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Sarah E. Greene

Washington University School of Medicine in St. Louis

Jerome S. Pinkner

Washington University School of Medicine in St. Louis

Erik Chorell

Washington University School of Medicine in St. Louis

Karen W. Dodson

Washington University School of Medicine in St. Louis

Carrie L. Shaffer

Vanderbilt University

See next page for additional authors

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Authors

Sarah E. Greene, Jerome S. Pinkner, Erik Chorell, Karen W. Dodson, Carrie L. Shaffer, Matt S. Conover, Jonathan Livny, Maria Hadjifrangiskou, Fredrik Almqvist, and Scott J. Hultgren



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Pilicide ec240 Disrupts Virulence Circuits in Uropathogenic *Escherichia coli*

Sarah E. Greene,^a Jerome S. Pinkner,^a Erik Chorell,^{a,b} Karen W. Dodson,^a Carrie L. Shaffer,^c Matt S. Conover,^a Jonathan Livny,^d Maria Hadjifrangiskou,^c Fredrik Almquist,^{b,e} Scott J. Hultgren^a

Department of Molecular Microbiology and Microbial Pathogenesis and Center for Women's Infectious Disease Research, Washington University School of Medicine in St. Louis, St. Louis, Missouri, USA^a; Department of Chemistry, Umeå University, Umeå, Sweden^b; Department of Pathology, Microbiology, and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA^c; The Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, Massachusetts, USA^d; Umeå Center for Microbial Research, Umeå University, Umeå, Sweden^e

ABSTRACT Chaperone-usher pathway (CUP) pili are extracellular organelles produced by Gram-negative bacteria that mediate bacterial pathogenesis. Small-molecule inhibitors of CUP pili, termed pilicides, were rationally designed and shown to inhibit type 1 or P piliation. Here, we show that pilicide ec240 decreased the levels of type 1, P, and S piliation. Transcriptomic and proteomic analyses using the cystitis isolate UTI89 revealed that ec240 dysregulated CUP pili and decreased motility. Paradoxically, the transcript levels of P and S pilus genes were increased during growth in ec240, even though the level of P and S piliation decreased. In contrast, the most downregulated transcripts after growth in ec240 were from the type 1 pilus genes. Type 1 pilus expression is controlled by inversion of the *fimS* promoter element, which can oscillate between phase on and phase off orientations. ec240 induced the *fimS* phase off orientation, and this effect was necessary for the majority of ec240's inhibition of type 1 piliation. ec240 increased levels of the transcriptional regulators SfaB and PapB, which were shown to induce the *fimS* promoter phase off orientation. Furthermore, the effect of ec240 on motility was abolished in the absence of the SfaB, PapB, SfaX, and PapX regulators. In contrast to the effects of ec240, deletion of the type 1 pilus operon led to increased S and P piliation and motility. Thus, ec240 dysregulated several uropathogenic *Escherichia coli* (UPEC) virulence factors through different mechanisms and independent of its effects on type 1 pilus biogenesis and may have potential as an antivirulence compound.

IMPORTANCE CUP pili and flagella play active roles in the pathogenesis of a variety of Gram-negative bacterial infections, including urinary tract infections mediated by UPEC. These are extremely common infections that are often recurrent and increasingly caused by antibiotic-resistant organisms. Preventing piliation and motility through altered regulation and assembly of these important virulence factors could aid in the development of novel therapeutics. This study increases our understanding of the regulation of these virulence factors, providing new avenues by which to target their expression.

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Address correspondence to Scott J. Hultgren, Hultgren@wusm.wustl.edu.

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Although antibiotics have drastically reduced mortality caused by infectious diseases, we now face the spread of resistant microorganisms, with epidemics of drug-resistant, as well as multidrug- and pan-drug-resistant, pathogens (1). Additionally, few new antibacterial drugs are currently in phase 2 or 3 clinical trials (2), limiting future treatment options. Thus, new strategies are needed to replace or augment current drug regimens and to find treatments that do not inspire rapid resistance. One approach is to develop antivirulence therapeutics that can treat disease, either alone or in synergy with existing antibiotics. Bacterial attachment is a critical initial step in the pathogenesis of many bacterial infections that allows the colonization of specific niches in the host. Antiadhesion therapeutics have been developed and shown to effectively prevent and/or treat infections in animal models (3). Here, we report on the antivirulence properties of compound ec240. We found that ec240 prevents the production of several

types of pili that play critical roles in colonization and biofilm formation by Gram-negative pathogens and simultaneously decreases their motility. Thus, we used this compound as a molecular scalpel to further dissect the complex circuitries that control the expression and biogenesis of these important virulence factors.

One large family of adhesive organelles are pili assembled by the chaperone-usher pathway, termed CUP pili. These pili are critical virulence factors in a wide range of pathogenic bacteria, including *Escherichia coli*, *Klebsiella*, *Pseudomonas*, *Haemophilus*, *Salmonella*, and *Yersinia* (4). CUP pili mediate adhesion to host and environmental surfaces, facilitate invasion of host tissues, and promote interaction of bacteria with each other to form biofilms. Each CUP gene cluster, organized in an operon, encodes pilin subunits, a tip adhesin, and a dedicated chaperone and usher that facilitate subunit folding and assembly. CUP pilin subunits have

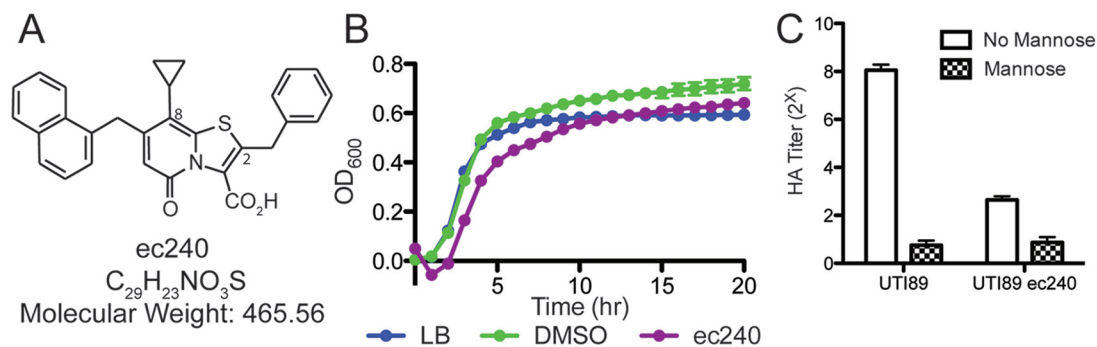


FIG 1 Pilicide ec240 affects type 1 pili. (A) Structure of ec240. (B) Growth curve of UTI89 in LB, LB-DMSO, or LB-250 μ M ec240. There is no significant difference in the final bacterial density under these conditions, as measured by Kruskal-Wallis test. (C) Type 1 piliation in UTI89 grown with DMSO or ec240 as measured by HA. Titers measured with no mannose and which are abrogated in the presence of mannose indicate agglutination mediated by type 1 pili. Titers in the presence of mannose indicate mannose-resistant or non-type-1-pilus-mediated agglutination. Error bars represent standard errors of the means. Averages shown are from at least three biological replicates.

an N-terminal extension and an incomplete immunoglobulin-like fold. Consequently, their folding requires their cognate periplasmic chaperone, which is composed of two Ig-like domains (5). The periplasmic chaperone binds to a pilin subunit, donating a β -strand to transiently complete the Ig fold of the subunit and facilitate its proper folding (6). Chaperone-subunit complexes then interact with their cognate outer membrane usher, which forms a gated channel and catalyzes pilus assembly, wherein the amino-terminal extension on each pilin subunit completes the Ig fold of its predecessor in the pilus, noncovalently linking pilin subunits together (7).

A recent analysis identified 458 CUP operons of 38 distinct CUP pilus types based on usher phylogeny (8). Single *Escherichia* genomes can have as many as 16 distinct CUP operons (8). Among the most studied of the CUP pili are the type 1, P, and S pili, each of which is often encoded by strains of uropathogenic *E. coli* (UPEC) and is associated with the ability of UPEC to establish urinary tract infections (UTIs) (9–11). Type 1 pili encoded by the *fim* operon are required for virulence in a murine cystitis model (9). The type 1 pilus tip adhesin FimH binds mannose-sylated glycoproteins expressed on the luminal epithelial surface of human and murine bladders, and this interaction facilitates bacterial colonization and invasion of bladder epithelial cells (12, 13). Upon internalization, UPEC can be expelled by innate defenses or escape into the host cell cytoplasm, where it can replicate into biofilm-like intracellular bacterial communities (IBCs), composed of $\sim 10^4$ cells, in a process dependent on type 1 pili (14–16). Although discovered in murine models of cystitis, IBCs have also been found in the urine of patients with UTIs (17). Thus, understanding type 1 pilus biology and how it is linked by regulatory cross talk with other CUP operons will further our understanding of clinical UPEC pathogenesis.

The UPEC genome typically contains 5 to 10 different complete CUP operons; however, a single UPEC cell usually expresses only one type of CUP pilus at a time (18). This suggests that UPEC strains with multiple CUP operons also possess intricate genetic circuitries that control the expression of CUP pili. Furthermore, pili are energetically expensive to produce and can be immunostimulatory, necessitating regulation of their expression. Each of the better-characterized UPEC CUP operons, type 1, P, and S pili, encoded by the *fim*, *pap*, and *sfa* operons, respectively, is phase

variable. Phase variation is a very common mechanism of virulence factor regulation in many bacterial species (19). Phase variation of the *fim* operon encoding type 1 pili depends on the recombinases FimB, FimE, and FimX. These recombinases can cleave the genome at 9-bp inverted repeat sequences flanking the *fimS* promoter, resulting in reorientation of the *fimS* promoter region into the phase on or off position (20–23). Phase variation of P and S pili does not involve chromosome rearrangement but rather depends on differential regulator binding and Dam methylation of the respective promoter regions (24, 25).

