Conversion of Commensal *Escherichia coli* K-12 to an Invasive Form via Expression of a Mutant Histone-Like Protein

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**ABSTRACT** The HU<sub>E38K, V42L</sub> mutant of the bacterial histone-like protein HU causes a major change in the transcription profile of the commensal organism *Escherichia coli* K-12 (Kar S, Edgar R, Adhya S, Proc. Natl. Acad. Sci. U. S. A. 102:16397–16402, 2005). Among the upregulated genes are several related to pathogenic interactions with mammalian cells, as evidenced by the expression of curli fibers, Ivy, and hemolysin E. When *E. coli* K-12/ HU<sub>E38K, V42L</sub> was added to Int-407 cells, there was host cell invasion, phagosomal disruption, and intracellular replication. The invasive trait was also retained in a murine ileal loop model and intestinal explant assays. In addition to invasion, the internalized bacteria caused a novel subversion of host cell apoptosis through modification and regulation of the BH3-only proteins Bim<sub>EL</sub> and Puma. Changes in the transcription profile were attributed to positive supercoiling of DNA leading to the altered availability of relevant promoters. Using the *E. coli* K-12/ HU<sub>E38K, V42L</sub> variant as a model, we propose that traditional commensal *E. coli* can adopt an invasive lifestyle through reprogramming its cellular transcription, without gross genetic changes.

**IMPORTANCE** *Escherichia coli* K-12 is well established as a benign laboratory strain and a human intestinal commensal. Recent evidences, however, indicate that the typical noninvasive nature of resident *E. coli* can be reversed under specific circumstances even in the absence of any major genomic flux. We previously engineered an *E. coli* strain with a mutant histone-like protein, HU, which exhibited significant changes in nucleoid organization and global transcription. Here we showed that the changes induced by the mutant HU have critical functional consequences: from a strict extracellular existence, the mutant *E. coli* adopts an almost obligate intracellular lifestyle. The internalized *E. coli* exhibits many of the prototypical characteristics of traditional intracellular bacteria, like phagosomal escape, intracellular replication, and subversion of host cell apoptosis. We suggest that *E. coli* K-12 can switch between widely divergent lifestyles in relation to mammalian host cells by reprogramming its cellular transcription program and without gross changes in its genomic content.
We previously isolated and characterized a gain-of-function mutant of the bacterial histone-like protein HU, HU<sub>E38K</sub>, V42L, which transformed the loosely organized nucleoid of E. coli K-12 into a condensed conformation (12, 13). The nucleoid remodeling was accompanied by major changes in the transcription program of the mutant bacterium (SK3842), resulting in dramatic changes in its morphology, physiology, and metabolism. Many of the changes in SK3842 (rod-to-coccoid morphological conversion, altered carbon utilization capabilities, and expression of cryptic virulence genes) were typical of some distinctive traits shown by commensal E. coli inside mammalian hosts, indicating a possible deviation from the typical E. coli K-12 behavior. Here we explore the mechanism by which HU<sub>E38K</sub>, V42L activates silent, pathogenicity-linked genes. We also characterize how the resulting physiology allows the mutant to interact “productively” with intestinal epithelial cells. The mutant serves as an in vitro locked-in model representing an alternate lifestyle that can be adopted by E. coli K-12 without gross genomic changes.

RESULTS

Mechanism of expression of pathogenicity-associated genes.

Previous biochemical and structural studies have demonstrated that DNA can wrap around wild-type HU multimers in a left-handed fashion, like histones in eukaryotes, generating negative superhelicity (13). We have also shown that a mutant HU<sub>α</sub>, HU<sub>E38K</sub>, V42L, multimer forms a right-handed spiral which generates positive superhelicity (14). Moreover, plasmid DNA extracted from wild-type E. coli is negatively supercoiled, but it is positively supercoiled when isolated from the mutant HU strain, SK3842 (13). It is commonly believed that the nature and amount of template DNA superhelicity dictate the functional efficiency of promoters (15). We surmised that the global change in the transcription profile in SK3842 is created by a change in the nature and extent of chromosomal superhelicity in the mutant. Our working model assumes that many pathogenicity-linked genes which are expressed only in SK3842 are transcribed because their promoters are available for transcription only when positively supercoiled. We tested this basic idea by studying the transcription of two representative genes having opposite transcription profiles in the wild type and in SK3842 in vitro: (i) the lac promoter, which is active in the wild type but not in the mutant, and (ii) the hlyE promoter (associated with pathogenicity), which behaves in the opposite manner. As expected, an in vitro transcription assay using negatively supercoiled DNA template showed that the lac promoter was active in the presence of cAMP and its receptor protein (CRP). The presence of HU<sub>α</sub> did not affect lac transcription in a significant way. However, addition of the purified HU<sub>E38K</sub>, V42L protein turned off not only lac transcription but also transcription from the control promoter, RNA1 (Fig. 1A). We used S-30 extract from a ΔhupAB strain for transcription of the hlyE promoter since standard in vitro transcription mix was not sufficient for its activity. From transcription from the hlyE promoter in vitro occurred very poorly with negatively supercoiled DNA template but became very active in the presence of HU<sub>E38K</sub>, V42L (Fig. 1A). These results clearly demonstrate that the differential effect of the HU<sub>E38K</sub>, V42L mutation on gene expression occurs directly at the promoters of the affected genes. We believe that HU<sub>E38K</sub>, V42L introduces segments of positive supercoiling into the plasmid DNA, allowing the hlyE promoter to become active. This was confirmed by using a hlyE promoter-containing plasmid, which was extracted and purified from SK3842 and shown to be positively supercoiled, for transcription. This template allowed a significant level of transcription from the hlyE promoter without the addition of any HU protein. Whereas the addition of wild-type HU<sub>α</sub> increased hlyE transcription marginally, addition of HU<sub>E38K</sub>, V42L increased hlyE transcription to a severalfold-higher level (Fig. 1B). Our results strongly suggest that expression of normally silent, pathogenicity-associated genes in E. coli requires positively supercoiled DNA.

