An *Escherichia coli* Effector Protein Promotes Host Mutation via Depletion of DNA Mismatch Repair Proteins

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ABSTRACT **Enteropathogenic *Escherichia coli* (EPEC) is an attaching and effacing (A/E) human pathogen that causes diarrhea during acute infection, and it can also sustain asymptomatic colonization. A/E *E. coli* depletes host cell DNA mismatch repair (MMR) proteins in colonic cell lines and has been detected in colorectal cancer (CRC) patients. However, until now, a direct link between infection and host mutagenesis has not been fully demonstrated. Here we show that the EPEC-secreted effector protein EspF is critical for complete EPEC-induced depletion of MMR proteins. The mechanism of EspF activity on MMR protein was posttranscriptional and dependent on EspF mitochondrial targeting. EPEC infection also induced EspF-independent elevation of host reactive oxygen species levels. Moreover, EPEC infection significantly increased spontaneous mutation frequency in host cells, and this effect was dependent on mitochondrially targeted EspF. Taken together, these results support the hypothesis that A/E *E. coli* can promote colorectal carcinogenesis in humans.**

IMPORTANCE **There is mounting evidence linking the gut microbiota with the induction of colorectal tumorigenesis. We previously described the downregulation of host cell mismatch repair (MMR) protein levels upon enteropathogenic *Escherichia coli* (EPEC) infection and speculated that this depletion may lead to an ablated DNA repair system. In this work, we identify EspF, a translocated EPEC effector protein, as one of the factors required for this phenotype and show that this effector protein must be targeted to the mitochondria in order to exert its effect. Furthermore, we found that the impaired mismatch repair system resulting from EPEC infection led to the generation of spontaneous mutations within host DNA at a site of microsatellite instability, a trait typical of colorectal tumors. Thus, this work provides a novel means by which enteric bacteria may promote colorectal carcinogenesis.**

Received 26 February 2013 **Accepted** 24 May 2013 **Published** 18 June 2013

Citation Maddocks ODK, Scanlon KM, Donnenberg MS. 2013. An *Escherichia coli* effector protein promotes host mutation via depletion of DNA mismatch repair proteins. mBio 4(3):00152-13. doi:10.1128/mBio.00152-13.

Invited Editor Harry Mobley, University of Michigan Medical School **Editor** Michele Swanson, University of Michigan

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Enteropathogenic *Escherichia coli* (EPEC) attaches intimately to intestinal epithelial cells and uses a type 3 secretion system (T3SS) to translocate multiple effector proteins into the host cell cytoplasm. Effectors induce cytoskeletal remodeling at the cell surface, forming actin pedestals on which the bacteria sit (1). EPEC effectors also induce a plethora of other host cellular changes. Multiple studies analyzing colonic mucosa samples from colorectal cancer (CRC) patients have demonstrated an association between adherent *E. coli* strains and CRC (2–4). In addition, evidence of a link between attaching and effacing (A/E) bacteria and cancer has been provided by the murine pathogen *Citrobacter rodentium*, which promotes tumorigenesis in $Apc^{min/+}$ mice (5) and facilitates chemically induced tumorigenesis (6). Whether the association between A/E bacteria and cancer is causal remains to be fully determined. The ability to modulate host protein expression raises the possibility that EPEC promotes oncogenic pathways in colonic epithelial cells. In support of this hypothesis, we recently described the ability of EPEC to deplete mismatch repair (MMR) proteins MSH2 and MLH1 in cultured colonic cells in a T3SS-dependent manner (2).

The MMR system corrects DNA base pair mismatches and insertion/deletion loops (IDL) caused by replication errors or DNA damaging agents. In mammalian cells, this system is orchestrated by protein heterodimers, termed the MutS and MutL complexes. MutSα is composed of the MSH2 and MSH6 proteins and represents the most abundant mismatch binding factor, while MutSβ is composed of MSH2 and MSH3. The MutL complexes contain MLH1 heterodimerized with either PMS2 (MutLα), PMS3 (MutLβ), or MLH3 (MutLγ). It is hypothesized that the MutS complexes recognize DNA base pair mismatches or IDL and then recruit MutL. A MutS/MutL conformation switch then results in the activation of exonuclease DNA degradation and eventual repair by DNA polymerase δ (reviewed in reference 7). Hence, MMR is ablated by loss of either one of the critical proteins (MSH2 or MLH1); this is exemplified by the rapid accumulation of spontaneous somatic mutations throughout the genome, particularly in long repeated sequences of 1 to 4 nucleotides, termed microsatellites, in the absence of either protein (8, 9). In addition to causing microsatellite instability (MSI), MMR disruption enhances somatic mutation of tumor suppressor genes such as *Apc* and *Tp53*,

which are mutated in the majority of CRC (10). Heritable MMR gene mutations are the cause of hereditary nonpolyposis colorectal cancer (HNPCC) (Lynch syndrome) (11).

MMR gene silencing, either by somatic mutation or promoter hypermethylation, also contributes to sporadic CRC development (12, 13). However, a significant proportion of tumors display MSI without accompanying MMR gene mutation or hypermethylation (14), suggesting that alternative causes of MMR disruption exist. In the present study, we investigated the mechanism by which EPEC depletes MMR proteins and sought to establish whether this effect increases host mutagenesis.