There is also cross regulation between CUP operons coordinating the expression of different CUP pili and flagella. Many regulators are involved in the control of type 1, P, and S pilus expression. Both P and type 1 pili are regulated by Lrp, HNS, IHF, and Crp, and type 1, P, and S pili are all regulated by Lrp and Dam (24–27). It has also been shown that the CUP regulators PapB, which controls P pilus (*pap*) expression, SfaB, which controls S pilus (*sfa*) expression, and SfaX, another regulator in the *sfa* operon, decrease the expression of type 1 pili (28–30). Type 1 pilus expression has also been shown to alter other CUPs. A phase locked on *fim* mutant, in which the *fimS* promoter cannot be reoriented into the off position, was shown to have decreased *pap* expression and motility (31, 32). Furthermore, SfaX and its homologue in the *pap* operon, PapX, were shown to decrease motility (30, 33). Thus, UPEC has complex networks coordinating the expression of these CUP pili and flagella.

Using our in-depth knowledge of CUP pilus assembly and the crystal structures of CUP chaperones and ushers, we have rationally designed compounds that block piliation. These small molecules, termed pilicides, have a ring-fused 2-pyridone backbone. Pyridone pilicides were initially designed to disrupt pilus assembly by preventing chaperone-subunit-usher interactions. Previously characterized compounds have been shown to bind to chaperones in a conserved hydrophobic domain known to interact with the usher, thus blocking pilus assembly by disrupting chaperone-subunit-usher interactions (34). UPEC grown with pilicides were significantly attenuated in (i) the ability to form type 1 pilus-dependent biofilms, (ii) their adherence to bladder epithelial cells *in vitro*, and (iii) their pathogenesis in a murine cystitis model (35, 36). Medicinal chemistry and the development of new synthetic methodologies have resulted in a new class of pilicide with a

smaller substituent in position 8 (Fig. 1A, e.g., cyclopropyl group) in combination with a substituent in position 2 (e.g., aryl group) in the central fragment (37–40). These compounds were characterized for the ability to inhibit type 1 pilus-dependent biofilms or the production of type 1 or P piliation on the cell surface (35–37, 40–42). A pilicide of this class, ec240 (Fig. 1A), is the most potent inhibitor of type 1 piliation to date, as assessed by hemagglutination assay (HA) (37, 40), and a very potent inhibitor of type 1 pilus-dependent biofilm formation, with a 50% inhibitory concentration of 7 μ M (37).

In this study, we investigated the effects of ec240 on prototypic UPEC strain UTI89, a cystitis isolate encoding 10 different CUP operons (43). RNA sequencing (RNA-Seq) and analyses with isobaric tags for relative and absolute quantification (iTRAQ) revealed that the most significant changes in the transcriptome and proteome in response to ec240 were related to CUP pilus expression. We discovered that growth in ec240 decreased type 1 piliation by affecting the phase variation of the *fimS* promoter, resulting in the phase off state and abolishing *fim* expression. This impact on the phase state likely contributed to the potency with which ec240 inhibits type 1 pili. Furthermore, transcriptional profiling revealed that ec240 increased the transcript levels of other CUP operons, specifically, those encoding S and P pili, and that this effect was independent of ec240 disruption of type 1 pilus expression. Interestingly, despite increased S and P pilus subunit transcript levels, ec240 prevents S and P piliation on the cell surface, indicating that ec240 posttranscriptionally blocks S and P pilus assembly. Thus, while ec240 blocks the assembly of S and P pili posttranscriptionally, possibly through a mechanism described for other pilicides, ec240 is also able to disrupt type 1 pilus regulation by inducing the *fimS* phase off state, increasing its ability to inhibit type 1 pili. We also discovered additional virulence pathways disrupted or affected by ec240, including decreased motility and altered siderophore synthesis. As ec240 disrupts multiple virulence pathways, it is a promising lead compound for development as an antivirulence therapeutic. Further investigation of its impact on the virulence of other Gram-negative pathogens could also be fruitful. This study also highlights the necessity of fully characterizing the global impact of bioactive small molecules on their target's biology, as potential therapeutics can have unpredicted effects, some of which may be therapeutically beneficial, while others may be detrimental.

RESULTS

ec240 disrupts type 1 pilus production. Here we analyzed the effects of the ring-fused 2-pyridone pilicide ec240 on UTI89 biology in general and on CUP expression in particular. ec240 (Fig. 1A) was not toxic to UPEC, causing only a slight lag in growth and not altering the final bacterial culture density (Fig. 1B). In addition to inhibiting type 1 pilus-dependent biofilms, ec240 inhibited the maturation of type 1 pilus-dependent biofilms when administered during biofilm formation (see Fig. S1 in the supplemental material). ec240 was also the most potent pilicide at inhibiting type 1 piliation, as quantified by HA (37, 40). In an HA, normalized bacteria are serially 2-fold diluted and the titer indicates the maximum dilution still capable of agglutinating guinea pig erythrocytes. Mannose acts as a competitive inhibitor of type 1 pilus-mediated adhesion; thus, when the HA is performed in the presence of mannose, agglutination by type 1 pili is prevented. UTI89 showed an HA titer of 2^8 when there was no mannose in the

assay but a titer of 2^1 when mannose was present (Fig. 1C); therefore, UTI89 demonstrated a mannose-sensitive HA (MSHA) titer of 2^8 . After growth in 250 μ M ec240, UTI89 exhibited an MSHA titer of 2^3 (Fig. 1C), indicating that growth in ec240 greatly decreased type 1 piliation on the cell surface. Lower doses of ec240 were also able to decrease type 1 piliation and the MSHA titer (see Fig. S2 in the supplemental material). The slight mannose-resistant HA (MRHA) titer of 2^1 in UTI89 grown with and without ec240 may be indicative of another pilus being expressed under these conditions but at the limit of detection. Thus, as the expression of type 1 pili is linked to that of other CUP pili, we investigated the global impact of ec240 on UPEC biology.

Transcriptional and proteomic responses to ec240. We assessed the transcriptional profile of UTI89 grown with 250 μ M ec240 or a dimethyl sulfoxide (DMSO) vehicle control to investigate the global effects of ec240 on UPEC biology. We utilized a relatively high concentration of ec240 in order to detail all possible “off-target” effects. RNA-Seq was conducted on triplicate biological samples grown under type 1 pilus-inducing conditions. We identified 52 gene transcripts that were altered ≥ 3 -fold when UTI89 was grown in ec240 rather than the DMSO vehicle control. Of these 52 genes, 15 had decreased transcript levels and 37 had increased transcript levels. The transcript levels of CUP pilus genes were affected by the pilicide ec240, as were the transcript levels of genes with a variety of different functions, including motility, siderophore synthesis and transport, metabolism, translation, gene regulation, and stress response (Fig. 2A). There were also some hypothetical genes with dysregulated transcript levels (Fig. 2A). As shown in the MA plot (a plot of the \log_2 of the ratio of abundances of each transcript between the two conditions [M] plotted against the average \log_2 of abundance of that transcript in both conditions [A]), the *pap* and *sfa* genes encoding P and S pili had the most upregulated transcript levels, while the *fim* and *flg* genes encoding type 1 pili and flagella had the most downregulated transcript levels (Fig. 2B), with *fim* transcripts downregulated 8- to 17-fold, *pap* transcripts upregulated 4- to 49-fold, and *sfa* transcripts upregulated 4- to 25-fold (Fig. 2C). Furthermore, CUP operon genes cumulatively make up 46% of the genes dysregulated by >3 -fold but 76% of the genes dysregulated by >10 -fold after growth in ec240. This indicates that under type 1 pilus-inducing conditions, the major effect of ec240 is the dysregulation of CUP pili.

We also measured the global effects of ec240 on proteins by conducting an iTRAQ analysis of UTI89 grown with DMSO or 250 μ M ec240 under the same conditions as used for RNA-Seq. This allowed us to identify 83 proteins with significantly altered abundance after growth in ec240 (50 proteins were increased and 33 proteins were decreased). By iTRAQ analysis, we identified altered levels of CUP proteins, as well as proteins involved in a variety of different functions (Fig. 2D). In regard to CUP proteins, growth of UTI89 in ec240 significantly decreased the expression of the FimC chaperone, FimD usher, FimF subunit, and FimH adhesin (Fig. 2E) and significantly increased the expression of the SfaE chaperone, SfaF usher, SfaH adhesin, PapA subunit, PapD chaperone, and PapG adhesin (Fig. 2E). Interestingly, as P pili can only be depolymerized into PapA subunits by boiling the pilus fibers in urea, the PapA detected by iTRAQ analysis likely represents the fraction of PapA that is in an unpolymerized state within the cell. Thus, ec240 decreased the transcript levels and abundance of Fim proteins but increased the transcript levels and abundance