Invasion of intestinal epithelial cells by SK3842. Since SK3842 demonstrated a dramatically altered gene expression profile, we undertook an investigation of its interaction with mammalian cells. Using transmission electron microscopy (TEM), we characterized the interaction of SK3842 with intestinal epithelial cells (Int-407). SK3842 efficiently invaded Int-407 cells, while the parental K-12 strain, MG1655, remained noninvasive (Fig. 2A). Within 1 h postinfection, there were numerous SK3842 cells enclosed in endocytotic vacuoles. TEM revealed that there were multiple membrane extensions from the surface of the epithelial cells (Fig. 2Bi) and many of the adhering bacteria were surrounded and engulfed by these protrusions (Fig. 2Bii), leading to the internal-
Internalization of SK3842 in prominent vacuoles (Fig. 2Biii). Using a gentamicin protection assay, we assessed the kinetics of cell invasion at different multiplicities of infection (MOIs). The mean invasive efficiency was 7.46% ± 0.492% with an MOI of 100 and 12.9% ± 1.003% with an MOI of 10 (Fig. 2C). As expected, MG1655 was noninvasive (P = 0.001). Remarkably, the invasion efficiency of SK3842 was higher than that of Salmonella enterica LT2, an enteric invasive pathogen used as a positive control (P = 0.009). Sequences of the bacterial entry event were further analyzed by fluorescence microscopy of Int-407 cells at different time points following the addition of 4′,6-diamidino-2-phenylindole (DAPI)-labeled SK3842 (Fig. 2D). Immediately following the addition of SK3842, large, prominent, circular dorsal ruffles were seen on the surface of the host cells (Fig. 2Dii), indicating a rapid reorganization of the cytoskeletal network in contrast to observations for control cells (Fig. 2Di). The large dorsal ruffles were transient in nature and gave way to smaller peripheral ruffles. SK3842 cells accumulated markedly in the ruffled region of the host cells (Fig. 2D).
host cell membrane (Fig. 2Diii). SK3842 cells were initially found to be closely apposed to the longer, wavy, membrane ruffles on the host cell surface (Fig. 2Div). Following this, smaller, actin-rich ruffles were seen to form loose, individual associations with the attached SK3842 cells (Fig. 2Dv). Ultimately, these encircling ruffles formed tight endocytic cups enclosing the bacterial cells, leading to their internalization (Fig. 2Dv). This shows that SK3842 induces rapid, reversible, and extensive rearrangement of the host cytoskeletal network prior to and during the process of its entry.  

**Invasion is a direct and reversible consequence of HUεE38K, V42L expression.** Previously we reported that the mutant phenotype of SK3842 can be rescued completely by overexpression of wild-type HUε (12). We used SK3842 carrying a wild-type HUε plasmid [SK382(pWTHU)] and induced wild-type HUε for various lengths of time to obtain cultures with different proportions of wild-type and mutant cells, ranging from 100% mutant (time zero) to 10 to 20% mutant (time 6 h postinduction). Following isopropyl-β-D-thiogalactopyranoside (IPTG) induction, the majority of the spherical SK3842(pWTHU) bacterial cells (time zero; Fig. 3Ai) assumed a larger ovoid shape (time 2 h; Fig. 3Aii), which then converted to short rods (time 4 h; Fig. 3Aiii) and finally to full-length rods (time 6 h; Fig. 3Aiv). Corresponding to the morphological conversion of spheres to rods, there was a time-dependent decline in the invasive ability of SK3842(pWTHU), lowering to 42.3% ± 9.5% of that of the control SK3842 culture after 6 h of IPTG induction (Fig. 3B). This result demonstrates that the invasive property of SK3842 is directly linked to the expression of HUεE38K, V42L and is reversible by overexpression of wild-type HUε.  