RESULTS

EPEC depletes MMR proteins posttranscriptionally. We previously reported that infection with wild-type EPEC strain E2348/69 induced a marked depletion of MSH2 and MLH1 protein levels in HT29 and SW480 colon cells. Given that promoter hypermethylation causes transcriptional silencing of MLH1, we speculated that MMR protein downregulation resulted from decreased transcription (2). Here we confirm that wild-type EPEC causes a dramatic depletion of MSH2 and MLH1 in HT29 cells after 4 to 5 h (Fig. 1A and B). Analysis of MSH6 levels also confirmed decreased expression of this protein at 6 h postinfection (see Fig. S1 in the supplemental material). As MSH6 stability is dependent on MSH2 expression (15), depletion of MSH6 may be a direct effect of infection or secondary to MSH2 depletion. Because of the importance of MSH2 and MLH1 for overall MMR competence, we focused our analysis on these two proteins. Treatment with gentamicin to kill EPEC after 6 h of infection fully restored MSH2 and MLH1 expression (Fig. 1A and B). Despite the dramatic drop in protein levels, quantitative real-time PCR (qRT-PCR) revealed that transcription of *MSH2* and *MLH1* was in fact elevated in response to infection (Fig. 1C). Elevated transcription corresponded with increased amounts of MSH2 and MLH1 protein in the postinfection period (Fig. 1B). We therefore conclude that loss of MMR proteins does not occur due to lower transcription. In contrast, the host cell appears to respond to EPEC infection by a compensatory increase in MMR gene transcription.

Adherence does not correlate with MMR protein depletion. We showed previously that a mutant of EPEC E2348/69 lacking a crucial component of the T3SS (Δ*espB* mutant) was unable to fully deplete MSH2 and MLH1, suggesting that MMR protein depletion is dependent either on intimate attachment itself or on an effector protein(s) translocated during intimate attachment (2). To explore this hypothesis, we infected HT29 cells with a panel of E2348/69 T3SS effector and related mutants.

Western blots revealed that effector mutants of EPEC E2348/69 had different effects on MSH2 (Fig. 2A) and MLH1 protein levels (data not shown). However, microscopic examination revealed that the mutants also had different adhesion properties. This made it difficult to discern whether specific effector proteins were causing the changes in host MMR protein. For this reason, we quantified the adherent and nonadherent bacteria in cocultures after 5 h and quantified MSH2 and MLH1 expression in the same cultures to determine the relationship between the numbers of adherent bacteria and the levels of MSH2 and MLH1 protein. Interestingly, for MSH2, we observed a nonlinear relationship, indicating a role for effector proteins in modulating the levels of this protein (Fig. 2B). Wild-type EPEC had the highest adherence levels. The Δ*espF* and Δ*map* strains were the only mutants that adhered at levels comparable to, albeit lower than, the levels of the wild type, with adherence of the other mutants being markedly lower (Fig. 2B; see Fig. S2 in the supplemental material).