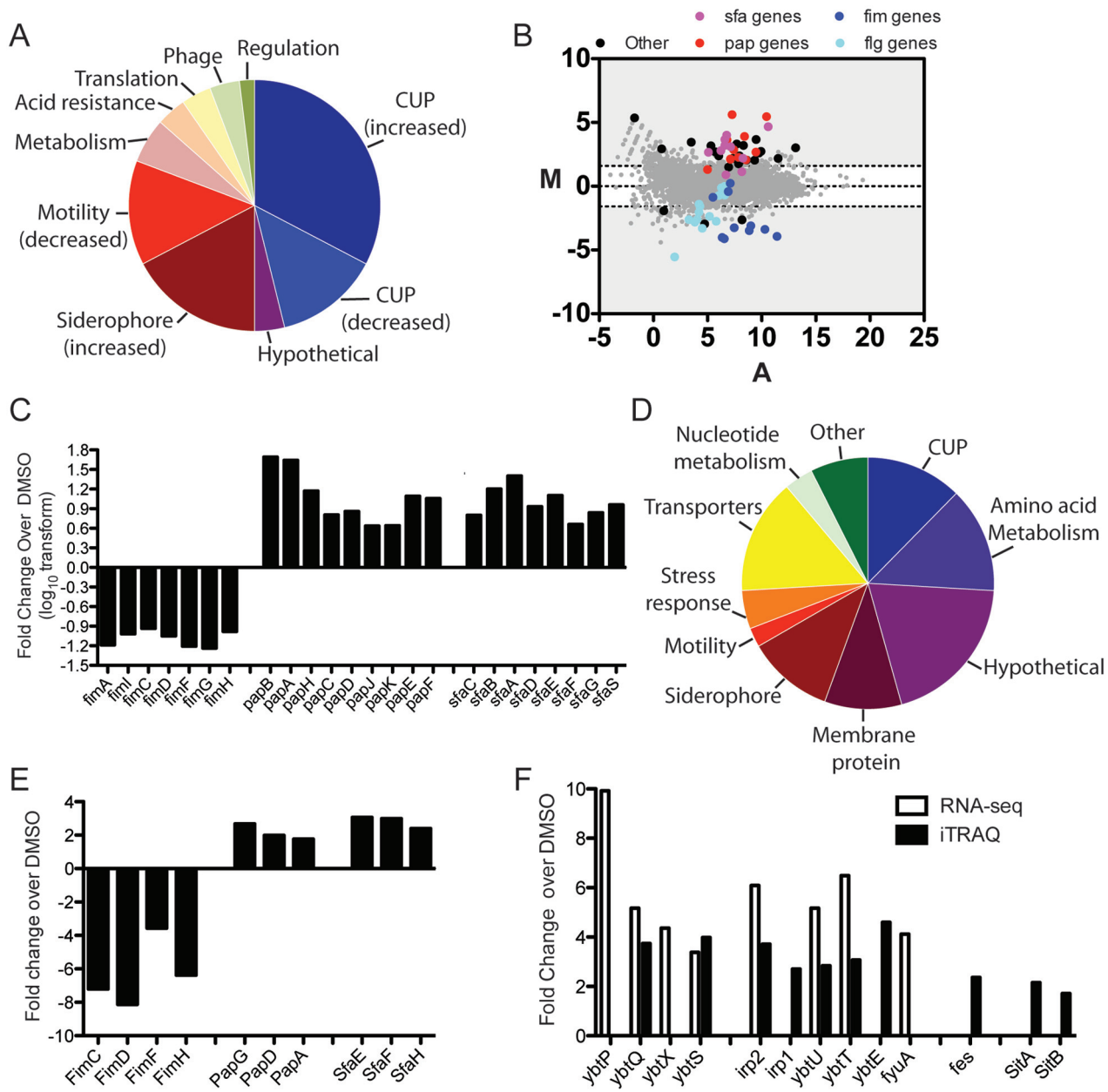


FIG 2 Global transcriptional and proteomic responses to ec240. (A) Genes whose expression is dysregulated by growth in ec240 rather than DMSO, as identified by RNA-Seq analysis. Genes were classified by using KEGG (<http://www.genome.jp/kegg/>) and EcoCyc (<http://www.ecocyc.org>). The genes in each functional category are shown as percentages of the total genes dysregulated. (B) MA plots of UTI89 gene abundance following treatment with ec240. Each dot represents an annotated UTI89 gene with the log₂ of its relative abundance in ec240 versus that in DMSO (M) plotted against the average log₂ of its abundance under both conditions (A). M and A values are based on data from three biological replicates under each growth condition. The dotted lines indicate 3-fold changes. (C) Fold changes in CUP gene transcripts altered in UTI89 grown with ec240 rather than the DMSO vehicle control, as determined by RNA-Seq. Genes are shown in their order within the operon. (D) Proteins dysregulated after growth in ec240 rather than DMSO, as determined by iTRAQ analysis. Proteins were classified by using KEGG (<http://www.genome.jp/kegg/>) and EcoCyc (<http://www.ecocyc.org>). The proteins in each functional category are shown as percentages of the total proteins dysregulated and include proteins with increased and decreased abundances. (E) Fold changes in CUP proteins altered in UTI89 grown with ec240 rather than the DMSO vehicle control, as determined by iTRAQ analysis. (F) Dysregulation by ec240 of siderophore-related and iron transport genes by both RNA-Seq and iTRAQ analysis. Shown are fold changes in transcript or protein levels in UTI89 grown in ec240 rather than DMSO. Genes are shown in their order within the operon.

of the Pap and Sfa proteins. The effect of ec240 on P and S pilus assembly on the cell surface is addressed below.

The RNA-Seq and iTRAQ analysis data confirmed that ec240 alters CUP pilus expression. We also found that UPEC physiology in general was changed. Several proteins involved in amino acid

biosynthesis and degradation have altered expression in the RNA-Seq and iTRAQ analyses. Furthermore, the 50S ribosomal genes *rpmJ* and *rpmE*, had 4.5- and 4.1-fold increased transcript levels, respectively, in the RNA-Seq analysis. Together, these changes could indicate an altered efficiency of mRNA translation and pro-

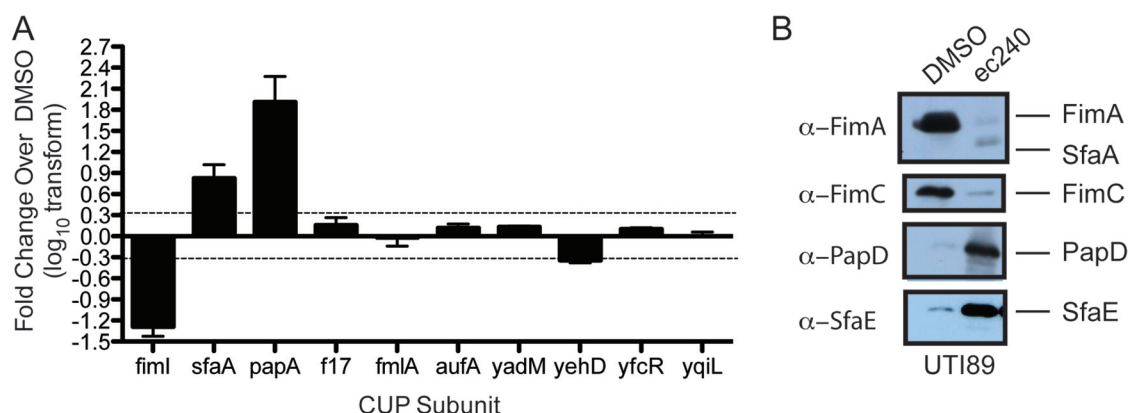


FIG 3 Pilicide ec240 alters CUP pilus expression. (A) qPCR of CUP pilin subunit transcripts in UTI89 grown with ec240. One subunit was used as a readout for the overall transcript level of the operon. Shown is a log transform of the fold change in the transcript level in UTI89 grown with ec240 compared to UTI89 grown with DMSO. Error bars represent standard errors of the means. Averages shown are from at least three biological replicates. Dotted lines indicates 2-fold changes in the transcript level for the qPCR data. (B) Immunoblot assay of CUP levels in UTI89 grown with DMSO or ec240. Antibodies against type 1 pili identified FimA and SfaA bands, while antibodies against PapD and SfaE identified PapD and SfaE, respectively.

tein production in response to growth in 250 μ M ec240. We also discovered that ec240 altered iron regulation. ec240 increased the transcript and protein abundance of several siderophore synthesis genes, including proteins for yersiniabactin synthesis and uptake (YbtESQTU, Irp1, and Irp2), as well as the enterochelin synthesis protein Fès and the iron transporter proteins SitAB (Fig. 2F), indicating that ec240 affects more than one iron uptake system. We have shown by quantitative real-time PCR (qPCR) that siderophore synthesis gene transcripts are increased >2-fold in UTI89 Δ *fim* grown with ec240 rather than DMSO, indicating that the effects of ec240 on siderophore expression are probably independent of its disruption of *fim* expression. Finally, the RNA-Seq and iTRAQ analyses demonstrated decreased expression of many flagellar and chemotaxis genes, an effect of ec240 further discussed below.

ec240 alters CUP pilus expression. RNA-Seq analysis demonstrated that ec240 alters *fim*, *sfa*, and *pap* transcript levels. However, the UTI89 genome encodes 10 CUP pilus operons, most with unknown functions. We therefore measured the transcript levels of subunit genes encoded at the 5' end of each of these CUP operons as surrogates for the overall expression of the operon. Using qPCR, we confirmed that growth in ec240 decreased *fimI* transcript levels but increased *papA* and *sfaA* CUP subunit transcript levels in UTI89 (Fig. 3A). However, the subunit transcript levels of the other 7 CUP operons in UTI89 (*f17*-like, *fml*, *auf*, *yad*, *yeh*, *yfc*, *yqi*) showed no significant changes after growth in pilicide, confirming the RNA-Seq results (Fig. 3A). The effect of ec240 on these systems was also confirmed by immunoblot assays with antibodies against the FimA major pilin, and the CUP chaperones FimC, SfaE and PapD. Growth of UTI89 in ec240 decreased FimA and FimC levels, while the levels of the PapD and SfaE chaperones were increased (Fig. 3B), further confirming the iTRAQ analysis data. The anti-FimA antibody also recognizes SfaA pilin, which can be distinguished from FimA by its smaller size. Thus, in the anti-FimA immunoblot assay, we found that even though *sfaA* subunit transcript levels and SfaE chaperone levels were increased by ec240, SfaA subunits were barely detectable (Fig. 3B). Thus, ec240 either blocked *sfaA* translation or more likely blocked S pilus assembly by inhibiting chaperone-subunit interactions, resulting in SfaA

degradation. Interestingly, while ec240 was identified as an inhibitor of type 1 pili, it more broadly impacted CUP pili, dysregulating type 1, P, and S pili. Surprisingly, it had divergent effects on these homologous systems.