**Escape of SK3842 from endosomes/phagosomes is hlyE-dependent.** For successful survival inside host cells, the internalized bacteria need to escape quickly from the phagosomes or modify
the phagosomes to prevent the formation of phagolysosomes. TEM revealed that the majority of intracellular SK3842 bacteria were free in the host cytosol 6 h postinvasion (Fig. 3C). When we used SK3842(ΔhlyE), the majority of the internalized bacteria remained phagosome bound even after 24 h (Fig. 3D) and some appeared to be partially degraded within the vacuoles (Fig. 3D, arrow). We confirmed the phagosomal escape of SK3842 using a chloroquine resistance assay. Chloroquine accumulates in endosomes and kills endosome-bound bacteria. There was no significant difference in the numbers of CFU of SK3842 recovered from gentamicin-treated cells and from cells treated with both chloroquine and gentamicin (Fig. 3E). This shows that most of the endocytosed SK3842 bacteria escaped rapidly from the endosomes into the host cytoplasm. In contrast, the recovery of SK3842(ΔhlyE) was significantly lower in the presence of both chloroquine and gentamicin than in the presence of gentamicin alone, indicating that hemolysin-deficient SK3842 was defective in phagosomal escape. This shows that the expression of the normally cryptic hemolysin gene in SK3842 is one of the major factors responsible for the phagosomal escape of SK3842.

SK3842 is internalized by a microfilament- and microtubule-based phagocytic pathway. To determine the contributions of host cell signaling and cytoskeletal components to SK3842 internalization, we used a series of chemical inhibitors. Invasion assays were performed in the presence of cytochalasin D (an actin polymerization inhibitor), colchicine (a microtubule polymerization inhibitor), chlorpromazine (an inhibitor of clathrin-mediated endocytosis), nystatin (an inhibitor of endocytosis via lipid rafts), amiloride (a blocker of macropinocytosis), mevastatin (a pan-Rho GTPase inhibitor), genistein (a tyrosine kinase inhibitor), staurosporine (a Ser/Thr kinase inhibitor), and wortmannin (a phosphatidylinositol-3 kinase inhibitor) (Fig. 4A). Cytochalasin D treatment virtually abolished SK3842 invasion, while colchicine reduced SK3842 invasion by 48%, indicating that the internalization of SK3842 is critically dependent on intact host cell microfilaments and to a slightly lesser extent on intact microtubules. Mevastatin and staurosporine strongly inhibited SK3842 entry, indicating that Rho GTPases, which are critical regulators of actin dynamics, and Ser/Thr kinases are crucial for entry of SK3842. Other drugs were ineffective in blocking SK3842 invasion of host cells. Thus, intact actin and microtubules, Rho GTPases, and Ser/Thr protein kinase C are some of the host components involved in SK3842 entry, culminating in an efficient cytoskeletal protein-based phagocytic process.

Curli fibers promote SK3842 internalization. The parental strain MG1655 is a canonical noninvasive organism lacking an apparent invasive apparatus. Therefore, it was important to identify the components of SK3842 that allow host cell entry. Based on SK3842 microarray data (unpublished results), we focused on two possible loci that have been shown to be involved in host cell invasion and showed differential expression from the parental strain, encoding curli fibers (csg) and Ivy lysozyme inhibitor (ivy) (4, 16, 17). Ivy aids in colonization of specific niches, and curli fibers are involved in host cell adhesion and invasion. We created an ivy null mutant by insertion mutagenesis; however, we failed to create a csg deletion strain. Instead, we used a synthetic β-breake peptide (NH2-QFGGGNPP-COOH; see reference 1) conjugated to a hexapeptide repeat of a major curli fiber protein, CsgA, to disrupt the assembly of curli fibers. Using SK3842(Δivy) or SK3842 grown in the presence of the breaker peptide, we found that the ivy deletion did not impact SK3842 invasion (P = 0.109) but curli breaker peptides caused a dose-dependent inhibition (P values of 0.006 and 0.004 for the 0.2 μM and 0.4 μM inhibitor concentrations, respectively) (Fig. 4B). Inclusion of the breaker peptide with SK3842(Δivy), however, resulted in a further reduction in SK3842 invasion capacity (P = 0.002). SK3842(ΔhlyE) showed no defect in host cell invasion. This result shows that curli fibers, which are constitutively activated in SK3842, play a major role in host cell invasion and Ivy possibly has a synergistic effect on curli fiber-mediated host cell invasion.

SK3842 replicates efficiently inside the host cell. We estimated the survival and/or replication of strain SK3842 in Int-407 cells by counting gentamicin-resistant intracellular bacteria at various time points after a 1-h infection period (Fig. 4C). The number of intracellular bacteria increased gradually over 14 h, reaching a peak of 300% ± 75% of the bacteria recovered at 1 h. From 14 to 24 h, there was no appreciable change. This result showed that SK3842, despite being a nontraditional invasive strain, could survive for at least 24 h in the host cell cytoplasm, replicate intracellularly, and resist being killed by the cellular machinery of the invaded cells.

Expression of certain bacterial virulence-associated genes is turned off intracellularly. The Hemolysin E is a cytolytic protein that potentially can destroy host cells. Since SK3842 survived intracellularly and showed no signs of cytotoxicity (see below), we investigated the expression of the hlyE gene, along with that of hupA and rrsB, at various times postinvasion by reverse transcription-PCR (RT-PCR) on total RNA from internalized SK3842 (Fig. 4D). Following SK3842 internalization, there was a sharp decline in hlyE expression, from robust expression 1 h post-invasion to complete absence after 24 h; hupA expression also showed a progressive decrease over time, but the loss was much less extensive. rrsB mRNA levels showed a gradual increase from 1 h to 24 h, consistent with the increase in the number of intracellular SK3842 bacteria. This strong downregulation of a potent cytotoxic gene in SK3842 after the completion of phagosomal escape explains why the mammalian cells remained intact even as the bacterial burden increased.

