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Identification of Genes Important for Growth of Asymptomatic Bacteriuria *Escherichia coli* in Urine

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***Escherichia coli* is the most important etiological agent of urinary tract infections (UTIs). Unlike uropathogenic *E. coli*, which causes symptomatic infections, asymptomatic bacteriuria (ABU) *E. coli* strains typically lack essential virulence factors and colonize the bladder in the absence of symptoms. While ABU *E. coli* can persist in the bladder for long periods of time, little is known about the genetic determinants required for its growth and fitness in urine. To identify such genes, we have employed a transposon mutagenesis approach using the prototypic ABU *E. coli* strain 83972 and the clinical ABU *E. coli* strain VR89. Six genes involved in the biosynthesis of various amino acids and nucleobases were identified (*carB*, *argE*, *argC*, *purA*, *metE*, and *ilvC*), and site-specific mutants were subsequently constructed in *E. coli* 83972 and *E. coli* VR89 for each of these genes. In all cases, these mutants exhibited reduced growth rates and final cell densities in human urine. The growth defects could be complemented in *trans* as well as by supplementation with the appropriate amino acid or nucleobase. When assessed *in vivo* in a mouse model, *E. coli* 83972*carAB* and 83972*argC* showed a significantly reduced competitive advantage in the bladder and/or kidney during coinoculation experiments with the parent strain, whereas 83972*metE* and 83972*ilvC* did not. Taken together, our data have identified several biosynthesis pathways as new important fitness factors associated with the growth of ABU *E. coli* in human urine.**

Urinary tract infections (UTIs) are among the most common bacterial infections in humans, affecting millions of people across the globe. It is estimated that there are more than 10 million cases of UTIs in Western Europe alone every year. UTIs also account for 25 to 40% of all nosocomial infections, making these infections an important medical and financial burden on our health care systems (17). Urinary tract infections affect primarily women; 40 to 50% of adult women will experience at least one UTI episode during their lifetime (8, 33). A UTI usually starts as a bladder infection but can, depending on the bacterial strain, ascend to the kidneys and may ultimately result in renal failure or dissemination to the bloodstream. The most important etiological agent of UTIs is *Escherichia coli*, which is associated with more than 80% of all such infections (34).

UTIs are classified into the following disease categories according to the focal point and severity of the infection: bacteriuria (the urine), cystitis (the bladder), pyelonephritis (the kidneys), and urosepsis (the blood). *E. coli* strains that cause cystitis and pyelonephritis have been studied extensively and are referred to as uropathogenic *E. coli* (UPEC). A subset of UPEC strains also cause urosepsis, and together with strains that cause neonatal meningitis and respiratory infection, these strains are collectively referred to as extraintestinal pathogenic *E. coli* (ExPEC) (18). In addition to well-documented symptomatic infections, many UTIs are asymptomatic. *E. coli* is also the major cause of asymptomatic bacteriuria (ABU); *E. coli* ABU strains cause few or no symptoms in the infected individual and establish bacteriuria in a commensal-like state. Despite the high prevalence of ABU, the molecular mechanisms associated with the ability of *E. coli* to establish this condition are not well understood. Previous studies have shown that many ABU *E. coli* strains fail to express key virulence factors normally associated with pathogenic UPEC strains, suggesting that this may constitute a mechanism of adaptation to long-term asymptomatic colonization of the human bladder (27, 32, 41, 40).

E. coli 83972 represents the prototypic ABU strain; it was originally isolated from a young Swedish girl who carried it asymptomatically for at least 3 years (4, 22). *E. coli* 83972 is well adapted for growth in the human urinary tract, where it can establish long-term bacteriuria (4, 15, 35). At the genome level, *E. coli* 83972 closely resembles the well-characterized and highly virulent UPEC isolate CFT073, suggesting that they may have a common ancestral origin (13, 19, 39). *E. coli* 83972 most likely represents a degenerated uropathogen which has lost the ability to produce a range of important virulence factors. It cannot adhere to bladder epithelial cells due to a lack of functional fimbriae and it does not activate host immune defenses during infection (20, 28). Nevertheless, *E. coli* 83972 grows well in human urine, with doubling times in the order of 25 to 45 min (12, 14, 27, 29). *E. coli* 83972 can also outcompete virulent UPEC strains in human urine and in the mouse bladder (29)—a phenotypic property that supports its use in the prophylactic treatment of patients who are refractory to conventional UTI therapy (5, 15). Extensive trials have suggested that deliberate colonization of the bladder with *E. coli* 83972 can prevent bacterial and fungal uropathogens from colonizing the urinary tract (5, 36–38). One common feature of *E. coli* 83972 and other ABU *E. coli* strains is the production of iron-chelating siderophores (23, 41, 42). Indeed, *E. coli* 83972 produces enterobactin, salmochelin, aerobactin, and yersiniabactin (42), and the genes responsible for the biosynthesis and transport of these mol-

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TABLE 1 Strains and plasmids used in the study

E. coli strain or plasmid	Relevant characteristics[a]	Reference
E. coli strains		
83972	OR:K5:H$^-$, ABU isolate	4
83972Nal	83972, nalidixic acid resistant	This study
83972Amp	83972, ampicillin resistant	7
VR89	ABU isolate	27
83972Δ*carAB*		This study
83972Δ*argE*		This study
83972Δ*argC*		This study
83972Δ*purA*		This study
83972Δ*metE*		This study
83972Δ*ilvA*		This study
83972Δ*ilvC*		This study
83972*carAB::kan*		This study
83972*argE::kan*		This study
83972*argC::kan*		This study
83972*purA::kan*		This study
83972*metE::kan*		This study
83972*ilvA::kan*		This study
83972*ilvC::kan*		This study
VR89*carAB::kan*		This study
VR89*argE::kan*		This study
VR89*argC::kan*		This study
VR89*purA::kan*		This study
VR89*metE::kan*		This study
VR89*ilvC::kan*		This study
Plasmids		
pACYC184	Cloning vector, Cam^r	
pVR9	*carAB* cloned into pACYC184, Cam^r	This study
pVR10	*argE* cloned into pACYC184, Cam^r	This study
pVR11	*argC* cloned into pACYC184, Cam^r	This study
pVR12	*purA* cloned into pACYC184, Cam^r	This study
pVR13	*metE* cloned into pACYC184, Cam^r	This study
pVR15	*ilvA* cloned into pACYC184, Cam^r	This study
pVR16	*ilvC* cloned into pACYC184, Cam^r	This study

[a] Cam^r, chloramphenicol resistance.

ecules are highly expressed during growth in the iron-limited conditions of the human urinary tract (26).

