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Escherichia coli Isolate for Studying Colonization of the Mouse Intestine and Its Application to Two-Component Signaling Knockouts

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The biology of *Escherichia coli* in its primary niche, the animal intestinal tract, is remarkably unexplored. Studies with the streptomycin-treated mouse model have produced important insights into the metabolic requirements for *Escherichia coli* to colonize mice. However, we still know relatively little about the physiology of this bacterium growing in the complex environment of an intestine that is permissive for the growth of competing flora. We have developed a system for studying colonization using an *E. coli* strain, MP1, isolated from a mouse. MP1 is genetically tractable and does not require continuous antibiotic treatment for stable colonization. As an application of this system, we separately knocked out each two-component system response regulator in MP1 and performed competitions against the wild-type strain. We found that only three response regulators, ArcA, CpxR, and RcsB, produce strong colonization defects, suggesting that in addition to anaerobiosis, adaptation to cell envelope stress is a critical requirement for *E. coli* colonization of the mouse intestine. We also show that the response regulator OmpR, which had previously been hypothesized to be important for adaptation between *in vivo* and *ex vivo* environments, is not required for MP1 colonization due to the presence of a third major porin.

Escherichia coli is one of the most extensively studied and best-characterized organisms. Its high growth rate, facile genetics, and simple nutritional requirements have made this bacterium an excellent model system for studying basic aspects of molecular biology and bacteriology and the primary host for DNA and protein engineering. The physiology of *E. coli* growth and survival under diverse conditions has been intensively studied, and a significant fraction of *E. coli* gene products and regulatory networks have been characterized. However, for such a well-studied organism, we know remarkably little about the biology of *E. coli* in its primary niche: the animal gastrointestinal tract.

E. coli is generally the most abundant aerobe in the intestines of warm-blooded vertebrates, although its numbers vary considerably with animal host and geography (1–3). As a species, this bacterium has a remarkable genetic diversity; the number of genes in common among fully sequenced isolates is less than half the number of genes in any individual strain (4–6). Some *E. coli* strains are pathogenic, depending on the host and site of infection (3, 7–9), and have been intensively studied to understand the factors controlling their virulence. However, the majority of *E. coli* strains associated with animals are believed to be part of the normal flora of the intestine, growing asymptomatically as commensals.

Most of our knowledge about *E. coli* colonization of the animal intestine comes from studies with streptomycin-resistant strains colonizing mice fed streptomycin continuously in their drinking water (10, 11). This streptomycin-treated mouse model has played a key role in the characterization of the growth of *E. coli* in the intestine and the identification of nutritional and metabolic requirements for colonization (10, 12–16). The model has been particularly effective because it not only overcomes colonization resistance—the barrier to establishing an infection in an animal whose microbial flora is unperturbed—but also enables colonization with strains that would otherwise be unable to compete with bacteria that are well adapted to the host (10, 11, 17). Streptomycin eliminates a significant portion of the microbial diversity in the mouse intestine (17) and enables *E. coli* to greatly expand the niches that it occupies. Indeed, the number of *E. coli* CFU in mouse feces in the streptomycin-treated mouse is 3 to 4 orders of magnitude higher than the number typically found in untreated mice (17). Thus, while this model system remains an invaluable tool for studying colonization, continuous streptomycin treatment may eliminate stresses or other environmental factors that emerge from increased competition with other bacteria or from attendant host responses.

We have developed a system for stable *E. coli* colonization of mice that does not require continuous antibiotic treatment, enabling the study of colonization without preventing growth of competing bacteria from the environment. The system is based on a genetically manipulable *E. coli* strain (designated MP1) that we isolated from a mouse. Streptomycin pretreatment is still necessary for MP1 to gain an initial hold in the intestine, but no subsequent treatment with antibiotics is needed for stable colonization.

We used this system to test the requirements for individual two-component signaling systems in intestinal colonization. Of the 32 histidine kinase-response regulator pairs identified in the MP1 genome, we found that only three (Cpx, Rcs, and Arc) are essential for colonization in competitions out to at least 20 days. Other two-component systems, many of which have been identified as important for infection by pathogenic *E. coli* or related bacteria, did not show strong colonization defects. We further demonstrate that MP1 expresses a third major porin, in addition

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to the classical porins OmpF and OmpC, which renders the EnvZ/OmpR two-component system nonessential for colonization. This work is the first systematic study of the role of two-component signaling in *E. coli* colonization of the intestine and introduces a valuable resource for studies of *E. coli* physiology and colonization as a commensal.

MATERIALS AND METHODS

E. coli HS was obtained from James Nataro, and Nissle 1917 was obtained from Dean Hamer.

Isolation of MP1. Fecal pellets from CD-1 mice (Charles River Laboratories) were resuspended in 10 mM $MgSO_4$, and dilutions were spread on MacConkey lactose agar plates (BD-Difco). Red colonies were picked and streaked on LB agar plates containing 40 μg/ml X-Gluc (5-bromo-4-chloro-3-indolyl-β-D- glucuronic acid) to identify cells expressing beta-glucuronidase. Dark blue colonies were picked and tested in a spot titer assay with the phage $P1_{vir}$ to screen for *E. coli* strains that are susceptible to P1 (18). Two of six colonies tested produced plaques. One of these was selected and named MP1.

Genome sequencing and analysis. The MP1 genome was sequenced by 454 pyrosequencing at the University of Florida ICBR Genomic Core facility. *De novo* assembly was performed with Newbler and produced 84 contigs with approximately 20× coverage. The phylogenetic group of MP1 was derived by the reconstruction of a maximum-likelihood tree from concatenated sequences of complete coding regions of 7 housekeeping genes frequently used for multilocus sequence typing (MLST) analysis of *E. coli*: *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*. The sequence type (ST) of each strain was based on the internal fragments of these 7 genes, as described in the MLST online database (http://mlst.ucc.ie/mlst/dbs/Ecoli) (19).

