RESULTS

Generation of a Tn*5* mutant library and identification of essential genes. To provide sufficient saturation density for the identification of *E. coli* K1 genes essential for growth *in vitro* and of those conferring fitness in a range of defined environments, approximately 300 individual pools, each with 1×10^3 to 5×10^3 transposon mutants of *E. coli* A192PP, were constructed and combined to form a library containing over 7.75×10^5 mutants. Linker PCR was performed on randomly selected mutants to confirm that Tn*5* had inserted into random genomic locations (see Fig. S1 in the supplemental material). TraDIS was performed on pooled but uncultured mutants to identify Tn*5* insertion sites within the 5.52-Mbp genome of A192PP (33). Sequences of indexed amplicons were determined, and 2×10^6 sequence reads containing Tn*5* were mapped onto the *E. coli* K1 A192PP genome. Reads mapped to 237,860 unique Tn*5* insertion sites and were distributed along the entire genome (Fig. 1A).

As the Tn*5* library contained a high transposon insertion density, genes with no or limited numbers of Tn*5* insertion sites are likely to be essential for growth in nutrient-replete media such as Luria-Bertani (LB) broth. We calculated insertion indices for each gene by normalizing the number of insertions in each gene by the gene length. Insertion index values for two technical replicates were highly correlated (Spearman's rho = 0.9589) (Fig. 1B). A density plot of insertion indices produced a bimodal distribution, with a narrow peak representing genes with no or a limited number of Tn*5* insertions and a broad peak containing genes with a large number of Tn*5* insertions (Fig. 1C); the former comprised genes that confer lethality when mutated, and the latter comprised genes that can be mutated without affecting bacterial viability. To identify genes that significantly lack Tn*5* insertions and therefore are essential for *in vitro* growth, gamma distributions from the density plot were used to determine $\log_2$ likelihood ratios. Examples of essential genes containing no or limited numbers of Tn*5* insertions are shown in Fig. 1D. A total of 357 genes were predicted to be essential for the *in vitro* growth of *E. coli* K1 A192PP, and these genes are shown in Table S1 in the supplemental material, together with KEGG (Kyoto Encyclopedia of Genes and Genomes) descriptors for genes involved in metabolic pathways.

COG (Clusters of Orthologous Groups) was used to identify the functional category of each gene essential for growth *in vitro* from the A192PP whole-genome sequence (BioProject accession number PRJEB9141). Genes involved in the ribosomal structure (11% of the total number of essential genes) and protein biosynthesis (15%) featured prominently and were significantly enriched in relation to their representation within the whole genome, as were genes encoding proteins for DNA replication (3%), cell wall (peptidoglycan and lipopolysaccharide) biosynthesis (6.25%), and membrane biogenesis (3%) (Fig. 2). Genes for protein secretion and export as well as ABC transporter genes were also well represented; the remaining essential genes were involved in a wide variety of cellular catabolic and anabolic functions. The list features 254 genes that were found by TraDIS (34) to be essential for the growth of an *E. coli* sequence type 131 (ST131) multidrug-resistant urinary tract isolate (from a total of 315 essential genes) in Luria broth. In a similar fashion, 253 genes determined to be essential for the growth of *E. coli* K-12 MG1655 in LB broth were also identified as being essential in the present study (Table S1); the K-12 study employed a comprehensive set of precisely defined, in-frame, single-gene deletion mutants (35) and not transposon insertion sequencing.

Maintaining Tn*5* library diversity. The polySia capsule is a major determinant of virulence in *E. coli* K1 and is central to the capacity of K1 clones to cause neonatal systemic infection (11, 36). PolySia biosynthesis imposes a substantial metabolic burden
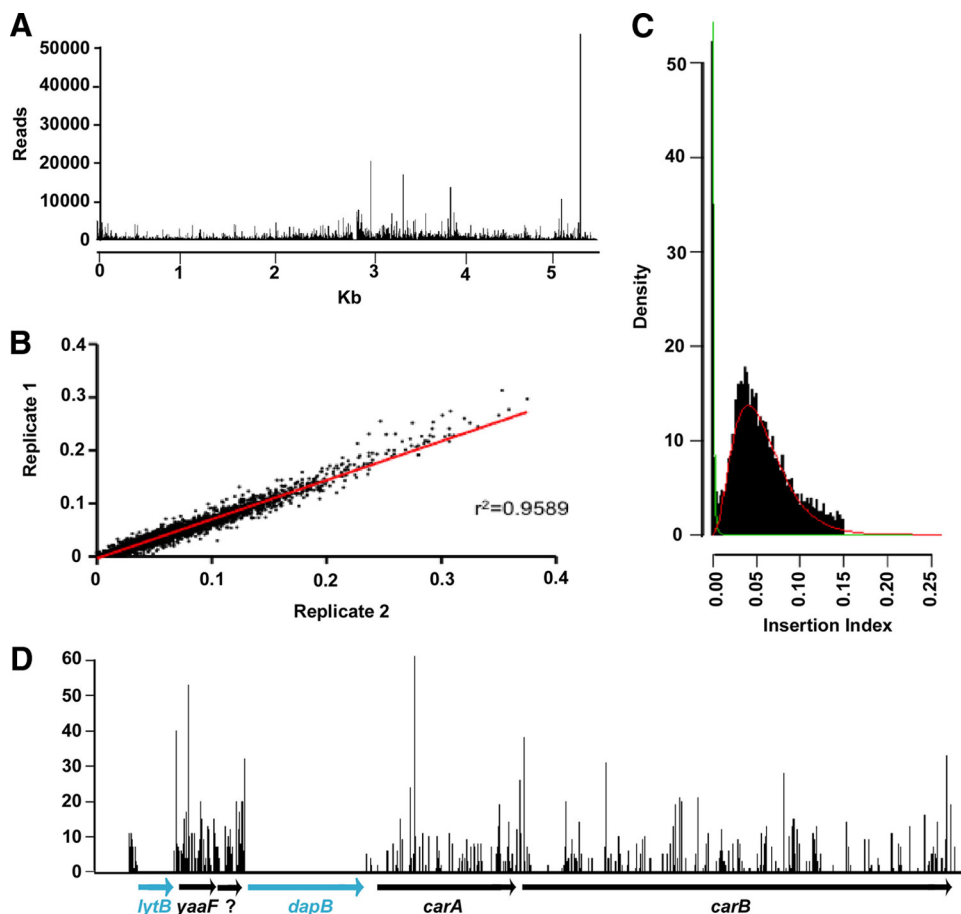
FIG 1 High-density transposon library for identification of genes essential for *in vitro* growth of *E. coli* K1 A192PP. (A) Distribution of Tn*5* insertions along the *E. coli* K1 A192PP genome. The number of sequence reads mapped to each single genomic location is plotted to show a representation of the entire genome. (B) Insertion index values for two biological replicates are strongly correlated. (C) Density plot showing the frequency of insertion index values for all genes. A bimodal distribution is evident, with the left peak representing "essential" genes in which a Tn*5* insertion is lethal for growth on selective Luria-Bertani agar; the right peak represents "nonessential" genes into which Tn*5* inserted without an induction of lethality. Green and red lines indicate gamma distributions used to estimate likelihood ratios and *P* values. (D) Tn*5* insertion site reads plotted to a 9-kb region of the *E. coli* A192PP genome. The height of each line on the *y* axis indicates the number of reads at each Tn*5* insertion site. The genes *lytB* and *dapB* possess no insertion sites, indicating that they are putative essential genes.

on producer strains (37). As TraDIS and other transposon insertion sequencing procedures generally employ growth in liquid medium for recovery and expansion of the output pool (38), we investigated the impact of batch culture on the expression of the K1 capsule within the Tn*5* library. The complete Tn*5* library was inoculated into LB broth and incubated for 8 h at 37°C, and the proportions of encapsulated and nonencapsulated A192PP bacteria were determined by susceptibility to the *E. coli* K1-specific bacteriophage K1E within the population. Nonencapsulated mutants initially comprised 4.66% of the bacterial population, but by the end of the incubation period, this value had risen to 98.24% (Fig. 3A). Growth rates in LB broth of *E. coli* A192PP and a nonencapsulated mutant of A192PP randomly selected from the Tn*5* library did not differ significantly (Fig. S2A).