Arguably, the ability to grow in human urine must be one of the prerequisites for ABU *E. coli* strains to establish long-term colonization of the human bladder. Bacterial metabolic processes are clearly important in relation to the persistence of *E. coli* strains in the urinary tract, with *E. coli* 83972 being an ideal strain for such studies, allowing for delineation between fitness and virulence. In this respect, we have previously shown, using transcriptomics, that a number of genes are upregulated in *E. coli* 83972 during growth *in vivo* in human urine compared to growth on laboratory medium (26). Here we have utilized *E. coli* 83972 and an additional ABU clinical isolate (VR89) to identify novel genes required for growth in human urine. Using a transposon mutagenesis approach, we show that genes involved in the biosynthesis of arginine (*argC*, *argE*, and *carAB*), methionine (*metE*), valine and leucine (*ilvC*), isoleucine (*ilvA* and *ilvC*), and adenine (*purA*) are important factors associated with the growth and fitness of ABU *E. coli* in human urine.

MATERIALS AND METHODS

Bacterial strains and growth medium. The strains used in this study are listed in Table 1. All strains were grown at 37°C on solid or in liquid modified Luria-Bertani (LB) medium or in pooled sterile filtered urine collected from at least three healthy female volunteers with no history of UTI or antibiotic use within the last 2 months. The urine was pooled, filter sterilized, and stored at 4°C until use (within 1 to 2 days). Antibiotics were added when required at the following concentrations: gentamicin, 5 μg/ml; kanamycin, 25 μg/ml; chloramphenicol, 16 μg/ml; ampicillin, 100 μg/ml; and nalidixic acid, 50 μg/ml. Amino acid and nucleobase supplements were added at the following concentrations: uracil, 50 μg/ml; arginine, 100 μg/ml; adenine, 50 μg/ml; isoleucine, 100 μg/ml; and valine, 100 μg/ml.

Growth experiments. Monocultures were grown in pooled human urine in 96-well microtiter plates inoculated to an optical density at 600 nm (OD_{600}) of 0.01 under shaking conditions (300 rpm on an orbital shaker) at 37°C. OD_{600} readings were acquired at 30-min intervals until stationary phase was reached. Each experiment included two to four replicates and was repeated at least three times. For competition experiments, an ampicillin-resistant variant of *E. coli* 83972 (*E. coli* 83972^{AMP}) was used instead of the wild-type *E. coli* 83972; *E. coli* 83972^{AMP} displayed a growth rate identical to that of wild-type *E. coli* 83972 under all conditions tested. Mixed cultures were inoculated into pooled human urine using a starting ratio of 1:1 and an initial OD_{600} of 0.05. Samples from each culture were extracted at the start of the cultivation, diluted, and cultured on LB plates with and without ampicillin to confirm the starting ratios of CFU. The cultures were then grown overnight at 37°C and 300 rpm on an orbital shaker. Samples were extracted after 16 h, diluted, and cultured on LB plates with and without ampicillin. Each competition experiment was performed in duplicate and repeated at least three times.

Transposon mutagenesis. Transposon mutant libraries were constructed using the mariner-based transposon system carried on plasmid pBT20 (21). Bacterial suspensions of the donor (*E. coli* SM10-λpir containing pBT20) and the recipients (*E. coli* 83972NalR and *E. coli* VR89NalR) were recovered from overnight plates and resuspended in LB broth. These suspensions were adjusted to OD_{600}s of 40 and 20, respectively, and mixed in a 1:1 ratio. Aliquots of 50 μl were spotted on a dry LB agar plate and incubated at 37°C for 6 h. The mating mixtures were subsequently recovered from the plate, resuspended in LB, and cultured on LB agar plates containing gentamicin and nalidixic acid. From these plates, the individual colonies were inoculated into microtiter plates containing LB broth or sterile filtered human urine. The plates were incubated statically overnight at 37°C and subsequently screened for mutants that could grow in LB broth but not in human urine. The insertion sites were determined using a semirandom PCR method (9).

Recombinant DNA techniques. Specific mutants of *E. coli* 83972 and *E. coli* VR89 were constructed using the λ-red recombinase-based gene inactivation system as previously described (6). The primers are listed in Table 2. Following amplification, the PCR products were transformed into *E. coli* 83972 and VR89 carrying the helper plasmid pKD46 and the transformations were plated on selective medium. Deletion mutants were verified by PCR (primers are listed in Table 2). The kanamycin resistance cassette was subsequently removed by use of the flippase-encoding plasmid pCP20 (6). The plasmids pVR9-13 and pVR15-16 were constructed by amplifying the relevant genes from CFT073 using the primers listed in Table 2. PCR fragments were digested with BamHI and SalI or SphI and cloned into pACYC184. The resulting seven plasmids were transformed into the mutants to enable complementation.

Mouse model of UTI. Female C3H/HeJ mice (8 to 10 weeks) were purchased from the Animal Resources Centre, Western Australia, and housed in sterile cages with *ad libitum* access to sterile water. The mouse model of UTI with competitive mixed infection was performed as previously described (1). Mice were inoculated with a mixture of 2.5×10^8 CFU of *E. coli* 83972^{AMP} and 2.5×10^8 CFU of each *E. coli* 83972 mutant, both grown for 20 h in LB medium. At 48 h after inoculation, urine was collected from each mouse and then mice were euthanized and the bladders and kidneys were excised. Bacterial numbers in the mouse urine,