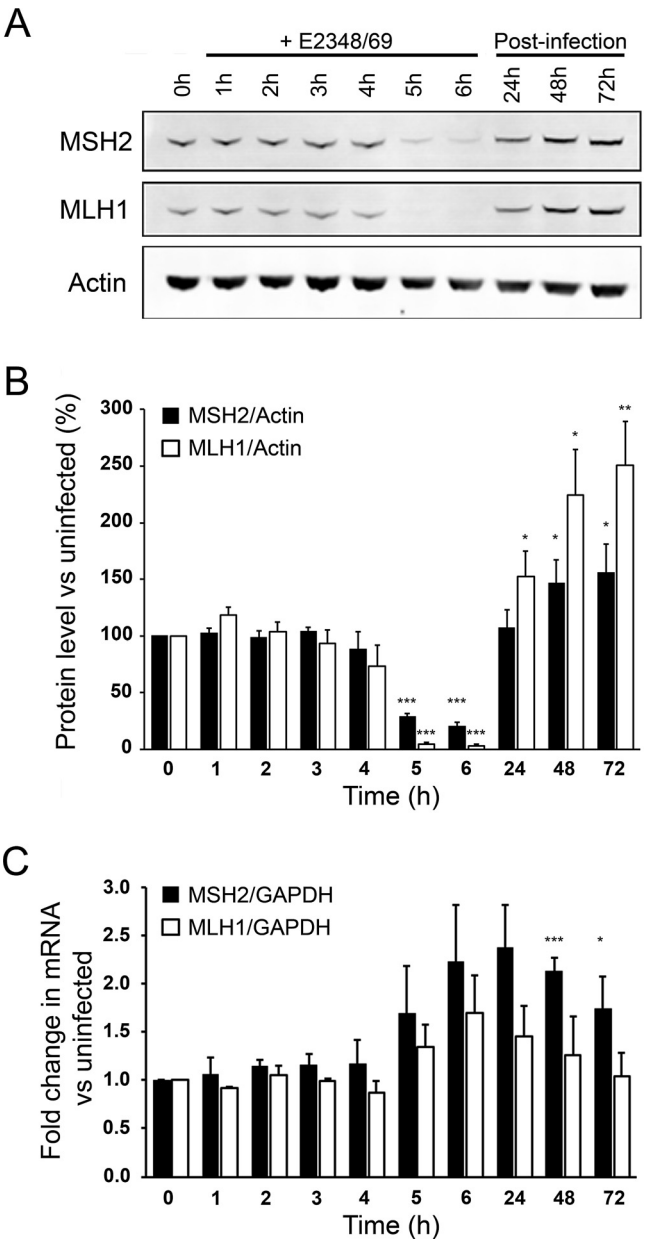

FIG 1 EPEC strain E2348/69 induces MSH2 and MLH1 protein depletion that is not due to transcriptional silencing. (A) Western blot showing MSH2 and MLH1 expression in HT29 cells cocultured with E2348/69 (wild-type EPEC) for up to 6 h. HT29 cells that were allowed to recover from infection were initially cocultured with strain E2348/69 for 6 h, then washed, and treated with antibiotics for up to 72 h postinfection. (B) MSH2 and MLH1 expression were quantitatively analyzed by secondary antibody infrared absorption analysis (Li-Cor Odyssey system) and corrected for loading using actin staining intensity. (C) Quantitative RT-PCR for *MSH2* and *MLH1* expression was performed on total mRNA extracts from the same cell populations used for protein analysis. Data are from three independent experiments, and values are means plus standard errors of the means (SEM) (error bars). The statistical significance of values compared to the value for control uninfected cells was assessed by Student's *t* test and indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

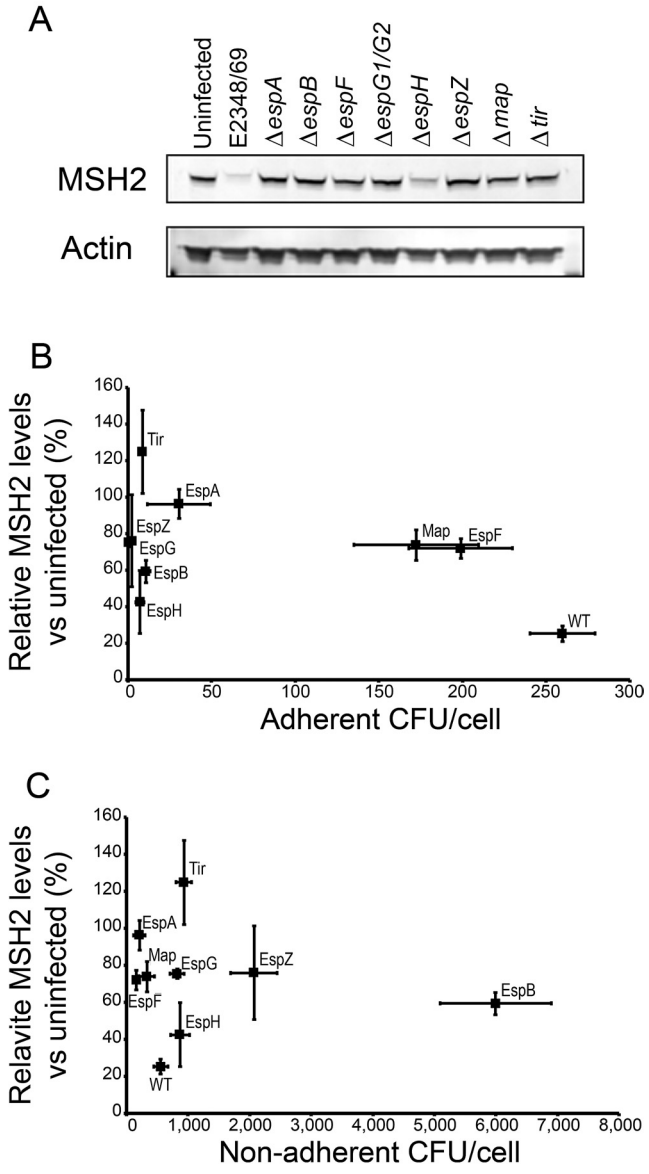

FIG 2 EPEC LEE effector mutants have differing abilities to adhere to HT29 cells and differing effects on MSH2 and MLH1 expression. (A) Western blot showing MSH2 expression in HT29 cells cocultured with EPEC strain E2348/69, effector protein mutants of E2348/69, and the Δ*espA* mutant (which is incapable of effector protein translocation) for 5 h. (B) HT29 cells were cocultured with EPEC E2348/69 (the wild-type [WT] strain), effector protein mutants of E2348/69, and the Δ*espA* mutant for 5 h, and bacterial attachment and MSH2 levels were quantified and plotted against one another. (C) Aliquots of the infection supernatant medium were diluted and spread onto agar plates to count nonadherent bacteria. The protein levels of MSH2 were plotted against the CFU of bacteria in the supernatant. Each experiment was repeated at least three times, and values are means (indicated by the markers) ± SEM (error bars).

It was clear that the number of adherent bacteria per cell did not predict the effect of infection on MMR protein expression. Despite having low adherence, the Δ*espH* mutant strain for example caused approximately 60% depletion of MSH2 levels (Fig. 2B), excluding this effector as a major contributor to MMR protein reduction. In contrast, the Δ*tir* mutant strain, which showed a level of adherence similar to that of the Δ*espH* mutant, actually caused increased MSH2 expression. Furthermore, the mutant strains with the highest levels of adherence, the Δ*espF* and Δ*map* mutants, induced only a modest (~25%) decrease in MSH2 expression (Fig. 2B). MLH1 was more sensitive to depletion than MSH2, with every strain inducing a large depletion in MLH1, ranging from 60 to 100% (Fig. S2).