SK3842 does not induce host cell death. Mammalian cell death by apoptosis or necrosis usually shares some common features, such as chromosomal DNA degradation and loss of membrane integrity. Chromosomal DNA extracted from Int-407 cells infected with different MOIs of SK3842 did not show any signs of fragmentation (Fig. 5A). SK3842-infected cells did not exhibit a significant difference in chromatin fragmentation (Fig. 5B) or lactate dehydrogenase (LDH) activity (Fig. 5C) from that of uninfected cells. SK3842-infected cells stained with Hoechst and propidium iodide (PI) did not show any nuclear condensation or fragmentation or any increase in membrane permeability indicative of apoptosis (Fig. 5D, bottom panel, versus Fig. 5D, middle panel) and showed no qualitative difference from control cells (Fig. 5D, top panel). Immunoblotting of SK3842-infected cell lysates confirmed that there was no cleavage of PARP into the 85-kDa active fragments or cytosolic release of cytochrome C for up to 24 h (Fig. 5E). Our results indicate that SK3842 infection does not induce any of the early events of apoptosis, such as cytochrome C release and PARP cleavage, or the final apoptotic indicators, such as nucleosomal DNA fragmentation and loss of membrane integrity.
Bim phosphorylation and degradation and Puma suppression are two major host cell responses to SK3842 invasion. The BclII family group of proteins, consisting of pro- and antiapoptotic members, contains the principal players determining the fate of a mammalian cell (18). We examined the cellular levels of antiapoptotic multidomain members (Bcl-2, Bcl-XL, and Mcl-1), proapoptotic multidomain proteins (Bax, Bak, and Bok), and proapoptotic BH3-only proteins (Bid, Bad, Puma, and Bim) at different time points following SK3842 invasion. There was no major change in the levels of most BclII group of proteins (Fig. 6A) except for two proapoptotic proteins, BimEL and Puma. There was a slight but reproducible increase in the antiapoptotic protein Mcl-1 in SK3842-infected cells. The changes in Puma and Bim were much more significant. Puma showed a sharp decline 14 h after infection and was barely detectable at 24 h. We observed an upward electrophoretic mobility shift in both BimEL and BimL (with BimEL being the predominant isoform), suggestive of increased phosphorylation. Since BimEL was the most prominent Bim isoform, we focused on it. The electrophoretic shift of BimEL was also accompanied by a gradual decline in the increase of phosphorylation. Incubation of SK3842-infected cell lysate with H9261-PPase resulted in the disappearance of the slower-migrating form of BimEL (Fig. 6B), confirming that the slower-migrating band represented the phosphorylated form of BimEL. Addition of the H9261-PPase inhibitor NaF prevented the appearance of the more rapidly migrating (dephosphorylated) form of BimEL. Since it has been reported that phosphorylation of BimEL leads to proteasome-mediated degradation of BimEL, we used the proteasome inhibitor MG132 to confirm the depletion of phosphorylated BimEL. Addition of MG132 led to accumulation of both
phosphorylated Bim and Puma in SK3842-infected cells (Fig. 6C), indicating that both phosphorylated Bim and Puma are proteasomally degraded in SK3842-infected cells. These results showed that SK3842 infection leads to major changes in at least 2 principal BH3-only proteins, Bim and Puma.

**SK3842 infection confers increased resistance to external apoptotic stimulus.** Since SK3842 entry did not result in host cell lethality, we wanted to check whether SK3842 infection could affect host cell susceptibility to external apoptotic signals. Treatment with staurosporine (a potent inducer of cell death) produced a much lower percentage of cells with apoptotic nuclei in SK3842-infected cells than in uninfected cells (Fig. 7A). SK3842-infected cells showed a significant reduction ($P = 0.03$) in staurosporine-induced apoptosis compared to uninfected cells by quantitative cell death enzyme-linked immunosorbent assay (ELISA) (Fig. 7B). There was also a marked reduction in proteolytic cleavage of PARP, caspase 3, and caspase 9, as well as the cytosolic level of cytochrome $c$, in SK3842-infected cells (Fig. 7C). Lower caspase 3 activity was confirmed by the reduced cleavage of Z-Asp-glu-val-asp-7-Amino-4-trifluoromethylcoumarin (Z-DEVD-AFC), a caspase substrate, by SK3842-infected cell lysate compared to that by uninfected cell lysate ($P = 0.01$) (Fig. 7D). These results, taken together, indicate that SK3842 infection inhibits the apoptotic response in host cells challenged with an external cytotoxic stimulus. Uptake of dead SK3842 or latex beads by host cells produced a significant increase ($P = 0.018$ and 0.004, respectively) in staurosporine-induced caspase 3 activity compared to that of live SK3842-infected cells (Fig. 7E). This shows that the inhibition of
apoptotic response in host cells requires endocytosis of live SK3842 bacteria. Roles of BimEL and Puma in mediating increased apoptosis resistance in SK3842-infected cells were tested. Staurosporine treatment of SK3842-infected cells induced dephosphorylation of BimEL and elevation of the Puma level over that of untreated SK3842-infected cells (Fig. 7F). However, the degree of BimEL dephosphorylation and the amount of Puma accumulation were still less than those in uninfected staurosporine-treated cells, accounting for the difference in the apoptotic response in these two cell cultures. Staurosporine treatment did not change the Mcl-1 level in uninfected Int-407 cells, implying that depletion of Mcl-1 does not play a major role in staurosporine-induced apoptosis in normal Int-407 cells. However, staurosporine caused an almost complete loss of the Mcl-1 protein in SK3842-infected cells, a finding which was unanticipated.