TABLE 2 Primers used in the study

Primer purpose and name	Sequence
Construction of deletion mutants	
carAB forward	GAGTGAATATTCTCTGGAGGGTGTTTTGATTAAGTCAGCGTAATGTGTAGGCTGGAGCTGCTTCG
carAB reverse	CAGGGTGGTGTCGTAATGCACTTTATATTGCAGCGCACTGCCATATGAATATCCTCCTTA
argE forward	GGGTGATCAGTTCGCGGGTGGGCTTGATAAACCTCGTCTCTAATGTGTAGGCTGGAGCTGCTTCG
argE reverse	TACCGCCATTTATCGAGATTTACCGTGCTCTGATTGCCACATATGAATATCCTCCTTA
argC forward	TTCAGCGCAAAGCAATGATGCGGGAAAGTTAATCTCCGATTAATGTGTAGGCTGGAGCTGCTTC
argC reverse	GCACCGCTTGTGCCGCCGCGCCTTTCAGTAAGTTGTCTTCCATATGAATATCCTCCTTA
purA forward	GACTGAACGGGCTAAATATGTTGTACGCTACCAGGGCGGTTAATGTGTAGGCTGGAGCTGCTTC
purA reverse	ACGCGTCGAACGGGTCGCGCAGAATCATGGTTTCAGTACCATATGAATATCCTCCTTA
metE forward	TATTGGGCGGGGAACTCCACGCGTGAAGAACTGCTGTAATGTGTAGGCTGGAGCTGCTTCG
metE reverse	GGCCAGCCGCGCGTTTTCAGGCCACAGTCCGGGTTGACCCCATATGAATATCCTCCTTA
ilvA forward	AAGAGCGGTACTACGTGCACCGGTTTACGAGGCGGCGCAGGTCTAATAGGCTGGAGCTGCTTCGAA
ilvA reverse	GGTCGCCAAGTTCGAACGCCGCCAGTACGCGCCCGTAGTCCATATGAATATCCTCCTTA
ilvC forward	CAGGGTAAAAAAGTAGTCATCGTCGGCTGTGGCGCACAGTAATGTGTAGGCTGGAGCTGCTTCG
ilvC reverse	AGCCGCGCAGTTTCTTACCTACCTGCTCAATCGCATGGCATATGAATATCCTCCTTA
Verification of deletion mutants	
dapA	TGCGAGATGTGCTTGATCT
carB	CATCGGCATTCAGCGCCAT
yijP	GTTTAGCCTGGACTTCTGTG
argB	ACGCCACCCAGTTTGATAA
yjeT	TGAATTCGACAATCTGGCTG
yjeB	TCACTCCACCAGCAATAATT
yigM	GTCAACCTGATACTTCGCTA
metE	TGCACCATGTTCGCCAGTG
ilvC	TAACCCGCAACAGCAATACG
k1, pKD4 (reference)	CAGTCATAGCCGAATAGCCT
k2, pKD4 (reference)	CGGTGCCCTGAATGAACTGC
kt, pKD4 (reference)	CGGCCACAGTCGATGAATCC
Complementation	
carAB forward	CGGCGGATCCGCTAACAGGAGGAATTAACCGTGAGTGAATATTCT
carAB reverse	GCGCGTCGACTTATTTGATCTGCGC
argE forward	CGGCGGATCCGCTAACAGGAGGAATTAACCATGAATATTGATACT
argE reverse	GCGCGCATGCTTAATGCCAGCAAAA
argC forward	CGGCGGATCCGCTAACAGGAGGAATTAACCATGTTGAATACGCTG
argC reverse	GCGCGTCGACTTAAATAAGAGACTG
purA forward	CGGCGGATCCGCTAACAGGAGGAATTAACCATGGGTAACAACGTC
purA reverse	GGCGGTCGACTTACGCGTCGAACGG
metE forward	CGGCGGATCCGCTAACAGGAGGAATTAACCATGACAATTCTTAAT
metE reverse	GCCGGTCGACTCATCCCCGACGCAA
ilvA forward	CGGCGGATCCGCTAACAGGAGGAATTAACCATGATGGCAGACTCA
ilvA reverse	GCGCGTCGACTTAACCCGCCAAAAA
ilvC forward	CGGCGGATCCGCTAACAGGAGGAATTAACCATGGCTAACTACTTC
ilvC reverse	GCGCGTCGACTTAACCCGCAACAGC

bladders, and kidneys were determined by plating on selective LB agar plates and by colony counts. The mixed strains were differentiated by their antibiotic resistance with *E. coli* 83972^{AMP} resistant to ampicillin and the *E. coli* 83972 mutants resistant to kanamycin. Competitive fitness indices were calculated by dividing the CFU/ml or CFU/0.1 g for each *E. coli* 83972 mutant by that of *E. coli* 83972^{AMP}. Differences in colonization between *E. coli* 83972^{AMP} and each of the mutants were tested using the Wilcoxon matched-pairs signed-rank test. All experiments were carried out in strict accordance with the recommendations in the Animal Care and Protection Act (25a) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (23a). Approval for mouse infection studies was obtained from the University of Queensland Animal Ethics Committee (SCMB/471/09/NHMRC [NF]).

Microarray analysis. The microarray results of the amino acid biosynthesis genes were extracted from a previously published transcriptomic analysis of *E. coli* 83972 during growth in pooled human urine and from urine specimens collected from three patients deliberately colonized with *E. coli* 83972 (26). GeneChip *E. coli* Genome 2.0 arrays (Affymetrix) were used for hybridization of the labeled cDNA. Three chips were hybridized with samples grown in three individual flasks in MOPS (morpholinepropanesulfonic acid) medium (including 0.2% glucose and 0.02% Casamino acids), three chips were hybridized with samples from cells grown in pooled human urine in three individual flasks, and nine chips were hybridized with samples obtained in triplicate from three individual patients; no pooling of samples was performed. The arrays were normalized, and fold changes during growth in urine compared to planktonic growth in MOPS were calculated.

Microarray data accession number. The supporting microarray data have been deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with accession number E-MEXP-584.

