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Murine Model of Chemotherapy-Induced Extraintestinal Pathogenic *Escherichia coli* Translocation

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***Escherichia coli* is a major cause of life-threatening infections in patients with neutropenia, particularly those receiving chemotherapy for the treatment of cancer. In most cases, these infections originate from opportunistic strains living within the patient's gastrointestinal tract which then translocate to major organ systems. There are no animal models that faithfully recapitulate these infections, and, as such, the host or bacterial factors that govern this process remain unidentified. We present here a novel model of chemotherapy-induced bacterial translocation of *E. coli*. Oral gavage of BALB/c mice with a clinical isolate of extraintestinal pathogenic *E. coli* (ExPEC) leads to stable and long-term colonization of the murine intestine. Following the induction of neutropenia with the chemotherapeutic drug cyclophosphamide, ExPEC translocates from the intestine to the lungs, liver, spleen, and kidneys with concomitant morbidity in infected animals. Translocation can also occur in mice bearing mammary tumors, even in the absence of chemotherapy. Translocation of ExPEC is also associated with an increase of the diversity of bacterial DNA detected in the blood. This is the first report of a chemotherapy-based animal model of ExPEC translocation in cancerous mice, a system that can be readily used to identify important virulence factors for this process.**

Cancer has been called the emperor of all maladies (1). Nearly 9 million people worldwide died last year due to complications from cancer, and the incidence is expected to double by 2030 (2). A hallmark of all cancers is the unchecked division of the body's own cells. As a result, dozens of modestly effective chemotherapeutic drugs have been developed that target fast-replicating cells. Nearly all chemotherapy for malignancy predisposes patients to an infection because it induces severe neutropenia (3–5). According to the CDC, 1 of every 10 cancer patients will require hospitalization for an infection during their chemotherapy. In 85% of these patients, the infection originates from microorganisms that comprise the patient's own microbiome, especially those that inhabit the gastrointestinal (GI) tract (6). Indeed, of the potentially 500 to 1,000 different species of bacteria that inhabit the GI tract, only a handful seem capable of causing such infections, with *Escherichia coli* being the Gram-negative organism most frequently isolated from the blood of bacteremic patients (7) and a serious cause of life-threatening infection in those with cancer (8). Such strains are referred to as ExPEC (extraintestinal pathogenic *E. coli*) strains. Strains of this type include those that cause urinary tract infections (commonly called uropathogenic *E. coli*, or UPEC, strains), which account for nearly 8 million infections every year in the United States alone (9, 10); one of the two strains that are the leading causes of neonatal meningitis (11, 12); those strains isolated in about one-third of all bloodstream infections (13); and strains that account for ~17% of all cases of severe sepsis (13). ExPEC strains display great genomic diversity with no singular molecular feature that distinguishes them, outside the fact that they are frequently isolated from extraintestinal sites and often harbor a complement of well-known virulence factors such as toxins, adhesins, or nutrient uptake systems. Such strains are also problematic in that they are a normal part of the human commensal flora and therefore are ubiquitous, and they can acquire resistance to commonly used antibiotics (13). Furthermore, there currently is no licensed vaccine against these or related *E. coli* pathotypes, and the use of antibiotics, although effective, can drive up resistance rates, especially if they are used prophylactically, as has been observed in cancer patients (14). Antibiotics also can purge the GI tract of protective intestinal commensals, thereby eliminating the so-called "colonization resistance" antagonism against enteric pathogens sometimes attributed to the gut flora (15). The prevention and treatment of these infections are not straightforward; there are no vaccines for any *E. coli* pathotype, and because of the highly variable pangenomic nature of the *E. coli* genome, and the propensity of these strains to be chronic colonizers of the GI tract, developing a vaccine specific enough to target only pathogenic strains and not harmless commensals is difficult (16). Development of a therapeutic, whether a vaccine, drug, or probiotic, will require knowledge of the factors that govern bacterial translocation and a model to test its efficacy.

The process by which bacteria that inhabit the gastrointestinal tract break free from this intestinal dwelling place to cause bloodstream or disseminated infections is called "bacterial translocation," a term coined in the 1960s by Wolochow and coworkers (17). In the 1980s, Berg and colleagues expanded the study of

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translocation and determined (i) that some, but not all, commensal species, including members of the enterobacteriaceae such as *E. coli* (18) and *Proteus mirabilis* (19) as well as Gram-positive bacteria such *Staphylococcus epidermidis* and *Enterococcus faecalis* (20), can translocate, and (ii) that the health status of the host and several other host factors, including the integrity of the epithelial barrier (21), the disruption of the equilibrium of intestinal microbiota with antibiotics (22), depletion of T cells (23, 24), injury (25), and treatment with immunosuppressive drugs (26), are risk factors for translocation. Despite much clinical and experimental knowledge about the risk factors that promote translocation in susceptible patients, the host and bacterial factors that underpin this process are unknown, as is the molecular mechanism. This is in part because there is no practical experimental model system that adequately mimics the pathophysiology of the process, from start to finish, in the neutropenic host.

Here, we describe the development of a murine model of bacterial translocation from the asymptomatically colonized GI tract in a chemotherapy- and cancer-dependent manner, as is observed in patients with malignancies. The model is robust in that neutropenia is observed, the frequency of translocation is high, multiorgan dissemination is observed, and the mice become ill from the developing infection. The utility of the model is validated with the discovery of a mutant strain that cannot translocate and cause systemic disease. Overall, this work lays a foundation to understand the factors and mechanisms that govern translocation.

MATERIALS AND METHODS

Experimental animals. BALB/c female, 8-week-old mice (Jackson Laboratories, Bar Harbor, ME) were used for all experiments. All mice received sterile food and water *ad libitum* and were housed individually in filtered cages postinfection (pi). All experiments were done with approval by Baylor College of Medicine's Institutional Animal Care and Use Committee.

Bacterial strains, cancer cell lines, and growth conditions. The strain used in this study was *E. coli* CP9 (serotype O4:K54:H5;F13,F14 [27]), which was isolated from a bacteremic patient (28, 29) and was a gift from James Johnson (University of Minnesota). This strain was chosen for this study because *E. coli* is the major identified Gram-negative organism that translocates (30–32) in neutropenic patients, it is often multidrug resistant (MDR) (33), and the strain was isolated from the blood of a patient (28). In addition, elegant work by Johnson and Russo and coworkers has characterized several virulence factors important for disease, and those researchers have performed subcutaneous injections of this strain into mice (34). Known virulence factors include IroN (the receptor for enterobactin), cytotoxic necrotizing factor 1 (CNF1), capsule, and hemolysin (34–36).

The strain was genetically modified by insertion of a chloramphenicol resistance and green fluorescent protein (GFP) gene by classical P1 phage transduction (37). *E. coli* MG1655 (K-12) was generously provided by Christophe Herman (BCM) and was similarly modified. Strains were stored at −80°C in Luria broth (LB)–20% (vol/vol) glycerol. All strains were grown overnight from a single isolate in LB at 37°C with aeration and diluted to the appropriate concentration the next day. Chloramphenicol (EMD Millipore, Darmstadt, Germany) was used to grow resistant strains at 10 μg/ml. EMT6 (ATCC CRL-2755) cells were grown in minimal essential media (Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA, USA) and 1% streptomycin (Gibco, Carlsbad, CA, USA) and a penicillin mixture (Thermo Fisher Scientific, Boston, MA, USA), to confluence at 37°C, disassociated in 1× 0.25% trypsin–EDTA (Thermo Fisher Scientific, Boston, MA, USA), and subcultured until enough cells were generated for injection. Strains were stored under cryogenic conditions in growth media–5% dimethyl sulfoxide (DMSO; Corning, Corning, NY, USA). The EMT6 cells are derived from a mouse mammary carcinoma that formed in BALB/cCRGL mice and were clonally expanded after being cultured *in vitro* (38).

Mouse model of bacterial translocation. CP9 *E. coli* and MG1655 *E. coli* cells were grown under the indicated conditions the day before infection. On the day of infection, the culture was centrifuged at 15,000 rpm and resuspended in 1× phosphate-buffered saline (PBS) twice. The optical density (OD) was measured using a spectrophotometer set to 600 nm, and the number of CFU delivered via gavage was calculated by correlation of the OD at 600 nm to the number of colonies after plating. Mice were subjected to gavage with 100 μl of a bacterial suspension with a sterile (20-gauge, 38-mm-long) flexible needle. Cyclophosphamide (Cytoxan [CTX]) (Baxter Healthcare Corporation, Deerfield, IL, USA) was dissolved in sterile water and diluted with filter-sterilized 1× PBS to a final concentration of 10 mg/ml, and the mice were given a total dose of 450 mg/kg of body weight (three 150-mg/kg doses administered at 1-day intervals) intraperitoneally (i.p.) at the indicated time points for each experiment. To graft mammary cancer into the mice, EMT6 cells were grown to confluence, subjected to trypsinization, and centrifuged at 4,000 rpm and 4°C for 5 min. Pelleted cells were kept on ice and resuspended in sterile 1× PBS. Numbers of viable cells per milliliter were determined by staining with trypan blue and counting with a hemocytometer. The culture was diluted to give mice 1×10^5 cells per mammary fat pad (number 4 right and left) as previous described (39). Animals were sedated under isoflurane gas (1 liter to 2.5 liters/min [O_2 at 55 lb/in^2]), the surgical area was shaved, and a subcutaneous incision was made below the midline. A Hamilton syringe (26 gauge, 30-degree bevel, 1 in. long) was used to inject 20 μl of cells in the number 4 right and left fat pads. Ketoprofen was given at 7 mg/kg to reduce pain prior to the incision, the wounds were closed using sterile, surgical staples, and the mice were observed daily for pain or complications. Following the procedure, the mice recovered well and showed no ill effects from the treatment and were not kept on additional pain medication.

Tumor growth was monitored daily, and measurements were made using vernier calipers. The volume of each tumor was calculated using a modified ellipsoid formula (width^2 × length × 0.5). Mice were euthanized once tumors reached 300 mm^3 to 500 mm^3 or 1,500 mm^3 to 1,700 mm^3 depending on the experimental conditions. Lung lobes were fixed in 10% buffered formalin and then embedded and stained with hematoxylin and eosin (H&E) for quantification of metastasis by the Pathology and Histology core at Baylor College of Medicine.