Predictably, the mutant strains with low numbers of adherent bacteria (Δ*espB*, Δ*espZ*, Δ*espH*, Δ*espG*, and Δ*tir* mutants) displayed high levels of nonadherent bacteria (Fig. 2C). Consistent with this trend, the wild-type strain and the Δ*map* and Δ*espF* mutant strains had the lowest numbers of nonadherent bacteria. There was no obvious correlation between the levels of nonadherent bacteria and MMR protein expression. Interestingly, while the Δ*espF* strain had the highest adherence levels of all the mutants; the amounts of MSH2 were relatively preserved compared with wild-type infection (71% ± 5.4% versus 25% ± 4.1% [Fig. 2B]). This result suggested that the locus of enterocyte effacement (LEE) effector EspF might play a major role in the EPEC infection-induced depletion of MSH2.

Complete host MMR protein depletion requires translocation and mitochondrial targeting of EspF. To further establish the importance of EspF in depleting host MMR protein, we investigated the pathway involved. T3SS effectors influence host cell biology by targeting specific organelles and by regulating the level and activity of host cell proteins. EspF has an N-terminal sequence that targets the mitochondria and the nucleolus, and EspF also localizes to the host plasma membrane (16). Here, we examined the role of EspF mitochondrial targeting using a version of the *espF* gene that encodes a Leu-to-Glu amino acid substitution at position 16 ($espF_{L16E}$); the resulting protein is inhibited in its ability to localize to mitochondria (17).

HT29 and SW480 cells were cocultured with wild-type EPEC (E2348/69), Δ*espF* mutant, Δ*espF* mutant complemented with plasmid-encoded *espF* (Δ*espF pespF*), and Δ*espF* mutant complemented with plasmid-encoded $espF_{L16E}$ (Δ*espF* $pespF_{L16E}$). As expected, Δ*espF* induced only a modest decrease in MSH2 and MLH1 compared to EPEC E2348/69 (Fig. 3A to C). Complementation of the Δ*espF* mutant with wild-type EspF restored its ability to deplete MMR proteins, further validating the role of EspF in depleting MMR protein. However, complementation with $EspF_{L16E}$ that translocates into the host cytoplasm but is deficient in mitochondrial targeting did not restore the ability to deplete host MMR proteins ($P < 0.001$ compared with the Δ*espF pespF* strain for both MSH2 and MLH1 from infected HT29 cells). EspF is rapidly targeted to the mitochondria (within 30 min), causing a change in mitochondrial membrane potential ($\Delta\Psi_m$) as early as 1 h postinfection (18, 19). To determine whether MMR protein depletion was solely due to mitochondrial disruption by the effector, we assessed the levels of MMR proteins following treatment with an uncoupling compound that causes mitochondrial membrane depolarization, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Cells were incubated for 7 h with increasing concentrations of CCCP from 1 μM to 50 μM, concentrations known to cause total loss of $\Delta\Psi_m$ within 1 h (20). Alterations to the mitochondria caused by CCCP failed to decrease the levels of MSH2 or MLH1 at any of the concentrations tested (see Fig. S3 in the supplemental material), demonstrating that loss of $\Delta\Psi_m$ is unlikely to be a mechanism by which EspF influences MMR protein expression.