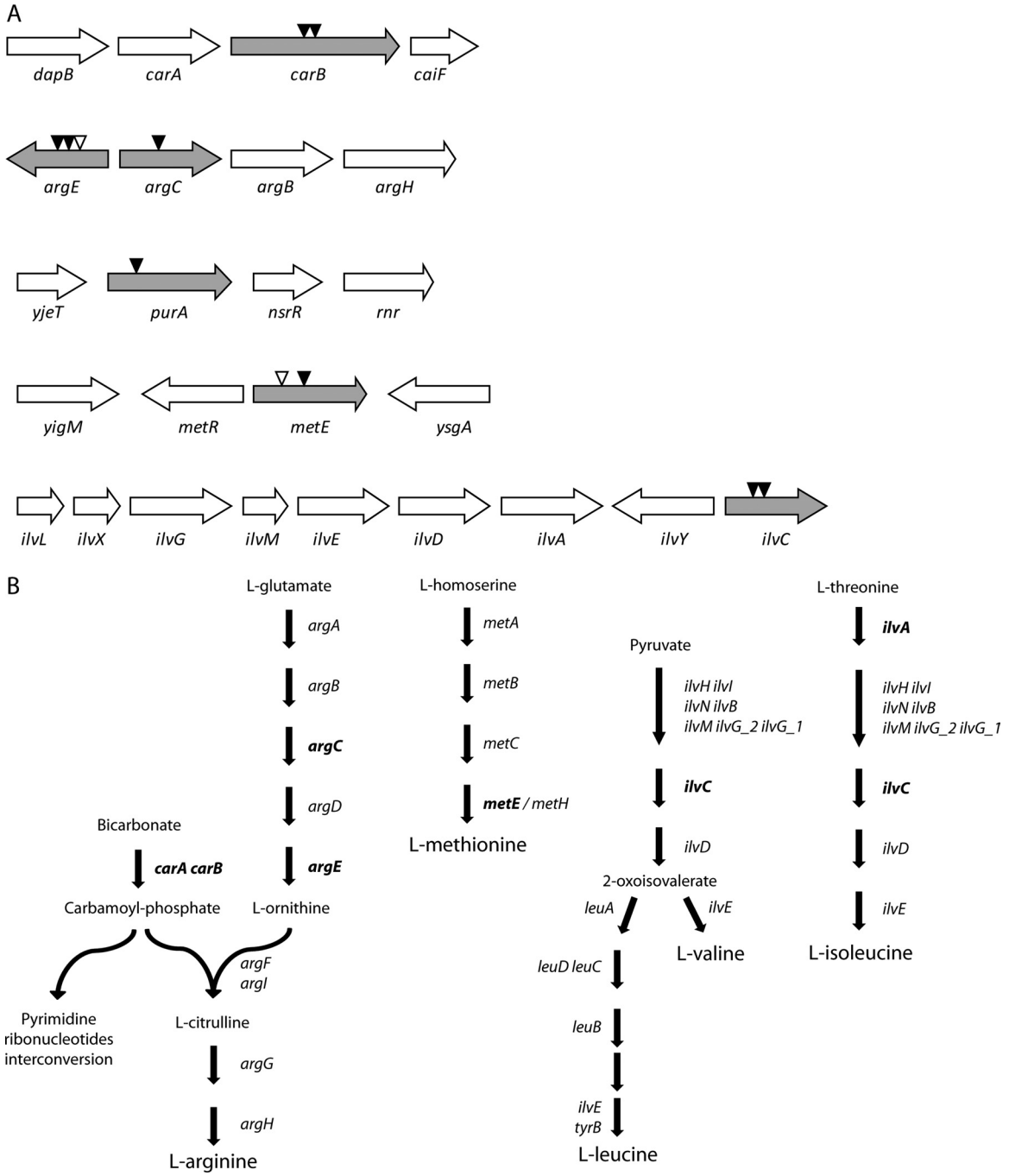

FIG 1 Growth mutants identified by transposon mutagenesis. (A) Identified insertion sites of the mariner transposon in the two *E. coli* strains 83972 (black triangles) and VR89 (white triangles). The illustrated gene order and orientations refer to the genomic context of *E. coli* MG1655. (B) Biosynthesis pathways for the amino acids arginine, methionine, leucine, valine, and isoleucine. The gene(s) encoding the enzyme responsible for each step is indicated. Genes that were mutated (knocked out) in *E. coli* strain 83972 are indicated in boldface.

RESULTS

Identification of mutants deficient in urine growth. A transposon mutagenesis approach was used to identify genes that are important for the growth of two well-characterized ABU *E. coli* strains in human urine, i.e., the prototypic *E. coli* 83972 and *E. coli* VR89. A total of 960 and 1,152 transposon mutants of *E. coli* strains 83972 and VR89, respectively, were screened based on the following criteria: attenuated growth in sterile filtered pooled human urine and normal growth in LB broth. In total, 11 mutants that displayed a >90% reduction in final cell density following overnight growth in pooled human urine were identified. In order to identify the genes of interest, the location of the transposon insertions was determined by semirandom PCR. As illustrated in Fig. 1A, the insertions were located in