The cultured Tn*5* library was avirulent, as determined by administration to P2 neonatal rat pups, whereas GI colonization with 2×10^6 to 6×10^6 CFU *E. coli* A192PP and the uncultured Tn*5* library was lethal. A similar colonizing inoculum of the cultured (8 h at 37°C) *E. coli* A192PP-Tn*5* library had no impact on survival, and all pups remained healthy over the 7-day observation period (Fig. 3B), even though all animals remained heavily colonized with K1 bacteria throughout the experiment (data not shown). Thus,
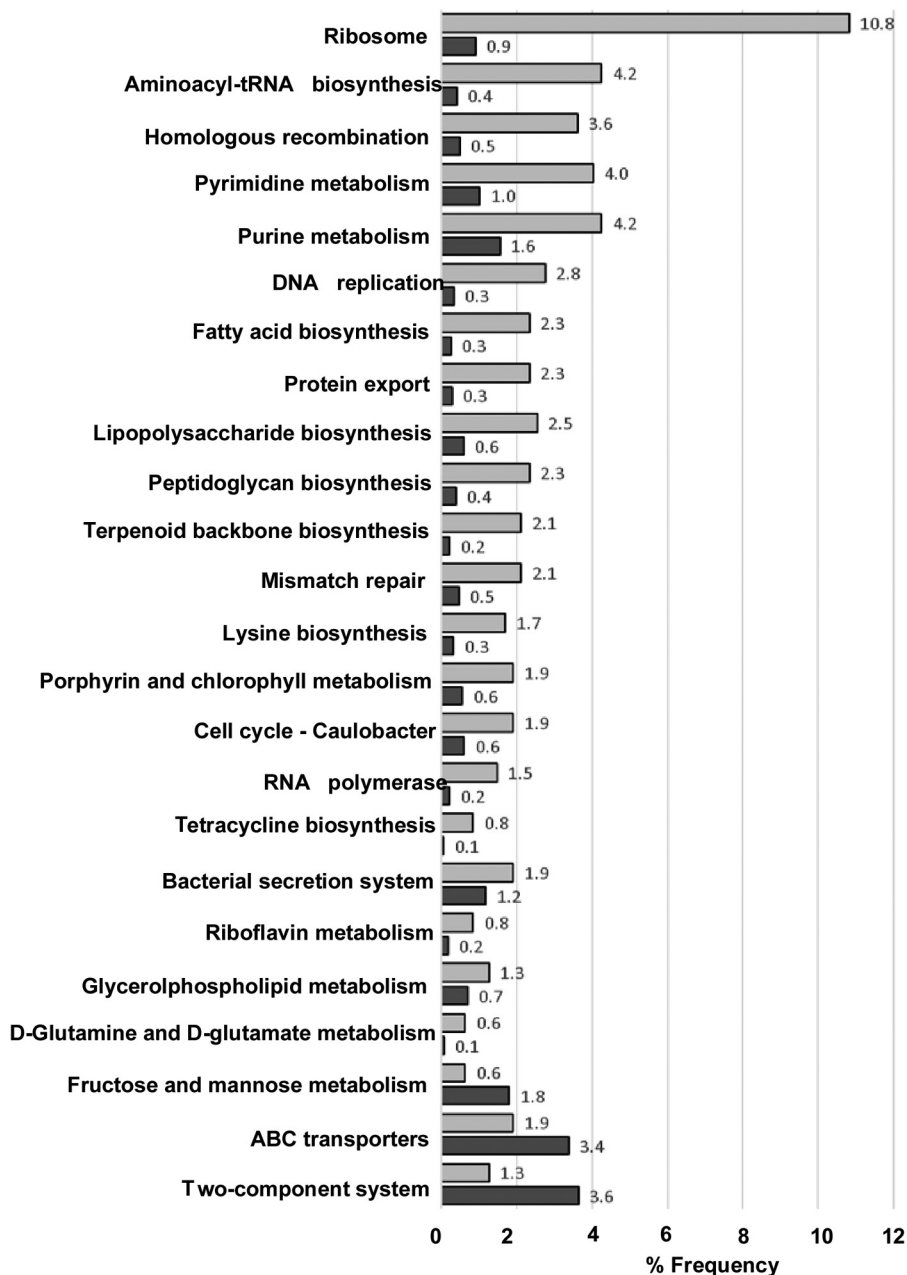
FIG 2 Essential *E. coli* A192PP genes in each selected KEGG functional ortholog (KO) category. Gene frequencies (light gray) (expressed as a percentage of essential coding DNA sequences [CDSs] for each category) are compared to their frequency within the whole genome (dark gray). KO = x/y, where y is the number of CDSs in the whole genome and x is the number of identified essential genes.

culture of the library prior to challenge resulted in the loss of phenotypic diversity and virulence. The complete Tn*5* library contained 2.81×10^5 unique Tn*5* insertions, of which 750 (2.66% of the bacterial population) possessed transposon insertions in genes determining capsule biosynthesis (data not shown). The probability that cultured sublibraries of more than 5×10^3 mutants contained a noncapsulated mutant was calculated to be ≥ 0.98 but was only 0.55 for sublibraries of 1×10^3 mutants. Low-complexity libraries of 10^3 mutants maintained virulence in P2 neonatal rat pups after culture, whereas more-complex libraries did not (Fig. S3), due to the absence of mutants lacking the capacity to express the polySia capsule within the inoculum. To minimize bias, in all subsequent experiments, libraries of sufficient complexity to
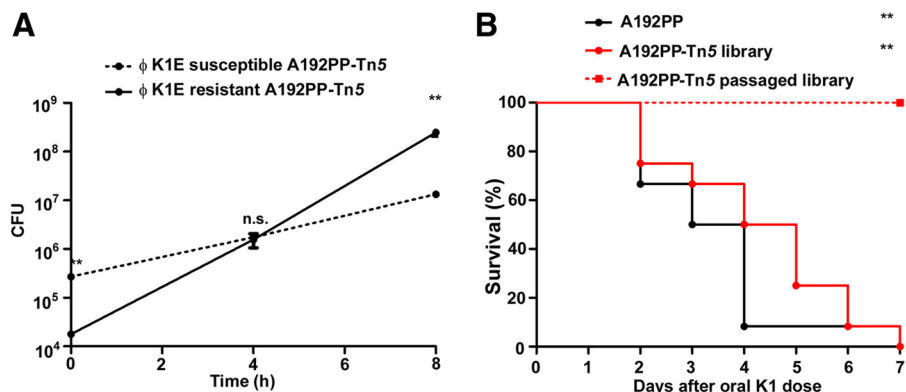
FIG 3 Culture of the *E. coli* K1 A192PP-Tn5 library results in loss of population diversity and enrichment of nonencapsulated mutants. (A) Changes in the proportions of encapsulated and noncapsulated *E. coli* A192PP bacteria during culture of the *E. coli* A192PP-Tn5 library in LB medium at 37°C (200 orbits/min) ($n = 3$) (± 1 standard deviation) ($P < 0.01$ by Student's *t* test). CFU of encapsulated and nonencapsulated bacteria were determined from the proportion of bacteria susceptible to the K1E bacteriophage. (B) Survival of P2 rats colonized with *E. coli* K1 A192PP, the uncultured *E. coli* A192PP-Tn5 library, and the cultured (LB broth for 8 h at 37°C) *E. coli* A192PP-Tn5 library. Pups ($n = 12$ for each group) were colonized with 2×10^6 to 4×10^6 CFU by the oral route. ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$ (as determined by a log rank [Mantel-Cox] test to compare the survival of the cultured library with those of the wild-type strain and the uncultured library).

contain multiple numbers of nonencapsulated mutants were used; for experiments utilizing neonatal rats, the period between colonization initiation and tissue harvesting was kept to a minimum, and tissue homogenates were cultured directly on selective agar plates with no intervening liquid culture step.

Genes required for GI colonization. *E. coli* A192PP bacteria colonize the small intestine of neonatal rats following oral administration of the bacterial bolus, with 10^7 to 10^8 K1 bacteria/g intestinal tissue persisting for at least 1 week (12, 13). Translocation of the neonatal pathogen to the blood compartment via the mesenteric lymphatic system occurs predominantly, and in all likelihood exclusively, across the epithelium of the midsection of the small intestine (MSI), even though the density of colonizing bacteria in this region of the GI tract is no higher than that within neighboring proximal small intestine (PSI) or distal small intestine (DSI) locations (12).