Construction of marked strains. The plasmid pAS07 (A. Siryaporn and M. Goulian, unpublished data) consists of the integration vector pCAH63 (20) with the *uidA* gene replaced with a sequence consisting of *tetR* and an operon fusion of *tetA* with *gfpmut3.1* (Clontech). The *tetR tetA* cassette was isolated by PCR from the transposon Tn*10* in the *E. coli* strain XL1-Blue (Stratagene). pML8 is pAS07 with *mcherry* (taken from pRSETb-mCherry [21]) in place of *gfpmut3.1*. The plasmids pAS07 and pML8 were integrated into the MP1 genome at the phage lambda attachment site and verified to be present in single copy by PCR as described in reference 20, resulting in MP13 and MP7, respectively. Both pAS07 and pML8 carry the genes *cat* and *tetA*, conferring resistance to chloramphenicol and tetracycline. However, for reasons that we do not understand, the *cat* gene does not provide high-level resistance to chloramphenicol in MP1.

Construction of deletion strains. Deletions were constructed by recombineering essentially as described in reference 22, except that electrocompetent cells were prepared by washing with an ice cold solution containing 20% glycerol and 1 mM unbuffered 3-(*N*-morpholino) propanesulfonic acid (MOPS) (23). For those response regulator genes in MP1 with at least 50 bp of flanking sequence identical to the sequence in *E. coli* K-12, we used the corresponding response regulator deletion from the Keio collection (24) as the template for the PCR. This made it possible in some cases to use long regions of homology on one or both sides of the response regulator gene without exceptionally long primers or extra PCR steps. In the few cases where the flanking sequence in MP1 was significantly different from the sequence in K-12 for at least one side of the gene, as well as for the genes that were not present in K-12, PCR primers with 40 to 50 bases of homology to the MP1 genome sequence were used with the template pKD13 (22). For these cases, gene knockouts were constructed in the same manner as for the Keio collection—using the same segment of pKD13 for the insertion and replacing all but the start codon and the last six codons of the targeted gene (24). Deletions were constructed in MP1 with selection on 35 μg/ml kanamycin, verified by PCR, and then moved into other strain backgrounds (e.g., MP13) by transduction with $P1_{vir}$. In cases where kanamycin sensitivity was required to move in a second deletion, the kanamycin resistance gene was removed with Flp recombinase by transformation with pCP20 as described in reference 22.

Construction of complementation strains. To complement the *arcA*, *rcsB*, and *cpxR* deletions, the corresponding genes with their promoters were isolated from MP1 genomic DNA by PCR with primers that added flanking BamHI and SacI restriction sites upstream and downstream of the gene, respectively. Following digestion with BamHI and SacI, the DNA segments were ligated into the (similarly digested) chromosomal integration plasmid pAH70 (20). The kanamycin resistance gene in the corresponding recipient strain (Δ*arcA*::*kan*, Δ*rcsB*::*kan*, or Δ*cpxR*::*kan*) was removed with pCP20 (22), and the complementing gene was inserted at the HK022 attachment site by transient expression of the phage integrase from plasmid pAH69 and verified to be in single copy as described in reference 20.

Inoculation of mice. All animal studies were carried out in accordance with animal protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Five-week-old CD-1 mice (Charles River Laboratories) were fed streptomycin and glucose in their drinking water, both at a concentration of 5 mg/ml, for 72 h. The drinking water was then replaced with water without antibiotic and glucose, and the mice were not given any further antibiotic treatment. After an additional 24 h, the mice were orally inoculated by gavage with ~10^9 cells in 100 μl phosphate-buffered saline (PBS), resuspended from an overnight culture as described below. For each strain, a single colony was picked from an LB agar plate containing kanamycin (35 μg/ml) and resuspended in 1 ml of LB (LB Miller medium; BD Difco). Eight microliters of this suspension was inoculated into 8 ml of LB and grown overnight (~16 h) at 37°C on a roller drum at ~40 rpm. A volume of this culture equal to 4.8 ml per unit of optical density at 600 nm (OD_{600}) of a 1:10 dilution of the overnight culture was spun down at 3,800 × *g* and 4°C, resuspended in 2 ml of ice-cold PBS, spun down again as described above, and resuspended in a volume of ice-cold PBS equal to 1/50 of the culture volume used for the first spin. The mutant and wild-type cell suspensions were mixed 1:1, and mice were orally inoculated with 100 μl. A portion of the remaining cell suspension was then serially diluted and plated on LB agar containing 15 μg/ml tetracycline to determine the input CFU. Mice were raised on standard laboratory rodent diet (LabDiet 5001).

Determination of *E. coli* CFU. Fresh feces were weighed and resuspended as a slurry in PBS, and serial dilutions were plated on LB containing 15 μg/ml tetracycline. Fluorescence images of plates were obtained using a homemade system as described previously (25). The competitive index (CI) was determined as [(GFP fluorescent CFU)/(mCherry fluorescent CFU)]/[(input GFP fluorescent CFU)/(input mCherry fluorescent CFU)], where the input CFU was determined from the inoculum.

Analysis of outer membrane proteins. Outer membranes were isolated using a modification of the Sarkosyl extraction protocol described in reference 26. Cells from 1 ml of an overnight culture grown in LB at 37°C were resuspended in 1 ml 10 mM HEPES (pH 7.4). The cells were then lysed by sonication and centrifuged at 6,000 × *g* for 4 min. The supernatant was then centrifuged at 50,000 × *g* for 1 h at 4°C. The pellet was resuspended in 400 μl 1% (wt/vol) *N*-lauroylsarcosine (Sarkosyl) in 10 mM HEPES (pH 7.4), incubated at 37°C for 30 min with shaking, and then centrifuged at 50,000 × *g* for 1 h at 4°C. The pellet was resuspended in 20 μl 10 mM HEPES (pH 7.4). Outer membranes were analyzed by SDS-PAGE with gels containing 6 M urea.