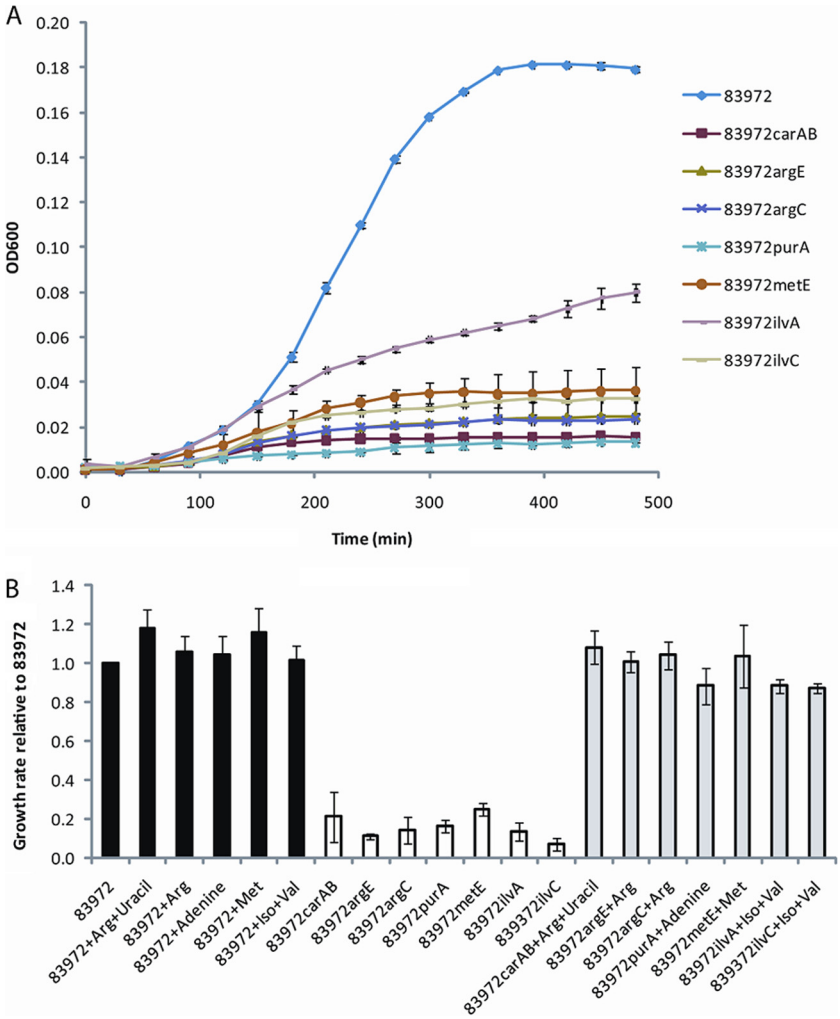

FIG 2 Growth of *E. coli* 83972 and its isogenic mutants in human urine. (A) The curves are shown as means of four replicates, and error bars indicate standard deviations (σ_{n-1}). (B) Growth rates of *E. coli* 83972 in human urine with and without addition of amino acids and/or nucleobases (black) and growth rates of its mutants in urine without (white) and with (gray) addition of amino acids and/or nucleobases. Growth rates are displayed as means of triplicates of independent experiments relative to the growth rate of *E. coli* 83972 grown in human urine with no addition of amino acids. Error bars indicate standard errors.

genes related to amino acid, purine, and pyrimidine biosynthesis. Insertions were identified within *carB*, *argE*, *argC*, *purA*, *metE*, and *ilvC*. The *carAB* operon encodes the carbamoylphosphate synthetase, an essential enzyme in the biosynthesis of arginine and an important component of the pyrimidine metabolism (Fig. 1B). The *metE* gene product is required for the terminal step of methionine biosynthesis (Fig. 1B). The *ilvC* gene product is involved in one of the last common steps in the isoleucine-valine biosynthesis pathways (Fig. 1B). The *purA* gene encodes adenylosuccinate synthase, which catalyzes the first committed step toward the *de novo* synthesis of adenine. Notably, four of the six genes with insertions were identified in several mutants independently and two of the six insertions, i.e., *argE* and *metE*, were found in both strain backgrounds (Fig. 1A). *argC* has previously been shown to be important for the growth and virulence of a UPEC strain (31).

Characteristics of mutants deficient in urine growth. To confirm the results obtained by the transposon mutagenesis screen, deletion mutants were constructed for each individual gene by λ-red-mediated homologous recombination. Six deletion mutants were made in each strain background (83972 and VR89) to validate the results. The resulting mutants were subsequently assayed for growth in sterile filtered pooled human urine. All of the mutants grew poorly in human urine (Fig. 2A). The six mutants in *E. coli* 83972 possessed a significantly reduced growth rate (7 to 25% of that observed for *E. coli* 83972) and achieved a significantly reduced final cell density (8 to 34% of that observed for *E. coli* 83972) (Fig. 2B and 3). Similar results were obtained in *E. coli* VR89 (data not shown).

IlvA catalyzes the first step in the isoleucine pathway and is not important for biosynthesis of valine or leucine. Therefore, it was expected that an *ilvA* mutant would be less attenuated for growth in urine than an *ilvC* mutant. In order to test this hypothesis, we constructed an *ilvA* mutant in *E. coli* 83972 and assessed its growth characteristics together with the *ilvC* mutant. As expected, when grown in urine, the *ilvA* mutant grew better than the *ilvC* mutant but was still significantly attenuated compared with the growth of the wild-type strain (Fig. 2A). The final cell density achieved by the *ilvA*

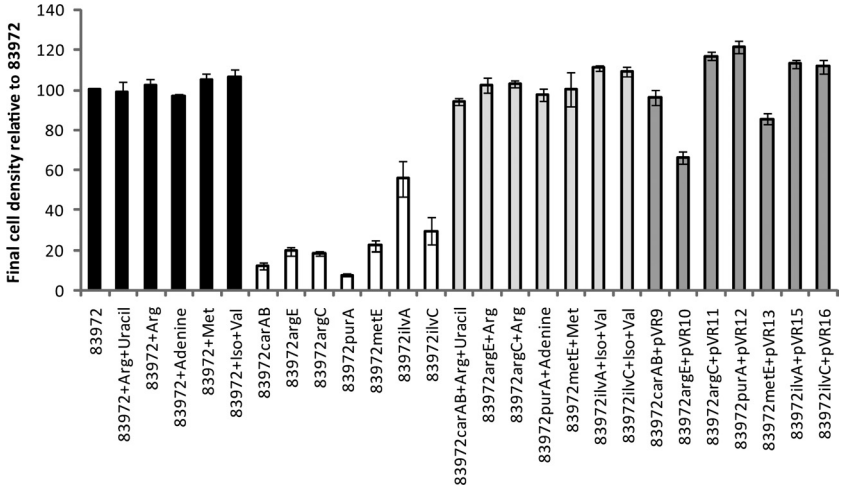

FIG 3 Final cell density of *E. coli* 83972 and its isogenic mutants in human urine supplemented with the appropriate amino acids and/or nucleobases or complemented with plasmid expressing the defunct gene. Black bars, wild-type *E. coli* 83972 with and without addition of amino acids and/or nucleobases; white bars, isogenic mutants; light gray bars, isogenic mutants with addition of amino acids and/or nucleobases; dark gray bars, isogenic mutants complemented with plasmids expressing genes corresponding to the defunct gene. Growth characteristics of the isogenic mutants and the corresponding mutant expressing the control vector pACYC184 were identical. Values are means of at least triplicates, and error bars indicate standard errors.

mutant was significantly higher than those of the other mutants and represented approximately 50% of that observed for the wild-type strain (Fig. 3).

Mutants deficient in urine growth can be complemented in *trans*. Each mutant generated was complemented with a plasmid expressing its respective nonfunctional gene. The final cell density following growth in pooled human urine was restored to wild-type levels for the *carAB*, *argC*, *purA*, *metE*, *ilvA*, and *ilvC* mutants (range of 86 to 121% of the final cell density of *E. coli* 83972) (Fig. 3). The *argE* mutant reached a final cell density that was 65% of that of *E. coli* 83972.

Mutants deficient in urine growth can be rescued by supplementation of amino acids. In order to assess whether the various mutants could be rescued from their growth deficiency by end product complementation, each mutant was grown in the presence of the amino acids or nucleobases for which it was auxotrophic. While the growth of *E. coli* 83972 was not significantly altered by the presence of the supplements (growth rates and final cell densities were 97 to 118% of those for growth in urine without supplements), the growth of the mutants was fully restored by the addition of amino acids and/or nucleobases. The growth rates of the mutants were restored to 88 to 108% of that of the wild type (Fig. 2B), while the final cell densities were 94 to 111% of that of the wild type (Fig. 3). This demonstrates that these amino acids and nucleobases are limiting in human urine and that the biosynthesis pathways of arginine, methionine, valine, uracil, adenine, and isoleucine are essential for efficient growth of *E. coli* 83972 in human urine.