**SK3842 invades intestinal mucosal cells both in vivo and ex vivo but exhibits no increase in virulence.** Given the limitations of cultured cell lines as reliable models for bacterial invasion and to confirm our hypothesis with a more relevant model, we used the murine intestinal ligated loop model and live murine intestinal implants to analyze the interaction of SK3842 with the intestinal mucosa. In the *in vivo* murine ligated intestinal loop assay, bacterial cultures were injected into sutured-off intestinal segments in anesthetized mice. The percentage of the initial bacterial inoculum recovered from intestinal mucosal cells 1 h postinoculation was 7.8% ± 3.6% for SK3842, as opposed to only 0.001% for MG1655 (Table 1). These data are consistent with the *in vitro* data using Int-407 cells. The number of SK3842 bacteria from the intestinal cells increased to 13.4% ± 2.7% after 3 h of inoculation, indicating that the number of internalized bacteria from intestinal mucosal cells not only remained undiminished but also increased marginally. Using an *ex vivo* model of bacterial invasion, we used live intestinal segments from mice to test the invasive capacity of SK3842. SK3842 showed a comparable invasive ability of 17.6% ± 8.6% for the intestinal implant tissue. Thus, the invasive phenotype of SK3842 is not restricted to *in vitro*-cultured cells but is also evident under *in vivo* and *ex vivo* conditions.

We wanted to check whether the invasive ability of SK3842...
SK3842 confers protection against staurosporine-induced cell death in host cells. (A) Nuclear morphology. Uninfected and SK3842-infected cells were treated with staurosporine, stained with Hoechst stain, and visualized under a fluorescence microscope to score the number of apoptotic nuclei (open circles). (B) Quantitation of DNA fragmentation. Relative apoptosis rates were determined in uninfected and SK3842-infected cells in response to staurosporine by chromatin fragmentation assay. "*" Represents a P value of <0.05 in comparison to results for staurosporine-treated control cells. (C) Status of principal apoptosis marker proteins. Cleavage of PARP, caspase 3, and caspase 9 and release of mitochondrial cytochrome c into the cytosol were determined in uninfected and SK3842-infected cells treated with staurosporine. Whole-cell extracts or cytosolic fractions were prepared and used for immunoblotting with the indicated antibodies. (D) Caspase 3 activity. The effect of SK3842 infection on caspase 3 activity was measured with DEVD-AFC as a substrate using lysates from uninfected and SK3842-infected cells treated with staurosporine. "*" Denotes a P value of <0.05 compared to results for staurosporine-treated control cells. (E) Effect of phagocytosis of killed SK3842 and latex beads on cytoprotection. Effect of heat-killed SK3842 (MOI of 100) and latex bead (1.1 μM) phagocytosis on staurosporine-induced caspase activation in Int-407 cells. After 24 h, cells were treated with staurosporine and lysed for measurement of caspase 3 activity using DEVD-AFC as a substrate. "*" denotes a P value of <0.05, and "**" denotes a P value of <0.01. (F) Effect on BclII proteins upon staurosporine treatment. Cell lysates from staurosporine-treated uninfected and SK3842-infected cells were used for immunoblotting with Bim, Puma, and Mcl-1 antibodies.
TABLE 1 Invasion of intestinal epithelial cells in vivo and ex vivo by SK3842

| Strain | % of invasion<sup>a</sup> | Ex vivo<sup>c</sup> | LD<sub>50</sub> |
|--------|--------------------------|-----------------|-------------|
|        | In vivo<sup>b</sup> | 1 h | 3 h |          |
| SK3842 | 7.8 ± 3.6 | 13.4 ± 2.7 | 17.6 ± 8.6 | 1.6 × 10<sup>8</sup> |
| MG1655 | 0.001 | 0.002 | 0.004 | 2.1 × 10<sup>8</sup> |

<sup>a</sup> Percentage of bacteria in the inoculum that invaded intestinal mucosal cells.

<sup>b</sup> Invasion assay done as per the ligated intestinal loop assay described in Materials and Methods.

<sup>c</sup> Invasion assay done as per ex vivo intestinal invasion assay described in Materials and Methods.

resulted in greater virulence in a mouse model. The 50% lethal dose (LD<sub>50</sub>) of MG1655 for intraperitoneal (i.p.) inoculation was 2.1 × 10<sup>8</sup> and was consistent with earlier observations about the virulence index of E. coli K-12. The LD<sub>50</sub> of SK3842 was comparable at 1.6 × 10<sup>8</sup> (Table 1), showing that the transition from an extracellular to an invasive form does not lead to enhanced lethality of SK3842.