Bacterial quantification of fecal and organ homogenates. Fecal pellets were removed from cages daily and weighed. Weighed fecal pellets (250 to 700 mg) were homogenized in 1× PBS. Homogenates were serially diluted and plated on agar/LB^{Chlor+}, and plates incubated overnight at 37°C with colonies were counted the next day. Organs were removed under sterile conditions immediately following CO_2 (2 liters/min, 5 min) euthanasia of animals. Organs were weighed and homogenized in 1× PBS with sterile blades. Intestines were washed of fecal contents with 1× PBS twice and then homogenized. Organ homogenates were plated on LB/agar^{Chlor+} and LB/agar incubated at 37°C overnight and colonies counted the next day. Assessment of disease severity was as follows: rough coat (score of 0 to 1), hunched posture (0 to 1), lethargy (0 to 1), and hyperpnea (0 or 1). Increments of increasing severity of disease were quantified in intervals of 0.5 except with hyperpnea, which was given a score of 1 (yes) or 0 (no). A Student's *t* test or a Tukey's test was used in conjunction with analysis of variance (ANOVA) to calculate statistical differences using Microsoft Excel 2013. Values were considered significant if a comparison of groups yielded a *P* value of less than 0.05.

Bacterial 16S rRNA gene analysis. Microbial DNA was extracted with a PowerSoil DNA isolation kit (MoBio) following the manufacturer's guidelines. The 16S rRNA gene V4 region amplicons (single index) were produced by PCR and sequenced on the MiSeq platform (Illumina) using the 2-by-250-bp protocol, yielding paired-end reads that overlapped ~247 bp (40). Following sequencing, raw BCL files were retrieved from the MiSeq platform and called into fastq files by Casava v1.8.3 (Illumina).

The read pairs were demultiplexed on the basis of unique molecular barcodes, filtered for PhiX using Bowtie2 v2.2.1, and reconstituted into two fastq files for each read using standard BASH. Sequencing reads were merged (allowing 4 mismatches per ≥50 bases) and processed using USEARCH v7.0.1001 (41). Sequences were demultiplexed using QIIME v1.8.0 (42) and then clustered using the UPARSE pipeline (41). Operational taxonomic unit (OTU) classification was achieved by mapping the UPARSE OTU table to the SILVA database (43). Abundances were recovered by mapping the demultiplexed reads to the UPARSE OTUs. A custom script constructed an OTU table from the output files generated in the previous two steps. The OTU table was used to calculate alpha diversity and beta diversity and provide taxonomic summaries and in a variety of other analyses built into QIIME that allowed characterization of individual samples and groups of samples based on alpha and beta diversity indices (44–46). Sequencing of V4 16S amplicons arising from the 39 blood samples evaluated yielded a total of 403,411 mergeable reads. Of these, 378,391 (93.6%) were mapped to the SILVA database at 97% identity. Normalization of the data set was set to 1,928 reads per sample in order to compare all the samples in the data set. For V4 16S amplicons arising from the 40 fecal samples evaluated, a total of 840,704 mergeable reads were recovered; of those reads, 771,244 (91.7%) were mapped to the SILVA database. A cutoff of 14,195 reads per sample was set to compare all the samples in the data set.

Generation of the mutant strains. *E. coli* CP9 was transformed with PKD46 (ampicillin resistant), which carries a λ red recombinase. Transformed colonies were selected on ampicillin-resistant plates. PCR products were generated using the knockout (KO) primers corresponding to regions flanking the gene of interest and FLP recombination target (FRT) sites using pBA169CM:FRT as the template DNA. The purified digested PCR product was transformed into competent cells of *E. coli* CP9:PKD46 grown in L-arabinose to induce recombineering enzymes. Transformed colonies were selected from chloramphenicol plates, isolated, and screened for recombineering product by PCR using AMP primers complementary to regions upstream and downstream of the insertion site of the chloramphenicol-resistant cassette. PKD46 was removed from the mutant strain by incubating streaked plates at 42°C overnight. Colonies were tested for ampicillin sensitivity to confirm removal of PKD46.

RESULTS

Establishing ExPEC colonization in the murine intestinal tract. We sought to generate an experimental model of bacterial translocation in the neutropenic host to address an important need in the cancer ward and for those receiving immunosuppressive drugs such as chemotherapy. A robust model should be consistent in this regard with clinical features observed in high-risk patients. This includes the stable colonization of the patient's GI tract with a bacterial species capable of translocation, the induction of translocation in response to an insult (in this case, chemotherapy) following neutropenia, the dissemination of the translocating species to major organ systems or the blood, and disease or morbidity that can lead to death of the host if the infection is left untreated. In addition, the model should be amenable to the development of cancer (after all, it is an underlining malignancy that causes the patient to seek care prior to many of these infections) and should also be consistent with the presence of bacterial DNA in the blood, a common feature in febrile neutropenic patients. ExPEC and CP9 were chosen as the study pathogen and strain, respectively, as outlined in Materials and Methods.

We first sought to determine if ExPEC CP9 could be processed by oral gavage and maintained in these mice and followed the experimental regimen shown in Fig. 1A. P1 transduction was used to insert a chloramphenicol resistance gene into CP9 so that ExPEC bacteria shed in feces could be selected from the intestinal bacterial flora on agar plates. The gavage of ExPEC CP9 into mice led to bacterial shedding in the feces for 15 days postinfection (dpi) and the detection of ExPEC in the small and large intestines, especially at gavage doses of between 10^5 and 10^9 cells (Fig. 1B and C). Doses lower than 10^5 did not yield any detectable colonies in the feces, indicating that gavage performed with doses above this level was best to achieve consistent colonization (Fig. 1B).

We next determined a chemotherapy regimen that would promote high levels of colonization of seeded ExPEC. We settled on the use of cyclophosphamide (CTX), an antitumor agent that alkylates DNA to kill rapidly dividing cells (47, 48) and is used to treat many types of cancers, including leukemia, myeloma, lymphoma, certain brain tumors, and retinoblastoma, as well as prostate and breast carcinomas (49). Either 10^9 or 10^5 ExPEC CP9 cells, the highest and lowest doses used in these colonization studies, were used for gavage of the mice, and CTX was given at 1, 3, and 5 dpi at human doses normalized to the weight of the mouse (Fig. 2A). Surprisingly, CTX treatment led to levels of ExPEC (at the 10^9 dose) being shed into feces that were approximately 4 orders of magnitude higher than those seen with untreated mice and promoted the retention of ExPEC even at the lowest dose of 10^5 CFU, a result which was not observed in the absence of CTX (Fig. 2B). In the absence of CTX, even high doses of ExPEC in the gavage treatments would steadily decline to a lower basal level of colonization at about 6 days. These data indicate that CTX, a chemotherapeutic drug commonly used to treat human cancers, can induce intestinal colonization by ExPEC at higher levels and for longer periods of time.

Chemotherapy induces bacterial translocation of colonized ExPEC. A successful model of bacterial translocation should demonstrate the movement of seeded intestinal bacteria from the GI tract to peripheral organs. We noticed that mice colonized with ExPEC that received chemotherapy became sick after a second dose of CTX. Hypothesizing that CTX-treated mice were ill because of ExPEC translocation, we subjected animals to gavage with 10^9 CFU of CP9 and administered chemotherapy as described in the Fig. 2 legend. Treatment with CTX once again led to higher levels of shed ExPEC at 8 dpi (Fig. 3A), with mice in this group becoming visibly ill as early as 4 dpi, a condition which progressively worsened until the animals reached a state of morbidity and were sacrificed (Fig. 3B). Necropsy of moribund mice indicated that ExPEC had translocated at high levels to the liver, lungs, kidney, and spleen in the CTX-treated mice, a condition rarely observed in mice colonized with ExPEC without chemotherapy (Fig. 3C). The higher levels of ExPEC in the intestinal segments in mice given chemotherapy confirmed that CTX induces the expansion of the populations of colonized ExPEC cells in these animals (Fig. 3D), a condition likely made possible by the highly immunosuppressive effects of CTX on circulating neutrophils (Fig. 3E), thus indicating that these mice were also neutropenic. Collectively, these data indicate that treatment of mice with the chemotherapeutic agent cyclophosphamide induces the translocation of ExPEC CP9 from the gastrointestinal tract to major organ systems, a process that correlates with severe neutropenia and a substantial reduction in the health of the mouse, which then leads to morbidity. A summary of the translocation of ExPEC to major organ systems over a wide range of CFU delivered into the animals via gavage is shown in Fig. 3F. It should be noted that that ExPEC cells derived from late-stage cultures, as opposed to those from the exponential phase, demonstrated a distinct trend toward higher

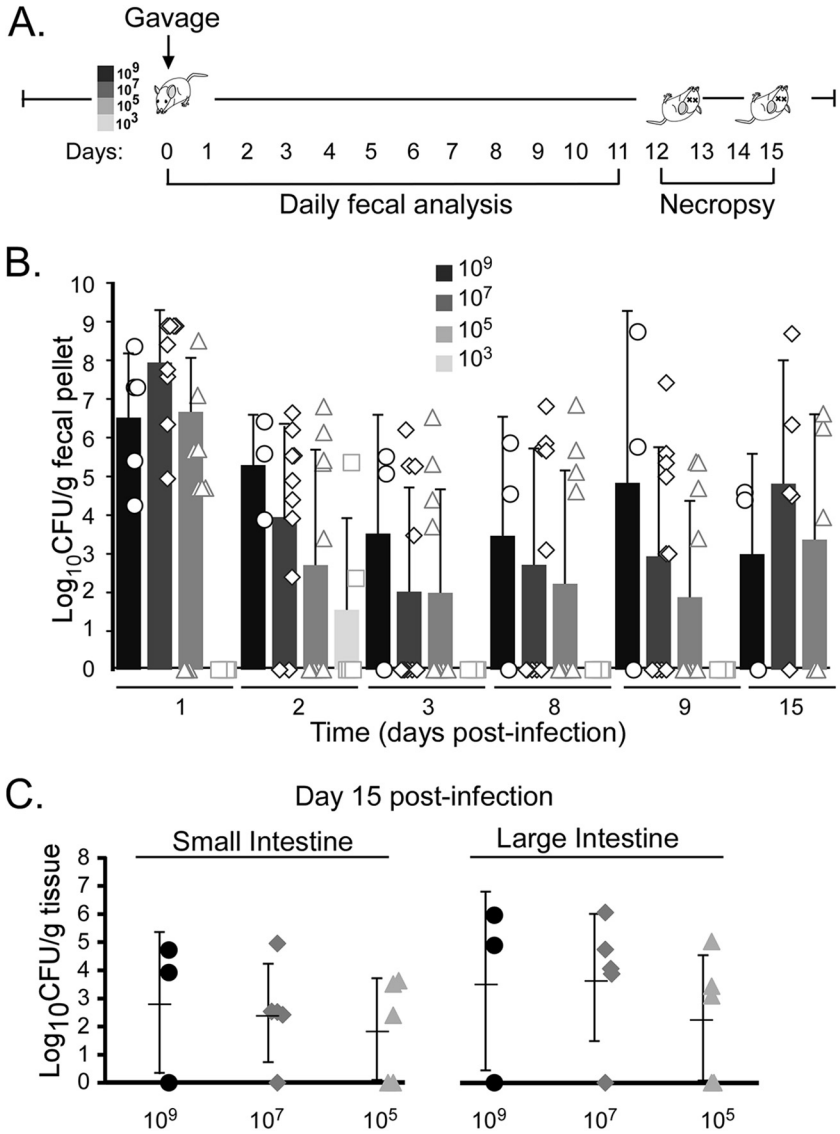


FIG 1 ExPEC strains colonize the mouse intestine. (A to C) BALB/c mice were subjected to oral gavage with ExPEC CP9 at different doses (10^9,10^7,10^5, and 10^3 CFU). (B) Intestinal colonization was monitored by plating fecal pellets on LB/agar^{Chlor+} and selecting for CP9 *E. coli* (chloramphenicol resistant). (C) Mice were euthanized on day 15, and intestinal tissue colonization was determined by sectioning and plating on LB/agar^{Chlor+}. Symbols represent individual mice within groups and are shaded accordingly. Error bars represent ± standard deviations, and *n* was at least 3.

levels of colonization. However, this effect was statistically significant only on the first day postgavage (data not shown).