Global gene expression analysis of genes involved in amino acid biosynthesis. We have previously examined the global gene expression profile of *E. coli* 83972 during growth in urine—both *in vitro* and *in vivo* (in human volunteers) (26). The expression profile of *E. coli* 83972 obtained during growth in urine was compared with that during exponential growth in minimal medium (MOPS-glucose including 0.02% Casamino acids). The expression levels of the genes involved in the biosynthesis of amino acids are displayed in Fig. 4. Of the six genes identified in our transposon mutagenesis screen, four were significantly upregulated during growth in urine compared to their levels during growth in minimal medium, i.e., *argC*, *argE*, *metE*, and *ilvC* were all induced 1.7- to 17-fold *in vivo* (Fig. 4). Indeed, the *metE* transcript was the 9th highest expressed *in vivo* of all 8,716 transcripts in the global gene expression analysis of *E. coli* 83972. It should also be noted that less than 5% of all 8,716 transcripts had absolute expression levels above 1,000, revealing that many amino acid biosynthesis genes were highly expressed in general. Of the 10 highest expressed amino acid-related genes *in vivo*, five were involved in biosynthesis of leucine, valine, and isoleucine and four were involved in biosynthesis of threonine, methionine, and lysine (Fig. 4). In contrast, genes involved in biosynthesis of phenylalanine, tyrosine, and tryptophan were among the lowest expressed of all amino acid-related genes in urine (9 of the 13 lowest among the 98 genes displayed in Fig. 4).

Interestingly, the mutations that significantly reduced growth in human urine (Fig. 1) were found among the amino acid pathways displaying the highest proportion of significantly upregulated genes (Fig. 4). In the Arg, Leu+Val+Iso, and Thr+Met+Lys pathways, 73%, 73%, and 52% of the genes, respectively, displayed significant upregulation in urine. Other pathways displaying a high proportion of induced genes were the Glu and Ala pathways (67% and 43%, respectively), while the remaining pathways contained a significantly lower proportion of induced genes (0 to 25%). Although the numbers of genes represented in the Glu and Ala pathways are comparably low, this may indicate that the biosynthesis of glutamate and alanine might also be important for growth in human urine. Taken together, the gene expression data support the growth profiles observed for our amino acid mutants and suggest that these amino acids are limiting in human urine.

Competition in pooled human urine. The severely attenuated growth phenotype displayed by the mutants suggested that they would be outcompeted by the parent *E. coli* 83972 strain during mixed growth in pooled human urine. To investigate this, we pitted an ampicillin-resistant version of *E. coli* 83972 (*E. coli*

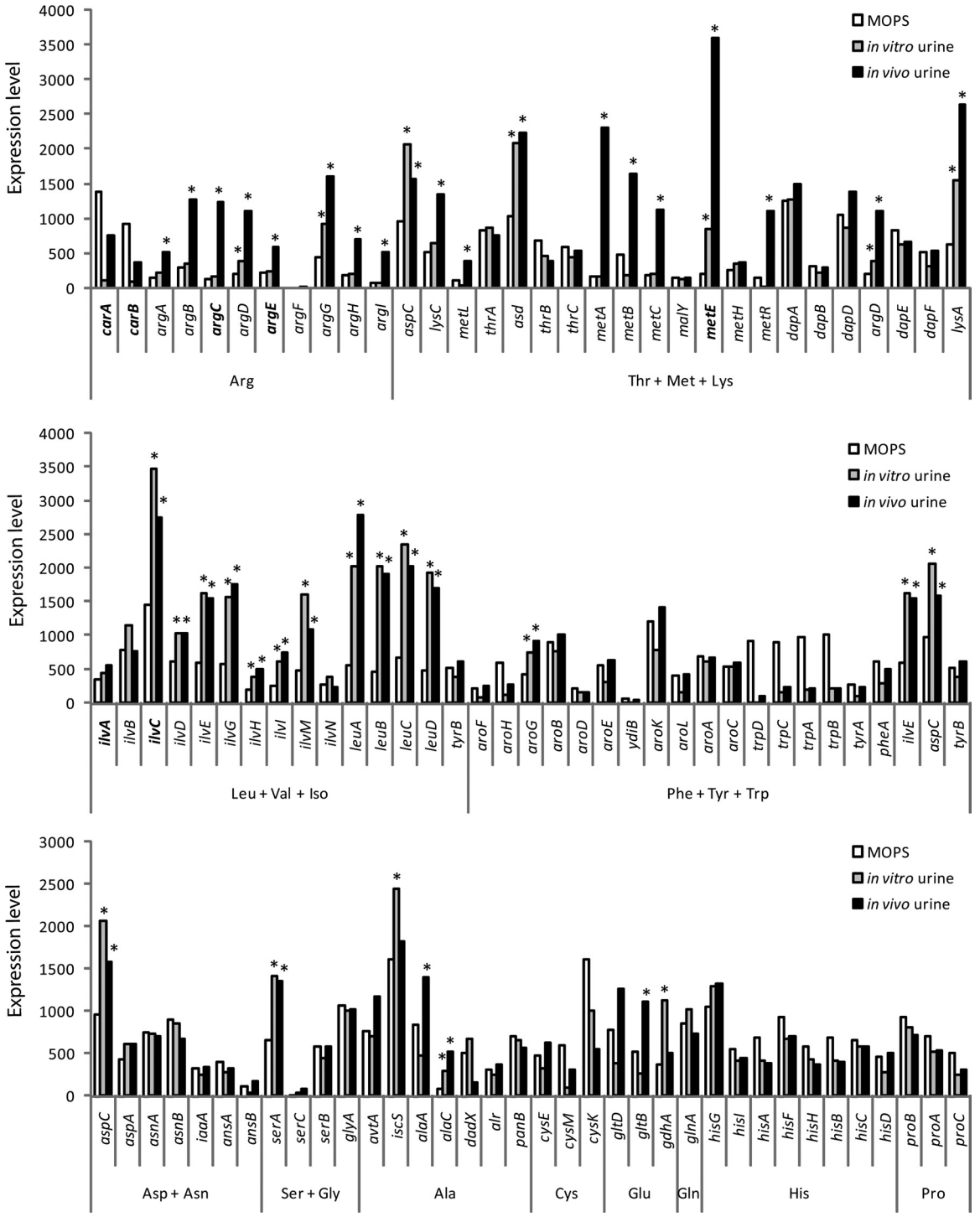

FIG 4 Expression levels of *E. coli* 83972 in minimal medium (MOPS) and human urine (*in vitro* and *in vivo*) of all genes involved in the biosynthesis of amino acids, as determined by microarray analysis. Genes that were mutated in *E. coli* 83972 are indicated in boldface. Asterisks indicate expression levels in human urine that were significantly higher than those in MOPS ($P < 0.05$).