Disrupted *fim* expression alters the expression of the other CUP pili. CUP pili are known to cross regulate each other. To investigate if the effects of ec240 on CUP pili were a direct consequence of its ability to disrupt *fim* expression, we deleted the *fim* operon and used qPCR to quantify the expression of the other nine CUP operons in UTI89 Δ *fim*, i.e., *sfa*, *pap*, *auf*, *fml*, *f17*-like, *yad*, *yeh*, *yfc*, and *yqi*. We found that *sfaA*, *papA*, and *f17*-like pilus subunit transcript levels were >2-fold higher in UTI89 Δ *fim* than in UTI89 after growth under type 1 pilus-inducing conditions (Fig. 4A). In order to quantify pilus production, we harvested pili from UTI89 and UTI89 Δ *fim*. Pilus preparations were depolymerized by boiling in urea and acid, and then individual pilin subunits were resolved by SDS-PAGE. Amino-terminal sequencing of the resolved major pilin subunits demonstrated that after growth under type 1 pilus-inducing conditions, UTI89 produced FimA, the type 1 pilus major subunit (Fig. 4B). Under the same conditions, UTI89 Δ *fim* produced PapA, the major subunit of P pili, and SfaA, the major subunit of S pili (Fig. 4B), confirming the qPCR results. However, we did not identify any *f17*-like pilin subunits. This could indicate that the *f17*-like pilin subunits were not translated, not polymerized into pili, or not harvested or resolved by this method. The Amino-terminal sequencing results indicated that the expression and assembly of other pili, specifically, S and P pili, were induced in UTI89 Δ *fim*, identifying new regulatory connections coordinating the expression of UPEC CUP pili.

HA titers were used to quantify CUP piliation. We found that, in contrast to UTI89, after growth under type 1 pilus-inducing conditions, the UTI89 Δ *fim* mutant produced HA titers of $2^{3.5}$ in the presence or absence of mannose (Fig. 4C), mirroring what has been shown for a conditional *fim* depletion strain (15). As FimH binds mannose, soluble mannose prevents erythrocyte agglutination by type 1 pili and therefore decreases HA titers mediated by type 1 pili. Thus, the *fim* operon deletion in the UTI89 Δ *fim* mutant resulted in the induction of a mannose-resistant pilus. On the basis of the increased *sfa* and *pap* expression in UTI89 Δ *fim*

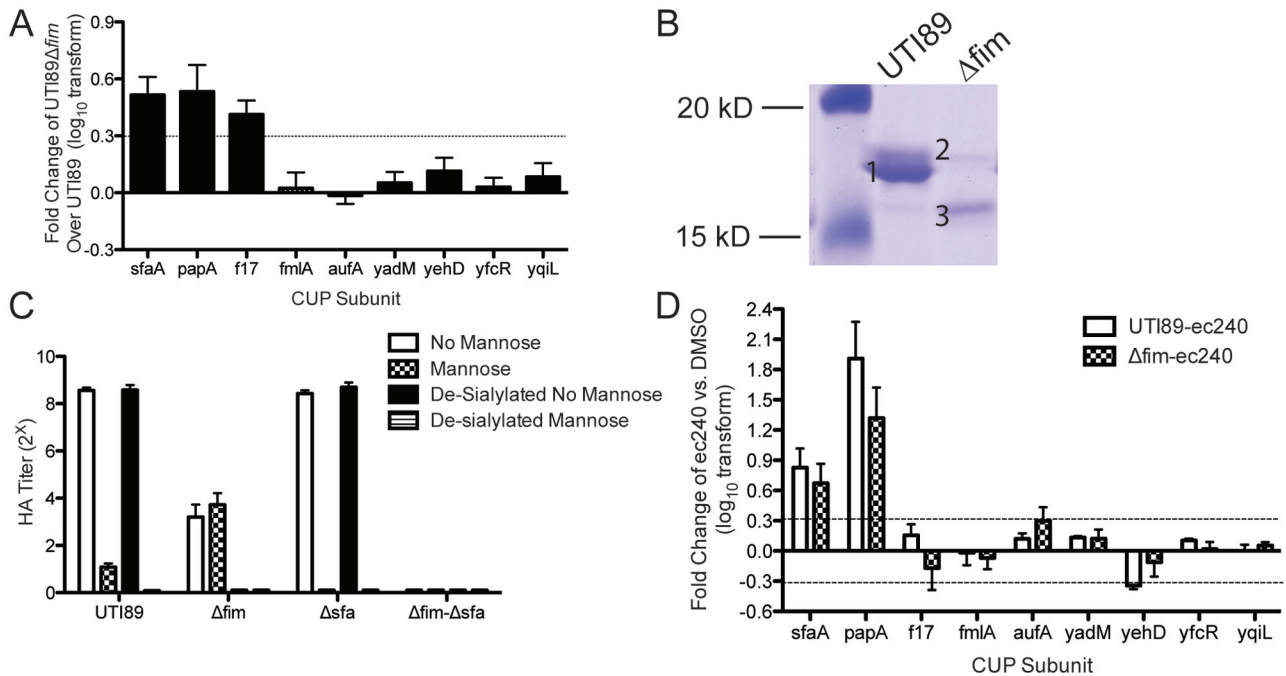


FIG 4 CUP pilus response to *fim* mutation. (A) qPCR of each CUP pilin subunit transcript in UTI89Δ*fim* as a readout of the overall transcript level of the CUP operon. Shown is a log transform of the fold change in the transcript level in UTI89Δ*fim* compared to UTI89. (B) Pili removed from the surface of UTI89 or UTI89Δ*fim* and depolymerized into pilin subunits were separated by SDS-PAGE and stained. Bands subjected to N-terminal sequencing are numbered, and the CUP pilin subunits they contain are FimA (band 1), PapA (band 2), and SfaA (band 3). (C) Type 1 piliation in UTI89 and CUP operon mutants as measured by HA titer and S piliation as measured by HA titer with desialylated guinea pig erythrocytes. (D) qPCR of each CUP pilin subunit transcript in UTI89 or UTI89Δ*fim* grown with ec240 as a readout of the overall transcript level of the CUP operon. Shown as a log transform of the fold change in transcript levels in each strain grown with ec240 compared to that strain grown with DMSO. Error bars represent standard errors of the means. Averages shown in are from at least three biological replicates. The dotted lines in panels A and D indicate 2-fold changes in the transcript level for the qPCR data.

(Fig. 4A and B) and the fact that P pili do not bind to guinea pig erythrocytes, we hypothesized that the mannose-resistant pili on the UTI89Δ*fim* mutant cell surface were S pili. Furthermore, UTI89Δ*sfa*, which is unable to produce S pili, lacks the low MRHA titer usually seen in UTI89 after growth under type 1 pilus-inducing conditions (Fig. 4C). Deletion of the *sfa* operon, creating UTI89Δ*fim*-Δ*sfa*, completely abolished the MRHA titer observed in the UTI89Δ*fim* mutant (Fig. 4C). The S pilus adhesin, SfaH, binds to sialic acid (44). To further confirm that the MRHA titer of UTI89Δ*fim* was mediated by S pili, we enzymatically removed sialic acid from the surface of the guinea pig erythrocytes used in the assay (44). The MRHA titer observed in the UTI89Δ*fim* mutant was abolished upon removal of the sialic acid receptor from the erythrocyte surface, while desialylation had no effect on UTI89 MSHA titers (Fig. 4C). These data confirmed that during growth under type 1 pilus-inducing conditions, deletion of the *fim* operon induces *sfa* expression and assembly of S pili in UTI89. Furthermore, HA titers of UTI89Δ*fim* provide an assay for quantifying S piliation.

Induction of CUP genes by ec240 is independent of its effects on *fim* gene expression. Because S and P pilus subunit transcript levels are increased in UTI89Δ*fim*, we investigated whether the ec240 effects on S and P subunit transcript levels were due to decreased *fim* expression. Interestingly, qPCR revealed a pronounced increase in the transcript levels of *sfaA* and *papA* in UTI89Δ*fim* in response to growth in ec240, relative to their levels in UTI89Δ*fim* grown with DMSO, the vehicle control (Fig. 4D). ec240 did not significantly alter the transcript levels of the other

CUP operons in UTI89Δ*fim*. Therefore, although ec240 decreased the *fim* transcript level, ec240 increased *pap* and *sfa* subunit transcript levels independent of its effects on *fim* expression.

Pilicide ec240 blocks CUP piliation. Although some of the original pilicides decreased P piliation, ec240 was selected on the basis of its ability to inhibit type 1 piliation and actually increased the transcript levels of the *sfa* and *pap* operons. This could limit the efficacy of ec240; it is not ideal to abolish one virulence factor while inducing other virulence factors. Therefore, we further investigated the effects of ec240 on S piliation by determining the HA titers of UTI89Δ*fim*, which produces S pili. After growth of UTI89Δ*fim* with ec240, we discovered greatly decreased MRHA titers (Fig. 5A), demonstrating that ec240 disrupted S pilus assembly, despite the increased *sfaA* transcript level observed after growth in ec240 (Fig. 4D and 5A).