Translocation of ExPEC can be induced in chronically colonized mice. We hypothesized that the long-term colonization (i.e., colonization in excess of the 8 days of colonization whose results are described above) of the GI tract would also lead to ExPEC translocation upon chemotherapy, a situation meant to mimic what is observed in humans, where the translocating bacterium may be a permanent inhabitant of the patient's GI tract. To test these ideas, we delivered 10^9 CFU of ExPEC CP9 or *E. coli* K-12 (MG1655) into mice and followed colonization by monitoring fecal shedding for 34 days. As demonstrated in Fig. 4A, ExPEC levels stabilized after a few days and remained moderate for the entire length of the experiment whereas *E. coli* K-12 levels steadily declined (or, in most cases, were not detected in the feces). Upon the initiation of chemotherapy on day 29 postinfection and necropsy of morbid mice on day 35 postinfection, most of the mice colonized with ExPEC demonstrated translocation of ExPEC CP9 whereas none of the mice subjected to gavage with *E. coli* K-12 displayed this property (Fig. 4B). Since colonization by K-12 is not as efficient as colonization by CP9, translocation in these experiments may have been a result of a composite of high levels of CP9 colonization and of this strain possessing specific factors that facilitate the occurrence of translocation. To differentiate between these two possibilities, we performed another series of experiments whereby mice were subjected to gavage with either ExPEC CP9 or MG1655 K-12, chemotherapy was started 1 day postinfection, and colonization and subsequent translocation were assessed. As demonstrated in Fig. S1A in the supplemental material, ExPEC CP9 and *E. coli* K-12 seemed to colonize the mouse at

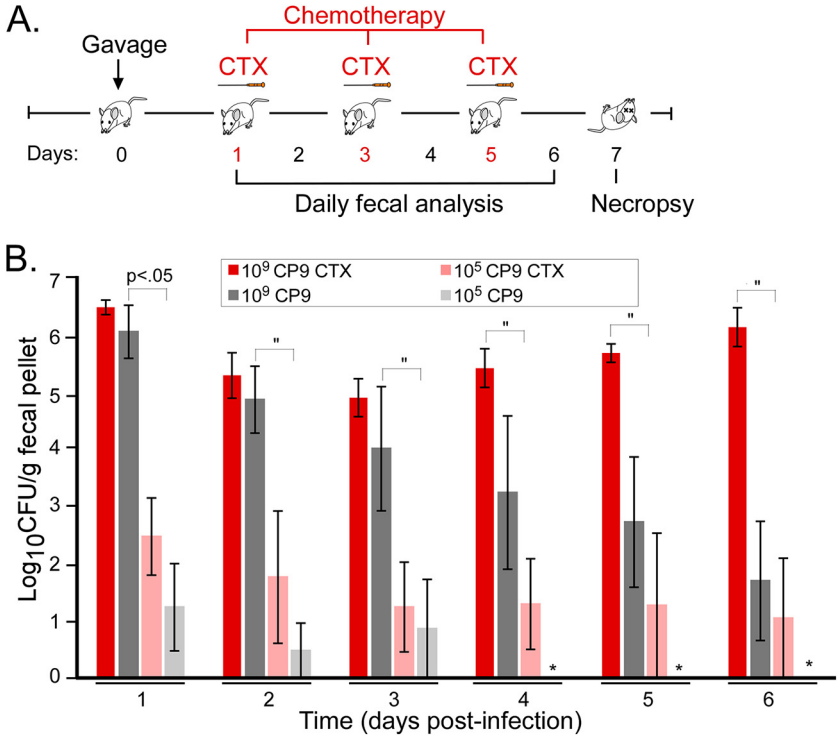
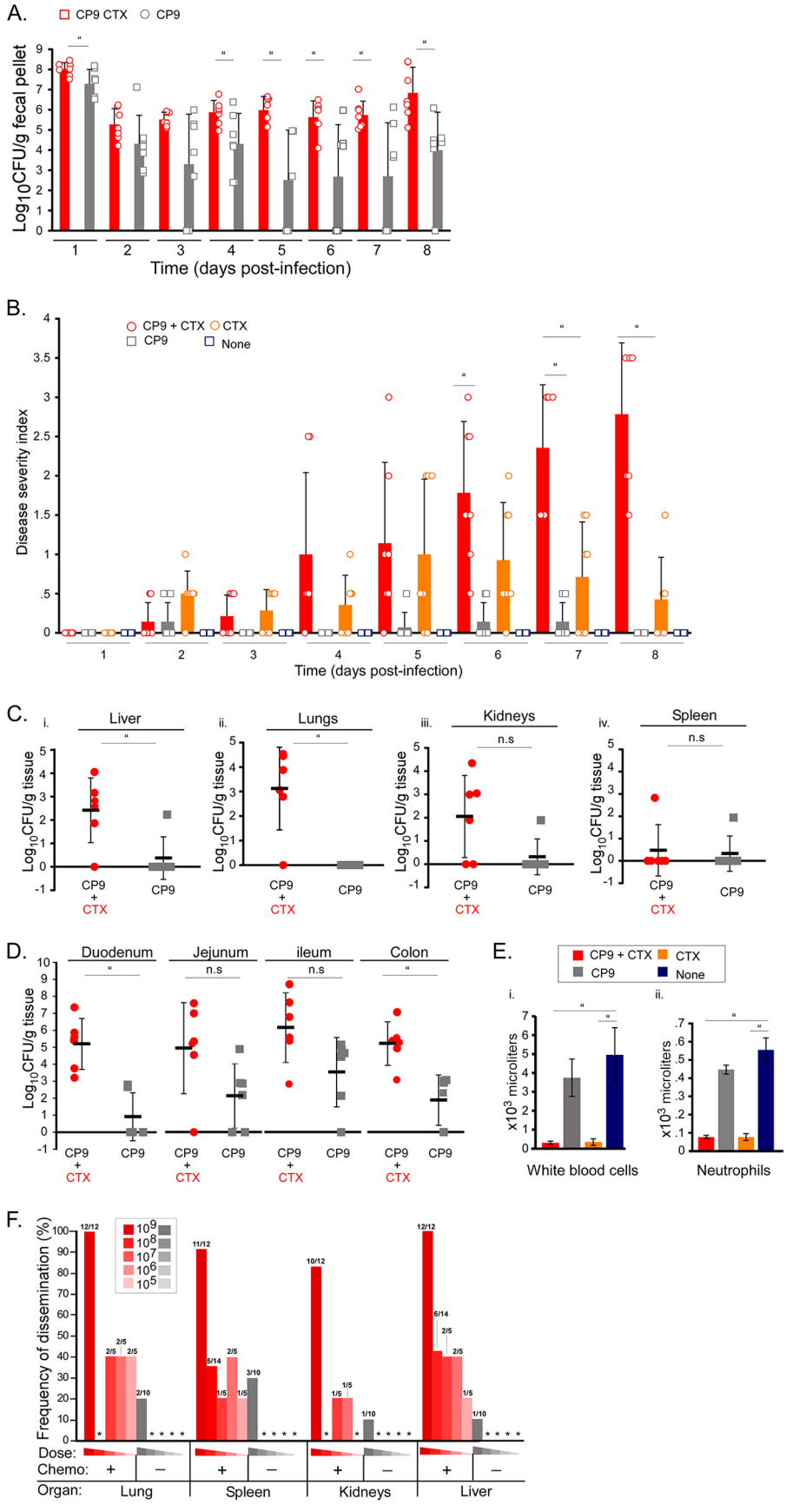


FIG 2 Chemotherapy leads to high levels of intestinal colonization. (A) BALB/c mice were subjected to oral gavage with ExPEC CP9 on day 0 and treated with CTX (150 mg/kg) on days 1, 3, and 5 postinfection or treated with PBS. (B) Mice were subjected to gavage with 1×10^9 or 1×10^5 CFU. Fecal pellets were plated daily on LB/agar^{Chlor+}. The red bars and pink bars represent mice treated by chemotherapy, and the dark-gray bars and light-gray bars represent untreated mice. Error bars represent ± standard errors, a double prime (″) denotes a significant ($P \leq 0.05$) difference between groups, a single asterisk (*) denotes no bacteria detected, and *n* was at least 5.

similar levels for the first 8 days. When mice were given chemotherapy, only the mice that were colonized with ExPEC showed evidence of translocation (7/7) whereas the K-12-colonized mice had no translocation (see Fig. S1B). These results suggest that the reason that there was little to no translocation observed in the original experiment with K-12 was that this strain somehow lacks the ability to translocate (relative to ExPEC). This implies that ExPEC generally harbors specific virulence factors that facilitate its translocation. In addition, these experiments indicate that it is experimentally achievable to induce translocation of ExPEC more than a month after gavage, an important point for building into this model such time-consuming steps as the development of cancer.