Mitochondria are a potential source of cellular reactive oxygen

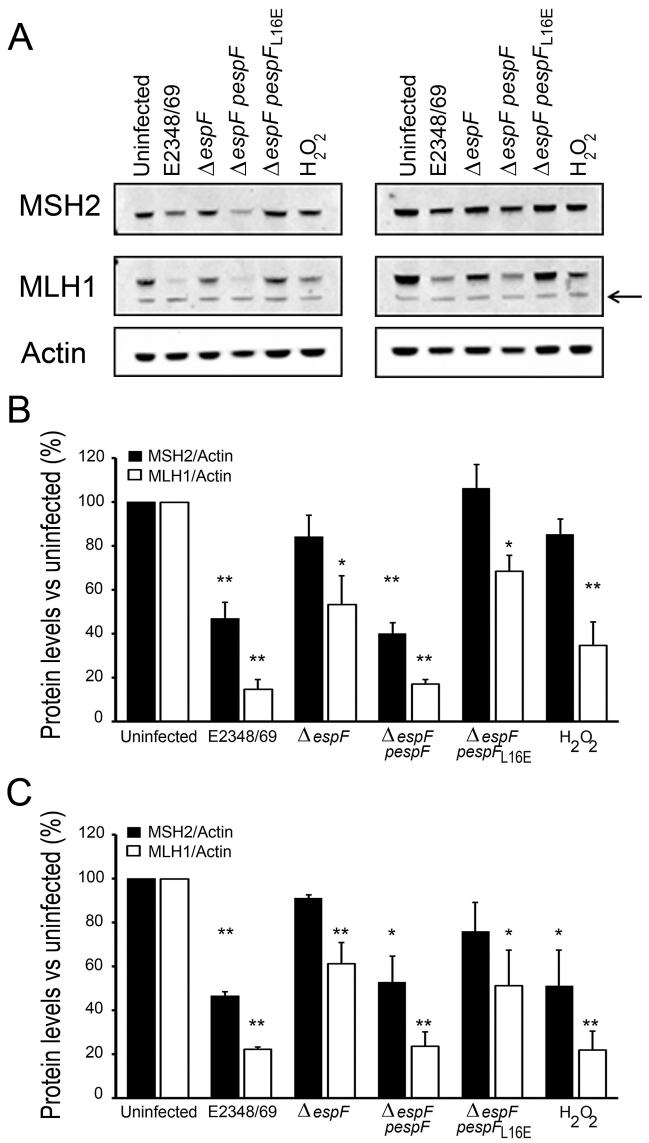

FIG 3 MMR depletion was dependent on mitochondrial targeting of EspF. (A) EspF-dependent depletion of MSH2 and MLH1 proteins in HT29 cells (left) and SW480 cells (right) was assessed by Western blotting following 5 h infection with EPEC strain E2348/69, Δ*espF* mutant, Δ*espF* mutant complemented with plasmid-encoded *espF* (Δ*espF pespF*), or Δ*espF* mutant complemented with plasmid-encoded $espF_{L16E}$ (Δ*espF* $pespF_{L16E}$). Cells were also treated with hydrogen peroxide (0.03% [vol/vol] H_2O_2) to induce oxidative stress. The black arrow indicates a cross-reactive band. (B and C) The expression of MSH2 and MLH1 in HT29 (B) and SW480 (C) cells were quantified using the Odyssey infrared imaging system and corrected for actin loading. Each experiment was repeated at least three times, and values are means (indicated by the markers) plus SEM (error bars). The statistical significance of values compared to the value for control uninfected cells was assessed by *t* test and indicated as follows: *, $P < 0.01$; **, $P < 0.001$.

species (ROS) (21). On the basis of evidence that oxidative stress depletes MMR protein levels in mammalian cells (22), we hypothesized that mitochondrial targeting of EspF might trigger elevated ROS levels, causing MMR protein depletion. To test whether the time scale and magnitude of MMR depletion caused by oxidative stress were comparable to EPEC infection, we treated cells with hydrogen peroxide (H_2O_2). H_2O_2 caused MSH2 and MLH1 protein depletion, which was similar in magnitude to that caused by E2348/69 (particularly in SW480 cells) over the same time course (Fig. 3A to C).

EPEC infection causes increased ROS levels in host cells independently of EspF. To determine whether EPEC infection induced a significant increase in ROS, cells were subjected to an infection time course and analyzed for ROS levels by flow cytometry (Fig. 4A). To assess the dependence of ROS elevation on EspF, the panel of EspF mutants was tested. Increased ROS levels were detected after 3 h of infection for all strains tested (Fig. 4B). EPEC infection induced a 4.1-fold increase in ROS at 5 h compared with uninfected cells. While the highest ROS levels were observed for the Δ*espF pespF* strain (4.9-fold increase after 5 h), this was followed by the Δ*espF* strain (4.7-fold increase after 5 h), indicating that ROS levels were not dependent on EspF. In addition, there was no significant difference between the Δ*espF pespF* and Δ*espF* $pespF_{L16E}$ strains, indicating that EspF mitochondrial targeting was not involved in ROS induction. Cells infected with the Δ*escN* mutant (which cannot assemble a functional T3SS) displayed the lowest levels of ROS (3.6-fold increase after 5 h), but this value was not significantly different from the value for wild-type EPEC (Fig. 4B). Together, these results indicate that EPEC infection induced a significant increase in host cell ROS. However, this ROS increase was independent of T3S, and therefore does not explain the role of EspF in depleting MMR proteins.

EPEC infection increases mutation frequency at a site of microsatellite instability. The failure to express functional DNA MMR proteins causes increased somatic mutation rate *in vivo* and strongly promotes colorectal carcinogenesis. Microsatellite DNA sequences are particularly susceptible to mutations normally corrected by the MMR system (9). MMR dysfunction therefore leads to accumulation of mutations in microsatellite sequences (and elsewhere in the genome), a phenomenon termed microsatellite instability (MSI). Interestingly, oxidative stress has been shown to enhance mutations in an MSI sequence within colonic cells deficient in MMR protein expression (23). With the knowledge that EPEC infection depletes MMR protein concomitant with elevated ROS levels, we sought to determine the functional consequences of these effects on host mutation frequency. To achieve this goal, we used a method that allows direct selection of cultured cells that have failed to repair spontaneously generated DNA mutations (24).

SW480 cells were stably transfected with a fusion gene containing a microsatellite sequence with 12 consecutive cytosine residues [$(C)_{12}$ TKBSD] or a control fusion gene without the microsatellite sequence (thymidine kinase-blasticidin deaminase [TKBSD]). The $(C)_{12}$ sequence is in frame with respect to the *TK* gene conferring ganciclovir sensitivity. Ganciclovir resistance results when a frameshift mutation occurs and is not repaired. The cells were infected for 6 h with EPEC strain E2348/69, Δ*espF* mutant, Δ*espF pespF* strain, or Δ*espF* $pespF_{L16E}$ mutant and subjected to ganciclovir selection. As expected, a low level of cell survival was observed in the TKBSD cells (Fig. 5) and in the uninfected $(C)_{12}$ TKBSD cells, indicating competent DNA repair in these cells (Fig. 5). In marked contrast, EPEC infection induced a significant increase in mutation at the $(C)_{12}$ site. Furthermore, this increase was EspF dependent, as determined by the significant decrease in mutation observed with the Δ*espF* strain versus the wild type. Restoration of EspF expression in the Δ*espF* strain restored the wild-type phenotype; however, complementation of Δ*espF* with $EspF_{L16E}$ did not,

