The Virulence Plasmid-Encoded *impCAB* Operon Enhances Survival and Induced Mutagenesis in *Shigella flexneri* after Exposure to UV Radiation

LAURA J. RUNYEN-JANECKY, MEI HONG,† AND SHELLEY M. PAYNE*

*Department of Microbiology and Institute for Cellular and Molecular Biology,
University of Texas at Austin, Austin, Texas 78712-1095*

Upon exposure to UV radiation, *Shigella flexneri* SA100 displayed survival and mutation frequencies comparable to those of *Escherichia coli* AB1157, which contains a functional UmuDC error-prone DNA repair system. Survival of SA100 after UV irradiation was associated with the presence of the 220-kb virulence plasmid, pVP. This plasmid encodes homologues of ImpA and ImpB, which comprise an error-prone DNA repair system encoded on plasmid TP110 that was initially identified in *Salmonella typhimurium*, and ImpC, encoded upstream of ImpA and ImpB. Although the *impB* gene was present in representatives of all four species of *Shigella*, not all isolates tested contained the gene. *Shigella* isolates that lacked *impB* were more sensitive to UV radiation than isolates that contained *impB*. The nucleotide sequence of a 2.4-kb DNA fragment containing the *imp* operon from *S. flexneri* SA100 pVP was 96% identical to the *imp* operon from the plasmid TP110. An SA100 derivative with a mutation in the *impB* gene had reduced survival following UV irradiation and less UV-induced mutagenesis relative to the parental strain. We also found that *S. flexneri* contained a chromosomally encoded *umuDC* operon; however, the *umuDC* promoter was not induced by exposure to UV radiation. This suggests that the *imp* operon but not the *umuDC* operon contributes to survival and induced mutagenesis in *S. flexneri* following exposure to UV radiation.

Shigella flexneri, a facultative intracellular bacterium, causes bacterial dysentery in humans (13, 28, 39). This pathogen faces numerous potentially stressful environments during its life cycle. These environments are encountered as *Shigella* is exposed to the external environment, as it transits through the human gastrointestinal tract, and during its growth within colonic epithelial cells. However, little is known about the response of *Shigella* to these potentially stressful environments.

One stress response that has been studied in detail in bacteria is the SOS response to DNA damage. Upon exposure to UV radiation or chemicals that damage DNA, *Escherichia coli* and other bacteria express a specific set of genes that are normally repressed by the LexA repressor binding to an SOS box in each promoter (11, 54). The initial signal of DNA damage, which is thought to be single-stranded DNA, activates RecA to RecA* (11). RecA* mediates the self-cleavage of the LexA repressor, leading to derepression of more than 20 genes, including *recA*, *lexA*, excision DNA repair genes, and the error-prone DNA repair genes *umuD* and *umuC* (11). RecA* also mediates the self-cleavage of UmuD into UmuD′, which is the form that mediates error-prone DNA repair (5, 32, 46).

The *E. coli umuDC* promoter, which contains a highly conserved SOS box, has a strong affinity for LexA. Thus, UV induction of the *umuDC* operon occurs only after most of the LexA repressor has been cleaved. Induction of *umuDC* expression results in an increased frequency of mutagenesis (10, 47). Current genetic and biochemical evidence supports the model in which a mutasome, composed of UmuD′-UmuC, RecA*, and DNA polymerase III, allows the bypassing of potentially

lethal lesions in the DNA during DNA replication (37). The interaction of the UmuD′-UmuC complex with DNA polymerase III is thought to result in relaxed fidelity, leading to the generation of mutations. Thus, the process of UV-induced mutagenesis is also known as error-prone DNA repair. Strains containing *umuDC* mutations are more sensitive to UV exposure than their isogenic parents, suggesting that error-prone DNA repair is important for survival following UV irradiation (3, 19).

The *umuD* and *umuC* genes constitute an operon located on the *E. coli* and *Salmonella typhimurium* chromosomes (20, 36, 49, 52). *umuDC* homologues also have been identified on several naturally occurring plasmids, and their gene products mediate error-prone DNA repair (56). These homologues include *impAB*, located on the large conjugative plasmid TP110, which was initially identified in *Salmonella typhimurium* (9, 26). Other plasmids from several different incompatibility groups also contain sequences with homology to the *imp* operon (27). The TP110 *imp* operon complements mutations in the *E. coli umuDC* operon, demonstrating that these operons are functionally similar (43).