Translocation of ExPEC occurs in cancerous mice. Bacterial translocation and subsequent bacteremia are major concerns in patients with cancer who are receiving immunosuppressive chemotherapy. We sought to further extend this translocation model beyond just chemotherapy-induced translocation to more accurately reflect what occurs in patient populations, i.e., that they first present with an underlying cancer and then receive chemotherapy. To test this idea, we surgically transplanted a breast carcinoma cell line (EMT6) into the mammary fat pad of mice (38). In this cancer model, EMT6 cells develop into large tumors, 2 weeks after implant, which can metastasize to the lungs, leading to the death of the animal. However, the cancer is treatable with cyclophosphamide, which has been shown to prevent tumor growth in animals (50). We first tested what would happen if mice received ExPEC after cancer, a situation designed to simulate the acquisition by a patient of an ExPEC strain during cancer care (Fig. 5A). Following the implant of cancerous EMT6 cells in the mouse mammary fat pad, we observed the rapid growth of tumors over ~5 weeks in both the left and right flanks in the absence of chemotherapy (Fig. 5B and C), with 5 of the 7 mice showing metastasis to the lungs (see Table S1 in the supplemental material). Cyclophosphamide completely inhibited the growth of tumors with a regimen of three doses (given on days 14, 16, and 18), with no metastasis to the lungs observed (Fig. 5B and C; see also Table S1). At day 24 postcancer, mice were subjected to gavage with ExPEC, chemotherapy was once again administered, and colonization, translocation, and disease were assessed. As shown in Fig. 5D, the levels of excreted ExPEC remained stable throughout the experiment in all test groups, and ExPEC could be found in the intestine as well (Fig. 5E). Surprisingly, in addition to the translocation expected for mice treated with chemotherapy, there was also substantial translocation in mice bearing tumors that did not receive CTX treatment (Fig. 5E), and all mouse groups approached a moribund state by day 8 after chemotherapy (Fig. 5F). This suggests not only that translocation can be induced in cancerous mice receiving chemotherapy, an interesting feature of our model, but also that the very process of cancer progression itself somehow influences the translocation of ExPEC in this model system. It should be noted that, whereas mice receiving cancer and chemotherapy treatment presented with higher levels of ExPEC colonization overall (as assessed by counts in the feces and intestinal tissue), there was a noticeably low level of translocation in the


A.
CP9 CTX
CP9
Log10CFU/g fecal pellet
Time (days post-infection)
B.
CP9 + CTX
CTX
CP9
None
Disease severity index
Time (days post-infection)
C.
i. Liver
ii. Lungs
iii. Kidneys
iv. Spleen
n.s
Log10CFU/g tissue
CP9 + CTX
CP9
D.
Duodenum
Jejunum
ileum
Colon
n.s
Log10CFU/g tissue
E.
CP9 + CTX
CTX
CP9
None
x10^3 microliters
White blood cells
Neutrophils
F.
Frequency of dissemination (%)
Dose:
Chemo:
Organ:
Lung
Spleen
Kidneys
Liver

spleen and kidneys. We do not yet understand the mechanism behind the latter process.

We next determined if translocation could be induced when ExPEC was already present in the intestine when cancer developed and chemotherapy was given, a situation that would resemble patient's being chronically colonized, acquiring cancer, and then receiving cancer therapy (see Fig. S2A in the supplemental material). Once more, mice developed palpable tumors that were treatable with cyclophosphamide (see Fig. S2B) and showed high levels of colonization by ExPEC (see Fig. S2C) and high levels of translocation to multiple organs in a chemotherapy-dependent manner (see Fig. S2E). This occurred despite the successful treatment of the cancer by chemotherapy (see Fig. S2B), where mice chronically colonized with ExPEC showed high levels of morbidity and translocation (see Fig. S2D and E). These results suggest that this system represents a cancer- and chemotherapy-induced model of ExPEC translocation from the GI tract of chronically colonized mice, a situation observed in high-risk patients.

ExPEC is associated with the presence of bacterial DNA in the blood. Polymicrobial translocation, the presence of multiple enteric bacterial species in the blood, is a common clinical finding in the chronically ill and is linked to many diseases, including liver failure, HIV infection, and cancer (51–53). We wondered whether, in our model system, we could detect the presence of bacterial DNA other than that of ExPEC in the blood of mice that had cancer and/or had been treated with chemotherapy. As such, we subjected the blood of mice from all the experimental test groups represented in Fig. S2 in the supplemental material to sequencing of the bacterial 16S rRNA gene, a technique used to determine the number of operational taxonomic units (OTUs) present in each sample (40), at 21 days postinfection. Somewhat surprisingly, the three test groups (of the seven assessed) with the highest number of OTUs present were the groups of mice that were initially colonized with ExPEC, with the group having the most OTUs present in the blood being the one that received chemotherapy (Fig. 6A). Interestingly, the mice colonized with ExPEC but not given chemotherapy or cancer, which showed very little ExPEC translocation overall (Fig. 3 and 4), had the second highest levels of detected OTUs of all of the bacterial enterics in the blood among the groups. Indeed, there was an increase in the diversity of bacterial DNA detected in the blood, as assessed by a Shannon diversity index analysis, in the test groups that had been colonized with ExPEC (Fig. 6B). In fact, the presence or absence of ExPEC was the only variable for which a clear separation of the groups was observed, as determined by principal component analysis (see Fig. S3A to C). This ExPEC-specific increase in bacterial diversity was not observed in feces (Fig. 6C), indicating that the specificity of this effect was at the level of entry into blood and not a consequence of dramatic changes in the composition of the flora that inhabitant the GI tract of these mice. Collectively, these findings suggest that ExPEC substantially increases the levels and diversity of bacterial 16S DNA in the bloodstream of ExPEC-colonized animals.

Validation of the model: the identification of a virulence factor necessary for translocation. The process of translocation has been referred to by Berg and colleagues as the sequence of events between colonization of the gastrointestinal tract and systemic, extraintestinal infection of distal tissues. The bacterial genes and virulence factors that mediate translocation have not been identified, largely because there has not been a suitable model that would reveal them as important for this process. Such factors would be good candidates for vaccines or targets for new therapeutics. We hypothesized that our model of intestinal translocation and concomitant bacteremia in the neutropenic host would have the sensitivity needed to identify bacterial genes that are important for this process. Reasoning that some known virulence determinants might be a good place to start, we used recombineering technologies to generate isogenic knockouts in *iroN*, which encodes the enterobactin receptor that is important for iron uptake in low-iron environments such as the vertebrate host (54), and *cnf1*, which encodes cytotoxic necrotizing factor 1, a potent cytotoxin that induces the rearrangement of the actin cytoskeleton (55). Both of these genes have been implicated in ExPEC virulence (34, 36). We also decided to make a strain of ExPEC that lacks *E. coli k1.3385* (*ecok1.3385*), a gene of uncharacterized function that likely encodes a secreted protein with mucinase activity (56, 57) and was previously reported to demonstrate broad protection against ExPEC in a screen for vaccine antigens (58). The gene product was subsequently named SslE (for "secreted and surface-associated lipoprotein from *E. coli*") (59, 60). Wild-type and mutant ExPEC strains were delivered via gavage into mice, which were treated with chemotherapy as described in the Fig. 2 legend, and the levels of ExPEC were assessed in the intestine and organs. As demonstrated in Fig. 7A and B, the levels of wild-type and mutant ExPEC shed into the feces and present in the different segments of the intestine were approximately equivalent, indicating that these genes do not alter the ability of ExPEC to colonize these mice. However, whereas wild-type, Δ*cnf1*, and Δ*iroN* strains showed comparable levels of translocation following chemotherapy, the use of ExPEC lacking *sslE* resulted in only 1 mouse of 10 showing dissemination to peripheral organs (Fig. 7C). In that mouse, only one organ (the liver) had a single colony of ExPEC (Fig. 7D). The strain lacking SslE grew just as well as wild-type ExPEC, did not show any loss of membrane permeability, and had no negative effect on the expression of downstream genes (data not shown). Taken together, these results indicate that *sslE* is somehow important for the multistep process of ExPEC translocation and, to our knowledge, is the first such factor identified in a

FIG 3 Chemotherapy induces the translocation of ExPEC. (A) BALB/c mice were subjected to oral gavage with ExPEC CP9 and treated with CTX (150 mg/kg; red bars) or left untreated (gray bars). ExPEC colonization was monitored by plating fecal pellets on LB/agar^{Chlor+} daily. (B) Disease severity in mice was monitored by daily observation as described in Materials and Methods. Control groups included uninfected mice treated (orange) or not treated (blue) with CTX. (C) Bacterial CFU levels in livers, lungs, kidneys, and spleens were determined by plating tissue on LB/agar^{Chlor+} and LB/agar. n.s, not statistically significant. (D) Intestinal tissue was similarly plated on LB/agar^{Chlor+} and assessed. (E) Levels of circulating neutrophils and white blood cells were determined by collecting blood in an endpoint cardiac stick and assessment in an Advia 120 hematology analyzer at the Comparative Medicine Pathology department at Baylor College of Medicine. (F) BALB/c mice were subjected to oral gavage with 5 different gavage doses (10^9 to 10^5) of ExPEC CP9 depending on the group and treated with CTX (150 mg/kg) on days 1, 3, and 5 postinfection or treated with PBS. The shaded red bars and pink bars represent mice treated by chemotherapy (Chemo), and the shaded dark-gray bars and light-gray bars represent untreated mice. Spread to organs was monitored on day 7. Error bars represent ± standard deviations, a double prime (″) denotes a significant ($P \leq 0.05$) difference between groups, and *n* was at least 5.