To test the effects of ec240 on P pilus assembly and piliation on the cell surface, we used the plasmid pFJ29 (45). UTI89 encodes a PapGIII adhesin that does not bind to human or guinea pig erythrocytes. However, pFJ29 encodes an isopropyl-β-D-thiogalactopyranoside-inducible *pap* operon from UPEC strain J96, which has a PapGI adhesin that binds to human erythrocytes. Using pFJ29, we demonstrated that ec240 decreased P piliation 2-fold, as measured by HA titer (Fig. 5B). Furthermore, there was a 50% decrease in PapA protein present after growth in ec240, while the levels of PapD chaperone remained constant, as determined by immunoblotting of whole-cell extracts with anti-PapA and anti-PapD antibodies (Fig. 5C). We propose that ec240 blocks P pilus assembly posttranscriptionally, leading to degradation and

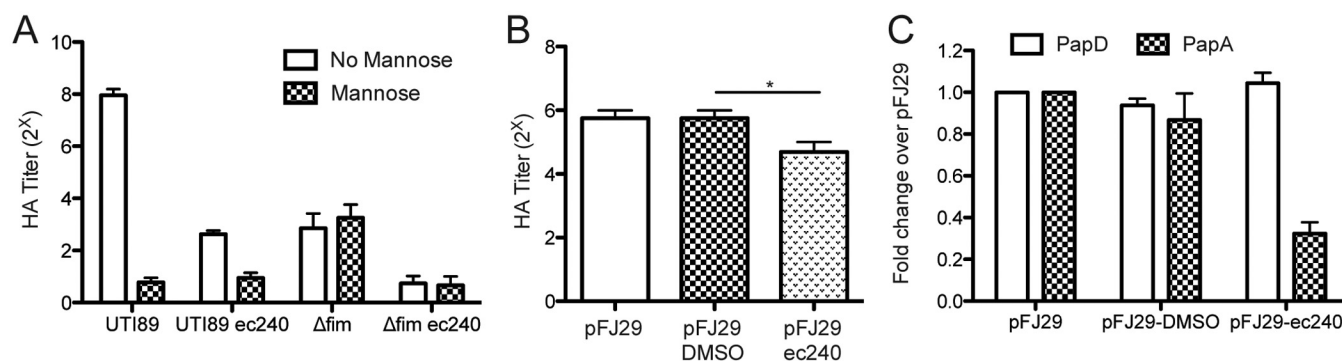


FIG 5 Pilicide ec240 alters CUP piliation. (A) Type 1 and S piliation as determined by HA titers. UTI89Δ*fim* produces S pili; thus, the HA titer of UTI89Δ*fim* grown with ec240 demonstrates ec240-reduced S piliation. (B) HA with human erythrocytes performed with C600/pFJ29 (encoding the *pap* operon from J96) grown alone or with DMSO or ec240. (C) ImageJ quantification of immunoblot assays quantifying PapA and PapD, shown as fold changes over C600/pFJ29. Error bars represent standard errors of the means. Averages shown are from at least three biological replicates. Statistically significant differences were determined by *t* test. *, *P* < 0.05.

decreased PapA levels (Fig. 5B). This explains the discrepancy between the highly increased *papA* transcript levels and only moderately increased PapA protein levels in UTI89 (Fig. 2) and indicates how ec240 decreased P piliation in a cell actively transcribing the *pap* operon (Fig. 5).

Mechanistic studies of ec240 action. Pilicides were originally designed to bind to the pilus chaperone and prevent chaperone-subunit-usher interactions to block proper pilus assembly (34). Therefore, we analyzed the effect that deleting the *fimC*, *sfaE*, or *papD* chaperone had on CUP expression, compared to the effects of ec240. Loss of the cognate chaperone for a CUP operon should prevent proper folding of the respective pilin subunits and their assembly into CUP pili. Like UTI89Δ*fim*, UTI89Δ*fimC* exhibited no MSHA but an MRHA titer of 2⁴, with similar titers in the presence and absence of mannose (Fig. 6A). The MSHA titer was restored to wild-type levels by the expression of *fimC* in *trans* (Fig. 6A). To further elucidate this defect, we examined *fim* expression. The *fimS* promoter element controlling the *fim* operon is invertible and switches between the phase on and off orientations. The phase state was quantified with a phase assay, by restriction endonuclease digestion of the PCR-amplified *fimS* promoter region, which results in different-sized DNA fragments corresponding to the phase on or off state (20). After growth under type 1 pilus-inducing conditions (46), the majority of the UTI89 population was in the *fimS* phase on position, while UTI89Δ*fimC* remained in the *fimS* phase off orientation and thus unable to express *fim* genes (Fig. 6B). Expression of *fimC* in *trans* in UTI89Δ*fimC* restored the phase on orientation (Fig. 6B). Thus, successful pilus assembly is needed for cells to turn phase on. However, the results for the P and S chaperone mutants were very different. We observed no significant difference in *papA* or *sfaA* subunit transcript levels between UTI89 and the UTI89Δ*papD* or UTI89Δ*sfaE* chaperone mutant, respectively, after growth under P or S pilus-inducing conditions (see Fig. S3 in the supplemental material). Thus, it is unlikely that ec240 induced increased *pap* or *sfa* transcript levels because of disruption of PapD or SfaE chaperone function. However, it is possible that a chaperone from one operon could complement the assembly defect of another operon, allowing for pilus assembly even in the Δ*papD* or Δ*sfaE* chaperone mutants. This has been demonstrated for the PapD chaperone, which can complement a Δ*fimC* chaperone mutant (47). Thus,

although *fim* expression is sensitive to pilus assembly, it appears that *sfa* and *pap* expression is not.

As mutation of the *fimC* chaperone altered the phase state and pilicides were originally designed to interfere with FimC function, we investigated if ec240 also affected the *fimS* phase orientation, possibly by inhibiting FimC function. Using a phase assay, we discovered that UTI89 grown in ec240 was *fimS* phase off, even after growth under type 1 pilus-inducing conditions (Fig. 6C). Lower concentrations of ec240 also induced the *fimS* phase off orientation (see Fig. S2 in the supplemental material). Furthermore, ec240 had similar effects on the phase regardless of whether the starting bacterial population was in the phase on or off orientation. Thus, ec240 could both keep phase off cells in the phase off orientation and switch phase on cells to the phase off orientation (Fig. 6C).

We hypothesized that the ec240-induced *fimS* phase off orientation was the result of disrupted chaperone function and pilus assembly defects, with subsequent negative feedback on *fimS*, as seen in UTI89Δ*fimC*. Therefore, we analyzed the ability of ec240 to decrease type 1 piliation in a phase locked on mutant. This *fim* phase locked on strain (LIR) has point mutations incorporated into the *fimS* left inverted repeat, such that the Fim recombinases can no longer reorient the *fimS* promoter (48). Surprisingly, ec240 had a minimal effect on type 1 piliation in the phase locked on strain (Fig. 6E). This was not the case for all pilicides with activity against type 1 pili. For example, pilicide ec342 decreased type 1 piliation in the phase locked on strain as well as in UTI89 (Fig. 6D and E). With qPCR, we demonstrated that the phase locked on strain produced the same level of *fim* subunit transcript after growth in ec240 or DMSO, indicating that ec240 had no effect on *fimS* promoter activity or transcript stability. Thus, it appeared that ec240 minimally disrupts type 1 pilus assembly in the LIR mutant and the effects of ec240 on type 1 piliation were primarily due to its ability to induce the *fimS* promoter into the phase off orientation. This effect on the *fimS* promoter could stem from ec240 altering phase state regulators; however, it is also possible that the small decrease in HA titer indicated a slight defect in FimC chaperone function, which could impact the *fimS* phase orientation, as seen in the UTI89Δ*fimC* mutant. Overall, the greater potency with which ec240 inhibits type 1 piliation than other pili-