Nucleotide sequence accession numbers. The whole-genome shotgun project for *E. coli* MP1 has been deposited at DDBJ/EMBL/GenBank under accession no. JEMI00000000. The version described in this paper is version JEMI01000000. The MP1 genome sequencing project is registered at NCBI under Bioproject ID PRJNA196008.

RESULTS

Isolation and characterization of MP1. We initially attempted to colonize mice with two human commensal *E. coli* strains, Nissle 1917 and HS (4, 27–29), without continuous selection with an

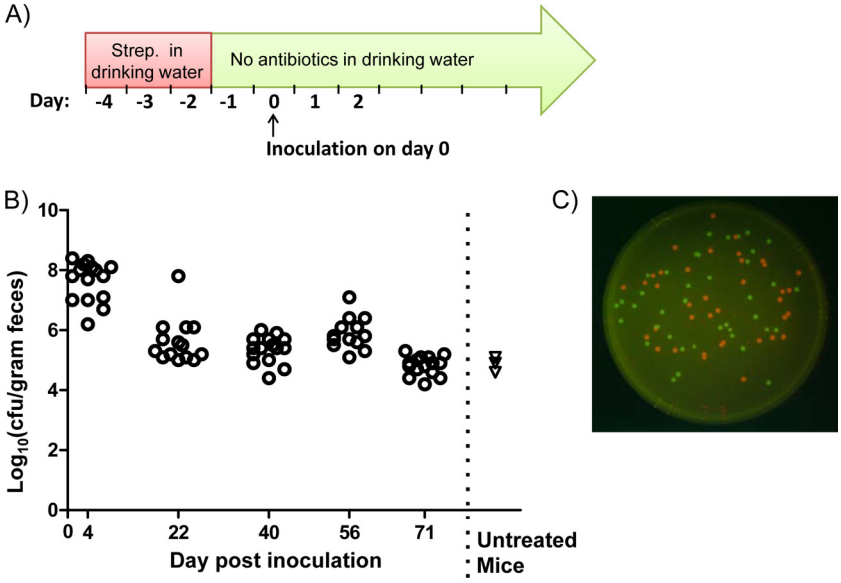

FIG 1 Colonization of mice with *E. coli* strain MP1. (A) Schematic of the colonization protocol. Mice were fed streptomycin (Strep.) in their drinking water for 72 h. The water with streptomycin was then removed and replaced with antibiotic-free water, and there was no further antibiotic treatment. After an additional 24 h, mice were orally inoculated with approximately 10^9 CFU of *E. coli* cells by gavage. (B) Circles show data for 16 mice colonized with MP7, an *mcherry*-marked MP1 derivative (MP1 att$_\lambda$::pML8). CFU were measured on the indicated days following inoculation. Triangles show measurements of total *E. coli* (Lac$^+$ colonies on MacConkey plates) for three mice that were not treated with streptomycin and were not exposed to MP1. (C) Colonies of MP7 and MP13, a *gfp*-marked MP1 derivative (MP1 att$_\lambda$::pAS07). The image is an overlay of red and green fluorescence images of colonies growing on LB agar containing 15 μg/ml tetracycline.

antibiotic. However, in both cases, we found that colonization was often relatively short-lived and showed significant mouse-to-mouse variability (data not shown). Furthermore, while we could move selectable markers from *E. coli* K-12 into Nissle 1917 and HS by transduction with bacteriophage P1$_{vir}$, we were unable to produce transducing particles, or infectious P1 virions, from either of these strains. Therefore, to find *E. coli* strains that are robust colonizers of the mouse gastrointestinal (GI) tract and are also convenient to manipulate genetically, we isolated *E. coli* from the feces of healthy mice and screened for strains that produce plaques with

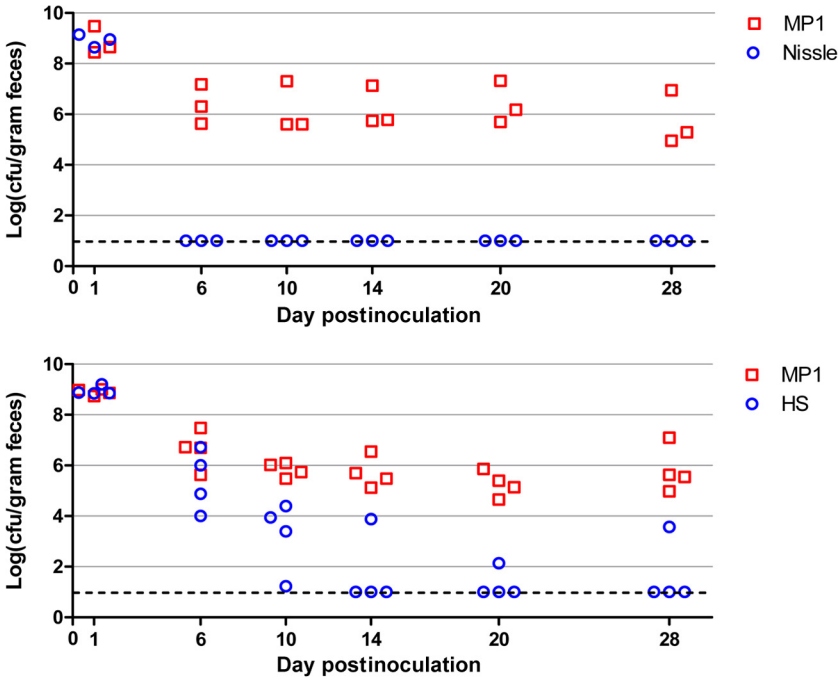

FIG 2 Mouse colonization competitions between MP1 and HS or Nissle. Each symbol represents CFU measurements for a single mouse, taken at the indicated days postinoculation. The dashed lines indicate the detection limit. The strains used for colonization were MP7 (MP1 att$_\lambda$::pML8), HS att$_\lambda$::pAS07, and Nissle att$_\lambda$::pAS07.