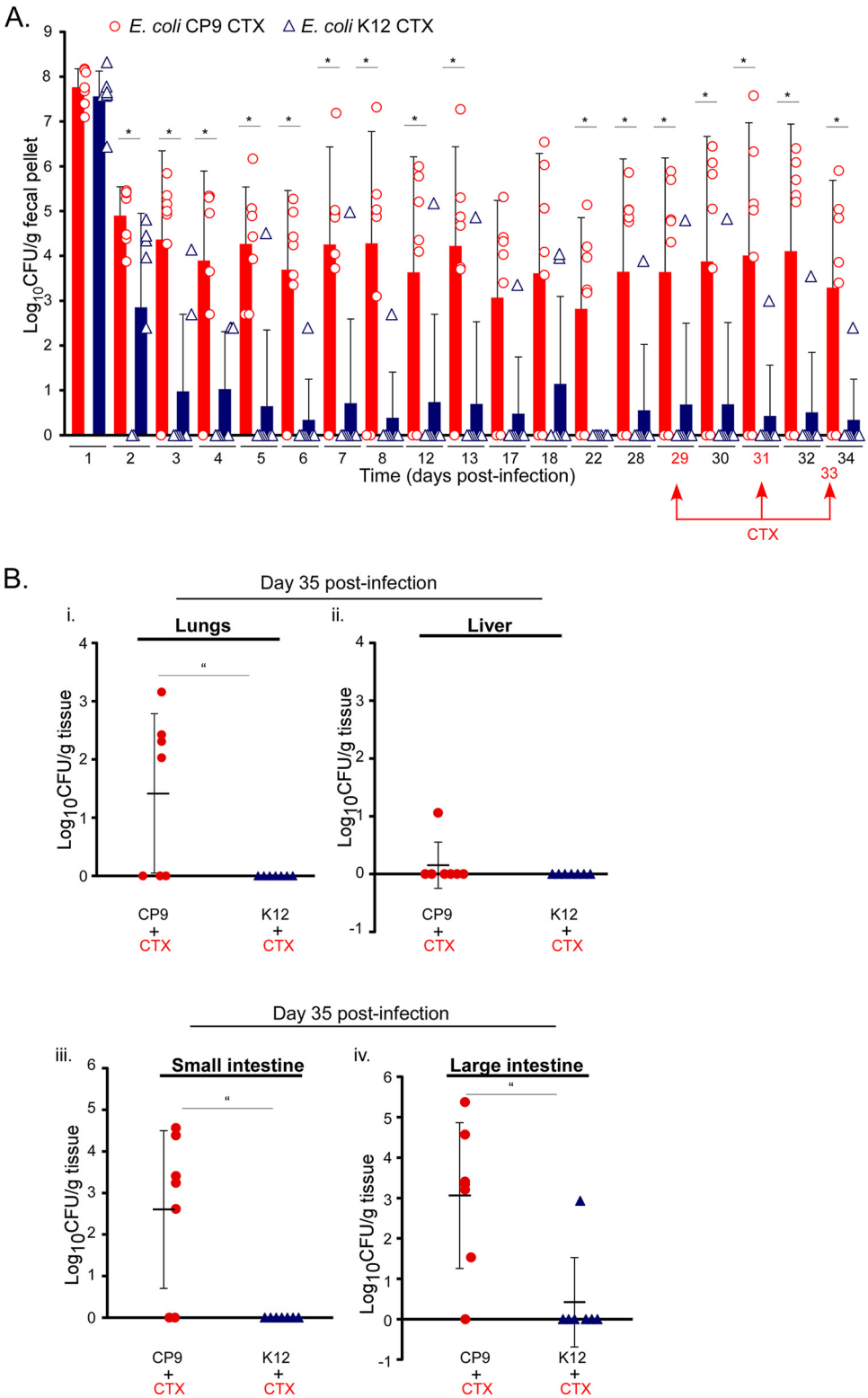


FIG 4 ExPEC translocation of chronically colonized mice. (A) BALB/c mice were subjected to gavage with 1×10^9 CFU of ExPEC CP9 (red bars, $n = 7$) or *E. coli* K-12 (blue bars, $n = 7$), and colonization was measured by plating fecal pellets on LB/agar^{Chlor+}. On day 29 postinfection, all mice were treated with CTX (150 mg/kg) at the intervals indicated. (B) Bacterial translocation was determined by plating the lung and liver on LB/agar^{Chlor+} and LB/agar. Error bars represent ± standard deviations, and a double prime (″) denotes a significant ($P \leq 0.05$) difference between groups.

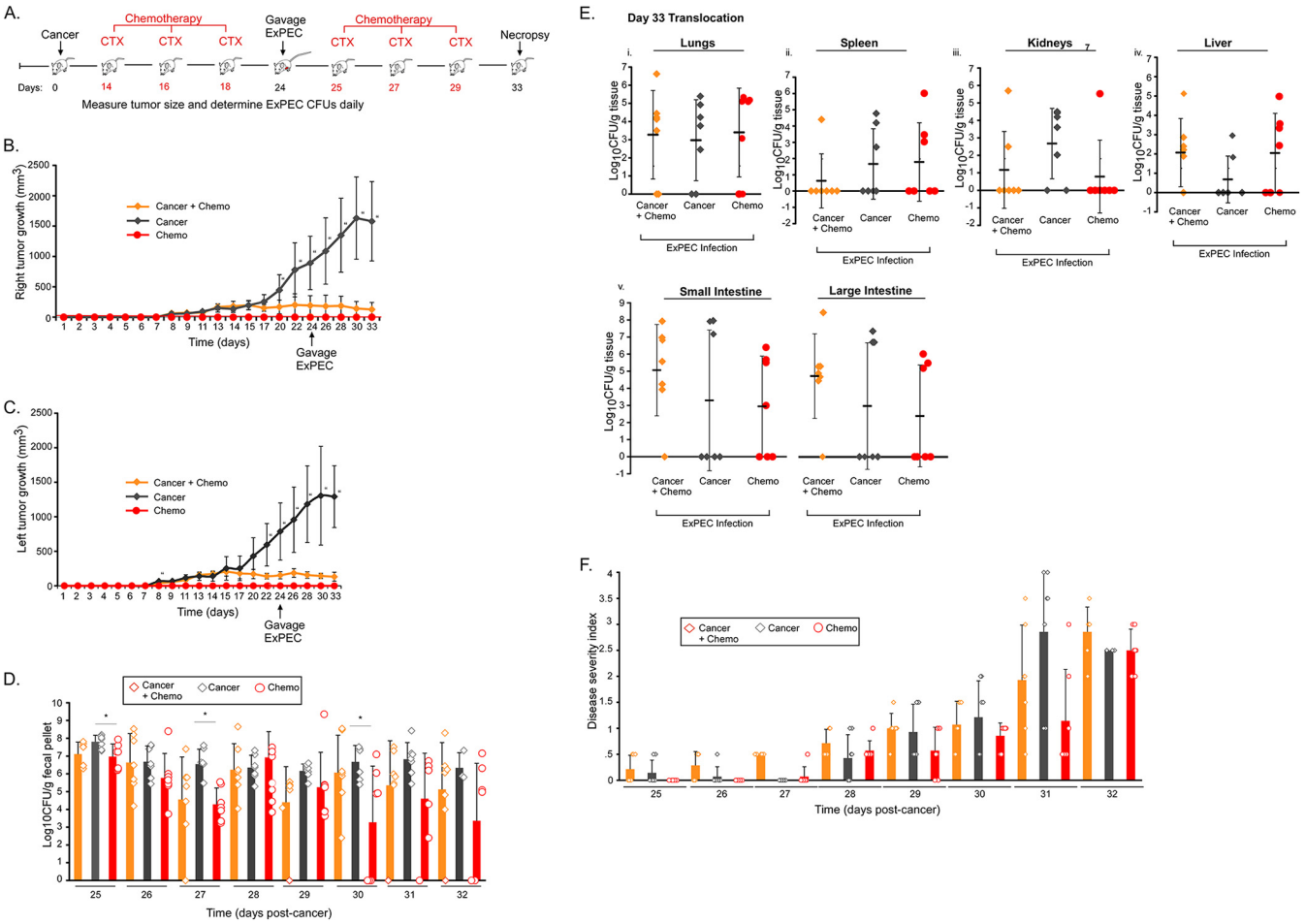


FIG 5 Establishment of ExPEC infection in a mouse model of mammary carcinoma. (A to C) BALB/c mice were orthotopically injected with EMT6 mammary carcinoma cells (10^5) (A) into the right (B) or left (C) mammary fat pad or were not subjected to injection. Mice were given CTX or left untreated on the days indicated in panel A, and tumor growth was measured by calculating the volume (in cubic millimeters) of the mass using vernier calipers. The error lines represent standard errors of the means of the data determined for the groups. Yellow, cancer (EMT6) with chemotherapy treatment (CTX); gray, cancer; red, chemotherapy. (D) On day 24, mice were subjected to oral gavage with 1×10^9 CP9 cells, and fecal pellets were collected and plated on LB/agar^{Chlor+} to enumerate CFU. (E) Mice were given another round of chemotherapy and euthanized, and CFU levels in the indicated organs were determined by plating on LB/agar^{Chlor+}. (F) Mouse health was monitored on day 1 and day 7 postinfection as described in Materials and Methods. Error bars represent ± standard deviations, a double prime (″) denotes a significant ($P \leq 0.05$) difference between groups, and n equals 7.

chronically colonized and chemotherapy-induced GI translocation model. These results also indicate that this model can be used to identify the mechanisms and factors involved in the development of the life-threatening systemic infection for *E. coli* that originates in the GI tract.

DISCUSSION

We demonstrate here a viable model of bacterial translocation that has many of the features of the human problem, including (i) the stable persistence of ExPEC in the GI tract of mice that remain asymptomatic throughout colonization, (ii) the induction of ExPEC translocation after administration of the widely used chemotherapeutic agent cyclophosphamide, (iii) a bacteremic, disseminated disease that results in ExPEC in the liver, spleen, kidneys, and lungs, (iv) morbidity associated with disseminated infection, (v) ExPEC translocation in mice with mammary tumors and in mice bearing tumors treated with cyclophosphamide, (vi) ExPEC-associated increases in the levels and diversity of bacterial DNA in the blood of chronically colonized mice, and (vii) the identification of a virulence factor important for this process, the putative mucinase SslE.

Berg and colleagues in the 1980s and 1990s performed extensive experimentation to explore the topic of translocation, using primarily rodents as the model system. This work was instrumental in defining many of the host factors that make the host susceptible to translocation, including suppression of the immune system with steroids (61), the loss of CD4 and CD8 T cells (24), the use of antibiotics that purge competing commensals (22), high bacterial loads in the cecum (62), mice that have tumors (63), diets without fiber (64), breakdown in the integrity of the gut wall (65), and hemorrhagic shock (66). The work here combined several of those features, as well as others, into one convenient model. Here, translocation was examined in the presence of an intact microbiome, in contrast to many earlier translocation studies where germfree or monoassociated mice were used. This is an important feature because it is likely that immunocompromised patients that

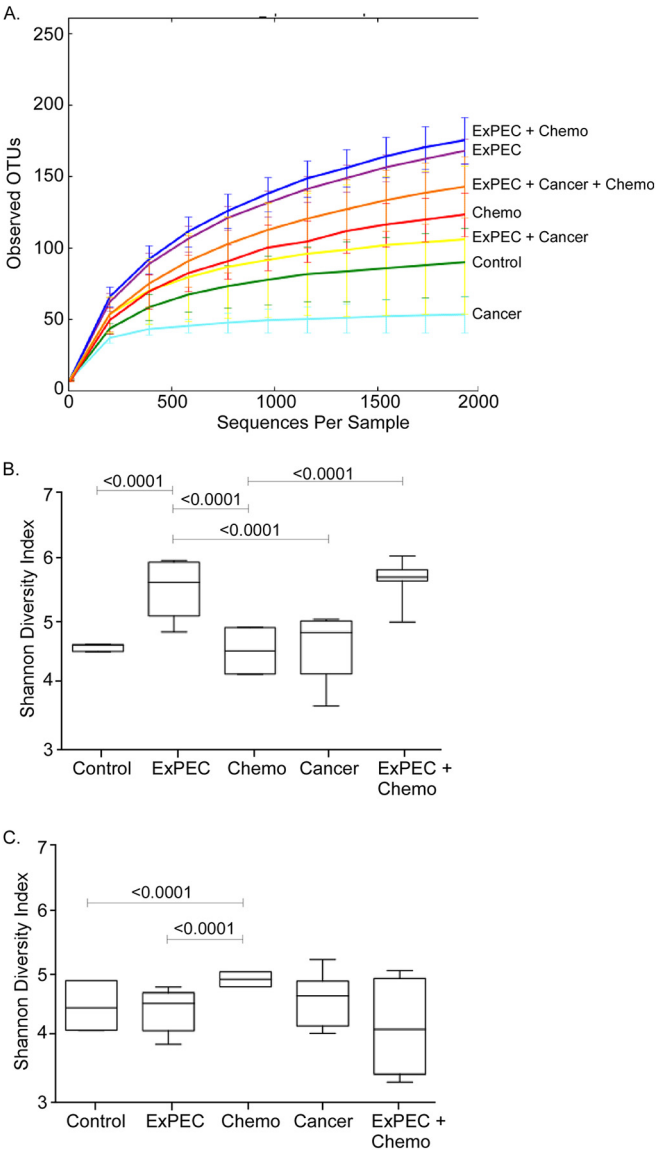


FIG 6 ExPEC promotes the translocation of enteric microbiota. (A) Rarefaction curves of phylogenetic diversity in blood samples representing the number of operational taxonomic units (OTUs) identified for each group. (B and C) Box plots of Shannon diversity index values constructed for blood samples rarefied to 1,896 sequences per sample (B) and fecal samples rarefied to 14,190 sequences per sample (C). A Mann-Whitney *U* test was employed to determine significance.