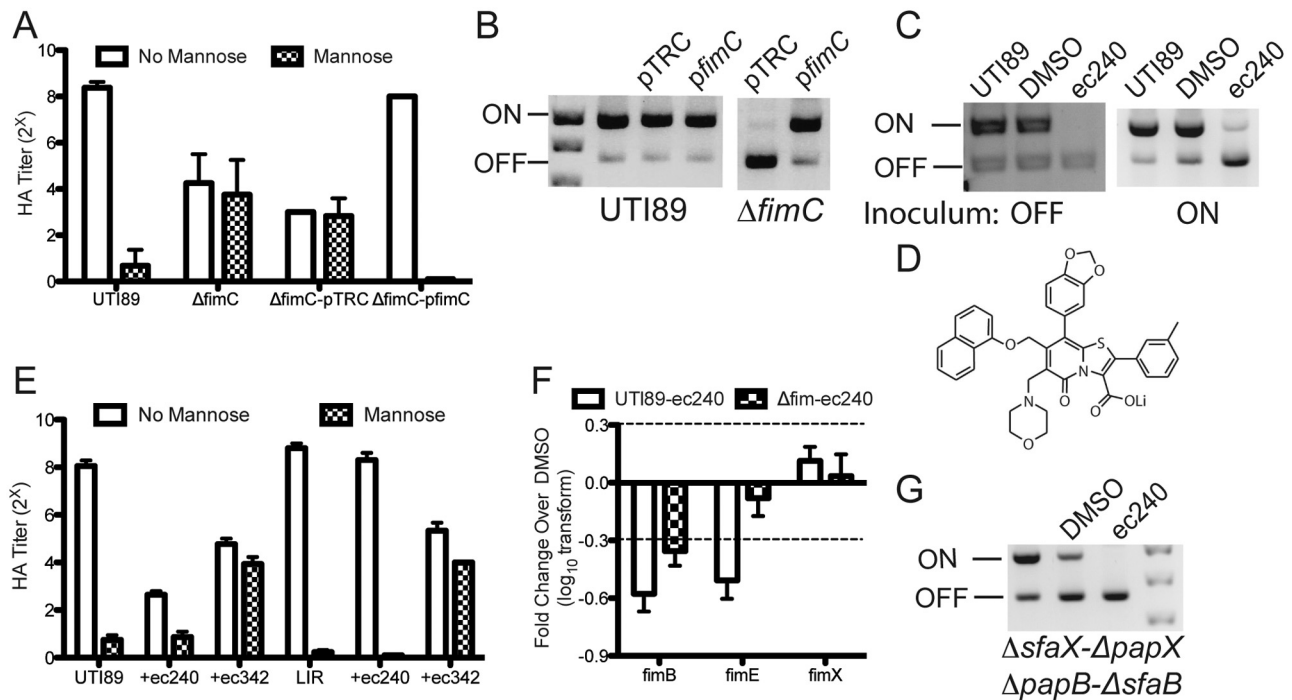


FIG 6 Mechanism of action of pilicide ec240. (A) HA titer of UTI89 and the UTI89 $\Delta fimC$ chaperone mutant, alone and transformed with the pTRC99a vector control or *pfimC* (pTRC99a-*fimC*). (B) Phase assay of UTI89 and the UTI89 $\Delta fimC$ chaperone mutant and each strain transformed with the pTRC99a vector control or *pfimC* (pTRC99a-*fimC*) (see Materials and Methods). (C) Phase off or on UTI89 inoculum was back diluted in LB, LB-DMSO, and LB-ec240. Shown is a phase assay after growth of the back-diluted culture under type 1 pilus-inducing conditions. (D) Structure of ec240. (E) Piliation of UTI89 or LIR (*fimS* phase locked on strain) grown under type 1 pilus-inducing conditions with DMSO, ec240, or ec342, as measured by HA. (F) qPCR of recombinase *fimB*, *fimE*, and *fimX* transcript levels in UTI89 or UTI89 Δfim grown with ec240, shown as the log transform of fold change in transcript levels in each strain grown with ec240 compared to each strain grown with DMSO. (G) Phase assay of UTI89 mutants after growth under type 1 pilus-inducing conditions, either alone or with DMSO or ec240. Error bars represent standard errors of the means. Averages shown in panels A, E, and F are from at least three biological replicates. Dotted lines indicate 2-fold changes in transcript levels for the qPCR data.

cides do may stem from its ability to induce UTI89 into the *fimS* phase off state, thereby preventing the expression of *fim* subunits.

The recombinases responsible for reorienting *fimS* between the on and off states are encoded by *fimB*, *fimE*, and *fimX*. *FimB* and *FimX* primarily turn the *fimS* promoter to the on orientation, while *FimE* changes *fimS* to the off orientation (21, 22). Furthermore, many inputs affect the phase state through modulation of recombinase expression or activity. Thus, we quantified recombinase transcript levels after growth in ec240 and found that UTI89 had >2-fold lower *fimB* and *fimE* transcript levels (Fig. 6F). A trend toward a decreased *fimB* transcript level was also observed in UTI89 Δfim grown with ec240 (Fig. 6F). Thus, the ability of ec240 to modulate the levels of the *fimB* and *fimE* recombinases may result in the induction of the *fimS* phase off orientation.

The effects of ec240 on *pap* and *sfa* transcripts are independent of *fim* disruption, as ec240 increased *pap* and *sfa* transcript levels even in UTI89 Δfim (Fig. 4D). *fim* expression impacts *pap* and *sfa* expression, but *pap* and *sfa* expression also modifies *fim* phase and expression. This CUP cross regulation occurs in part via *PapB* and *SfaB*, which are *pap* and *sfa* transcriptional regulators encoded at the beginning of their respective operons that can both induce the off orientation of the *fimS* switch (28, 29). Furthermore, both show significantly increased expression in UTI89 grown with ec240 (see Fig. S4 in the supplemental material). *papB* is also increased in UTI89 Δfim after growth in ec240 (see Fig. S4). *SfaX*, a regulator encoded at the end of the *sfa* operon, has also been

shown to turn UPEC phase off and to decrease transcription from the *fimS* promoter (30). The *SfaX* homologue in the *pap* operon, *PapX*, has not been shown to have the same effects on type 1 pili; however, it shows some functional homology to *SfaX*, as both decrease motility (33). Thus, increased expression of *PapB*, *SfaB*, *SfaX*, and possibly *PapX* may explain the *fimS* phase off orientation and decreased *fim* expression observed in response to ec240. To investigate if this is the sole mechanism of action of ec240, we generated a deletion mutant strain, UTI89 $\Delta sfaX$ - $\Delta papX$ - $\Delta papB$ - $\Delta sfaB$, lacking all four of these regulators. Treatment of UTI89 $\Delta sfaX$ - $\Delta papX$ - $\Delta papB$ - $\Delta sfaB$ with ec240 induced the *fimS* phase off orientation (Fig. 6G). Thus, while ec240 may work in part by inducing the regulators *PapB*, *SfaB*, and *SfaX*, which are known to facilitate the off orientation of *fimS*, there exists an alternative pathway by which ec240 carries out its mechanism of action on type 1 pilus expression.

Pilicides alter flagellar expression and regulation. In addition to affecting CUP pili, ec240 alters other virulence factors, including motility. Flagella are an important virulence factor in many Gram-negative bacteria, and they are important for UPEC pathogenesis (49, 50). Flagella are large and complex surface organelles that can be immunogenic and require significant energy to assemble. Therefore, there is intricate regulation controlling flagellar expression, with the master regulator *FlhDC* stimulating the transcription of class 2 genes for structural components and the regulators *FliA* and *FlgM* controlling the class 3 structural components

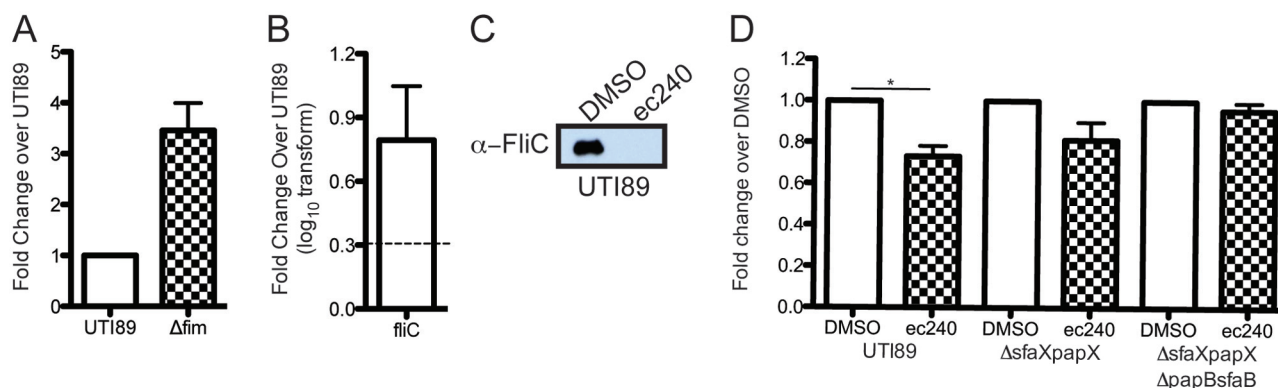


FIG 7 Effects of pilicide ec240 on motility. (A) Swimming motility in soft agar of UTI89 Δfim as determined by the fold change in the diameter of the growth zone compared to that of UTI89 after the growth of each strain under type 1 pilus-inducing conditions. (B) qPCR of *fliC* transcript levels in UTI89 Δfim , shown as a log transform of the fold change relative to UTI89 after growth under type 1 pilus-inducing conditions. (C) Immunoblot assay of flagellar levels in UTI89 grown with DMSO and ec240 with anti-H7 antibody. (D) Swimming motility in soft agar of UTI89 or UTI89 mutants grown with ec240 as determined by the fold change in the diameter of the growth zone compared to that of UTI89 or UTI89 mutants grown with the DMSO vehicle control. *, $P < 0.05$. Statistically significant differences were determined by one-sample t test. Error bars represent standard errors of the means. The dotted lines in panel B indicate 2-fold changes in transcript levels for the qPCR data.

(51). In CFT073, locking the *fimS* promoter phase off was previously shown to have no effect on motility, after growth under motility-inducing conditions (31). However, here we demonstrated that UTI89 Δfim had greater motility (Fig. 7A) and greater expression of *fliC* (Fig. 7B) than UTI89 after growth under type 1 pilus-inducing conditions. Thus, under conditions where UPEC make type 1 pili, disruption of *fim* expression stimulates motility, further indicating that type 1 pili and flagella are inversely regulated, as well as controlled by environmental signals.

The inverse cross regulation of type 1 pilus and flagellum expression was decoupled by the pilicide ec240, which decreased the expression of both *fim* and the flagellar structural genes from class 2 (*flgBCDEF*, *fliF*) and class 3 (*fliC*), as determined by RNA-Seq analysis. We used qPCR to confirm the expression levels of flagellar regulators and structural and chemotaxis genes in UTI89 and found that ec240 decreased the transcript levels of these genes and that it had a larger effect on the UTI89 Δfim mutant (see Fig. S5 in the supplemental material). Furthermore, immunoblot analysis revealed decreased FliC after growth of UTI89 in ec240 (Fig. 7C). Importantly, we also demonstrated that ec240 decreased UTI89 swimming motility *in vivo* (Fig. 7D). Thus, the pilicide ec240 is able to decouple the regulation coordinating the expression of type 1 pili and flagella in order to simultaneously decrease type 1 piliation and flagellar motility. We hypothesized that the effects of ec240 on motility might be due to increased expression of SfaX and PapX, which are known to decrease motility (30, 33). Growth of UTI89 $\Delta sfaX$ - $\Delta papX$ in ec240 no longer significantly decreased motility compared to that in the DMSO control, although a slight reduction remained (Fig. 7D). However, ec240 had no effect on the motility of UTI89 $\Delta sfaX$ - $\Delta papX$ - $\Delta papB$ - $\Delta sfaB$ (Fig. 7D). Therefore, deletion of *sfaB* and *papB* in the context of *sfaX* and *papX* deletion abolished the activity of ec240 on motility, suggesting a possible role for SfaB and PapB in the control of motility in response to ec240.