Few attempts have been made to determine the genes or gene products required by *E. coli* K1 for colonization of the GI tract (39). To prevent a loss of diversity of the *E. coli* K1 A192PP-Tn5 library, we minimized the period of colonization before sampling the *E. coli* K1 population of the MSI. The colonizing *E. coli* K1 population in proximal, middle, and distal regions of the small intestine did not expand beyond 4 h after the initiation of colonization (Fig. 4A); GI tissues were therefore excised at this time point. To identify mutants with a decreased capacity to colonize the MSI, P2 rats were fed 1×10^9 CFU of an *E. coli* K1 A192PP-Tn5 library containing 2×10^5 mutants, the pups were sacrificed after 4 h, and *E. coli* K1 bacteria in the MSI were enumerated. The bacterial load in rats colonized with the Tn5 library was comparable to that in rats colonized with the wild-type strain (data not shown). MSI tissues from four rats were pooled, homogenized, and cultured on LB agar containing kanamycin to ensure that the mutant frequency was not overestimated by the inclusion of measurements of DNA from dead bacteria; Kan^r colonies were then pooled, DNA was extracted, and the fitness of each mutant was determined by TraDIS. Input and output pools each comprised 2×10^5 CFU, and the ratios of input to MSI read counts were expressed as $\log_2$ fold changes. A wide distribution of fitness scores (40) was detected (Fig. 4B). The majority of transposon insertions did not have a strong negative or positive effect on colonization of the MSI. A total of 387 transposon insertions, within 167 genes, showed significantly decreased normalized read counts between input and output pools (negative $\log_2$ fold change and $P < 0.05$) (see Table S2 in the supplemental material). Of the 387 insertion
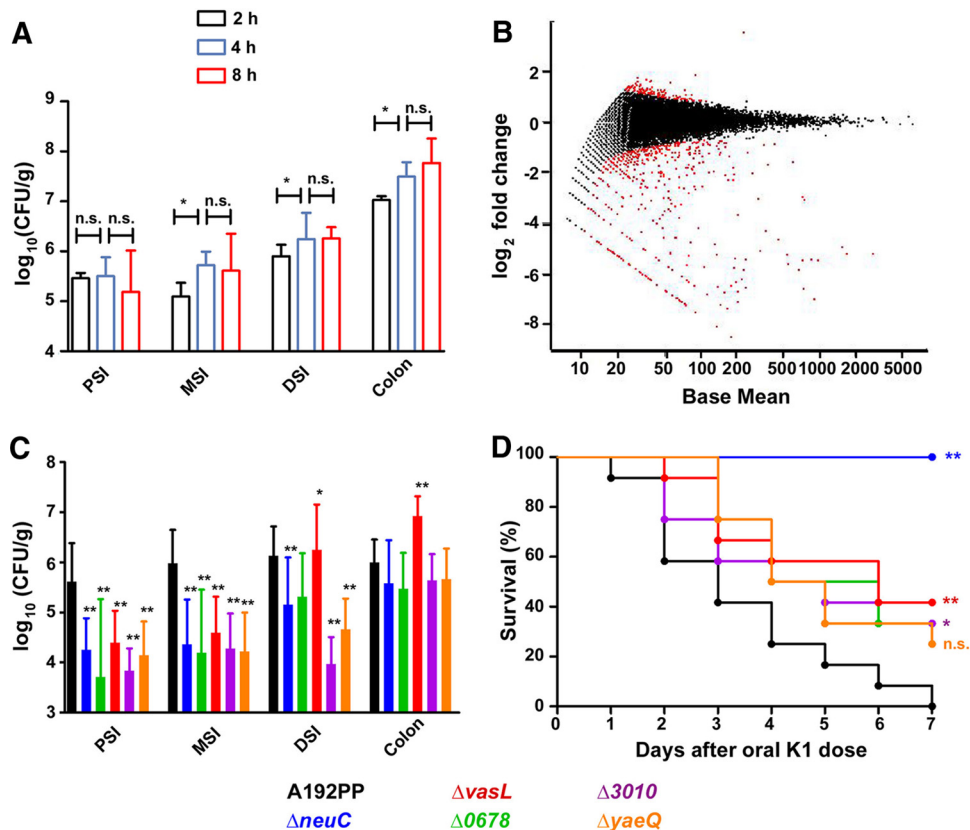
FIG 4 Identification of genes promoting GI colonization of *E. coli* A192PP in neonatal rats by using a high-density transposon library. (A) Colonization of PSI, MSI, DSI, and colon after oral administration of 2×10^6 to 6×10^6 CFU *E. coli* K1 A192PP to P2 pups. (B) Log$_2$ fold change and average Tn5 insertion site read abundance for each gene after MSI colonization of P2 rat pups ($n = 4$) over a 4-h stabilization period, expressed as an MA plot. An inoculum containing 2×10^4 unique *E. coli* K1 A192PP-Tn5 mutants was prepared, and 1×10^9 CFU were administered orally. *E. coli* colonies (2×10^5) were recovered from the inoculum (input pool) and from MSI homogenates (output pool) by culture on LB agar containing 50 μg/ml kanamycin. Red data points represent Tn5 insertion sites determined as being differentially expressed in the output pool compared to the input pool by using a negative binomial test with a false discovery rate of 0.1. (C) Colonization of P2 rat intestine by *E. coli* K1 A192PP and single-gene mutants. Bacteria (2×10^6 to 4×10^6 CFU) were administered orally to P2 rats ($n = 12$/group). Pups were sacrificed, and the *E. coli* K1 burden in intestinal sections was enumerated 24 h after the initiation of colonization. Parent and mutant strain CFU values were compared by using Student's t test (*, $P < 0.05$; **, $P < 0.01$). (D) Survival of P2 rats colonized with *E. coli* K1 A192PP and single-gene mutants. Bacteria (2×10^6 to 4×10^6 CFU) were administered orally to P2 rats ($n = 12$/group). ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$ (as determined by a log rank [Mantel-Cox] test).

sites, 180 were not detectable in the output pool, demonstrating a complete loss in the output pool. Many of these transposon insertion sites occurred within the same gene (Table S2). For example, within the *neuC* gene, 70 unique transposon insertion sites were identified as being lost during colonization. Transposon-interrupted genes were identified as being important for colonization of the MSI and were grouped into seven arbitrary categories: (i) genes encoding surface structures, including pili; (ii) genes encoding secretory components; (iii) genes involved in intermediary metabolism; (iv) stress response genes; (v) cytoplasmic membrane (CM)-located genes; (vi) genes for iron acquisition; and (vii) others and hypothetical genes.

High proportions of mutations associated with a decreased MSI-colonizing capacity were located in genes affecting the biosynthesis of surface structures (Table S2). A few genes were involved in lipopolysaccharide (LPS) biosynthesis (*yrbH* and *yiaH*) and outer membrane (OM) protein biosynthesis (*ompG* and *ycbS*), but the majority affected the polySia capsule, with genes of the *neu* operon (41) accounting for 194 of the 387 colonization-attenuated mutants. There is some evidence that capsular polysaccharides may promote adhesion to biological and nonbiological surfaces during biofilm forma-

tion (37), but there has been little or no consideration of a role for capsules as mediators of GI colonization.

A limited number of genes associated with type II and IV secretion were identified as being required for colonization of the MSI; these multiprotein complexes translocate a wide range of proteins and protein complexes across host membranes (42, 43) and are implicated in adherence and intestinal colonization by enterohemorrhagic *E. coli* in farm animals (44). Genes for the assembly of pilus proteins, including some carried on the *tra* locus, which are likely to be located on plasmids that initiate conjugation, were also linked to colonization; pili are virulence factors that may mediate attachment to and infection of host cells (45). Colonization by both commensals and pathogens is dependent on nutrient scavenging, sensing of chemical signals, and regulation of gene expression as the bacteria adapt to a new and potentially hostile environment that in the case of *E. coli* K1 appears to rely on stress response genes such as *yhiM* (which encodes a protein aiding survival at low pH), the heat shock protein genes *clpB* and *yrfH*, as well as DNA repair genes. A large number of genes encoded enzymes involved in the metabolism of sugars (e.g., *gcd*, *rpiR*, and *glgC*), amino acids (*dadX*, *metB*, and *tdcB*), fatty acids (*yafH* and *fixA*), growth factors (*bisC*, *yigB*, and *thiF*), and other secondary metabolites (*yicP*). Transporters and permeases involved in central intermediary metabolism were also featured prominently: these proteins included permeases of the major facilitator superfamily (YjiZ), the hexose phosphate transport protein UhpT, the carnitine transporter CaiT, and a range of CM-located sugar transporters. Of note was the impact of a mutation of the *fucR* L-fucose operon activator on colonization; fucose is abundant in the GI tract, and the fucose-sensing system in enterohemorrhagic *E. coli* regulates colonization and controls the expression of virulence and metabolic genes (46). The availability of free iron is severely limited in the GI tract, and ingestion of iron predisposes an individual to infection (47); the importance of iron acquisition for *E. coli* K1 during GI colonization is reflected in the requirement for a number of genes related to iron uptake (e.g., *feoB* and *fepA*).