undergo translocation harbor a relatively intact intestinal microbiome, although, depending on their condition or treatment, it certainly may be imbalanced. Furthermore, a human bloodstream isolate (ExPEC CP9) was used which has now is been sequenced (unpublished data), thereby allowing the investigator to identify the virulence factors that may mediate translocation by simply screening for mutant strains that fail to translocate. This differs from the use of the nonpathogenic C25 strain used in previous studies. This feature is important because available data from characterized *E. coli* strains suggest that some isolates do contain a complement of key virulence factors (67). Perhaps the most significant addition, however, is that the model can be easily manipulated to include a growing tumor (here, a mammary carcinoma) that is responsive to chemotherapy (here, cyclophosphamide), as a result of which translocation is induced upon treatment of the tumor. This feature allows the investigator to examine translocation in the context of what a patient, having first been diagnosed with a malignancy and then undergoing a chemotherapy regimen, would be experiencing in the clinic.

Very little is understood regarding the underlying mechanisms that govern translocation. Issues remain concerning the role of the intestinal commensals in translocation (i.e., whether the composition of the GI microbiome predisposes one to translocation, and, if so, how), the mechanism by which translocating bacteria access the epithelium and subsequently pass through it, and the bacterial genes that are required for each step in the process (mucus clearance, adherence, invasiveness, survival in the blood, etc.). Regarding the route of translocation, some have suggested that intestinal macrophages phagocytose bacteria in the gut and transport them to the mesenteric lymph nodes (MLN) (68). There, the bacteria may gain access to the thoracic duct and enter the bloodstream to spread to other organs or tissues (69). Other work suggests that the hepatic portal vein is the dominant route of dissemination, with as much as 75% of all translocation occurring via this route (70). The routes of entry into the blood may also be different for different species of bacteria. For example, whereas *E. coli* and *P. mirabilis*, both of which are normal commensals of the GI tract, were found translocated to the MLN segments nearest to the intestinal segment that they colonized at the highest levels, the pathogen *Salmonella enterica* serovar Typhimurium translocated to every area of the MLN independently of its levels in the different segments of the intestine (71). Whereas macrophage-mediated transport of bacteria to the MLN and disruption of physical GI barrier by trauma or inflammation have been proposed as ways bacteria may translocate, it is also likely that different species have different mechanisms by which they transverse the host epithelium. These include direct invasion of the epithelium and paracellular transport through junctional complexes. Having viable models of translocation for the most frequently observed enterics associated with bloodstream infections, including the strains of *E. coli* reported here, will go a long way toward enhancing the possibility of applying the newest and most sensitive tracking methodologies to determine the exact route by which ExPEC enters the systemic circulation, as well as to begin to answer some of the questions already posed.

Compared to the work examining the host side of translocation, there is a considerable dearth of knowledge concerning the bacterial genes or factors that are necessary for this process. These include the mechanism by which bacteria move through a GI epithelial barrier, the surface proteins needed to engage the epithelium, the genes needed by the bacterium to resist host-specific killing processes, and the genes needed for survival and growth in blood and off-site tissues. While assessing whether the model system could be used to identify mutants in *E. coli* that lacked the ability to translocate, it was discovered that *sslE*, but not *cnf1* or *iroN*, was important for the process of translocation. This is important for two main reasons. First, it demonstrates that the model can be used to identify nontranslocating phenotypes. Knowing the genes involved in this process will allow one to develop vaccines, inhibitors, or probiotic strains that prevent translocation. Second, SslE is emerging as an important virulence factor for pathogenic *E. coli*. SslE encodes a 1,528-amino-acid protein

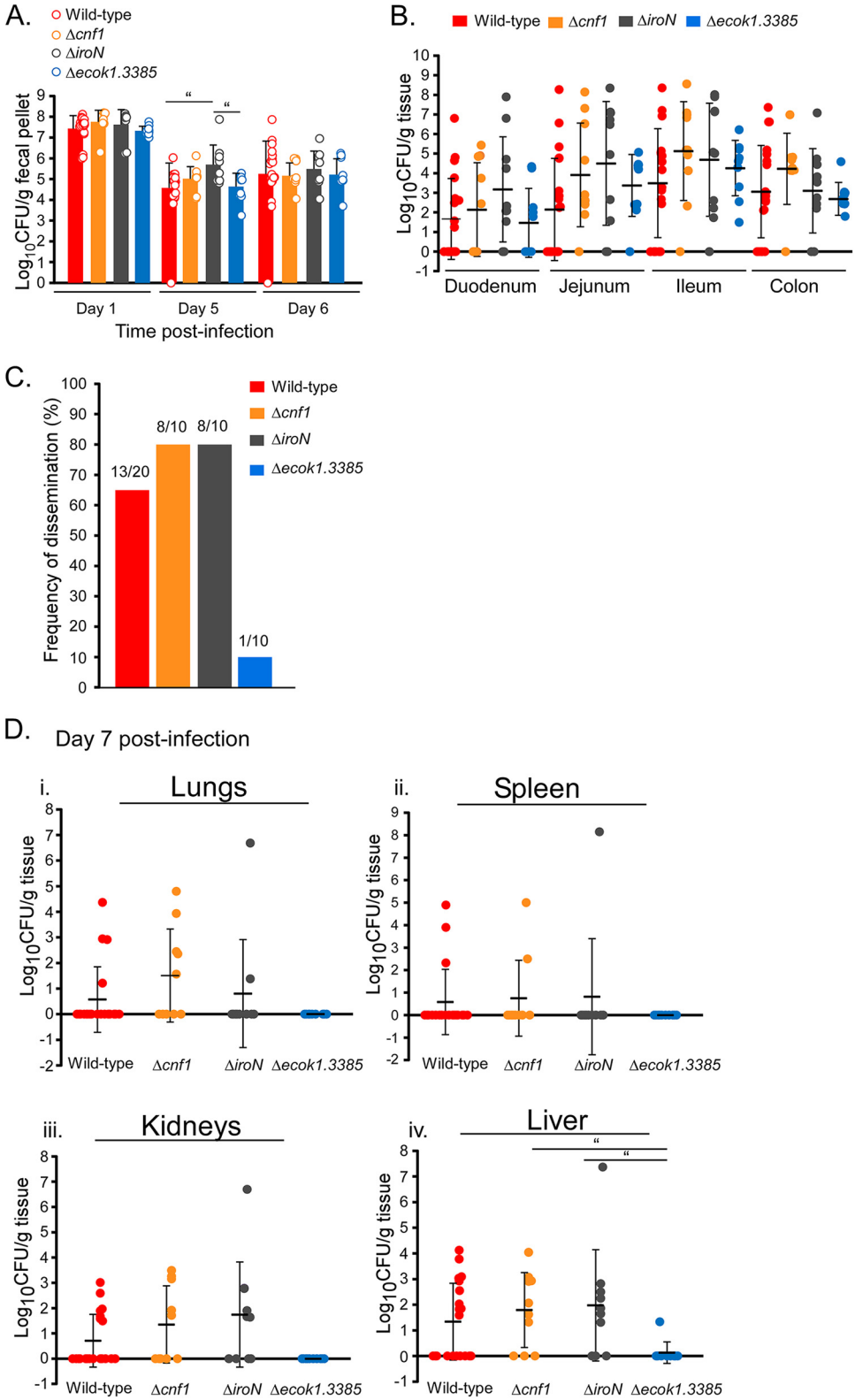


FIG 7 SslE is required for ExPEC translocation. (A) BALB/c mice were subjected to oral gavage with ExPEC CP9 wild-type or mutant strains at a dose of 1×10^9 CFU. Colonization was measured by plating fecal pellets on LB/agar^{Chlor+}. (B) Following treatment with CTX on days 1, 3, and 5, mice were euthanized on day 7 postinfection and intestinal tissue homogenates were plated on LB/agar^{Chlor+} media. (C) Translocated CP9 levels were determined by plating organs on LB/agar^{Chlor+}. Numbers above the bars represent the ratio of the number of mice with translocated CP9 bacteria in organs to the total number of mice in the group. (D) CFU levels of translocated CP9 bacteria were determined by plating organs on LB/agar^{Chlor+}. Error bars represent ± standard deviations, a double prime (″) denotes a significant ($P \leq 0.05$) difference between groups, and *n* was at least 10.

that was initially identified in a reverse vaccinology screen for *E. coli* proteins that protect against intraperitoneal or intravenous injection of ExPEC (58). Follow-up studies also demonstrated the efficacy of the vaccine in a murine model of ascending urinary tract infection (57). The gene encoding this protein is found in a majority of ExPEC strains and is localized to both the outer membrane (59) and medium, its secretion being dependent on the type II secretion system (T2SS) (58, 72). SslE is annotated as a M60-like metallopeptidase domain located between amino acids 1090 and 1386, the latter being widely distributed in bacteria associated with mucosal surfaces (73). The M60-like domain of SslE cleaves the intestinal mucins Muc 2 and 3, an activity that facilitates the access of heat-labile enterotoxin (LT) from enterotoxigenic *E. coli* (ETEC) to intestinal enterocytes (56) and is necessary for the passage of ExPEC through a synthesized mucin matrix (57, 74). Given this knowledge, it is possible that the lack of translocation observed for ExPEC devoid of *sslE* is because this strain cannot degrade the thick mucus barrier to gain access to the underlying intestinal epithelium. Future studies will seek to resolve the nature of this phenotype and to determine how it may be used to further development of an *E. coli* vaccine to protect against these types of infections.