**DISCUSSION**

Invasion of host cells is often the first step adopted by many pathogenic bacteria to initiate their virulence process. However, except for the small subset of enteroinvasive E. coli strains, host cell invasion is not a preferred mode of interaction by even pathogenic E. coli (19). We previously showed that an HU mutant E. coli laboratory strain, SK3842, expresses many silent pathogenesis-linked genes (12). We also demonstrated that the HU<sup>E38K, V42L</sup> mutant generates positive superhelicity while wild-type HU creates negative supercoiling both in vitro and in vivo (13). The results reported here showing that the hlyE promoter retains its active status in plasmids isolated from SK3842 and remains responsive only to HU<sup>E38K, V42L</sup> and not to wild-type HU<sup>α</sup> confirm that HU<sup>E38K, V42L</sup> modulates the physical architecture of promoters of cryptic, pathogenicity-related genes. This is consistent with the model of positive supercoiling generated by HU<sup>E38K, V42L</sup>, most likely in a segmental fashion, which allows transcription of this pathogenesis-associated genes. These promoters are silent when DNA is negatively supercoiled, as in the wild-type cell. The change in physical and biochemical attributes of the E38K V42L mutation were previously shown to correlate with widespread morphological and physiological changes in SK3842. In this present study, we have demonstrated that the cellular changes resulting from HU<sup>E38K, V42E</sup> are responsible for a major shift in traditional E. coli K-12—epithelial cell interaction dynamics: from a strictly extracellular, noninvasive behavior, SK3842 adopts an efficient intracellular mode of existence. Entry and postinternalization events of SK3842 follow many of the archetypal maneuvers of such invasive pathogens as Shigella and Salmonella: (i) epithelial cell entry by actin-based invasion, (ii) phagosomal escape, and (iii) intracellular replication (20). Activation of at least two major, normally quiescent virulence determinants is responsible for this invasive phenotype: curli fibers for host cell entry and hemolysin E for phagosomal escape. Hemolysin E expression is strongly repressed following the phagosomal escape of SK3842, indicating an active bacterial strategy to limit cytotoxicity intracellularly. Abolition of the invasive phenotype with reversal of SK3842 to its wild-type form proved that SK3842 behavioral changes are directly linked to its mutant physiological characteristics and not induced by any unrelated genomic alterations. Functional divergence from the canonical host-microbe interaction, without any gross genotypic variation, has been reported in some cases (21, 22). But to our knowledge this is the first instance where mutation in an architectural protein has resulted in an extracellular commensal bacterial species adopting an invasive phenotype. Others have shown that ectopic overexpression of curli fibers triggers host cell entry (16) and overexpression of hemolysin E imparts a hemolytic phenotype to E. coli K-12 (23). This indicates that despite their functional inconsequence under normal conditions, these genes encode proteins with valid physiological effects and possibly fulfill certain indispensable cellular demands during atypical situations. The present study provides an empirical linkage between global nucleoid remodeling-linked physiological changes in E. coli K-12 and a functional shift in behavioral pattern with regard to host cells. There are known bacterial architectural proteins, like H-NS in the enterobacteriaceae and Lsr2 in Mycobacterium (24–26), which undergo structural and functional changes in response to host cues and act as master switches to manipulate bacterial virulence by changing the global gene expression profile. It is possible that HU, the most abundant and well-conserved bacterial architectural protein, can also assume different structural configurations in response to specific host environmental cues, and the HU<sup>E38K, V42L</sup> protein conformation and resultant cellular changes are representative of the host-induced changes in a select residential bacterial population.