GI tract-colonizing capacity and virulence of single-gene mutants. To investigate the contribution of the polySia capsule to the colonization of the neonatal rat GI tract, we disrupted the *neuC* gene of *E. coli* A192PP using bacteriophage λ Red recombinase to produce a capsule-free mutant as judged by resistance to *E. coli* K1-specific phage K1E. We also produced other single-gene mutants for genes identified by the TraDIS GI screen: *vasL* (encoding a type IV secretion system protein), *yfeC* (predicted to form part of a toxin/antitoxin locus), and two genes with unknown function, *yaeQ* and A192PP_3010 (the latter is present in genomes of other extraintestinal *E. coli* pathogens, including IHE3034, UTI89, RS218, PMV-1, and S88). Growth rates of these mutants, in particular the capsule-negative *neuC* mutant (see Fig. S2B in the supplemental material), were indistinguishable from that of the *E. coli* A192PP parent in LB medium. All mutants were examined for their capacity to colonize the GI tract and cause lethal infection in P2 rat pups (Fig. 4C and D).

The *E. coli* A192PP parent strain or single-gene mutants (2×10^6 to 6×10^6 CFU) were administered orally to P2 rats; all members of a litter of 12 pups received the same strain. Pups were sacrificed 24 h after initiation of colonization, and *E. coli* K1 bacteria in the small intestine (PSI, MSI, and DSI) and colon were enumerated. The capacity of all mutants to transit through the upper portion of the alimentary canal, pass through the stomach, and colonize the small intestine was markedly inferior to that of the wild-type strain (Fig. 4C). Reductions in colonization of the PSI, MSI, and DSI by the mutants, including *E. coli* A192PPΔ*neuC*::*kan*, were significant, with the only exception being colonization of the DSI by A192PPΔ*yfeC*::*kan*, with no significant difference between the parent and mutant. Interestingly, no increases in the numbers of viable A192PPΔ*neuC*::*kan*, A192PPΔ*vasL*::*kan*, A192PPΔ*3010*::*kan*, and A192PPΔ*yaeQ*::*kan* bacteria recovered from the colon were noted to compensate for reductions in the colonization of the small intestine. There was a significant increase in the colonic burden of viable A192PPΔ*yfeC*::*kan* bacteria compared to that of bacteria of the

parent strain. We established previously (12) that *E. coli* A192PP transits to the blood circulation via the mesenteric lymphatic system by exploiting a vesicular pathway through the GI epithelium only at the MSI. As numbers of mutant bacteria colonizing this region of the small intestine were much reduced compared to those of the parent strain, we determined the capacity of the single-gene mutants to elicit lethal systemic infection following GI colonization by oral administration of 2×10^6 to 6×10^6 bacteria at P2 (Fig. 4D). Four of the five mutants (A192PPΔ*neuC::kan*, A192PPΔ*vasL::kan*, A192PPΔ*3010::kan*, and A192PPΔ*yfeC::kan*) displayed significantly reduced lethal potential compared to that of the A192PP parent. The loss of capsule (A192PPΔ*neuC::kan*) resulted in a complete loss of lethality over the 7-day observation period. The administration of A192PPΔ*vasL::kan* elicited a lethal response in 41.6% of pups; 33.3% and 25% of pups survived after receiving A192PPΔ*3010::kan* and A192PPΔ*yfeC::kan*, respectively, at P2. For A192PPΔ*yaeQ::kan*, 75% of pups succumbed to lethal infection, but this did not reach levels of significance compared to the 100% lethality engendered by the A192PP parent ($P >$ 0.05). Overall, these data indicate that the TraDIS screen efficiently identified genes important for MSI colonization that impact pathogenic potential.

A bottleneck to infection in the neonatal rat prevents identification of genes for translocation across the gastrointestinal epithelium. Our initial intention was to exploit the high degree of susceptibility of P2 neonatal rats to systemic infection, sepsis, and meningitis following oral administration of an effective dose of *E. coli* A192PP bacteria in order to determine all genes required to enable the neonatal pathogen to overcome previously defined (12–15) physical and immunological barriers to invasion of the blood circulation and dissemination to the meninges. However, previous studies indicated that relatively few *E. coli* K1 bacteria migrate from colonized sites within the GI tract to the blood (10), constraining the genetic diversity of the translocated bacterial population and eliminating genotypes from the translocated gene pool in a stochastic manner that does not reflect the fitness of individual genes to contribute to genotypes with invasive potential (32). We therefore determined if there were bottlenecks that would compromise the identification of mutants with an attenuated capacity to translocate from the GI tract to the blood compartment; if any experimental bottlenecks are narrower than the complexity of the *E. coli* A192PP Tn*5* library, many relevant transposon insertion mutants will be lost entirely by chance (38). Furthermore, the existence of a restrictive bottleneck would limit the complexity of the library that could be used for TraDIS evaluation of populations colonizing the MSI (input pool) and reaching the blood (output pool).

We constructed the *E. coli* A192PPΔ*lacZ* mutant by bacteriophage λ Red recombineering and confirmed that there was no significant difference in lethal potential between *E. coli* A192PP and the *lacZ* mutant (Fig. 5A). We then used mixtures of parent and mutant bacteria to investigate the existence of bottlenecks that restrict translocation to the blood compartment. A 1:1 mixture (total of 2×10^6 to 4×10^6 CFU) of *E. coli* A192PP and A192PPΔ*lacZ* was administered orally to P2 rat pups, the animals were sacrificed after 24 h, and GI tissue homogenates were plated for the quantification of bacteria of each strain. The competitive index (CI), the ratio of input A192PP and A192PPΔ*lacZ* bacteria to output A192PP and A192PPΔ*lacZ* bacteria, was calculated for excised PSI, MSI, DSI, colon, and mesenteric lymphatic tissues and for blood. CI values for the PSI, MSI, DSI and colon were not significantly different from 1 (one-sample *t* test), indicating that the composition of the colonizing inoculum was maintained in each rat pup (Fig. 5B). However, there was more heterogeneity in CI values of bacterial populations from the blood, and in five pups, only one strain could be recovered from the blood (the parent strain only for four animals and A192PPΔ*lacZ* only for one animal). The highly restrictive bottleneck between GI epithelial transport and entry into the blood circulation supports the argument that the reduced virulence of the complete, cultured library in comparison to that of less-complex sublibraries (Fig. S3) is due at least in part to a reduced likelihood that a fully virulent mutant would randomly escape capture by the mesenteric lymphatic system. The presence of
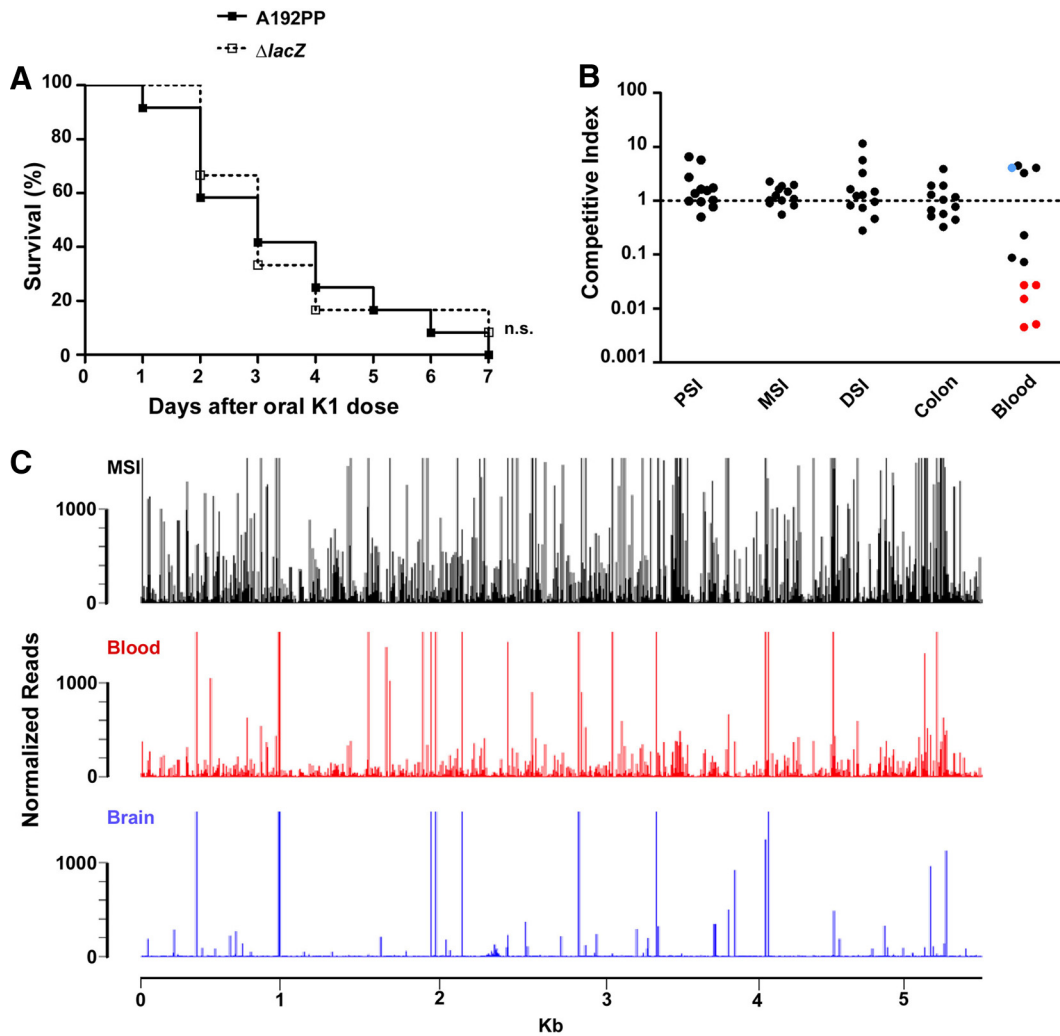
FIG 5 Bottleneck to systemic infection in the neonatal rat. (A) Survival of rats colonized at P2 by oral administration of *E. coli* K1 A192PP or A192PPΔ*lacZ*::*kan* (n = 12 pups for both groups). ns, nonsignificant; *, *P* < 0.05; **, *P* < 0.01 (as determined by a log-rank [Mantel-Cox] test). (B) Competitive indices of intestinal colonization and gut-to-blood transit of *E. coli* K1 A192PP and A192PPΔ*lacZ*::*kan*. A 1:1 mixture of *E. coli* K1 A192PP and A192PPΔ*lacZ*::*kan* (total of 2 × 10⁶ to 4 × 10⁶ CFU) was administered orally to P2 pups. After 24 h, ratios of A192PP and A192PPΔ*lacZ*::*kan* bacteria were enumerated in segmented GI tissues and in the blood, as indicated, using selective media. Data for animals in which only *E. coli* K1 A192PP or A192PPΔ*lacZ*::*kan* was detected in the blood are shown in red and blue, respectively, indicating the existence of a bottleneck to infection. (C) Loss of diversity of *E. coli* K1 A192PP-Tn*5* populations recovered from the blood (red) and brain (blue) following translocation from the GI tract (black).