There is increasing evidence that the translocation of endogenous microbiota is a common event in those who are very ill (51, 52, 75). The contribution of these translocating species to disease onset or progression, as well as how their byproducts or secreted toxins alter the immune response or host physiology, is currently unknown. The use of 16S rRNA gene sequencing as a method to characterize the "translocating microbiome" is an attractive area of study and will shed light on the details of the species that enter the blood and that may be difficult to grow by traditional microbiological methods. In this regard, while developing a model of ExPEC translocation, we also observed 16S rRNA gene evidence that members of the host endogenous microbiota were also present in the blood of these mice. In addition to the presence of microbial DNA in mice receiving chemotherapy (an expected finding that has been widely reported in immunosuppressed patients), there was a marked increase in both the diversity and the numbers of bacterial products in mice that were stably colonized by ExPEC. Given that the fecal commensals did not show this expansion and increase, these results suggest that ExPEC itself can stimulate or otherwise aid in the movement of bacteria or their products into the blood. A histological examination of the duodenum, jejunum, ileum, and large intestine did not identify any definitive morphological or inflammatory lesions that could specifically explain how ExPEC and/or other enterics breached the intestinal epithelial barrier (data not shown). Considering the number of diseases associated with translocation and the prevalence of ExPEC as a major translocating pathotype, this finding is worthy of further investigation and will be the topic of additional studies in the laboratory.

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REFERENCES

1. **Mukherjee S.** 2010. The emperor of all maladies: a biography of cancer. Simon and Schuster, New York, NY.
2. **Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F.** 9 October 2014, posting date. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer http://dx.doi.org/10.1002/ijc.29210.
3. **Hilali F, Ruimy R, Saulnier P, Barnabe C, Lebouguenec C, Tibayrenc M, Andremont A.** 2000. Prevalence of virulence genes and clonality in *Escherichia coli* strains that cause bacteremia in cancer patients. Infect Immun **68:**3983–3989. http://dx.doi.org/10.1128/IAI.68.7.3983-3989.2000.
4. **Aapro MS, Cameron DA, Pettengell R, Bohlius J, Crawford J, Ellis M, Kearney N, Lyman GH, Tjan-Heijnen VC, Walewski J, Weber DC, Zielinski C; European Organisation for Research and Treatment of Cancer (EORTC) Granulocyte Colony-Stimulating Factor (G-CSF) Guidelines Working Party.** 2006. EORTC guidelines for the use of granulocyte-colony stimulating factor to reduce the incidence of chemotherapy-induced febrile neutropenia in adult patients with lymphomas and solid tumours. Eur J Cancer **42:**2433–2453. http://dx.doi.org/10.1016/j.ejca.2006.05.002.
5. **Crawford J, Dale DC, Lyman GH.** 2004. Chemotherapy-induced neutropenia: risks, consequences, and new directions for its management. Cancer **100:**228–237. http://dx.doi.org/10.1002/cncr.11882.
6. **Huang E.** 2000. Internal medicine: handbook for clinicians, resident survival guide. Scrub Hill Press, Arlington, VA.
7. **Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis **39:**309–317. http://dx.doi.org/10.1086/421946.
8. **Samet A, Sledzinska A, Krawczyk B, Hellmann A, Nowicki S, Kur J, Nowicki B.** 2013. Leukemia and risk of recurrent *Escherichia coli* bacteremia: genotyping implicates *E. coli* translocation from the colon to the bloodstream. Eur J Clin Microbiol Infect Dis **32:**1393–1400. http://dx.doi.org/10.1007/s10096-013-1886-9.
9. **Talan DA, Stamm WE, Hooton TM, Moran GJ, Burke T, Iravani A, Reuning-Scherer J, Church DA.** 2000. Comparison of ciprofloxacin (7 days) and trimethoprim-sulfamethoxazole (14 days) for acute uncomplicated pyelonephritis in women: a randomized trial. JAMA **283:**1583–1590. http://dx.doi.org/10.1001/jama.283.12.1583.
10. **Hooton TM, Stamm WE.** 1997. Diagnosis and treatment of uncomplicated urinary tract infection. Infect Dis Clin North Am **11:**551–581. http://dx.doi.org/10.1016/S0891-5520(05)70373-1.
11. **de Louvois J.** 1994. Acute bacterial meningitis in the newborn. J Antimicrob Chemother **34**(Suppl A):61–73. http://dx.doi.org/10.1093/jac/34.suppl_A.61.
12. **Murphy SL.** 2000. Deaths: final data for 1998. Deaths: final data for 1998. Natl Vital Stat Rep **48:**1–105.
13. **Russo TA, Johnson JR.** 2003. Medical and economic impact of extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. Microbes Infect **5:**449–456. http://dx.doi.org/10.1016/S1286-4579(03)00049-2.
14. **Cometta A, Calandra T, Bille J, Glauser MP.** 1994. *Escherichia coli* resistant to fluoroquinolones in patients with cancer and neutropenia. N Engl J Med **330:**1240–1241. http://dx.doi.org/10.1056/NEJM199404283301717.
15. **Hentges DJ, Stein AJ, Casey SW, Que JU.** 1985. Protective role of intestinal flora against infection with *Pseudomonas aeruginosa* in mice: influence of antibiotics on colonization resistance. Infect Immun **47:**118–122.
16. **Rasko DA, Rosovitz MJ, Myers GS, Mongodin EF, Fricke WF, Gajer P, Crabtree J, Sebaihia M, Thomson NR, Chaudhuri R, Henderson IR, Sperandio V, Ravel J.** 2008. The pangenome structure of *Escherichia coli*: comparative genomic analysis of *E. coli* commensal and pathogenic isolates. J Bacteriol **190:**6881–6893. http://dx.doi.org/10.1128/JB.00619-08.
17. **Wolochow H, Hildebrand GJ, Lamanna C.** 1966. Translocation of microorganisms across the intestinal wall of the rat: effect of microbial size and concentration. J Infect Dis **116:**523–528. http://dx.doi.org/10.1093/infdis/116.4.523.
18. **Berg RD, Garlington AW.** 1979. Translocation of certain indigenous

bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. Infect Immun **23:**403–411.
19. **Steffen EK, Berg RD, Deitch EA.** 1988. Comparison of translocation rates of various indigenous bacteria from the gastrointestinal tract to the mesenteric lymph node. J Infect Dis **157:**1032–1038. http://dx.doi.org/10.1093/infdis/157.5.1032.
20. **Berg RD.** 1995. Bacterial translocation from the gastrointestinal tract. Trends Microbiol **3:**149–154. http://dx.doi.org/10.1016/S0966-842X(00)88906-4.
21. **Berg R.** 1992. The scientific basis of the probiotic concept, vol 3. Chapman and Hall, London, United Kingdom.
22. **Berg RD.** 1981. Promotion of the translocation of enteric bacteria from the gastrointestinal tracts of mice by oral treatment with penicillin, clindamycin, or metronidazole. Infect Immun **33:**854–861.
23. **Owens WE, Berg RD.** 1980. Bacterial translocation from the gastrointestinal tract of athymic (nu/nu) mice. Infect Immun **27:**461–467.
24. **Gautreaux MD, Deitch EA, Berg RD.** 1994. T lymphocytes in host defense against bacterial translocation from the gastrointestinal tract. Infect Immun **62:**2874–2884.
25. **Parks DA, Bulkley GB, Granger DN, Hamilton SR, McCord JM.** 1982. Ischemic injury in the cat small intestine: role of superoxide radicals. Gastroenterology **82:**9–15.
26. **Berg RD.** 1983. Bacterial translocation from the gastrointestinal tracts of mice receiving immunosuppressive chemotherapeutic agents. Curr Microbiol **8:**285–292. http://dx.doi.org/10.1007/BF01577729.
27. **Johnson JR, Russo TA, Scheutz F, Brown JJ, Zhang L, Palin K, Rode C, Bloch C, Marrs CF, Foxman B.** 1997. Discovery of disseminated J96-like strains of uropathogenic *Escherichia coli* O4:H5 containing genes for both PapG(J96) (class I) and PrsG(J96) (class III) Gal(alpha1-4)Gal-binding adhesins. J Infect Dis **175:**983–988. http://dx.doi.org/10.1086/514006.
28. **Russo TA, Guenther JE, Wenderoth S, Frank MM.** 1993. Generation of isogenic K54 capsule-deficient *Escherichia coli* strains through TnphoA-mediated gene disruption. Mol Microbiol **9:**357–364. http://dx.doi.org/10.1111/j.1365-2958.1993.tb01696.x.
29. **Russo TA, Singh G.** 1993. An extraintestinal, pathogenic isolate of *Escherichia coli* (O4/K54/H5) can produce a group 1 capsule which is divergently regulated from its constitutively produced group 2, K54 capsular polysaccharide. J Bacteriol **175:**7617–7623.
30. **Gudiol C, Bodro M, Simonetti A, Tubau F, Gonzalez-Barca E, Cisnal M, Domingo-Domenech E, Jimenez L, Carratala J.** 2013. Changing aetiology, clinical features, antimicrobial resistance, and outcomes of bloodstream infection in neutropenic cancer patients. Clin Microbiol Infect **19:**474–479. http://dx.doi.org/10.1111/j.1469-0691.2012.03879.x.
31. **Ani C, Farshidpanah S, Bellinghausen Stewart A, Nguyen HB.** 2015. Variations in organism-specific severe sepsis mortality in the United States: 1999–2008. Crit Care Med **43:**65–77. http://dx.doi.org/10.1097/CCM.0000000000000555.
32. **Velasco E, Byington R, Martins CA, Schirmer M, Dias LM, Goncalves VM.** 2006. Comparative study of clinical characteristics of neutropenic and non-neutropenic adult cancer patients with bloodstream infections. Eur J Clin Microbiol Infect Dis **25:**1–7. http://dx.doi.org/10.1007/s10096-005-0077-8.
33. **Pitout JD.** 2012. Extraintestinal pathogenic *Escherichia coli*: a combination of virulence with antibiotic resistance. Front Microbiol **3:**9. http://dx.doi.org/10.3389/fmicb.2012.00009.
34. **Johnson JR, Porter SB, Zhanel G, Kuskowski MA, Denamur E.** 2012. Virulence of *Escherichia coli* clinical isolates in a murine sepsis model in relation to sequence type ST131 status, fluoroquinolone resistance, and virulence genotype. Infect Immun **80:**1554–1562. http://dx.doi.org/10.1128/IAI.06388-11.
35. **Russo TA, Wang Z, Davidson BA, Genagon SA, Beanan JM, Olson R, Holm BA, Knight PR, III, Chess PR, Notter RH.** 2007. Surfactant dysfunction and lung injury due to the *E. coli* virulence factor hemolysin in a rat pneumonia model. Am J Physiol Lung Cell Mol Physiol **292:**L632–L643. http://dx.doi.org/10.1152/ajplung.00326.2006.
36. **Johnson JR, Clermont O, Menard M, Kuskowski MA, Picard B, Denamur E.** 2006. Experimental mouse lethality of *Escherichia coli* isolates, in relation to accessory traits, phylogenetic group, and ecological source. J Infect Dis **194:**1141–1150. http://dx.doi.org/10.1086/507305.
37. **Thomason LC, Costantino N, Court DL.** 2007. *E. coli* genome manipulation by P1 transduction. Curr Protoc Mol Biol **79:**V:1.17:1.17.1–1.17.8. http://dx.doi.org/10.1002/0471142727.mb0117s79.
38. **Rockwell SC, Kallman RF, Fajardo LF.** 1972. Characteristics of a serially transplanted mouse mammary tumor and its tissue-culture-adapted derivative. J Natl Cancer Inst **49:**735–749.
39. **Prinz RD, Willis CM, Viloria-Petit A, Kluppel M.** 2011. Elimination of breast tumor-associated chondroitin sulfate promotes metastasis. Genet Mol Res **10:**3901–3913. http://dx.doi.org/10.4238/2011.December.8.9.
40. **Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R.** 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J **6:**1621–1624. http://dx.doi.org/10.1038/ismej.2012.8.
41. **Edgar RC.** 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods **10:**996–998. http://dx.doi.org/10.1038/nmeth.2604.
42. **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R.** 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods **7:**335–336. http://dx.doi.org/10.1038/nmeth.f.303.
43. **Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO.** 2013. The SILVA ribosomal RNA gene database project: improved data processing and Web-based tools. Nucleic Acids Res **41:**D590–D596. http://dx.doi.org/10.1093/nar/gks1219.
44. **Lozupone C, Hamady M, Knight R.** 2006. UniFrac—an online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinformatics **7:**371. http://dx.doi.org/10.1186/1471-2105-7-371.
45. **Chao A, Chazdon RL, Colwell RK, Shen TJ.** 2006. Abundance-based similarity indices and their estimation when there are unseen species in samples. Biometrics **62:**361–371. http://dx.doi.org/10.1111/j.1541-0420.2005.00489.x.
46. **Shannon CE.** 1948. A mathematical theory of communication. Bell Syst Tech J **27:**379–423. http://dx.doi.org/10.1002/j.1538-7305.1948.tb01338.x.
47. **Brock N.** 1996. The history of the oxazaphosphorine cytostatics. Cancer **78:**542–547.
48. **Suchitra Ku Panigrahy SJ, Archana Tiwari.** 2011. Therapeutic use of cyclophosphamide and its cytotoxic action: a challenge for researchers. J Pharm Res **4:**2755–2757.
49. **Moore MJ.** 1991. Clinical pharmacokinetics of cyclophosphamide. Clin Pharmacokinet **20:**194–208. http://dx.doi.org/10.2165/00003088-199120030-00002.
50. **Twentyman PR.** 1977. Sensitivity to cytotoxic agents of the EMT6 tumour in vivo: tumour volume versus in vitro plating. 1. Cyclophosphamide. Br J Cancer **35:**208–217. http://dx.doi.org/10.1038/bjc.1977.28.
51. **Fouts DE, Torralba M, Nelson KE, Brenner DA, Schnabl B.** 2012. Bacterial translocation and changes in the intestinal microbiome in mouse models of liver disease. J Hepatol **56:**1283–1292. http://dx.doi.org/10.1016/j.jhep.2012.01.019.
52. **Merlini E, Bai F, Bellistri GM, Tincati C, d'Arminio Monforte A, Marchetti G.** 2011. Evidence for polymicrobic flora translocating in peripheral blood of HIV-infected patients with poor immune response to antiretroviral therapy. PLoS One **6:**e18580. http://dx.doi.org/10.1371/journal.pone.0018580.
53. **Zhang J, Mi L, Wang Y, Zhang D.** 2012. Detection of bacterial DNA in serum from colon cancer patients: association with cytokine levels and cachexia. J Cancer Ther Res **1:**19–23. http://dx.doi.org/10.7243/2049-7962-1-19.
54. **Russo TA, McFadden CD, Carlino-MacDonald UB, Beanan JM, Barnard TJ, Johnson JR.** 2002. IroN functions as a siderophore receptor and is a urovirulence factor in an extraintestinal pathogenic isolate of *Escherichia coli*. Infect Immun **70:**7156–7160. http://dx.doi.org/10.1128/IAI.70.12.7156-7160.2002.
55. **Rippere-Lampe KE, O'Brien AD, Conran R, Lockman HA.** 2001. Mutation of the gene encoding cytotoxic necrotizing factor type 1 (cnf(1)) attenuates the virulence of uropathogenic *Escherichia coli*. Infect Immun **69:**3954–3964. http://dx.doi.org/10.1128/IAI.69.6.3954-3964.2001.
56. **Luo Q, Kumar P, Vickers TJ, Sheikh A, Lewis WG, Rasko DA, Sistrunk J, Fleckenstein JM.** 2014. Enterotoxigenic *Escherichia coli* secretes a highly conserved mucin-degrading metalloprotease to effectively engage intestinal epithelial cells. Infect Immun **82:**509–521. http://dx.doi.org/10.1128/IAI.01106-13.
57. **Nesta B, Valeri M, Spagnuolo A, Rosini R, Mora M, Donato P, Alteri CJ, Del Vecchio M, Buccato S, Pezzicoli A, Bertoldi I, Buzzigoli L,**

Tuscano G, Falduto M, Rippa V, Ashhab Y, Bensi G, Fontana MR, Seib KL, Mobley HL, Pizza M, Soriani M, Serino L. 2014. SslE elicits functional antibodies that impair in vitro mucinase activity and in vivo colonization by both intestinal and extraintestinal *Escherichia coli* strains. PLoS Pathog **10:**e1004124. http://dx.doi.org/10.1371/journal.ppat.1004124.

58. **Moriel DG, Bertoldi I, Spagnuolo A, Marchi S, Rosini R, Nesta B, Pastorello I, Corea VA, Torricelli G, Cartocci E, Savino S, Scarselli M, Dobrindt U, Hacker J, Tettelin H, Tallon LJ, Sullivan S, Wieler LH, Ewers C, Pickard D, Dougan G, Fontana MR, Rappuoli R, Pizza M, Serino L.** 2010. Identification of protective and broadly conserved vaccine antigens from the genome of extraintestinal pathogenic *Escherichia coli*. Proc Natl Acad Sci U S A **107:**9072–9077. http://dx.doi.org/10.1073/pnas.0915077107.
59. **Baldi DL, Higginson EE, Hocking DM, Praszkier J, Cavaliere R, James CE, Bennett-Wood V, Azzopardi KI, Turnbull L, Lithgow T, Robins-Browne RM, Whitchurch CB, Tauschek M.** 2012. The type II secretion system and its ubiquitous lipoprotein substrate, SslE, are required for biofilm formation and virulence of enteropathogenic *Escherichia coli*. Infect Immun **80:**2042–2052. http://dx.doi.org/10.1128/IAI.06160-11.
60. **Yang J, Baldi DL, Tauschek M, Strugnell RA, Robins-Browne RM.** 2007. Transcriptional regulation of the yghJ-pppA-yghG-gspCDEFGHIJKLM cluster, encoding the type II secretion pathway in enterotoxigenic *Escherichia coli*. J Bacteriol **189:**142–150. http://dx.doi.org/10.1128/JB.01115-06.
61. **Berg RD, Wommack E, Deitch EA.** 1988. Immunosuppression and intestinal bacterial overgrowth synergistically promote bacterial translocation. Arch Surg **123:**1359–1364. http://dx.doi.org/10.1001/archsurg.1988.01400350073011.
62. **Steffen EK, Berg RD.** 1983. Relationship between cecal population levels of indigenous bacteria and translocation to the mesenteric lymph nodes. Infect Immun **39:**1252–1259.
63. **Penn RL, Maca RD, Berg RD.** 1985. Increased translocation of bacteria from the gastrointestinal tracts of tumor-bearing mice. Infect Immun **47:**793–798.
64. **Spaeth G, Berg RD, Specian RD, Deitch EA.** 1990. Food without fiber promotes bacterial translocation from the gut. Surgery **108:**240–246.
65. **Morehouse JL, Specian RD, Stewart JJ, Berg RD.** 1986. Translocation of indigenous bacteria from the gastrointestinal tract of mice after oral ricinoleic acid treatment. Gastroenterology **91:**673–682.
66. **Deitch EA, Bridges W, Baker J, Ma JW, Ma L, Grisham MB, Granger DN, Specian RD, Berg R.** 1988. Hemorrhagic shock-induced bacterial translocation is reduced by xanthine oxidase inhibition or inactivation. Surgery **104:**191–198.
67. **Vigil PD, Stapleton AE, Johnson JR, Hooton TM, Hodges AP, He Y, Mobley HL.** 2011. Presence of putative repeat-in-toxin gene tosA in *Escherichia coli* predicts successful colonization of the urinary tract. mBio **2:**e00066-11. http://dx.doi.org/10.1128/mBio.00066-11.
68. **Wells CL, Maddaus MA, Simmons RL.** 1987. Role of the macrophage in the translocation of intestinal bacteria. Arch Surg **122:**48–53. http://dx.doi.org/10.1001/archsurg.1987.01400130054008.
69. **Wells CL, Maddaus MA, Erlandsen SL, Simmons RL.** 1988. Evidence for the phagocytic transport of intestinal particles in dogs and rats. Infect Immun **56:**278–282.
70. **Mainous MR, Tso P, Berg RD, Deitch EA.** 1991. Studies of the route, magnitude, and time course of bacterial translocation in a model of systemic inflammation. Arch Surg **126:**33–37. http://dx.doi.org/10.1001/archsurg.1991.01410250037005.
71. **Gautreaux MD, Deitch EA, Berg RD.** 1994. Bacterial translocation from the gastrointestinal tract to various segments of the mesenteric lymph node complex. Infect Immun **62:**2132–2134.
72. **Peterson KM, Mekalanos JJ.** 1988. Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization. Infect Immun **56:**2822–2829.
73. **Nakjang S, Ndeh DA, Wipat A, Bolam DN, Hirt RP.** 2012. A novel extracellular metallopeptidase domain shared by animal host-associated mutualistic and pathogenic microbes. PLoS One **7:**e30287. http://dx.doi.org/10.1371/journal.pone.0030287.
74. **Valeri M, Rossi Paccani S, Kasendra M, Nesta B, Serino L, Pizza M, Soriani M.** 2015. Pathogenic *E. coli* exploits SslE mucinase activity to translocate through the mucosal barrier and get access to host cells. PLoS One **10:**e0117486. http://dx.doi.org/10.1371/journal.pone.0117486.
75. **Mi L, Lin J, Zheng H, Xu X, Zhang J, Zhang D.** 2012. Bacterial translocation contributes to cachexia from locally advanced gastric cancer. Hepatogastroenterology **59:**2348–2351. http://dx.doi.org/10.5754/hge11810.