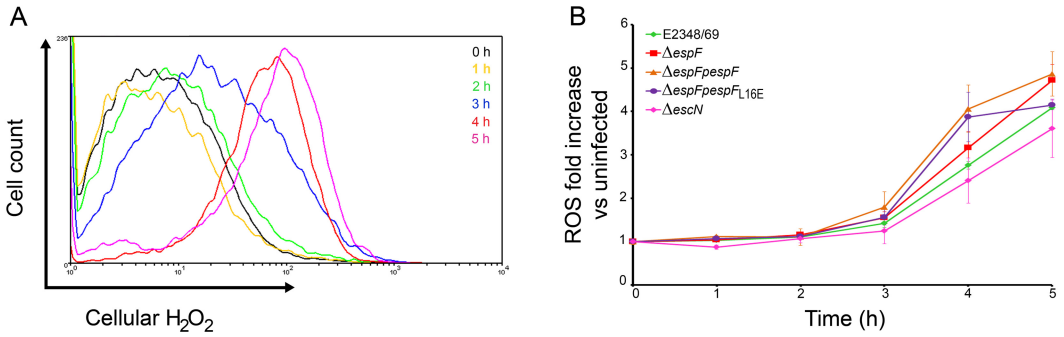

FIG 4 EPEC infection induces an increase in ROS. (A) HT29 cells were infected with EPEC strain E2348/69, and the levels of ROS were determined by flow cytometry over time. (B) Infection of HT29 cells with EPEC strain E2348/69, Δ*espF* mutant, Δ*espF pespF* strain, Δ*espF pespF*$_{L16E}$ strain, or Δ*escN* mutant all resulted in elevated ROS levels after 3 h of infection. Data are from three independent experiments. The data points in panel B are means ± SEM (error bars).

demonstrating that the effect was dependent on mitochondrial targeting.

DISCUSSION

In the present study, we confirmed the prior observation that EPEC depletes MMR protein in colonic epithelial cells. We established that this effect did not occur via transcriptional silencing, as host cells responded to EPEC infection with increased *MSH2* and *MLH1* transcription, suggesting a possible compensatory response. This result demonstrates that MMR protein depletion is an EPEC-directed effect that the host cells attempt to counteract. Given these results and the rapidity of the effect, it seems likely that infection modulates MMR protein expression via a posttranslation mechanism. We demonstrated that the EPEC-secreted effector EspF was required for complete EPEC-induced MMR protein depletion and that the mitochondrial targeting motif of EspF was essential for this effect.

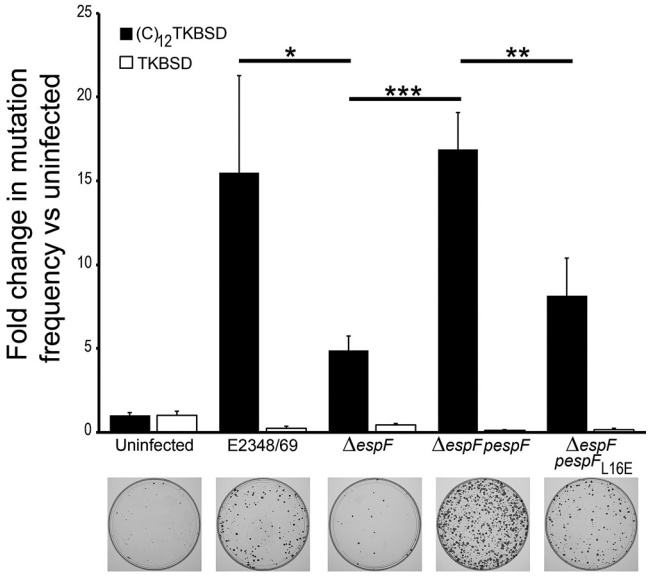

FIG 5 The EPEC effector EspF increases mutation frequency at a microsatellite DNA site. SW480 cells transfected with a reporter fusion gene containing a $(C)_{12}$ microsatellite sequence were subjected to infection with EPEC strain E2348/69, Δ*espF* mutant, Δ*espF pespF* strain, or Δ*espF pespF*$_{L16E}$ strain (black bars). Cells transfected with the fusion gene without the microsatellite sequence acted as a negative control (white bars). Examples of each of the $(C)_{12}$TKBSD cell plates are displayed below their respective bars in the graph. Data are from six experiments, and values are means plus SEM. The statistical significance of values was assessed by *t* test and indicated as follows: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

Oxidative stress is reported to cause MMR protein depletion (22), and we successfully validated this mechanism in our model using H_2O_2. We hypothesized that mitochondrial targeting of EspF might specifically enhance ROS generation in host cells and that elevated ROS levels would explain the enhanced ability of EspF-expressing strains to deplete MMR proteins. Contrary to our expectations, EspF-competent strains did not have an enhanced ability to promote host cell ROS generation. All of the strains we tested caused elevated ROS levels, suggesting that this effect does not require the T3SS. While ROS are not the mechanism by which EspF specifically depletes MMR protein, they may explain the residual MMR protein depletion caused by almost all strains tested. Intracellular ROS can cause oxidative modifications to both proteins and DNA (25, 26). The increased sensitivity to EPEC infection of MLH1 compared to MSH2 was a consistent trend in our experiments. Analysis of MLH1 reveals that it contains an oxidation susceptible motif (27) not present in MSH2. Furthermore, H_2O_2 caused a more marked depletion of MLH1 than MSH2. On the basis of this evidence, we speculate that infection-induced ROS contributes to MLH1 depletion by EPEC but that EspF is required for maximal depletion of MSH2, which is more resistant to ROS than MLH1 is.