Delay or prevention of epithelial cell apoptosis after bacterial infection allows invading bacteria time to adapt to the intracellular environment before invading deeper mucosal layers and to maintain their residential niche (27). Invasion by SK3842 does not trigger host cell death—a phenomenon which is perhaps imperative for SK3842’s survival and intracellular replication. SK3842 infection also confers significant apoptosis resistance against external apoptotic stimuli, such as staurosporine. Two critical cellular events underlying the SK3842-infected host cell survival are phosphorylation-driven Bim<sub>α</sub> degradation and downregulation of Puma. Of all the BH3-only proteins, only Bim and Puma (along with t-Bid) can engage with and antagonize every single prosurvival Bcl-2 molecule (28). Loss of Bim and Puma renders cells resistant to chemotherapy drugs, gamma irradiation, and cytokine deprivation (29). Bim and Puma have also been shown to be involved in apoptosis resistance in host cells harboring such obligate intracellular microbes as Chlamydia (30). The fact that SK3842 infection degrades two of the most potent, multispecific prodeath proteins probably accounts for the fact that infected host cells do not display any sign of cell death even after 24 h and up to 96 h (data not shown). This is not the case for traditional invasive bacteria, such as Salmonella and Shigella, which cause apoptosis of the host epithelial cells after a delay of 12 to 18 h (31). One of the major prosurvival proteins, Mcl-1, also shows a slight upregulation in SK3842-infected cells. Mcl-1 interacts with a high affinity to the BH3-only proteins Bim, Puma, and Bid but also selectively interacts with the “effector” proapoptotic protein Bak (32). The major prosurvival role of Mcl-1 is postulated to be linked to its sequestration of Bak on the outer mitochondrial membrane, preventing Bak oligomerization. In SK3842-infected cells, Bim and Puma are not available for interaction due to their phosphorylation status and/or disappearance. Therefore, a much
larger pool of Mcl-1 is available to sequester Bak from initiating apoptosis. The increased available Mcl-1 pool coupled with the slight increase in its cellular level is probably responsible for the improved apoptosis resistance of SK3842-infected cells. Upon staurosporine treatment of SK3842-infected cells, increased levels of unphosphorylated Bim and Puma disrupt the Mcl-1–Bak interaction and unleash Bak for initiation of the apoptosis cascade. But since the levels of Bim and Puma are still much lower than those in uninfected, staurosporine-treated cells, these cells showed lower indices of apoptosis. One paradoxical observation was the complete abrogation of the Mcl-1 level in staurosporine-treated SK3842-infected cells. We are not sure of the reason for this phenomenon, but it is possible that the proteasomal degradation system is more active in SK842-infected cells and that Mcl-1, unthetered from its binding partner Bak, is subjected to much more rapid degradation than in normal cells. Invasiveness is a trait almost uniquely associated with pathogens and host cell lethality. The fact that SK3842 engenders a well-defined survival program in the host cell and shows no increase in virulence in an animal model signifies that SK3842 not only is an unconventional derivative of extracellular E. coli but also follows a noncanonical intracellular relationship with the host cell.

Apart from enteroinvasive E. coli, there have been reports of even other E. coli pathovars exhibiting invasive behavior under in vitro conditions. Enteropathogenic and enterohemorrhagic strains have been reported to invade intestinal epithelial cells in vitro (33, 34). However, these strains do not show intracellular replication and phagosomal escape or invade human intestinal explants in vivo (35) and thus do not appear to be specifically adapted for intracellular survival. In contrast, SK3842 not only exhibits classical invasive properties in vitro but also retains its invasive phenotype under in vivo (ileal loop assay) and ex vivo (intestinal explants assay) conditions, indicating that its invasive trait is an integral cellular attribute and hence is of possible physiological significance. Not all invasive E. coli strains require dedicated invasive machinery. Adhesive-invasive E. coli (AIEC), associated with the genesis of Crohn’s disease, shows invasive properties similar to those of SK3842 (36). AIEC strains also lack specific virulence determinants, and the genes which have been implicated in AIEC pathogenesis are present in commensal K-12 strains, giving rise to the idea that only changes in the gene expression profile or minor sequence variations in commensal E. coli strains generate the virulence potential of AIEC strains (37). SK3842 is morphologically and physiologically completely distinct from AIEC strains, probably reflecting the fact that there are diverse modes and consequences of extracellular E. coli adopting an invasive lifestyle in the absence of any external genetic flux. Further work using SK3842 as an in vitro model to study the molecular events associated with a change in mammalian cell–E. coli K-12 interaction and a traceable in vivo model for analyzing commensal bacteria in invasive modes can provide valuable insights into host-microbe dynamics and potential routes of divergence from the canonical code.

**MATERIALS AND METHODS**

**Bacterial strains and cell line.** SK3842 and its growth conditions have been described previously (12). MG1655(hlyE) and MG1655(Δhly) were created by inserting a cat cassette within the hlyE gene and a kan cassette within the rxy gene by recombineering using the method of Yu et al. (38). Primers used for these experiments were as follows: for MG1655(ΔhlyE), GAGGCGAATGATTATGACTGAAATCGTTGCAGA TAAAACGGGTAGATGAATTTAAAAACGCAATCGTGCAGGAAAGA TCACTTCG (forward primer) and TCAAGACTCTGGATCTCCTAAAGA GTGTTCTTTTACCTGTTTCTTCTGATACCTATTCAACGGACCAT AGACATAAGCG (reverse primer); and for MG1655(Δhly), GGAGGTT AAAAACATGGCGAAGATAAGCTCGGAGGAAATGATT7TTAAGG CTTAGGACAGAAGCCAGACGG (forward primer) and TAATTTAA ATAATAAGCCATCCGGATGTTTTTACCGTCTGGGTCAACCGCTC AGAAGAATCCTGCAAGAACG (reverse primer). The mutant HUα gene was transduced into these two strains by P1 transduction to convert them to SK3842(hlyE) and SK3842(Δhly) (39). To make the in vitro transcription plasmid for hlyE, plasmid SK302(hlyE) was constructed by cloning a fragment of DNA stretching from −474 bp to +82 bp relative to the hlyE transcript start site at the EcoRI-HindIII sites of the pBR322 vector. SK761(λc) was constructed by cloning 390 bp of the lac promoter (−314 to +76) into the EcoRI and PstI sites of the transcriptional vector pSA508 (40). The Int-407 cell line (human embryonic intestine; ATCC, CCL 6) was obtained from the American Type Culture Collection. This cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mm glutamine, and 100 U/ml penicillin-streptomycin in a humidified incubator in an atmosphere of 10% CO2 at 37°C.