significant bottlenecks between the GI tract, blood circulation, and brain was confirmed by determination of the complexity of recovered Tn*5* library populations from these sources (Fig. 5C).

   Identification of *E. coli* K1 A192PP genes required for survival in human serum. Systemic infection in neonatal rats is likely to be maintained only if *E. coli* A192PP bacteria survive in the blood circulation. Due to the limited exposure to antigens *in utero* coupled with deficits in adaptive immunity, neonates depend on innate immunity for protection against infection. The complement system provides frontline innate defense against Gram-negative bacterial infection, and the polySia capsule in turn enables *E. coli* K1 to avoid successful complement-mediated attack by host immune mechanisms. To obtain insights into *E. coli* K1 pathogenesis during the invasive phase of infection, and in light of restrictions placed on the neonatal rat model with regard to the use of TraDIS by the gut-to-blood bottleneck, we used the *E. coli* A192PP Tn*5* library to investigate genes essential for A192PP fitness in pooled normal human serum,
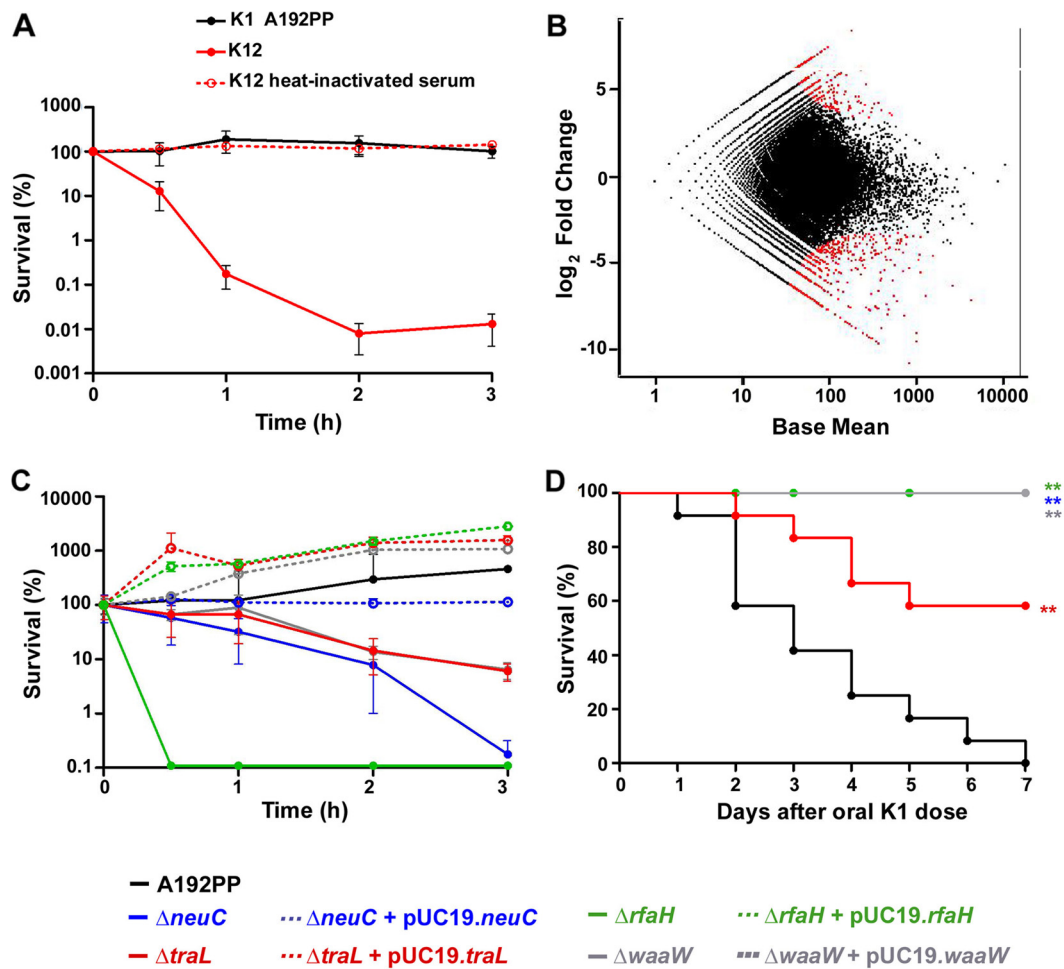
FIG 6 Identification of genes contributing to the complement resistance of *E. coli* A192PP by using a high-density transposon library. (A) Survival of *E. coli* A192PP and *E. coli* K-12 strain MG1655 in 22% pooled human serum. The latter was used a serum-susceptible control ($n = 3$); error bars represent ranges of values. (B) Log$_2$ fold change and average Tn5 insertion site read abundance for each gene after incubation of 1×10^6 CFU containing 2×10^4 unique *E. coli* K1 A192PP-Tn5 mutants in 22% pooled human serum for 3 h at 37°C. Colonies (2×10^5) were obtained by culture of diluted aliquots on LB agar containing 50 μg/ml kanamycin. The inoculum served as the input pool. Red data points represent Tn5 insertion sites determined as being differentially expressed in the output pool compared to the input pool using a negative binomial test with a false discovery rate of 0.1. (C) Survival of 1×10^6 *E. coli* K1 A192PP bacteria and single-gene mutants in 22% normal human and heat-inactivated (56°C for 30 min) serum. The final volume of the reaction mixture was 1.5 ml ($n = 3$); error bars represent ranges of values. (D) Survival of P2 rats colonized with *E. coli* K1 A192PP and single-gene mutants. Bacteria (2×10^6 to 4×10^6) were administered orally to P2 rats ($n = 12$/group). ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$ (as determined by a log rank [Mantel-Cox] test).

a reliable and plentiful source of all soluble components of the three complement pathways (48).

E. coli A192PP is resistant to the bactericidal action of human serum (Fig. 6A). A portion of the A192PP-Tn5 library containing 2×10^4 mutants (1×10^9 CFU) was incubated in either 30% human serum or 30% heat-inactivated serum (final volume of 375 μl) at 37°C for 3 h. Kan^r bacteria in the input and output pools (each with 2×10^5 bacteria) were collected, DNA was extracted from each pool, and transposon insertion sites were sequenced. A wide distribution of fitness scores was detected (Fig. 6B). Mutation of 97 genes (negative log$_2$ fold change and $P < 0.05$) resulted in decreased survival in normal serum but not in heat-inactivated serum (Fig. 6C; see also Table S3 in the supplemental material).