Preliminary analysis of the response of *Shigella* growing in the intracellular environment led to the identification of an *S. flexneri* DNA fragment homologous to the 3′ end of the *impB* gene encoded on the plasmid TP110 (15). In this article, we describe the identification and characterization of the virulence plasmid-encoded *impCAB* genes and the chromosomally encoded *umuDC* genes in *S. flexneri*. Our data suggest that the *imp* operon but not the *umuDC* operon contributes to survival and induced mutagenesis in *S. flexneri* following UV irradiation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. All strains were maintained at −80°C

* Corresponding author. Mailing address: Department of Microbiology and Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX 78712-1095. Phone: (512) 471-9258. Fax: (512) 471-7088. E-mail: payne@mail.utexas.edu.
† Present address: Chiron Corporation, Emeryville, CA 94608-2916.

TABLE 1. Strains and plasmids

Strain or plasmid	Characteristics	Reference or source[a]
E. coli strains		
DH5α	Standard lab cloning strain	38
SM10λ*pir*	*pir*R6K	51
AB1157		16
1107-81	Enteroinvasive	J. H. Crosa, Oregon University of the Health Sciences
550-3076	Enteroinvasive	J. H. Crosa, Oregon University of the Health Sciences
Shigella strains		
SA100	*S. flexneri* wild-type serotype 2a	35
SA102	SA100 derivative lacking the virulence plasmid pVP	T. Daskaleros
SM100	Streptomycin-resistant SA100 derivative	S. Seliger
2457	*S. flexneri* serotype 2a	S. Formal
8-2031	*S. flexneri* serotype 2b	TDH
M90T	*S. flexneri* serotype 5	P. Sansonetti
229272	*S. flexneri* serotype 5	TDH
SD125	*S. dysenteriae* serotype 1	B. A. D. Stocker
O-4576	*S. dysenteriae* serotype 1	TDH
O-1392	*S. boydii* serotype 5	TDH
224860	*S. boydii* serotype 7	TDH
PB66	*S. sonnei*	D. Winsor
1-1245	*S. sonnei*	TDH
Plasmids		
pVP	220-kb virulence plasmid from SA100	35
pWKS30	Low-copy-number cloning vector	55
pIMP	pWKS30 containing the *imp* operon from SA100 on a 2.4-kb PCR product	This study
pGTXN3	Transcriptional fusion vector containing the promoterless mutant 3 *gfp* gene from Cormack et al.	This study
pLR13	pGTXN3 carrying a 479-bp DNA fragment containing the SA100 *umuDC* promoter	This study
pLR20	pGTXN3 carrying a 479-bp DNA fragment containing the AB1157 *umuDC* promoter	This study

[a] TDH, Texas Department of Health.

in tryptic soy broth (TSB) plus 20% glycerol. *E. coli* strains were routinely grown in Luria broth (L broth) or on Luria agar (L agar) plus antibiotics at 37°C, and *Shigella* spp. strains were grown in L broth or on TSB agar plus 0.1% Congo red dye and antibiotics at 37°C. Strains containing *gfp* fusions were grown in low-salt L broth, which contains 5 g of NaCl/liter instead of 10 g/liter. Antibiotics were used at the following concentrations: 250 μg of carbenicillin/ml, 30 μg of chloramphenicol/ml, and 200 μg of streptomycin/ml.

Recombinant DNA methods. Plasmids smaller than 20 kb were isolated with the QIAprep Spin Miniprep kit (Qiagen, Santa Clarita, Calif.). The method of Kado and Liu (18) was used to isolate the 220-kb virulence plasmid from *S. flexneri*. Isolation of DNA fragments from agarose gels was performed with the QIAquick Gel Extraction kit (Qiagen) or the GeneClean kit (Bio 101, Vista, Calif.).

For matings, overnight cultures of the donor and recipient strains were washed once in phosphate-buffered saline (PBS) and resuspended at a concentration of approximately 10^{10} bacteria per ml. Twenty microliters of each culture was mixed, and the mixture was spotted onto Luria agar. After a 6-h incubation at 37°C, the cells from the plate were resuspended in 1 ml of PBS and plated on L agar or TSB agar plus 0.1% Congo red dye containing the appropriate antibiotics.