83972^{AMP}) against wild-type *E. coli* 83972 (control) and each mutant using a 1:1 inoculation ratio. The number of CFU of each strain was then enumerated after 16 h of growth. The growth of *E. coli* strains 83972 and 83972^{AMP} was identical, indicating the suitability of *E. coli* 83972^{AMP} for these assays. In the mixed competition assays, six of the seven mutants were significantly outcompeted by *E. coli* 83972^{AMP}, which constituted 83 to 100% of the final population after 16 h of growth (Student's paired *t* test, $P < 0.01$) (Fig. 5). The exception was *E. coli* 83972*ilvA*, which was not outcompeted by *E. coli* 83972^{AMP}.

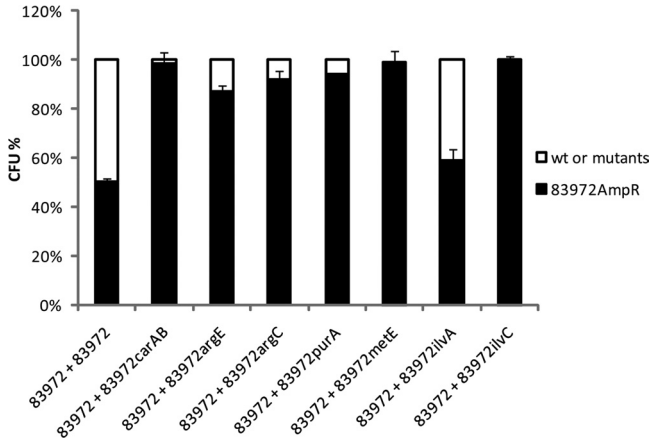

FIG 5 Competition experiment in human urine cultures between *E. coli* 83972 and its mutants, mixed 1:1 at the starting point. After 16 h of growth under hydrodynamic conditions at 37°C, the number of CFU of each strain in the mixed biofilm population was determined. The data shown are the means of three independent experiments in different batches of urine, and error bars indicate standard errors. wt, wild type.

Contribution of genes required for growth in human urine to colonization of the mouse urinary tract. To assess the role of the genes identified in our transposon mutagenesis screen on colonization of the mouse urinary tract, we examined the ability of the wild-type and mutant strains to survive in competitive-colonization experiments. Mice were coinoculated with wild-type and mutant strains (*viz.*, 83972*carAB*, 83972*argC*, 83972*metE*, and 83972*ilvC*) in a 1:1 ratio. In the mouse model, *E. coli* 83972*carAB* was significantly outcompeted by *E. coli* 83972 in the bladder ($P = 0.0156$) and kidney ($P = 0.0313$) of infected mice (Fig. 6). Of the remaining mutants, only the *E. coli* 83972*argC* strain exhibited a colonization defect; this strain was significantly outcompeted by *E. coli* 83972 in the kidney ($P = 0.0313$) of infected mice. Interestingly, no difference in total bacterial counts was observed between *E. coli* 83972 and any of the mutants in the urine of infected mice (Fig. 6A).

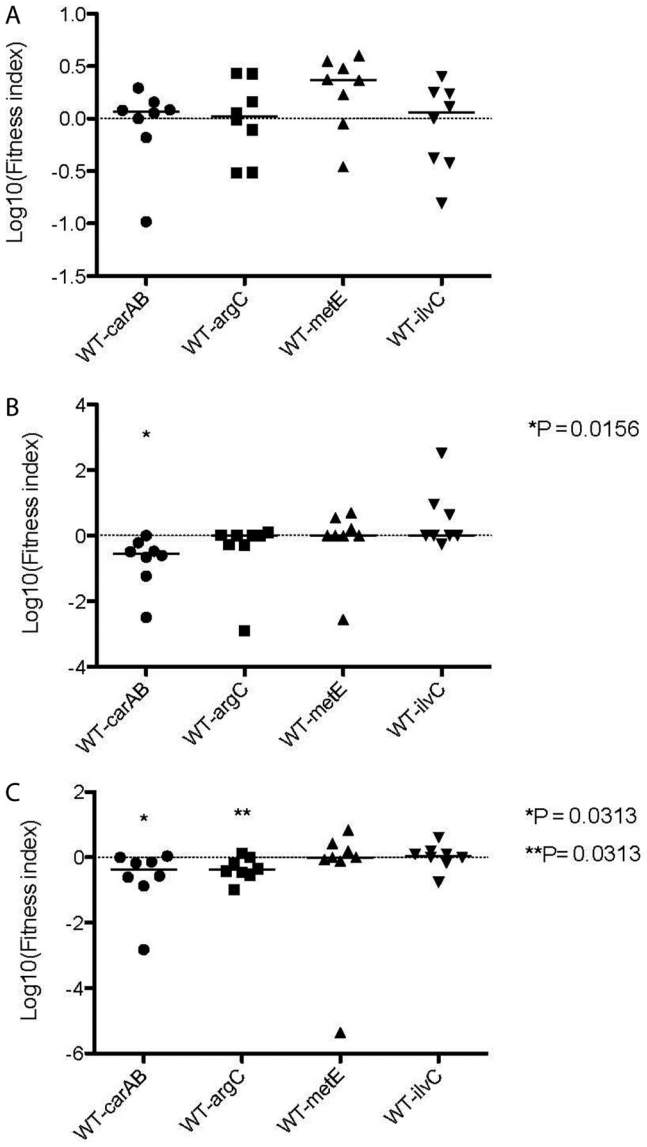

FIG 6 Competitive colonization of mouse urine, bladder, and kidneys. *E. coli* 83972^{AMP} and each mutant were mixed at a 1:1 ratio and inoculated into a group of eight mice. Urine (A), bladders (B), and kidneys (C) were collected 48 h after inoculation and processed for quantitative colony counts. Asterisks indicate significant differences in colonization.