A high proportion of genes identified in the TraDIS screen as contributing to resistance encoded cell surface constituents. It is well established that the polySia capsule protects *E. coli* K1 from complement attack (16, 17), and three mutations in the *kps* capsule gene

cluster compromised serum survival. The central region of the cluster contains the *neu* genes that direct the biosynthesis, activation, and polymerization of the *N*-acetylneuraminic acid building block of polySia. *neuC* encodes the UDP *N*-acetylglucosamine 2-epimerase that catalyzes the formation of *N*-acetylmannosamine (49), and the *O*-acetyltransferase *neuD* acetylates monomeric neuraminic acid at carbon position 7 or 9 (50). KpsM is a component of the multimeric ATP-binding cassette transporter involved in the translocation of the polySia capsule through a transmembrane corridor to the cell surface (41, 51). A disruption of the genes encoding these proteins will prevent polySia expression (41); the interruption of *rfaH*, identified in the TraDIS screen, will also prevent capsule expression, but its loss will have a more profound effect on the surface topography of *E. coli* A192PP, as this transcriptional antiterminator is required for the expression of operons that direct the synthesis, assembly, and export of LPS core components, pili, and toxins in addition to the capsule (52, 53). Indeed, survival in serum is dependent on antitermination control by RfaH (54). Another identified gene that impacts capsule formation was *bipA*; BipA is a tyrosine-phosphorylated GTPase that regulates a variety of cell processes, including some associated with virulence, through the ribosome (55, 56). Other genes involved in LPS biosynthesis and pilus formation were also identified: *waaW* encodes a UDP-galactose:(galactosyl) LPS alpha1,2-galactosyl-transferase involved in the synthesis of the R1 and R4 LPS core oligosaccharides (57), and *wzzE* encodes a polysaccharide copolymerase that catalyzes the polymerization of LPS O-antigen oligosaccharide repeat units into a mature polymer within the periplasmic space in readiness for export to the cell surface (58). Both mutations will prevent the attachment of LPS O-antigen side chains to the core oligosaccharide of LPS. The 16 genes that specify pilus synthesis that were identified in the screen included the majority of the genes of the *tra* locus.

The TraDIS screen identified a range of proteins that are embedded in the OM (Fig. 6C), none of which had been previously implicated in complement resistance. These proteins are likely to influence the topography of the bacterial surface. Of the remaining genes with an assigned function, the majority were involved in cell metabolism and the stress response; it is well established that metabolic processes are intimately associated with the process of complement-mediated killing of bacteria (59, 60). To verify the screen, we constructed four single-gene mutants of *E. coli* A192PP by bacteriophage λ Red recombineering. Genes with roles in LPS synthesis (*rfaH* and *waaW*), capsule synthesis (*neuC*), and pilus assembly (*traL*) were mutated; none of these mutants showed any reduction in growth rates in LB broth. All mutants displayed significant reductions in complement resistance following incubation in pooled human serum (Fig. 6D). *E. coli* A192PPΔ*rfaH* was exquisitely susceptible, with no colonies being detected after 30 min. The viability of A192PPΔ*neuC* was also compromised, with a log reduction in viability over the 3-h incubation period of 3-fold. Killing of A192PPΔ*traL* and A192PPΔ*waaW* was less marked, but these mutations significantly reduced viability. Complementation of the mutants with the functional gene introduced on a pUC19 vector completely restored resistance in all cases (Fig. 6D). These genes also contributed to lethality in P2 neonatal rats (Fig. 6E). The lethal capacity of A192PPΔ*neuC*, A192PPΔ*rfaH*, and A192PPΔ*waaW* was completely attenuated in comparison to that of *E. coli* A192PP; 42% of pups administered A192PPΔ*traL* succumbed to systemic infection (all $P < 0.01$).

DISCUSSION

Systemic infection with meningeal involvement arises spontaneously after GI colonization of neonatal rats with a high proportion of *E. coli* K1 isolates, and the pathway to infection mirrors to a large extent that of natural infections in the human host. In contrast to models of bacterial infection that create artificial pathogenesis by bypassing some or all of the barriers to infection by the injection of a bacterial bolus directly into the blood circulation, the neonatal rat model provides an opportunity to investigate the progress of the pathogen as it transits from gut to blood to brain in a stepwise fashion. TraDIS and other transposon sequencing methods enable the simultaneous and rapid

determination of the fitness contribution of every gene under a given condition and therefore have the potential to enable the identification of genes that are essential for, or significantly contribute to, each step of the infection process. However, stochastic loss will become evident if each mutant in the input pool does not have an equal chance to overcome the physical, physiological, and immunological barriers presented by the host (61). This was clearly the case with the epithelial transit of *E. coli* A192PP, with evidence that on occasion systemic infection arose due to only one viable bacterial cell entering the blood circulation (Fig. 5B), and complements other studies showing single-cell or low-cell-number bottlenecks in models of severe infection (62–64). As translocation from colonization sites within the MSI to the blood was not amenable to analysis by TraDIS, we determined genes essential for survival in the presence of complement, a major component of the innate immune system that protects against extracellular systemic pathogens (17).

The high density of transposon insertions into random genomic positions along the entire *E. coli* A192PP chromosome, with minimal insertional bias (Fig. 1A), enabled the identification of genes essential for growth in nutrient-replete LB medium. Of the 357 *E. coli* A192PP genes considered essential, orthologues of 254 (from 315) were previously identified using TraDIS in a multidrug-resistant uropathogenic strain of *E. coli* ST131 grown in LB broth (34), and 253 were identified in an *E. coli* K-12 strain (35), confirming the existence of a core set of essential genes in *E. coli*. As anticipated, a high proportion of these genes encoded enzymes involved in a range of key metabolic functions such as carbohydrate, protein, and nucleobase metabolism, and the remainder were associated with essential functions such as transport, cell organization, and biogenesis.

During characterization of the *E. coli* A192PP mutant library, we examined the impact of culture in liquid medium on the expression of the polySia capsule, which places large demands on cell energy expenditure, as lengthy incubation times before marker selection may decrease library complexity (38). Unexpectedly, we found that prolonged culture of the library enriched the proportion of nonencapsulated mutants (Fig. 3A). We anticipated that the loss of capsule would enable the nonencapsulated mutants to grow at a higher rate than those of capsule-replete mutants and the wild type and to outcompete capsule-bearing library members. However, the growth of a nonencapsulated mutant selected at random from the library was virtually identical to, and not significantly different from, that of the *E. coli* A192PP parent strain (see Fig. S2A in the supplemental material). There was also no difference in the climax populations of the strains at the end of the logarithmic phase of growth. In a similar fashion, the growth curve for a *neuC* single-gene mutant was identical to that of *E. coli* A192PP (Fig. S2B). *neuC* is involved in the synthesis of the *N*-acetylneuraminic acid monomeric unit of polySia and, as a consequence, is unable to elaborate the capsule. It is clearly impractical to evaluate the growth kinetics of every distinct nonencapsulated mutant in the Tn*5* library, but it currently appears that differences in growth rates of individual library members cannot explain the highly reproducible enrichment that we observed. Indeed, the use of transposon insertion libraries is predicated on the assumption that there are no significant differences in the growth rates of individual mutants. At present, the basis of the loss of mutants expressing capsule in TraDIS library cultures cannot be readily explained.

A sublibrary of 2×10^5 mutants was used to establish genes involved in GI colonization. To minimize bias due to any outgrowth of nonencapsulated mutants on the GI epithelium, we harvested *E. coli* K1 from the MSI after 4 h, by which time maximal CFU had been achieved; bacteria were plated directly on solid medium to further avoid outgrowth. Bias due to this restricted timeline is likely to be low, as the majority of genes involved in adhesion and complement resistance are expressed constitutively. TraDIS identified the polySia capsule as a major determinant of GI colonization associated with *E. coli* K1. There is little or no evidence from the literature that capsules of Gram-negative bacteria enhance GI colonization; indeed, it has been reported that they interfere with adhesive interactions by obstructing the binding of underlying surface

molecules to mucosal surfaces (65, 66). The *E. coli* A192PPΔ*neuC*::*kan* single-gene mutant displayed a reduced capacity to colonize the MSI (Fig. 4C), although it should be borne in mind that passage through the upper alimentary canal and stomach may impact the number of mutant bacteria gaining access to the small intestine. In this context, it should be noted that capsular exopolysaccharide protects *E. coli* from the environmental stress of stomach acid (67).