**Quantitative invasion assay.** Invasion efficiency was tested by a gentamicin protection assay as performed in established protocols. Int-407 cells were incubated with SK3842 at different MOIs for 1 h, followed by the addition of gentamicin, containing culture medium (100 μg/ml). At various times postinfection, cells were washed and lysed with lysozyme-buffered saline (PBS) containing 0.5% Triton X-100. Appropriate dilutions of the lysed solution were plated on agar plates, and the number of viable bacteria was determined. Unless mentioned otherwise, the MOI used was 1:100, infection time was for 1 h prior to incubation in gentamicin–DMEM, and incubation time was 24 h following 1 h of infection. The MOI used for MG1655 and LT2 was 1:100.

**Chloroquine resistance assay.** Phagosomal escape was evaluated with a chloroquine resistance assay (41). Briefly, infected Int-407 cells were incubated in the presence of gentamicin (100 μg/ml) with or without chloroquine (100 μg/ml) for an additional 3 h. The cells were subsequently lysed and plated to determine the number of intracellular bacteria surviving the drug treatment.

**Transcription assays.** For in vitro transcription of lacP, supercoiled DNA template (2 nM) was preincubated with or without proteins at 37°C in a 45-μl reaction mixture containing 20 mM Tris acetate (pH 7.8), 10 mM magnesium acetate, 100 mM potassium glutamate, 1 mM ATP, and 1 mM dithiothreitol (DTT). When needed, 0.1 mM CAMP, 50 nM CRP, and either 160 nM HUα or HUαΔESBK, V421 were added before the addition of 20 nM RNA polymerase. After incubation for 5 min, transcription was initiated by the addition of 5 μl of NTP mix containing 0.1 mM GTP, 0.1 mM CTP, 0.01 mM UTP, and 20 μCi of [α-32P]UTP (3,000 Ci/mmol) (ICN). Reactions were terminated after 10 min by addition of an equal volume of RNA loading buffer (80% [vol/vol] deionized formamide, 1× Tris-borate-EDTA [TBE], 0.025% bromophenol blue, 0.025% xylene cyanol). The reactions were analyzed on an 8% polyacrylamide-urea gel followed by autoradiography. When required, 160 nM HUα or HUαΔESBK, V421 was added before RNA polymerase. For hlyE transcription, S-30 extract from DM0100 was prepared according to the method of Zubay (42). Standard S-30 transcription reactions were carried out in a final volume of 50 μl, and reaction mixtures contained Tris acetate (20 mM, pH 8.0), magnesium acetate (10 mM), potassium glutamate (300 mM), ammonium acetate (30 mM), DTT (1 mM), 5 U RNasin, ATP (2 mM), CTP (0.5 mM), GTP (0.5 mM), and UTP (0.05 mM) added in a 2.5× master mix. Three hundred nanomolar HUα or HUαΔESBK, V421, and 360 μg of the S-30 protein in 15 μl were added to 20 μl of concentrated master mix. After preincubation on ice for 30 min, 30 nM plasmid DNA, isolated from either MG1655 or SK3842, was added to the reaction mix. After 10 min of incubation at 37°C, 20 μCi of [α-
tissue specimens were washed twice with ice-cold PBS to eliminate residual lethal dose (LD50), five groups of five mice/group were inoculated i.p. with contained at least one Peyer’s patch. A 100-ml volume of bacterial suspension (in normal saline) containing 10⁸ CFU was injected into the closed proximal to the ileocecal junction for each mouse. Each ligated loop was tied at the center, and 2 ml of the bacterial suspension was injected into it. The ligated intestines were removed and cut open. Tissue specimens were thoroughly washed with sterile PBS to eliminate mucus and debris and then soaked for 3 h at 37°C in a gentamicin solution (200 µg/ml). The tissue specimens were washed twice with ice-cold PBS to eliminate residual gentamicin and then homogenized in 500 µl of 0.5% Triton X-100, and bacterial titers were determined by plating 100 µl of homogenates on LB-spectinomycin agar plates.

**Ex vivo intestinal invasion assay.** Ex vivo invasion assays were conducted with MG1655 and SK3842 (with spectinomycin markers) cultures using a procedure described elsewhere (45). Groups of five BALB/c mice were deprived of food for 24 h, after which they were euthanized and the small intestines were removed and placed in DMEM-fetal bovine serum (FBS). One-inch sections were cut from the ileal region, and the lumen was thoroughly washed with 1× PBS. One end was tied off, and 100 µl of bacterial culture containing 10⁵ CFU was injected into the intestinal section. The other end was tied off, and the tissue was placed in DMEM-FBS and allowed to incubate for 1 h at 37°C. The ends were then cut, and the tissue was washed with gentamicin in DMEM-FBS (200 µg/ml). After washing, the intestine was opened longitudinally and allowed to incubate in gentamicin–DMEM-FBS for 1 h at 37°C. The intestinal tissue was then washed three times to remove all traces of gentamicin, homogenized in 2 ml of 0.5% Triton X-100, diluted, and plated on LB-spectinomycin plates.

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