DISCUSSION

Our knowledge of the factors that are involved in the virulence of UPEC has advanced significantly over the last few decades. We currently have a relatively good understanding of the repertoire of adhesins, invasins, and toxins that are necessary for uropathogenicity (24). However, our knowledge of the physiological factors required for growth and survival of *E. coli* in human urine, and in the urinary tract, is limited. Relatively few reports have addressed this important issue compared to the number of studies on virulence. Nevertheless, the importance of metabolic processes in the persistence and colonization of the urinary tract by bacterial strains is increasingly appreciated (2, 3). Urine is a complex growth medium. It contains significant amounts of urea and creatinine but is iron limiting. Urine has also been reported to contain essential amino acids, purines, and pyrimidines (25). However, it is unclear if these are available in concentrations that are sufficient to support growth in the absence of *de novo* growth factors for synthesis. Previous reports have suggested that the biosynthesis of arginine and guanine is important for growth and virulence of uropathogens (31). In addition, a study by Hull and Hull suggested a requirement for glutamine, serine, leucine, methionine, phenylalanine, proline, and uracil (16).

Transcriptomic analyses of *E. coli* growing in human urine from patients and *in vitro* have provided valuable information about the nutritional requirement of UTI strains (11, 26, 29). In order to understand how ABU strains grow and persist in the urinary tract and to complement our transcriptomic data, we analyzed transposon mutant libraries of *E. coli* ABU strains 83972 and VR89. Screening for mutants deficient in urine growth identified six genes required for efficient growth in human urine. The mutants were defunct in genes involved in the biosynthesis of arginine (*carB*, *argC*, and *argE*), methionine (*metE*), valine, isoleucine, and leucine (*ilvC*), as well as adenine and uracil (*carB* and *purA*). Two genes, i.e., *argE* and *metE*, were identified indepen-

dently in both strain backgrounds. All six mutants, in both *E. coli* strain 83972 and VR89, had severely affected growth rates (7 to 25% of that of the wild type) and reached a significantly reduced final cell density (8 to 34% of that of the wild type) compared with those of their isogenic parent strain. These six mutants were also easily outcompeted by the wild-type strain during competition experiments. Amino acid or nucleobase supplementation readily rescued the growth deficiency of the mutants, demonstrating that these growth factors are limiting in human urine.

To determine whether the metabolic genes identified through transposon mutagenesis were required for the growth and fitness of *E. coli* ABU strains during colonization of the urinary tract, four mutants were assayed in an ascending UTI mouse model, *viz.*, *E. coli* 83972*carAB*, 83972*argC*, 83972*metE*, and 83972*ilvC*. In these experiments, there was no significant difference in the numbers of mutant and wild-type cells recovered from the urine of infected mice. These findings may result from differences in the composition of human and mouse urine. It is also possible that the continuous replenishment of nutrients in the urine of mice (unlike the *in vitro* batch culture), as well as growth in the proximity of epithelial cells and cellular debris, may contribute to these differences.

Some differences were observed with respect to bladder and kidney colonization of the *E. coli* mutants 83972*argC* and 83972*carAB*. The mutant strain *E. coli* 83972*argC* was impaired in the colonization of the kidneys, while *E. coli* 83972*carAB* was outcompeted in both the bladder and the kidneys. This correlates well with previous studies of an *argC* mutant of UPEC isolate CP9 which was also significantly impaired in colonization of the mouse kidneys but not in colonization of the bladder (31). We note that our study involved the use of Toll-like receptor 4 (TLR4)-deficient mice, a characteristic which likely accounts for the ability of *E. coli* 83972 to colonize the kidneys. Interestingly, a previous study showed that *argG* does not significantly impact the growth and persistence of UPEC strain CFT073 in the mouse UTI model (2). This may suggest that different steps in the arginine biosynthesis pathway have different effects on the fitness of UPEC strains *in vivo*.

Several studies have demonstrated that iron is an important limiting factor during growth in urine and that most *E. coli* strains that colonize the urinary tract, including ABU isolates, have numerous iron acquisition systems (10, 29). *E. coli* 83972 possesses functional yersiniabactin-, enterobactin-, aerobactin-, salmochelin-, and citrate-dependent iron transport systems (39, 42). Many of the genes involved in these systems are highly upregulated during growth in urine (14, 29). As many of these systems are functionally redundant with respect to iron acquisition, it was not unexpected that our mutagenesis screen did not identify any iron acquisition genes. Russo et al. found several urine-upregulated genes (i.e., *iroN* and *ure1*) that were not essential for growth in urine (30). This demonstrates the value of combining different experimental approaches, namely, mutagenesis and transcriptomics.

Although our libraries were not saturated, our findings indicate that the growth advantage displayed by *E. coli* 83972 compared with the growth of pathogenic strains is not related to unique biosynthetic pathways. Many of the same nutrients appear to be required for both pathogenic (3, 16, 30, 31) and asymptomatic (present study) isolates. This is interesting in terms of delineating between fitness *per se* and virulence. We note that the *argC*, *ilvA*, *ilvC*, and *metE* genes were also significantly upregulated (1.7- to 15-fold) in the pyelonephritis isolate CFT073 grown in human urine compared to their levels in minimal medium (data extracted from a previous microarray study [14]). This indicates that the genes identified as important for growth of the two ABU *E. coli* strains in the present study might also be important for growth and colonization of UPEC isolates.

Based on genomic sequencing, *E. coli* 83972 differs surprisingly little from highly pathogenic UTI strains, suggesting that genetic adaptation to commensalism through gene loss and point mutations—rather than distinct biosynthetic pathways—may explain its superior growth characteristics (28, 39). Many virulence factors, fimbriae in particular, are costly to produce. It remains to be determined if the loss of key virulence factors can increase fitness for growth in urine; this may very well be the case for *E. coli* 83972.

In conclusion, our results point to the classification of several biosynthesis pathways as bona fide bacterial fitness factors. Such findings may offer a novel target for the development of future antimicrobial agents. Compounds that specifically inhibit one or more metabolic pathways may have potential for the treatment of symptomatic urinary tract infections.

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