While the exact mechanism by which EspF depletes MMR protein remains to be fully elucidated, the functional consequences of this effect were dramatic: we are the first to report that EPEC infection causes increased mutation frequency in host cells and that these mutations are substantially dependent on translocation and mitochondrial targeting of EspF. Interestingly, the only other EPEC effector mutant that displayed properties similar to those of the Δ*espF* strain was the Δ*map* mutant. Like the Δ*espF* mutant, the Δ*map* mutant showed high adherence and inability to induce complete MMR protein depletion. Map is also targeted to host mitochondria where it too causes a loss of $\Delta\Psi_m$ (28). However, we found no evidence that loss of $\Delta\Psi_m$ causes depletion of MMR proteins. In the future, it would be interesting to investigate the properties of a Δ*espF* Δ*map* double mutant or to ectopically express single EPEC effectors in host cells to help delineate the specific contributions of each effector.

Several other previously described activities of EPEC effectors

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Mutation(s)	Gene carried on plasmid	Reference
Bacterial strains[a]			
E2348/69 (EPEC 0127:H7)			37
CVD452	Δ*escN*		38
UMD872	Δ*espA*		39
UMD864	Δ*espB*		40
UMD874	Δ*espF*		41
Δ*espG* Δ*orf3* mutant	Δ*espG* (Δ*espG1* Δ*espG2*)		42
Δ*espH* mutant	Δ*espH*		43
MK34	Δ*espZ*		44
JAC719	Δ*tir*		45
SE882	Δ*map*		46
Plasmids			
pJN61		*espF*	19
pJN61L16E		$espF_{L16E}$	This study
pcDNA3-TKBSD			24
pcDNA3-$(C)_{12}$TKBSD			24

[a] All bacterial strains were derived from EPEC strain E2348/69.

also have the potential to promote cancer; in particular, infection can modulate host cell survival. EPEC effectors NleH1 and NleH2 can block apoptosis (29). EPEC-induced epidermal growth factor receptor (EGFR) activation also enhances the survival of infected cells; activated EGFR signaling is a feature of many cancers (30). Inhibition of host cell death also provides a rationale for EPEC effector-induced MMR disruption. Oxidative stress causes a variety of DNA lesions that are detected and repaired by the MMR pathway. However, when MMR proteins encounter high levels of DNA damage, they invoke a proapoptotic response via p53 to eliminate the damaged cell (31). By causing MMR protein depletion, EPEC could prevent the apoptotic response that may otherwise be triggered by the effects of EPEC-induced ROS on host DNA.

EPEC infection is a leading cause of infantile diarrhea in the developing world. In developed countries, EPEC is no longer considered a serious public health problem and is not routinely sought in clinical laboratories. Contemporary epidemiological data for EPEC in adults is therefore lacking. However, studies in Europe and Australia reveal that EPEC is carried by ~2.5 to 10% of healthy children (32, 33). Independent studies using tissue samples from adults with CRC demonstrate an association between mucosally adherent *E. coli* and CRC (2–4). Immunohistochemical analysis of human colonic mucosa cocultured with EPEC demonstrates that EPEC can enter colonic crypts and attach to epithelial cells in the proliferative compartment (2). The ability to asymptomatically colonize humans and to interact with proliferative cells in the crypt niche provides the biological context for EPEC to influence tumorigenesis. That EPEC can elevate ROS, suppress DNA repair, and increase mutation frequency in host cells provides a compelling mechanism by which these bacteria could promote CRC development.

CRC is a leading cause of cancer-related death worldwide. The majority of cases occur due to somatic mutations rather than inherited mutations, but the exact causes of the somatic mutations that initiate and drive sporadic CRC remain poorly defined. The importance of gene-environment interactions in causing CRC has been highlighted; however, conclusive research in this area is lacking (34). Mounting evidence suggests a causal link between bacterial infection and CRC and the potential contribution of pathogenic bacteria to CRC development has been noted (5, 6, 35).

Our data suggest that EPEC infection could promote somatic mutations (particularly in microsatellite DNA sequences) in colonic epithelium that does not feature genetic or epigenetic MMR silencing. Tumors developing in this way would be likely to show signs of MSI and critically, mutations in MSI sequences within oncogenes/tumor suppressor genes. Frameshift mutations in the tumor suppressor *APC* have been detected in tumors that do not display MMR gene mutation or hypermethylation (36). Many colorectal tumors display MSI that is not accompanied by MMR gene mutations or hypermethylation. The concept that alternative mechanisms account for MMR inactivation in a significant proportion of colorectal tumors is therefore well established (22, 23, 36).

Our findings provide a strong rationale for further investigation of A/E *E. coli*, particularly EPEC, in the context of colorectal carcinogenesis. Epidemiological studies in humans will be critical to establish a causal relationship. However, standard stool sample testing may not be an effective means to identify A/E bacteria due to their probable low levels in asymptomatic carriers. For this reason, we suggest analysis of tissue samples, e.g., biopsy samples or tissue samples removed during colorectal surgery.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Prior to infection, EPEC bacteria were cultured overnight in LB broth and appropriate antibiotics at 37°C. Overnight cultures were then diluted 1:50 in Dulbecco modified Eagle medium (DMEM) with nutrient mixture F-12 (DMEM/F12) with 5% fetal bovine serum (FBS) and maintained at 37°C for 1 h without agitation to activate the bacteria.

Tissue culture. Human colorectal cancer cell lines HT29 and SW480 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Cells were maintained at 37°C with 5% CO_2 in DMEM/F12 medium (Gibco Invitrogen, NY) supplemented with 10% fetal bovine serum (Benchmark FBS; Gemini Bio-Products, CA). As the FBS brand/lot can have a considerable effect on bacterial growth rate (2), a single lot number of FBS was used for each set of experiments to provide consistency. For *in vitro* infections, cells were seeded in 12-well or 24-well plates (Corning, NY) and grown to a confluent monolayer.