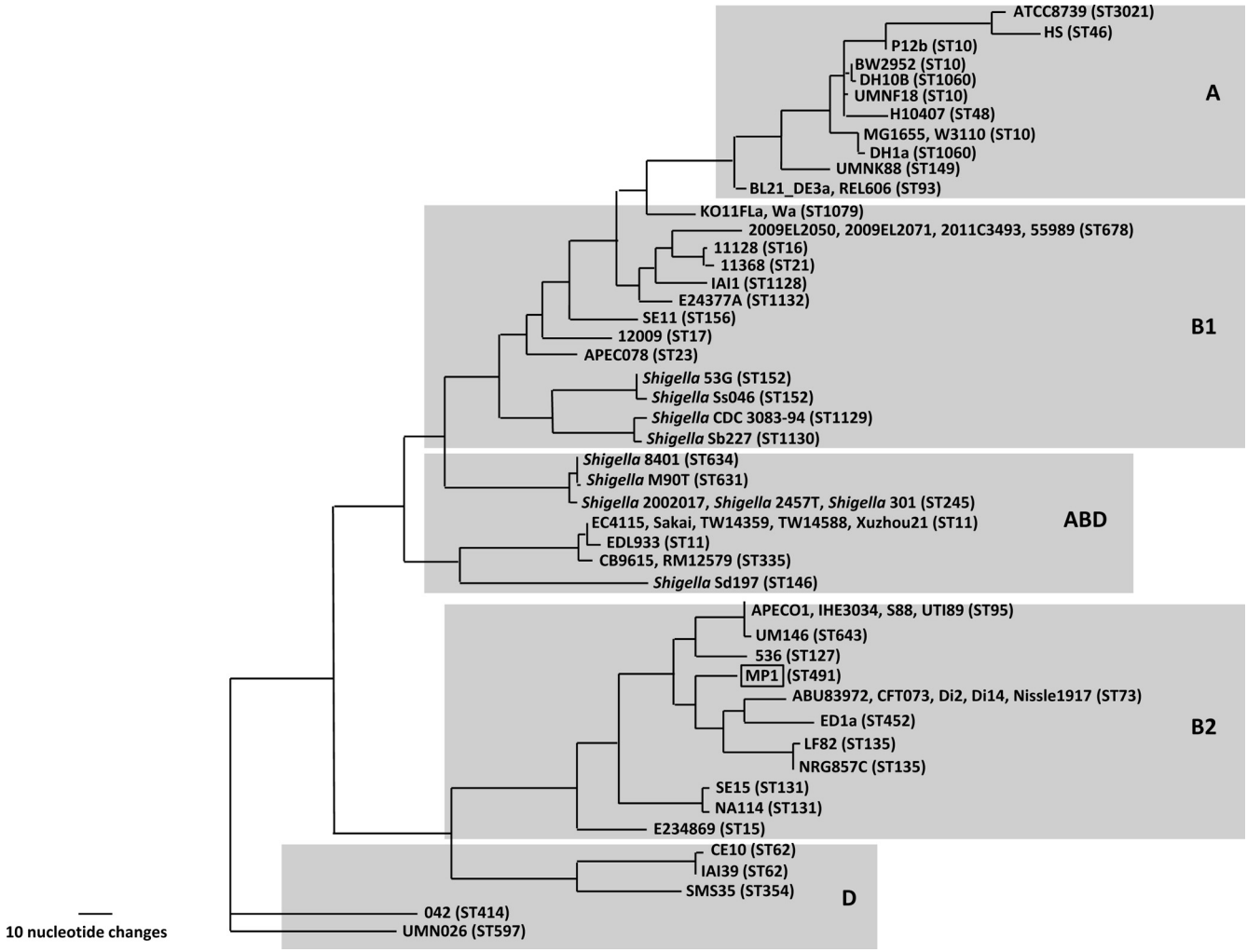

FIG 3 MLST-based tree of 67 *E. coli* strains. The gray boxes denote each phylogenetic group, and the ST of each strain is indicated.

bacteriophage $P1_{vir}$ in a soft-agar assay. After testing two isolates for their colonization potential, we settled on one strain, which we named MP1.

To follow colonization, we constructed fluorescently marked derivatives of MP1 using a tightly regulated expression system based on the tetracycline repressor and resistance genes *tetR* and *tetA* from the transposon Tn*10*. Operon fusions *tetA-mcherry* or *tetA-gfp*, together with *tetR*, were integrated at the lambda phage attachment site. (MP1 is λ^- and has the $attB_\lambda$ sequence found in *E. coli* K-12.) In the absence of tetracycline, mCherry and GFP expression are tightly off. In the presence of tetracycline, however, fluorescence is easily detectable in colonies growing on agar plates (Fig. 1C) (30).