Other cell surface structures that are likely to have an impact on adhesion to and colonization of the mucosal layer associated with the MSI were identified by TraDIS. Pili are established mediators of adhesion of *E. coli* to the host epithelium, although a large proportion of the evidence comes from enterotoxigenic and enteropathogenic strains (68, 69). LPS and OM protein-encoding genes were also implicated, as were genes involved in the stress response, reflecting ongoing adaptation to a new and hostile environment. The involvement of genes encoding metabolic enzymes, including some for anaerobic respiration, equates to increases in bacterial cell numbers in the anaerobic environment of the small intestine, and for iron acquisition genes, this reflects the low availability of intestinal luminal iron (47, 70). Genes encoding some components of type II and type IV secretion systems were found at decreased frequencies in the output pool. Members of these gene categories were also identified previously by Martindale et al. (39) as being necessary for GI colonization by *E. coli* K1 fecal isolate RS228 using signature-tagged mutagenesis; no genes found in that study were identified in the present study, in spite of the close genetic relatedness of the strains employed.

The intestinal lumen represents a potentially important portal of entry for pathogens into the host through adhesion, invasion, or disruption of the epithelial barrier (71). In neonatal rats, *E. coli* K1 induces no detectable disruption of barrier integrity but exploits an intracellular pathway to access the submucosa (12). Only small numbers of bacteria breach the mesenteric lymphatic barrier in an apparently random fashion (Fig. 5), and this precludes analysis by TraDIS. To accumulate data on genes and gene products facilitating invasion and survival/replication in the blood circulation, we examined essentiality for avoiding complement-mediated bactericidal effects. Although not all *E. coli* K1 isolates from cases of systemic infection are resistant to complement, resistance is encountered more frequently among K1 and K5 capsular types than among other K types (72); *E. coli* O18:K1 strains (such as A192) are in turn resistant more often than are other O:K serotype combinations (73) due to the capacity of the polySia capsule to prevent complement activation. It is assumed, but not established, that the polySia capsule surrounding susceptible strains does not completely mask either OM-located activators of complement or lipid domains on the outer surface of the cell that are targets for OM intercalation of the C5b-9 membrane attack complex, the entity responsible for bacterial killing (59). In addition, long and numerous LPS O-antigen side chains are necessary but not sufficient to enable the target cell to avoid complement killing (74), and they are able to bind the C1 inhibitor to arrest classical or lectin pathway activation at the early C1 stage (75). The importance of these structures for the complement resistance of *E. coli* K1 is supported by the decreased frequency of key LPS and capsule genes in the output pool along with a large number of OM-embedded proteins.

A small number of OM proteins, such as TraT and Iss, have been implicated as being determinants of complement resistance (74), but they have been introduced into low-resistance backgrounds in high copy numbers; their role in the intrinsic resistance of clinical isolates is unclear, and no mechanisms have been invoked to account for increases in resistance. The insertion of large numbers of protein molecules into the OM may fortuitously alter the biophysical properties of the bilayer, reducing the surface area and fluidity of lipid patches that are essential for the binding and assembly of the C5b-9 membrane attack complex. The identification of a range of OM proteins as being putative complement resistance determinants by TraDIS creates an opportunity to systematically investigate their precise function through the generation of single-gene mutants, and we intend to pursue this line of investigation. We suggest that the

architecture of the external surface of the OM, together with other more-external macromolecular structures such as polysaccharide capsules, influences the capacity of the pore-generating C5b-9 complex to perturb the integrity of the OM. Thus, the surface of susceptible strains contains a sufficient number of exposed lipid domains to facilitate C5b-9 generation and penetration, whereas the spatial and temporal organization of the OM of resistant bacteria is dominated by supramolecular protein assemblages to a degree where insufficient hydrophobic domains are available to act as C5b-9 assembly and binding sites, and this state persists throughout the growth cycle. The data that we have generated in this study are compatible with this hypothesis. An array of metabolic genes emerged as being essential for the maintenance of the complement-resistant phenotype (Fig. 6D) and may be indicative of repair processes invoked due to complement attack. The exposure of resistant *E. coli* to complement results in a minor perturbation of membrane integrity and metabolic homeostasis (76, 77), and C5b-9 intercalation into the OM has profound effects on cellular metabolic parameters (60).

TraDIS was also employed by Phan and coworkers to define the serum resistome of a globally disseminated, multidrug-resistant clone of *E. coli* ST131 (34). Those researchers identified, and in most cases validated, 56 genes that contributed to the high level of complement resistance displayed by this pathogen. In a fashion similar to that in our study, genes involved in the synthesis and expression of cell surface components were prominent. A number of genes contributing to LPS biosynthesis, such as those of the *waa* operon, the *wzz* locus, and *rfaH*, were common to both studies, as was the gene encoding the intermembrane protein AcrA. Genes of the plasmid-borne *tra* locus, which we determined to be components of the *E. coli* A192PP serum resistome, were not present in *E. coli* ST131 (34), but other OM-located proteins may fulfill a similar role in reducing the fluidic properties of the bilayer. In contrast to the well-established role of the *E. coli* K1 polysialyl polymer in the prevention of complement activation, no capsule genes were identified as components of the serum resistome of *E. coli* ST131, but different ST131 isolates express different capsule types due to extensive mosaicism at the capsule locus (78), and these uronic acid-containing polymers are unlikely to prevent complement activation (75). Thus, the different strategies employed by the two strains to prevent successful complement attack, together with differences in bacterial surface composition and topography, probably explain variations in the serum resistomes of these related pathogens.

In summary, we identified *E. coli* K1 genes required for growth in standard laboratory liquid medium and for colonization of the GI tract of P2 neonatal rat pups. Both data sets provide insights into the biology of K1 neuropathogens and could provide the basis for drug discovery programs for the identification of selective antibacterial or colonization-inhibiting agents. In our rodent model, the stochastic nature of invasion of the blood and probably brain prevented TraDIS analysis of gene essentiality for crossing gut epithelial and choroid plexus borders, but some indication of genes necessary for survival in blood was obtained from analyses of output pools after incubation of *E. coli* A192PP in human serum, a potent source of complement.

MATERIALS AND METHODS

Ethics statement. Animal experiments were approved by the Ethical Committee of the University College London (UCL) School of Pharmacy and the United Kingdom Home Office and were conducted in accordance with national legislation.

Bacteria and culture conditions. *E. coli* strain A192PP was obtained by serial passage of *E. coli* A192 (serotype O18:K1), isolated from a patient with septicemia (79), in P2 neonatal rats as described previously (11). Carriage of the polysialyl K1 capsule was determined with phage K1E (80): colonies were streaked onto Mueller-Hinton (MH) agar, 10 μl of a phage suspension containing ~10^9 PFU/ml was dropped onto each streak, the plates were incubated overnight at 37°C, and the proportion of encapsulated bacteria within cultures was quantified by comparing the ratio of phage-susceptible to phage-resistant colonies. *E. coli* A192PP single-gene mutants (Table 1) were constructed by using bacteriophage λ Red recombination (81); the oligonucleotides employed for the construction of targeted mutants, for the confirmation of targeted mutants, and for the construction of complemented mutants are shown in Tables S3 to S5 in the supplemental material. All bacteria were cultured in LB medium and on LB agar at 37°C; media were supplemented with either 100 μg/ml ampicillin or 50 μg/ml kanamycin as required.

TABLE 1 Strains used in this study

Strain	Description
E. coli K1	
A192PP	O18:K1:H7; virulent in neonatal rat model of infection
A192PPΔ*lacZ::kan*	*lacZ* mutant of A192PP; Kan[r]
A192PPΔ*neuC::kan*	*neuC* mutant of A192PP; Kan[r]
A192PPΔ*neuC::kan*/pUC19.*neuC*	Complemented A192PPΔ*neuC::kan*; Kan[r] Amp[r]
A192PPΔ*rfaH::kan*	*rfaH* mutant of A192PP; Kan[r]
A192PPΔ*rfaH::kan*/pUC19.*rfaH*	Complemented A192PPΔ*rfaH::kan*; Kan[r] Amp[r]
A192PPΔ*traL::kan*	*traL* mutant of A192PP; Kan[r]
A192PPΔ*traL::kan*/pUC19.*traL*	Complemented A192PPΔ*traL::kan*; Kan[r] Amp[r]
A192PPΔ*vasL::kan*	*vasL* mutant of A192PP; Kan[r]
A192PPΔ*waaW::kan*	*waaW* mutant of A192PP; Kan[r]
A192PPΔ*waaW::kan*/pUC19.*waaW*	Complemented A192PPΔ*waaW::kan*; Kan[r] Amp[r]
A192PPΔ*yaeQ:kan*	*yaeQ* mutant of A192PP; Kan[r]
A192PPΔ*0678::kan*	0678 mutant of A192PP; Kan[r]
A192PPΔ*3010::kan*	3010 mutant of A192PP; Kan[r]
E. coli K-12	
MG1655	F^{−} λ[−] Δ*ilvG rfb-50 rph-1*
MG1655Δ*lacZ::kan*	*lacZ* mutant of MG1655; Kan[r]

Tn5 library construction. The EZ-Tn5 ⟨KAN-2⟩ Tnp transposome (Epicentre Biotechnologies) was introduced in *E. coli* A192PP by electroporation. Transformants were selected by growth on LB plates containing 50 μg/ml kanamycin overnight. Pools of 1×10^3 to 5×10^3 colonies were collected and frozen at −80°C in phosphate-buffered saline (PBS) containing 20% glycerol. Aliquots of individual pools were combined to create larger populations of mutants of up to 7.75×10^5 mutants. Genomic DNA was extracted from 1-ml cultures by using the PurElute bacterial genomic kit (Edge Biosystems) according to standard protocols.