Isolation of stable transfectants. pcDNA3-TKBSD and pcDNA3-$(C)_{12}$TKBSD were prepared as previously described (24) and used to transfect SW480 cells using the Xfect transfection reagent (Clontech, Palo Alto, CA). Two days posttransfection, selection was initiated by the addi-

tion of 10 μg/ml blasticidin S (EMD Millipore Corp., Billerica, MA). After 2 to 3 weeks, stable clones were isolated and additionally propagated with 100 μg/ml blasticidin S. To ensure thymidine kinase expression, clones were also subjected to ganciclovir (Sigma-Aldrich, St. Louis, MO) treatment (30 μM), and sensitivity was confirmed prior to experimentation.

***In vitro* coculture.** Activated bacterial cultures were added to confluent cell monolayers at a multiplicity of infection (MOI) of 30:1. After variable periods, cells were grown with 200 μg/ml gentamicin, 200 IU/ml penicillin, and 200 μg/ml streptomycin to kill bacteria and allow the cells to recover after infection. Whole-cell protein extracts were prepared from cell pellets after lysis in 1% Triton X-100 (in phosphate-buffered saline [PBS]) or radioimmunoprecipitation assay (RIPA) buffer, supplemented with Complete protease inhibitor cocktail (Roche Diagnostics, Germany). For qRT-PCR analysis, RNA was extracted from cell pellets using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions.

Quantification of mutation frequency. To determine the frequency of mutation within the $(C)_{12}$ microsatellite instability site of the TKBSD fusion gene, transfected cells we re infected for 6 h in DMEM/F12 medium containing 5% FBS. After infection, the cells were cultured for 24 h in DMEM/F12 medium containing 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (complete DMEM [cDMEM]) and 200 μg/ml gentamicin (Invitrogen) and cultured an additional 24 h with cDMEM supplemented with 100 μg/ml gentamicin. Forty-eight hours postinfection, infected and control uninfected cells were cultured in cDMEM containing 10 μg/ml gentamicin with or without 30 μM ganciclovir. After 7 days, total cell survival was calculated for the cells that did not receive ganciclovir selection. The ganciclovir selection medium was replaced every second day, and selection was continued for 14 days. After selection, the cells in petri dishes were fixed with 4% formaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich), and the numbers of colonies were counted. The numbers of colonies were determined as a ratio of the cell survival. Cells transfected with TKBSD were subjected to the same treatment and acted as negative controls for the acquisition of mutations.

Western blotting. Whole-cell protein extracts were resolved through precast 4 to 12% gradient Novex-NuPAGE gels (Invitrogen) and transferred to Immobilon-FL membranes (Millipore, MA). Blots were probed with mouse anti-MSH2 (Ab-2) (clone FE11) (diluted 1:500) (EMD Biosciences), mouse anti-MLH1 (diluted 1:1,000) (BD Biosciences, CA), and rabbit anti-beta-actin AC-15 (diluted 1:5,000) (Sigma-Aldrich) primary antibodies. IRDye 680- and IRDye 800-conjugated secondary antibodies diluted 1:15,000 (Li-Cor Biosciences, NE) were then applied. Infrared signals were detected and quantified using the Odyssey imaging system (Li-Cor Biosciences).

qRT-PCR. Quantitative real-time PCR (qRT-PCR) reactions were performed using Stratagene brilliant II SYBR green QRT-PCR (quantitative reverse transcription-PCR) master mix with low ROX dye (Agilent Technologies, CA) in a Stratagene Mx3005P instrument (Agilent Technologies, CA). The following primers were used: MSH2 forward, 5′-CAGTATATTGGAGAATCGCA; MSH2 reverse, 5′-AGGGCATTTGTTTCACC; MLH1 forward, 5′-GATTACCCCTTCTGATTGACA; MLH1 reverse, 5′-ACTGAGGCTTTCAAAACA; GAPDH forward (GAPDH stands for glyceraldehyde-3-phosphate dehydrogenase), 5′-CGGAGTCAACGGATTGGTCGTAT; and GAPDH reverse, 5′-AGCCTTCTCCATGGTGGTGAAGAC.

Flow cytometry. After infection, the cells were resuspended in PBS containing the cell-permeant ROS indicator dye CM-H_2DCFDA [5- (and 6-)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester] (10 μM) (Invitrogen) and incubated with the probe for 30 min at 37°C in a 5% CO_2 incubator. Oxidation of the probe resulted in a deacetylated fluorescent product that was detected using a BD LSR II flow cytometer (BD Biosciences). The relative amounts of ROS were determined by geometric mean fluorescence intensity (MFI).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00152-13/-/DCSupplemental.

Figure S1, TIF file, 1.1 MB.
Figure S2, TIF file, 0.2 MB.
Figure S3, TIF file, 1.9 MB.

ACKNOWLEDGMENTS

We thank Greg Foster for providing pJN61L16E and Josef Jiricny for kindly donating pcDNA3-TKBDS and pcDNA3-$(C)_{12}$TKBSD. We thank David Harrison and Scott Bader for the original inspiration for this work.

This work was supported by awards R21 CA141038 and R01 AI32074 from the National Institutes of Health. O.D.K.M. was supported by a United Kingdom Fulbright-AstraZeneca Research Scholarship.

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