To overcome colonization resistance, mice were pretreated with streptomycin in their drinking water (5 mg/ml) for 72 h but received no additional streptomycin following this treatment. After an additional 24 h to allow clearance of antibiotic, mice were orally inoculated (intragastrically) with $\sim 10^9$ CFU of *E. coli* cells (Fig. 1A). With this protocol, we found that MP1 colonizes mice to at least 71 days—the longest interval that we tested—with a colonization level in the range of 10^5 to 10^6 CFU per gram of feces (Fig. 1B). We also verified that MP1 is significantly better than Nissle or HS at colonizing mice (Fig. 2). In competition experiments with MP1, Nissle was below the detection limit by 6 days postinfection. HS was a better colonizer than Nissle but was below the detection limit in 3 of 4 mice by 14 days. We note that the colonization levels measured for MP1 (10^5 to 10^6 CFU/gram feces) are comparable to the levels we measured for *E. coli* in the normal flora of untreated mice (Fig. 1) and are consistent with previous reports (for example, see reference 17).

MP1 genome. We assembled 84 contigs from 454 pyrosequencing of the MP1 genome. Multilocus sequence typing (MLST) (19) assigns MP1 to the sequence type ST491. At present, two other strains in the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli) belong to ST491, and both are human isolates: ROAR372, a B2 commensal isolated in France (31), and G110b, a Lac^- enteroaggregative *E. coli* (EAEC) strain isolated in Nigeria (Iruka Okeke, personal communication). A phylogenetic tree for 67 genomes based on the sequences of the 7 housekeeping genes used for MLST places MP1 squarely within the B2 phylogenetic group and closest to the aggregative and invasive *E. coli* (AIEC) strains LF82 (32) and NRG 857C (33), the urinary pathogenic *E. coli* (UPEC) strains CFT073 (34) and D i14 and D i2 (35), and the human commensal strain ED1a (5) (Fig. 3).

TABLE 1 List of potential ExPEC-associated virulence factors and their presence or absence in MP1[a]

Factor type or function	Gene	Result[b]
Fimbriae/adhesins	*fimH*	+
	fmlA (c1936)	+
	c2395	+
	yadN	+
	aufA	+
	ygiL	+
	yfcV	+
	pixC	+
	ppdD	+
	yehA	+
	csgA	+
	sfaS	−
	papG	−
	focH	−
	draC	−
	iha	−
Toxins	*picU*	±[c]
	hlyA	−
	cnf1	−
	cdtA	−
	sat	−
	astA (EAST1)	−
Iron acquisition	*irp1*	+
	fyuA	+
	chuA	+
	entF	+
	iutA	−
	iucA	−
	iroN	−
	ireA	−
	cvaC	−
Protectins	*kpsMT* group III	−
	kpsMT group II	−
	traT	−
	ibeA	−
Miscellaneous	*ompT*	+
	iss	+
	aslA	+
	usp	−

[a] Potential ExPEC-associated virulence genes were taken from references 88 and 89.
[b] +, the gene is present and the coding sequence is intact; −, the gene is absent; ±, the gene is present but not functional.
[c] Based on the current MP1 genome sequence, the *picU* gene has a frameshift caused by a single base deletion.

The B2 phylogenetic group has a high representation of extraintestinal pathogenic *E. coli* (ExPEC). More generally, the genomes of B2 strains, whether or not they are ExPEC, often encode significant numbers of ExPEC virulence factors (36, 37). MP1 is no exception; its genome encodes many proteins that are potentially associated with extraintestinal virulence, especially adhesins and iron acquisition systems (Table 1).

MP1 also harbors an 8.5-kb plasmid that carries the colicin gene *colY* (38). Consistent with colicin production, MP1 colonies produced zones of clearing in a soft-agar overlay with *E. coli* K-12 strain MG1655, and this bactericidal activity was lost when MP1 was cured of the plasmid (data not shown).

Two-component system knockouts. We applied this new model system to study the effects of disrupting individual two-component signaling response regulators on MP1 colonization of mice. The *E. coli* K-12 strain MG1655 has 30 histidine kinase-response regulator pairs that have been shown or predicted to function as two-component signaling systems (39–41). From the MP1 genome sequence, we identified 32 two-component systems (31 histidine kinase-response regulator pairs plus the CheA/CheB/CheR chemotaxis proteins), 30 of which are shared with MG1655. Of the two that are not found in MG1655, one is the KguS/KguR two-component system, which was recently identified as a regulator of α-ketoglutarate utilization in *E. coli* CFT073 and other UPEC strains (42), and the other is orthologous to the PgtB/PgtA system from *Salmonella*, which is associated with regulation of phosphoglycerate transport (43, 44).

Response regulator gene deletions were constructed by recombineering and moved into an MP1 derivative marked with *gfp* (MP13) by P1 transduction. Competitions were performed against an MP1 derivative marked with *mcherry* (MP7). Competitive indices determined from mouse feces after at least 20 days following inoculation are shown in Fig. 4. Of the 32 deletions, three (*arcA*, *cpxR*, and *rcsB*) resulted in severe colonization defects, with competitive indices that were below our detection limit of 10^{-4} for all or virtually all of the mice. To verify that the observed colonization defects were due to the absence of the targeted response regulators, we tested whether integration of the genes at an ectopic site complemented the deletions. The *arcA*, *cpxR*, and *rcsB* genes with their native promoters were integrated at the bacteriophage HK022 attachment site in the chromosome in the respective deletion strains. Integration at this site, which is between *torS* and *torT*, may disrupt TorS/TorR signal transduction. However, a *torR* deletion did not show a detectable colonization defect (Fig. 4). For all three cases, restoration of the response regulator gene complemented the deletion, indicating that the observed colonization defects from the deletions are due to loss of the ArcA, CpxR, and RcsB response regulators (Fig. 5).