Linker PCR of Tn5 insertion sites. Linker PCR was used to test individual transformant colonies and to confirm individual random-insertion events. DNA (2.5 μg) was digested with the AluI restriction enzyme (Promega) and purified by using a MinElute PCR purification kit (Qiagen). A linker, formed by annealing of oligonucleotides 254 (5'-CGACTGGACCTGGA-3') and 256 (5'-GATAAGCAGGGATCGGAACC TCCAGGTCCAGTCG-3'), was ligated to purified fragments (50 ng) with a Quick ligation kit (NEB). Linker PCR was performed with linker- and transposon-specific oligonucleotides (258 [5'-GATAAGCAGGGATC GGAACC-3'] and 5'-GCAATGTAACATCAGAGATTTTGAG-3', respectively) by using a HotStart *Taq* Master-mix kit (Qiagen) and thermocycling conditions of 95°C for 5 min; 35 cycles of 94°C for 45 s, 56°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. The resulting amplicons were separated on 1.5% agarose gels at 100 V for 60 min.

Illumina sequencing. For sequencing of Tn5 insertion sites, approximately 2 μg of genomic DNA was degraded to ~500-bp fragments by ultrasonication using a Covaris instrument. Fragments were end repaired and A tailed by using the NEBNext DNA library preparation reagent kit for Illumina sequencing (NEB). The adapters Ind_Ad_T (ACACTCTTTCCCTACACGACGCTCTTCCGATC*T, where * indicates phos-phorothionate) and Ind_Ad_B (GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTC) were an-nealed and ligated to DNA fragments. PCR was performed with the transposon- and adapter-specific primers Tn-FO (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGGGGATCCTCTAGAGTCGACCTGC-3') and Adapt-RO (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACACTCTTTCCCTACACGACGCTCTTCC GATC-3'). Tn-FO and Adapt-RO contain a forward overhang and a reverse overhang for indexing of amplicons by Nextera index primers (Illumina). PCR was performed by using a HotStart *Taq* Mastermix kit (Qiagen) and thermocycling conditions of 95°C for 5 min; 22 cycles of 94°C for 45 s, 56°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. The resulting amplicons were separated on 1.5% agarose gels at 70 V for 90 min, and those between 150 and 700 bp were selected and purified by using a QIAquick gel extraction kit (Qiagen). Samples were indexed with oligonucleotides from the Nextera XT index kit (Illumina) by using HotStart ReadyMix (Kapa Biosystems) and with thermocycling conditions of 95°C for 3 min; 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 72°C for 5 min applied. Indexed amplicons were purified by using the AMPure XP system (Agencourt). The final concentration of samples was confirmed by using Qubit dsDNA BR assays (ThermoFisher Scientific). Indexed amplicons were sequenced on an Illumina Mi-Seq platform as 151-bp paired-end reads according to the manufacturer's protocol (Illumina).

Bioinformatic and statistical analyses. Raw sequence reads that passed Trimmomatic quality control filters (82) and contained the Tn5 transposon were mapped to the *E. coli* K1 A192PP reference genome (14) by using Bowtie (83), permitting zero mismatches and excluding reads that did not map to a single site. The reference genome assembly contains open reading frames (ORFs) located on contigs that were mapped to the IHE3034 chromosome and ORFs located on other contigs that are likely to map to plasmids and other mobile genetic elements. An in-house pipeline based on the SAMtools (http://samtools.sourceforge.net/) and BCFtools toolkits was utilized for the alignment files to determine insertion sites and coverage. To identify essential and nonessential genes, the insertion index was calculated for each gene by dividing the number of unique insertions in the gene by the gene length. Observed insertion index values were

fitted to a bimodal distribution with a gamma distribution (or an exponential distribution for genes with no observed insertion sites) corresponding to essential and nonessential genes. The $\log_2$ likelihood, and corresponding *P* values, of each gene belonging to essential or nonessential sets was calculated by using R software. To compare the fitnesses of individual mutants in input and output populations, reads were normalized and tested for differential base means by calculating $\log_2$ fold changes and corresponding *P* values at a false discovery rate of 0.1 using DESeq with R software.

Colonization and infection of neonatal rats. Timed-birth Wistar rat pup litters (usually *n* = 12) were purchased from Harlan UK, delivered at P2, and colonized on the same day. Pups were retained throughout each experiment with the natural mothers in a single dedicated cage under optimal conditions (19°C to 21°C, 45 to 55% humidity, 15 to 20 changes of air/h, and a 12-h light/dark cycle) and were returned to the mother immediately after colonization. Mothers had unrestricted access to standard rat chow and water. The procedure was described in detail previously (84). In brief, all members of P2 rat pup litters were fed 20 μl of mid-logarithmic-phase *E. coli* bacteria (2×10^6 to 6×10^6 CFU unless otherwise stated) from an Eppendorf micropipette. GI colonization was confirmed by culture of perianal swabs on MacConkey agar, and bacteremia was detected by MacConkey agar culture of blood taken postmortem. Disease progression was monitored by daily evaluation of symptoms of systemic infection, and neonates were culled by decapitation and recorded as dead once a threshold had been reached: pups were regularly examined for skin color, agility, agitation after abdominal pressure, the presence of a milk line, temperature, weight, and behavior in relation to the mother. Neonates were culled immediately when abnormalities for three of these criteria were evident. After sacrifice, GI tissues were excised aseptically without washing, the colon was separated, and the SI was segmented into 2-cm portions representing proximal, middle, and distal small intestinal tissues. Tissues were then transferred to ice-cold phosphate-buffered saline and homogenized. Bacteria were quantified by serial dilution culture on MacConkey agar supplemented with 25 μg/ml kanamycin as appropriate. The presence of *E. coli* K1 was confirmed with phage K1E: 20 lactose-fermenting colonies were streaked onto MH agar, 10 μl of a phage suspension containing $\sim10^9$ PFU/ml was dropped onto each streak, and the plates were incubated overnight. *E. coli* K1 bacteria were quantified by multiplying the total CFU by the proportion of K1E-susceptible colonies. In all cases, at least 19 colonies were susceptible to the K1 phage; *E. coli* K1 was never found in samples from noncolonized pups.

Susceptibility to human serum. Serum was obtained from healthy volunteers and used immediately. Bacteria were grown to late logarithmic phase in LB broth in an orbital incubator (minimum of 200 orbits/min), and 500 μl of the culture was removed, washed twice with gelatin-Veronal-buffered saline plus magnesium and calcium ions (pH 7.35) (GVB^{++}), and suspended in an equal volume of GVB^{++}. Fresh human serum was diluted 1:3 in GVB^{++} and prewarmed to 37°C. Bacterial suspensions and serum solutions were mixed 1:2 to give a final concentration of $\sim10^7$ CFU/ml and incubated at 37°C for 3 h in a total volume of 125 μl containing 22% serum. Surviving *E. coli* bacteria were quantified by serial dilution and incubation on LB agar overnight. Prewarmed, heat-inactivated (56°C for 30 min) serum served as a control.

Accession number(s). Raw read data for all transposon insertions have been deposited in the European Nucleotide Archive (ENA). All files are located at https://www.ebi.ac.uk/ena/data/view/PRJEB24291; accession numbers are as follows: ERR2235345 and ERR2235346 for the identification of essential genes for replicates 1 and 2, ERR2235567 for the input population, ERR2235568 for the output population of rat MSI genes, ERR2235569 for the output population of serum-exposed *E. coli* A192PP, and ERR2235570 for the output population of bacteria exposed to heat-inactivated serum.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00698-17.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 4, PDF file, 0.8 MB.

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