DISCUSSION

CUP pili are a diverse group of extracellular adhesive fibers assembled by the chaperone-usher pathway that contain different ad-

hesins that bind to different receptors with stereochemical specificity and mediate attachment, invasion, and biofilm formation in a variety of niches and host tissues. Many Gram-negative bacteria encode multiple CUP pili, and many of these pili are disease associated. The genome of the clinical isolate UTI89 carries 10 CUP pilus operons (43). These include type 1, P, and S pili, which are all associated with the ability of UPEC to establish UTIs (9–11). Additionally, S pili are associated with neonatal meningitis (52). Small molecules targeting CUP pili have the potential to be developed into therapeutics for the possible treatment and/or prevention of bacterial infections such as UTIs (3, 35). Pilicides are low-molecular-weight compounds that were designed to block piliation. The pilicide ec240 was identified as a potent inhibitor of type 1 pili; however, it was not known whether ec240 has broad-spectrum activity against other CUP pili or how it might affect the expression of other virulence factors. Thus, in this work, we characterized the activity of ec240 on CUP expression in UTI89 and its effects on other virulence traits of the organism. Our analysis of ec240 provided important insights into how such antivirulence molecules can short circuit genetic pathways controlling the expression of multiple virulence factors, thus revealing antivirulence properties beyond inhibition of the expression of a specific CUP pilus. Further, this analysis has demonstrated how antivirulence therapeutics can serve as molecular scalpels to further dissect the molecular networks interconnecting virulence factor expression.

CUP pili have complex regulation coordinating their expression. Type 1 pilus phase switching is mediated by the recombinases FimB, FimE, and FimX (22, 23), but numerous environmental signals and regulators affect the phase switching rates of the *fimS* promoter and promoter activity when *fimS* is in the phase on orientation (24, 26, 30, 53, 54). We have now demonstrated that the *fimS* phase state and *fim* expression are also sensitive to successful pilus assembly. Furthermore, there are extensive networks that interconnect CUP pili with the control of other virulence factors, especially flagella. Bacteria alternate between the production of pili to colonize a specific environment and the production of flagella to swim to new environments and therefore need regulatory circuits to accomplish these lifestyle choices. For

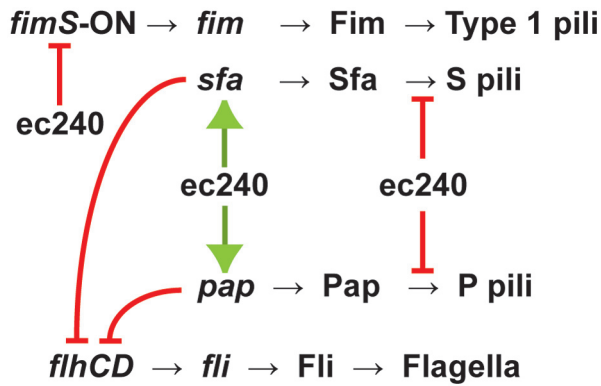


FIG 8 Model of pilicide ec240 action on CUP pili and flagella.

example, type 1 pili, encoded by the *fim* operon, have phase-variable expression mediated by the inversion of its promoter element (20). It has been shown that a phase on *fim* mutant of UPEC strain CFT073 has decreased P pilus transcripts and flagellar expression (31, 32). Furthermore, several regulators in the S and P operons decrease the expression of type 1 pili and flagella (28–30, 33), thus coordinating the types of extracellular appendages that are expressed by a cell at a given time. The presence of redundant regulators to coordinate the production of different CUP pili and flagella indicates the necessity of producing these virulence factors only in specific niches and host tissues. Here we present evidence of additional regulatory circuitries interconnecting the expression of type 1 pili with other CUP pili and flagella. We discovered that UTI89Δ*fim* has increased *pap*, *sfa*, *fli*-like, and *fliC* transcript levels and increased flagella and S and P pili on the cell surface. CUP pili are known to mediate tissue tropisms through binding to different receptors, and thus, such a compensatory response could increase UPEC fitness by allowing it to bind to other receptors found in that environment or enabling some of the population to colonize new niches or host tissues.

The pilicide ec240 was designed to prevent CUP pilus assembly and was found to decrease type 1 pilus-dependent biofilm formation (36). ec240 is one of the most potent pilicides described to date at inhibiting type 1 piliation as measured by HA titers. We found that ec240 results in the *fimS* promoter adopting the phase off orientation, thus preventing the transcription of *fim* genes (Fig. 8). Since ec240 has a minimal effect on type 1 pilus biogenesis in the *fimS* phase locked on mutant, we argue that it works primarily by inducing the phase off orientation of the *fimS* promoter switch, which may explain its greater potency than other pyridone compounds. The *fimS* phase off orientation may stem from slight defects in FimC chaperone function or could be due to the effects of ec240 on *fim* regulators. Growth in ec240 increased the expression of several negative regulators of type 1 pili, SfaB, PapB, and SfaX, which likely contribute to the ability of ec240 to turn UPEC *fimS* phase off. However, growth of UTI89Δ*sfaX*-Δ*papX*-Δ*papB*-Δ*sfaB* in ec240 resulted in the *fimS* phase off configuration, indicating that ec240 may also impact other regulators of *fim* expression. It is also possible that ec240 controls the *fimS* phase state by impacting both FimC chaperone function and the levels of phase regulators. We are further investigating other pathways affected by ec240 that may contribute to the *fim* phase effects. For example, the global regulator Crp has been previously shown to be involved

in the control of the expression of type 1 and P pili (24, 26). We find that type 1 pilus expression is decreased in a Δ*crp* mutant; however, ec240 does not alter the expression of type 1, P, or S pili in the Δ*crp* deletion mutant compared to that in the DMSO control, indicating that Crp may be involved in mediating ec240 control of CUP pili. If the effects of ec240 on *fim* are indeed mediated by several redundant regulators, it should be more difficult for UPEC to evolve resistance to the effects of ec240, increasing its therapeutic potential.

Given the complex regulatory circuits coordinating CUP pilus and flagellum expression, we used RNA-Seq and iTRAQ to analyze the global effects of ec240 on wild-type UTI89 and UTI89Δ*fim*. These analyses revealed that ec240 dysregulated a variety of biological processes. The largest changes induced by ec240 in UTI89 were dysregulation of CUP pili and flagella, specifically, increased expression of S and P pili and decreased expression of type 1 pili and flagella. Additionally, ec240 was found to impact iron regulation within the cell, including siderophore synthesis genes and iron transporters. Iron is an essential element, and UPEC produces a variety of iron-scavenging compounds called siderophores to harvest iron from their environment. During the formation of IBCs inside murine bladder host cells, UTI89 up-regulates the expression of several siderophore synthesis genes (55), further indicating their importance in UPEC UTIs. It is possible that ec240 alters a regulator of siderophores or results in decreased intracellular levels of iron. We are further investigating this effect in order to fully understand the virulence profile exhibited by UPEC after growth in ec240. We also clarified the effects of ec240 on CUP pili. The increased S and P subunit transcript levels were not due to disrupted *fim* expression, as we found that ec240 increased S and P subunit transcripts even in the UTI89Δ*fim* mutant. Thus, ec240 activates S and P transcript levels independent of its effects on *fim* expression, and we are further investigating this ec240 activity. Although no function other than inversion of the *fimS* switch has been attributed to the recombinases, it would be interesting to investigate if they have an impact on the expression of other CUP pili, as seen for several other CUP regulatory proteins.

ec240 prevents type 1, P, and S piliation, which is associated with UTIs and other infections (9–11, 52). ec240 is very similar in structure to the pilicide 5d, which was crystallized with the PapD chaperone and which bound to a conserved hydrophobic patch on the chaperone known to mediate chaperone-subunit-usher interactions (36, 37). Thus, we propose that ec240 blocks S and P pilus assembly, as demonstrated for other similar pilicides (36, 37), (Fig. 8). However, ec240 controls the *fimS* phase state and therefore type 1 piliation. It is possible that ec240 induces a slight defect in FimC chaperone function, which then impacts *fimS* orientation, or that ec240 controls *fimS* phase by altering the levels of phase regulators. Overall, it appears that ec240 has different mechanisms for inhibiting these homologous pili. The biological impact of ec240 inhibition of all three of these UTI-associated pili needs to be investigated in animal models of UTI to further evaluate the therapeutic potential of ec240.

In addition to the effects of ec240 on S and P pili, we demonstrated that ec240 was able to disrupt the regulatory connections between type 1 pili and flagella. Although flagellar expression is induced in UTI89Δ*fim*, where type 1 pili are not expressed, ec240 leads to decreased expression and production of both type 1 pili and flagellar proteins. We have shown that growth in ec240 in-

creased the expression of the P and S operons, which include PapX and SfaX, regulators that are known to decrease motility (30, 33). However, ec240 remained capable of inhibiting motility in UTI89 Δ sfaX- Δ papX and thus the effects on flagella were not mediated solely by the activity of PapX and SfaX. However, in UTI89 Δ sfaX- Δ papX- Δ papB- Δ sfaB, the effect of ec240 on motility was abolished, indicating a possible new role for SfaB and PapB in motility regulation. The importance of all four of these regulators in mediating the effects of ec240 on motility should decrease the rate at which UPEC can evolve resistance to this compound, as resistant mutants would need to have inactivated all four of these regulators.