EnvZ/OmpR and porins in MP1. We were surprised that an *ompR* deletion did not have a strong effect on colonization. In the absence of OmpR, the classical porins OmpF and OmpC are not expressed, which greatly reduces outer membrane permeability and the rate of nutrient uptake (45–49). We compared the high abundance outer membrane proteins of MG1655 and MP1 by urea–SDS-PAGE and found that the two strains have different outer membrane protein profiles. In particular, MP1 and MP1 Δ*ompR* show a strong band not present in MG1655 (Fig. 6), suggesting at least one additional MP1 outer membrane protein is highly expressed in cells growing in LB at 37°C. We hypothesized that this protein could be a major porin in MP1 not present in MG1655 that provides a significant component of the outer membrane permeability in *ompR*-null strains. To test this hypothesis, we compared the sensitivity of MG1655 and MP1 to the beta-lactam antibiotic cefoxitin. Deletion of *ompR* in K-12 strains confers cefoxitin resistance due to decreased outer membrane permeability from the loss of OmpC and OmpF expression (50), (Table 2). In contrast, MP1 Δ*ompR* is cefoxitin sensitive (Table 2), which is consistent with additional porins contributing to outer membrane permeability. To look for a porin that provides cefoxitin access to the periplasm in MP1 Δ*ompR*, i.e., in the absence of the porins OmpF and OmpC, we moved selected kanamycin resistance insertions from the *E. coli* K-12 Keio collection (24) into

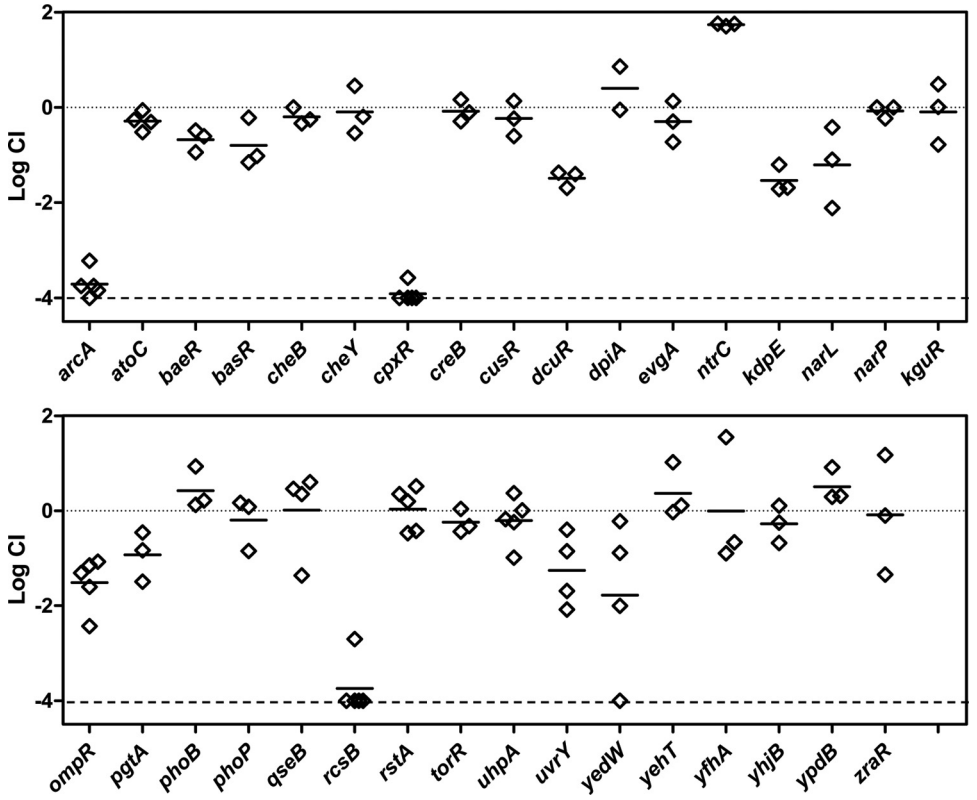

FIG 4 Mouse colonization competitions between response regulator deletion strains and wild type. The competitive index (CI) is shown for competitions with the indicated response regulator deletion. Response regulator deletions were in the strain MP13 (MP1 att$_\lambda$::pAS07) and competed against the marked wild-type strain MP7 (MP1 att$_\lambda$::pML8). Each symbol represents measurements for a single mouse, taken at least 20 days postinoculation, and the horizontal bars indicate the geometric means. The lower dashed line is the detection limit (10^{-4}).

MP1 Δ*ompR* by P1 transduction and screened for resistance to 15 μg/ml cefoxitin. We chose kanamycin insertions in genes that are near candidate porin genes in MP1 (identified by sequence similarity to OmpF). This strategy was predicated on the assumption that only a single porin locus was responsible for cefoxitin permeation across the outer membrane in MP1 Δ*ompR* and that there would be sufficient flanking homology for P1 transduction to replace the locus with a K-12 chromosomal segment with reasonable efficiency. Two transductants that we tested were cefoxitin resistant, and the chromosomal regions for both were linked to two candidate porin genes that are within 25 kb of each other. Based on sequence similarity and chromosome location, one of the genes is *yedS*, a pseudogene in MG1655 that is intact in MP1 and in some other *E. coli* isolates, including CFT073. The second gene encodes a protein that is 90% identical to NmpC, a porin in MG1655 that is encoded by a gene within the cryptic prophage

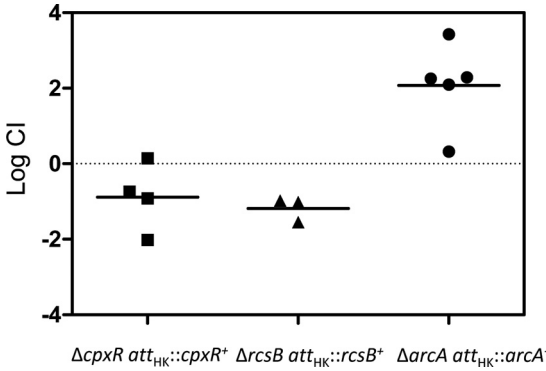

FIG 5 Complementation of *arcA*, *rcsB*, and *arcA* deletions in mouse colonization competition assays. The complemented genes were inserted at the phage HK022 attachment site, as described in Materials and Methods. Each symbol represents an individual mouse, and the bars indicate the geometric means. The competitive indices were determined from the numbers of CFU in feces at 21 days (*cpxR* and *arcA*) or 14 days (*rcsB*) postinoculation.

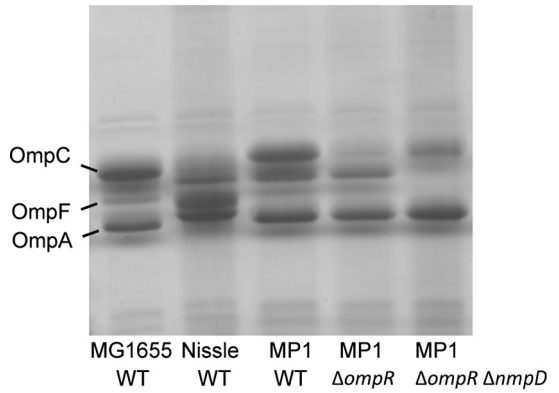

FIG 6 Outer membrane profiles for wild-type (WT) strains and the indicated mutants. Outer membranes were resolved by urea–SDS-PAGE followed by staining with Coomassie brilliant blue.

TABLE 2 Cefoxitin susceptibility

Strain	Growth on cefoxitin (15 μg/ml)
MG1655	No
MG1655 *ompR*	Yes
MP1	No
MP1 Δ*ompR*	No
MP1 Δ*ompR* Δ*yedS*	No
MP1 Δ*ompR* Δ*nmpD*	Yes
MP1 Δ*nmpC*	No

DLP12 that is disrupted by an insertion sequence (51, 52). Since this porin gene in MP1 is located in a different region of the chromosome, we have named it *nmpD*, to avoid confusion with *E. coli* K-12 *nmpC*. Several strains closely related to MP1, including CFT073, LF82, and NRG 857C, have both *nmpC* (without the insertion sequence) and *nmpD* (32–34). In particular, NmpD from MP1 has a predicted amino acid sequence that is identical to that of the protein C2348, encoded in the CFT073 genome. We deleted *nmpD* and *yedS* in MP1 Δ*ompR* and tested the sensitivity of these strains to cefoxitin. MP1 Δ*ompR* Δ*yedS* failed to grow on 15 μg/ml cefoxitin, but MP1 Δ*ompR* Δ*nmpD* was resistant (Table 2). In addition, comparison of urea–SDS-PAGE gels of MP1 and MP1 *nmpD* outer membranes indicates that NmpD is an abundant protein in the outer membrane (Fig. 6). In mouse competitions we found that MP1 Δ*nmpD* has a relatively mild colonization defect, similar to MP1 Δ*ompR*, but the double-deletion strain MP1 Δ*ompR* Δ*nmpD* is severely compromised in its ability to colonize mice (Fig. 7). Taken together, these results suggest that that NmpD is a major porin in the MP1 outer membrane that makes a significant contribution to outer membrane permeability and does not require OmpR for expression.

DISCUSSION

MP1 provides a new model system for studying *E. coli* colonization of the mouse gastrointestinal tract. This strain does not require continuous antibiotic treatment or the use of germfree mice, enabling studies of growth and survival in complex polymicrobial environments. MP1 can be manipulated with the standard genetic techniques that have proved so valuable for working with laboratory *E. coli* strains, including P1 transduction and recombineering. Therefore, this system can be used to engineer strains not only to study *E. coli* physiology but also to monitor physical, chemical, and biological properties of the mouse large intestine and to create defined perturbations of this environment.

We found that pretreating mice with streptomycin prior to inoculation was necessary to ensure reproducibly stable colonization from a single inoculation. This pretreatment presumably renders specific niches in the intestinal tract accessible to MP1 by eliminating pre-existing bacteria, especially facultative anaerobes (10, 17, 53). Previous studies suggest that the normal flora returns by 5 to 6 days following streptomycin treatment (54, 55). Our observation that within a week MP1 colonization stabilizes at 10^5 to 10^6 CFU/gram of feces—a colonization level comparable to *E. coli* counts in untreated mice (Fig. 1) (17)—is consistent with a rapid return of competing flora.

MP1 is in the B2 *E. coli* phylogenetic group (reviewed in reference 3), and is closely related to several ExPEC and human commensal strains (Fig. 3). Furthermore, as with many other B2 group *E. coli* strains, MP1 contains a considerable number of genes associated with ExPEC virulence (Table 1). We cannot rule out the possibility that MP1 is a bona fide ExPEC strain, capable of causing infection at extraintestinal sites in addition to colonizing the GI tract as a commensal (8, 9, 37). Interestingly, Nissle 1917, a B2 commensal strain closely related to MP1, colonized mice more poorly than *E. coli* HS, a member of phylogenetic group A that is quite distant from MP1 (Fig. 2–3). This suggests that fitness in the mouse GI tract depends on elements of the pangenome that are not necessarily shared by otherwise closely related strains (4, 5) and is consistent with previous studies demonstrating that individual species are often colonized by *E. coli* from multiple phylogenetic groups (2, 56).

To study the role of specific signal transduction systems in colonization, we constructed individual knockouts of the response regulators from histidine kinase-response regulator pairs identified in the MP1 genome and performed competitions against a marked derivative of wild-type MP1 in mouse colonization experiments. Interestingly, severe defects were observed only for three response regulator deletions: *arcA*, *cpxR*, and *rcsB*. The defect for Δ*arcA* is the most easily interpreted of the three, as the ArcB/ArcA two-component system is important for controlling the transition from aerobic to microaerobic or anaerobic growth (57). Thus, the requirement for *arcA* likely reflects the low-oxygen environment of the large intestine, where *E. coli* proliferates. Previous studies using the streptomycin-treated mouse model also identified a requirement for *arcA* and demonstrated that proper respiratory control is critical for efficient colonization (14, 16).