As important virulence factors, CUP pili are optimal targets for antivirulence compound development, and several such therapeutics have been developed. The structure of the FimH adhesin bound to mannose (36) has been used to rationally design molecules, termed mannosides, that block FimH function by binding in the FimH mannose-binding pocket. Mannosides are potent inhibitors of biofilm formation *in vitro* and can prophylactically prevent acute UTI and treat chronic UTI in a mouse model when delivered orally (3). Furthermore, vaccines that stimulate an immune response to pilus adhesins can protect against cystitis in mouse models (56) and pyelonephritis in a primate model (57). Unlike these other antivirulence therapeutics, pilicide ec240 can simultaneously disrupt the production of type 1, P, and S pili, as well as flagella. Thus, ec240 holds promise for the development of a therapeutic for treating UPEC UTIs. As other UPEC isolates may have modified CUP regulatory pathways or different CUP pilus structures, further studies are needed to determine if ec240 functions similarly in other UPEC isolates. These pili are also involved in a variety of other infections mediated by Gram-negative bacteria, and future work will establish their efficacy against different organisms. To develop this compound into a therapeutic, further studies are needed to assess its pharmacokinetics and pharmacodynamics and to determine the concentration at which it accumulates in the bladder or other potential sites of infection. Mannosides are very different in structure from ec240, but it has been shown that they are present in the urine of mice at $\geq 100 \mu\text{M}$ after oral or intraperitoneal dosing at 100 or 10 mg/kg, respectively, indicating that it is possible for small molecules to be present in the urine at these levels (3). Although most of the experiments reported here were conducted at $250 \mu\text{M}$, we also observed some of these phenotypes at lower concentrations of ec240 (see Fig. S2 in the supplemental material), so this compound could be therapeutically active even if it cannot reach $250 \mu\text{M}$ at the site of infection. Overall, molecules like ec240 could be therapeutically beneficial and can be used as chemical probes to further dissect the regulatory networks that fine-tune piliation and virulence factor expression.

MATERIALS AND METHODS

Pilicides. Pilicides ec240 and ec342 were synthesized as previously described (37, 39).

Bacterial strains. Mutant strains were constructed with the λ Red recombinase system (58). The strains used in this study are detailed in Table S1 in the supplemental material.

Bacterial growth conditions. Unless otherwise stated, strains were grown under type 1 pilus-inducing conditions, i.e., static incubation in Luria broth (LB) at 37°C for 24 h and then subculturing at 1:1,000 in LB for an additional 24-h static incubation at 37°C (2×24 h) (46). For P pilus-inducing conditions, bacteria were grown on tryptic soy agar plates

at 37°C for 2×24 h (59). For S pilus-inducing conditions, bacteria were grown on LB plates with 40 mM NaCl at 37°C (60). Growth was in the pilicide at $250 \mu\text{M}$ or in the DMSO vehicle control at 0.5%, unless otherwise stated. Strains were grown in ampicillin at $100 \mu\text{g/ml}$, where needed.

Swimming motility. Swimming motility was assessed after growth in type 1 pili-inducing conditions by the diameter of growth in 12 ml of LB 0.25% agar in six-well plates.

Immunoblot analyses. UTI89 or isogenic mutants were grown under type 1 pilus-inducing conditions with DMSO or $250 \mu\text{M}$ ec240. Normalized cells were processed for SDS-PAGE. Membranes were probed with antisera for FimCH, FimA, PapD, SfaE, or H7 (BD-Difco) at 1:10,000.

RNA extraction and qPCR. qPCR for CUP transcript levels was conducted with samples of UTI89 or isogenic deletion mutants grown under type 1 pilus-inducing conditions. RNA was extracted from flash-frozen bacterial cell pellets according to the manufacturer's protocol (Qiagen, Zymo Research). RNA was treated with Turbo DNase (Ambion) according to the manufacturer's protocol and then tested for complete removal of DNA by PCR with 16S or GyrA primers. RNA was reverse transcribed with random primers (Invitrogen) and Superscript II reverse transcriptase (Life Technologies). For qPCR, 100 ng of a cDNA or RNA negative control that was not reverse transcribed was used as a template for qPCR with SYBR green according to the manufacturer's protocol (Bio-Rad). qPCR was conducted with primers specific to CUP subunit genes or other target genes and the *gyrA* housekeeping gene. All primers were validated for qPCR efficiency. Relative fold change was determined by the Pfaffl method, where fold change = $2^{\Delta\Delta\text{CT}}$ target (control - sample) / $2^{\Delta\Delta\text{CT}}$ ref (control - sample) (61). We used technical replicates for each gene tested and at least three biological replicates for each experiment.

Generation of cDNA libraries for RNA-Seq. Triplicate UTI89 samples were grown under type 1 pilus-inducing conditions in the presence of $250 \mu\text{M}$ ec240 or the DMSO control. Total RNA was isolated as described above. Illumina cDNA libraries were generated by using a recently developed multiplexed library construction protocol called RNAtag-Seq (Alexander A. Shishikin et al., submitted for publication). Briefly, bar-coded adapters were ligated directly to the total RNA, tagging each sample with a unique sequence identifier. The bar-coded samples were then pooled, and all subsequent steps of library construction were conducted with the pool. These steps included (i) rRNA depletion with RiboZero (Epicenter), (ii) first-strand cDNA synthesis with a primer complementary to a constant region of the bar-coded adapter, (iii) ligation of an adaptor to the 3' end of the cDNA, and (iv) PCR amplification of the cDNA with primers fused to Illumina sequencing adapters. These libraries were sequenced with the Illumina HiSeq 2000 sequencing system to generate 25 base paired-end reads.

RNA-Seq analysis. Reads were aligned with the UTI89 chromosome and pUTI89 (RefSeq NC_007946 and NC_007941, respectively) by using BWA version 5.9 (62). Gene annotations were from RefSeq and Rfam (63). The overall fragment coverage of genomic regions corresponding to features such as open reading frames and rRNAs was conducted by using bioinformatic pipelines developed in house as previously described (64). Differential-expression analysis was conducted with DESeq (65).

Hemagglutination assays. Bacteria were grown under type 1 pilus-inducing conditions in the presence of $250 \mu\text{M}$ ec240 or the DMSO control. Pilus expression was assessed by HA as previously described (66) in bacterial cultures normalized to an optical density at 600 nm (OD_{600}) of 1 and guinea pig erythrocytes normalized to an OD_{640} of 2. The experiment was conducted in parallel with phosphate-buffered saline (PBS)–4% mannose. To quantify pilus binding to sialic acid, guinea pig erythrocytes were enzymatically desialylated by incubation in neuraminidase from *Arthrobacter ureafaciens* (EY Labs) for 2 h at 37°C while rocking (44). The erythrocytes were pelleted at 3,000 rpm, the enzyme was removed, and the erythrocytes were resuspended in PBS to an OD_{640} of 2. P pilus HAs were conducted after growth overnight at 37°C while shaking in LB with $250 \mu\text{M}$ ec240 or the DMSO control. HAs were conducted with bacterial

cultures normalized to an OD₆₀₀ of 1 and human erythrocytes normalized to an OD₆₄₀ of 1.9.

Phase assays. Bacterial cells were grown under type 1 pilus-inducing conditions in the presence of 250 μ M ec240 or the DMSO control. Phase primers flanking the *fimS* promoter were used to PCR amplify the *fimS* region of the UTI89 genome, the PCR product was digested with *Hinf*I for 2 h at 37°C, and then the DNA bands were resolved on a 2% agarose gel (20). Band intensities were quantified by ImageJ.

Amino-terminal sequencing. Bacterial cultures of UTI89 or isogenic mutants were grown under type 1 pilus-inducing conditions. One liter of bacterial culture was pelleted by centrifugation at 8,100 rpm for 12 min at 4°C, and the bacteria were resuspended in 25 ml of 4 mM Tris, pH 8. Pili were removed from the cell surface by blending at speed 7 in an Omni-mixer (Sorvall) for 2 min, twice. The suspension was centrifuged at 16,000 \times g, and the supernatant containing the pili was decanted and salt precipitated with 300 mM NaCl and 100 mM MgCl₂. Pili were collected by centrifugation at 21,000 \times g and resuspended in 1 mM Tris, pH 8. Pili were depolymerized by boiling in 50 mM HCl and 4 M urea for 10 min and then neutralized with 50 mM NaOH, subjected to SDS-PAGE to separate pilin subunits, transferred to polyvinylidene difluoride, stained with 1% Coomassie blue R-250 in 40% methanol, and destained in 50% methanol. Subunit bands were excised, and protein identities were determined by N-terminal sequencing (Midwest Analytical Inc.).

Absolute quantitation of protein level differences by iTRAQ mass spectrometry. UTI89 was grown under type 1 pilus-inducing conditions in the presence of DMSO (vehicle) or 250 μ M ec240. Bacteria were pelleted by centrifugation at 6,000 rpm for 10 min, and cells were acidified in order to dissociate type 1 pilus subunits as described above. Bacteria were mechanically disrupted in radioimmunoprecipitation assay buffer containing Complete protease inhibitor (EDTA free; Roche). Total cell lysates were subjected to five pulses of sonication at a 20% amplitude (Sonic Dismembrator; Fisher Scientific), followed by incubation with constant rotation at 4°C for 2 h to solubilize membrane proteins. Insoluble cellular debris was then removed by centrifugation at maximum speed for 10 min. The total protein concentration of each sample was determined by bicinchoninic acid assay (Pierce), and 100 μ g of total protein per sample was processed for iTRAQ mass spectrometry as previously described (67, 68), with some modifications (see Text S1 in the supplemental material). Autovalidation was performed such that peptide assignments to mass spectra were designated valid following an automated procedure during which score thresholds were optimized separately for each precursor charge state and the maximum target-decoy-based false-discovery rate (FDR) was set to 1.0%. To obtain iTRAQ protein ratios, the median was calculated for all of the peptides assigned to each protein. Frequency distribution histograms for log₂ protein ratios were obtained with GraphPad Prism 6.0. Log₂ ratios were then fitted to a normal distribution by least-squares regression. The mean and standard deviation derived from the Gaussian fit were used to calculate *P* values that were subsequently corrected for multiple comparisons by the Benjamini-Hochberg (BH) method (68). Proteins with a BH FDR *P* value of <0.05 were defined as significantly changed.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02038-14/-/DCSupplemental>.

Text S1, DOCX file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 3.1 MB.
Figure S2, TIF file, 9.5 MB.
Figure S3, TIF file, 3.2 MB.
Figure S4, TIF file, 4 MB.
Figure S5, TIF file, 3.9 MB.

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