The colonization defects observed for the Cpx-null and Rcs-null strains suggests that *E. coli* cells experience significant envelope stress in the GI tract. The CpxA/CpxR two-component system is stimulated by various perturbations to the cell envelope, and many of the genes regulated by CpxR encode secreted proteins that repair or degrade misfolded proteins in the periplasm (58–60). This envelope stress-responsive system is also important for the assembly of pili and other surface structures that are required for infection by pathogenic *E. coli* and for host colonization by other bacteria (61–67). The RcsC/RcsD/RcsB signal transduction system is also associated with envelope stress (68, 69). The specific inducing signals for this system are not well characterized. However, many of the conditions associated with Rcs stimulation perturb the cell envelope. In addition, RcsB-regulated genes affect cell surface properties such as capsule and other surface structures and confer resistance to envelope perturbants such as lysozyme

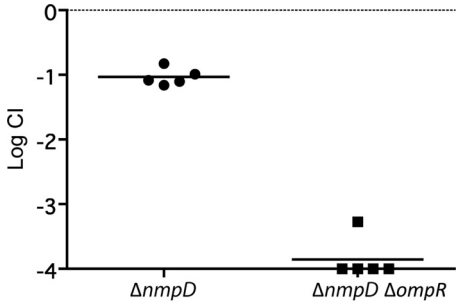

FIG 7 Mouse colonization competitions between WT MP1 and the Δ*nmpD* and Δ*nmpD* Δ*ompR* strains. Each symbol denotes an individual mouse, and the bars indicate the geometric means. The competitive indices were determined from numbers of CFU in feces at 21 days postinoculation.

and some antimicrobial peptides (68–73). The colonization defects associated with *cpxR* and *rcsB* strains may indicate chemical or physical properties of the GI tract that damage the *E. coli* cell envelope and require the Cpx and Rcs systems for protection. It is also possible that colonization requires specific macromolecules on the cell surface that depend on the Cpx or Rcs systems for regulation or efficient assembly. Dysregulation of any of these pathways may impair *E. coli* growth and survival in the mouse intestine.

It is striking that 30 of the 33 response regulator deletion strains that we tested did not exhibit severe colonization defects. Many of these response regulators have been associated with colonization or virulence of various pathogens (for example, see references 74, 75, 76, 77, 78, and 79). It is particularly noteworthy that an *ompR* deletion does not show a strong colonization defect. The EnvZ/OmpR system is a key component of the complex network that differentially regulates the major porins OmpF and OmpC (80, 81). It has been hypothesized that this network enables *E. coli* cells to sense and adapt to *in vivo* and *ex vivo* environments (49). This model posits that OmpF, the higher-permeability porin, will be expressed in low-nutrient, low-toxin environments that are likely to be encountered outside the host, whereas OmpC, the lower permeability porin, will be expressed in the intestine, where levels of nutrients as well as toxins are likely to be high. Deletion of *ompR* abrogates expression of both OmpF and OmpC (82), significantly reducing outer membrane permeability and nutrient uptake (47–49). However, studies of outer membrane permeability in Δ*ompR* strains have mainly focused on *E. coli* K-12 and B/r backgrounds. We found that MP1 expresses a third major porin, NmpD, that is not regulated by OmpR and that accounts for the ability of MP1 Δ*ompR* to colonize mice. Thus, whether or not EnvZ/OmpR plays a role in distinguishing between environments inside and outside animal hosts, this signaling system is not essential for colonization. Furthermore, the results suggest the differential regulation of OmpF and OmpC may have other physiological consequences in addition to modulating outer membrane permeability.

Interestingly, studies of MG1655 colonization in both germfree and streptomycin-treated mouse models have found that adaptation of this strain to the mouse intestine selects for *envZ* missense mutations that produce high levels of OmpR phosphorylation (83–85). We have not looked for the emergence of such mutants in our mouse colonization experiments. However, since MP1 was isolated from mice, it would be surprising if such a mutation increased the fitness of this strain in the mouse intestine under our colonization conditions.

Several response regulator deletion strains showed average competitive indices between 10^{-1} and 10^{-2} (Fig. 4), which may indicate mild colonization defects. In addition, results for one deletion (Δ*ntrC*) suggest a possible fitness advantage. However, further studies with more mice and more time points will be required to determine whether these effects are significant. Of course, laboratory competition experiments will always have limited sensitivity and will be unable to detect small but evolutionarily significant fitness defects. It is also likely that many two-component systems do not confer any selective advantage in the colonization experiments described here because these systems are not activated. For example, the robust colonization by *cusR* and *phoB* deletions suggests that the concentration of copper ions is relatively low and that there is ample inorganic phosphate in the large intestine (86, 87).

More generally, if the primary environment where MP1 proliferates is the gastrointestinal tract, then this bacterium may have evolved so that its natural set point is adapted to the average conditions it encounters in this niche. Hence, many of the two-component systems in MP1 may not be required for colonizing the intestine of a healthy well-fed mouse but are instead critical for adaptation to fluctuations in this environment—e.g., changes in diet, host microbial flora, or physiological state of the mouse—as well as transitions to other environments within or outside the animal. Further studies of *E. coli* colonization of the mouse gastrointestinal tract, and the response to perturbations associated with entrance, persistence in, and exit from the animal, or expansion to extraintestinal niches, promise to provide new insights into the physiology and evolution of signal transduction systems in *E. coli* and the role of these systems in interactions with the host.

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