UV irradiation survival assays. Bacteria were grown at 37°C with aeration in L broth containing the appropriate antibiotics to an optical density of 650 nm (OD_{650}) of 0.5 to 0.9, pelleted by centrifugation, and resuspended in PBS at a concentration of 10^8 bacteria per ml. Aliquots of the concentrated bacteria (0.4 ml) in 16-mm petri dishes were irradiated with UV light at doses indicated in the figures (0 to 40 J/m^2), and the number of cells that survived UV irradiation was quantitated by plate counts on L agar. Statistical analyses of the data were performed using the ANOVA statistics package in Microsoft Excel 97 (Microsoft Corporation, Redmond, Wash.).

Mutagenesis assays. Bacteria grown to an OD_{650} of 0.5 to 0.9 were pelleted, resuspended in PBS to a concentration of 10^9 bacteria per ml, and irradiated with UV light at a fluence of 10 J/m^2. The number of cells that survived UV irradiation was quantitated by plate counts on L agar, and the number of cells that acquired a UV-induced mutation conferring rifampin resistance was determined essentially as described by Sedgwick and Goodwin (41). Statistical analyses of the data were performed using the ANOVA statistics package in Microsoft Excel 97 (Microsoft Corporation).

PCR procedures. All PCRs were carried out using *Pfu* polymerase (Stratagene Cloning Systems, La Jolla, Calif.) in the reaction buffer supplied by the manufacturer supplemented with 250 μM each dNTP and 1 μM primers. Bacterial

cultures (1 to 2 μl) grown overnight, washed once in PBS, and diluted 10-fold were used as the template per 100-μl reaction. The reactions were initially incubated at 95°C for 5 min, and the PCR was then done according to the specific conditions for each reaction. Thirty cycles were carried out for all reactions. Each cycle consisted of a 1-min denaturation step at 95°C, a 1-min annealing step at 3 to 5°C below the melting temperature for the primers, and an extension step of 2 min per kb to be amplified at 72°C. The primers were impB1 (5′CACTCGA TGAACTGAACC3′), impB2 (5′TTTCCCGTTTCATTTGCC3′), impB3 (5′C ACAGCAGGCATACAGCC3′), upstream primer (5′AATTCTCCTCTCACAT GCGG3′), downstream primer (5′GGTGCTTTGCAATCTGCTG3′), umuDCP1 (5′GATCTAATGCTCCATCTGCG3′), umuDCP2 (5′CGCGGGAGATCCGCA GGC3′), and umuDC3 (5′GCCGCTATATTTATTTGACCC3′). For inverse PCR, plasmid DNA from SA100 was digested with *Hin*cII and *Eco*RV and ligated with T4 DNA ligase. The inverse PCR was carried out by using 2 μl of the ligation reaction and primers impB2 (5′TTTCCCGTTTCATTTGCC3′) and impB5 (5′TGCTCTCGCCTTCGTATA3′) at an annealing temperature of 50°C.

Sequence analysis of the *imp* operon. For determining the nucleotide sequence of the *imp* operon from *S. flexneri* SA100 pVP, a 2.4-kb PCR product containing the operon and generated as described above was used as the template. For determining the nucleotide sequence of the 3′ end of the *imp* operon from *S. flexneri* 8-2031, a subclone of a cosmid that contained *impB* from 8-2031 (7) was used as the template for sequencing. Nucleotide sequences were determined by the Molecular Biology Sequencing Facility at the University of Texas at Austin. The DNA that was generated in the sequencing reactions was labeled with the dRhodamine Dideoxy-terminator Cycle Sequencing kit (Perkin-Elmer Co., Applied Biosystems Division, Foster City, Calif.) and analyzed with an ABI Prism 377 DNA sequencer (Perkin-Elmer Co., Applied Biosystems Division).

Construction of an *impB* mutation in *S. flexneri* by allelic exchange. The allelic exchange vector pHM5 was constructed as follows. pGP704, which replicates in λ*pir* lysogens but not in SM100 (29), was linearized with *Sma*I and ligated to a 1.9-kb *Eco*RV fragment containing the *sacB* gene, which encodes sucrose sensitivity, from pMTL-sac#4 (57). A 2-kb PCR product containing part of the *imp* operon and amplified with the primers impB3 and impB2 (see the description of the PCR procedures above) was cloned into the vector pWKS30 (55) linearized with *Eco*RV to generate pLR25. A 1.6-kb fragment containing a chloramphenicol resistance gene (*cam*) was isolated from pMA9, a derivative of pNK2884 (21), by digestion with *Hin*dIII followed by treatment with the Klenow fragment of DNA polymerase I. This *cam* cassette was inserted into the *Eco*RV site in the *impB* gene on pLR25 to generate pLR25::Cm. The *imp* operon with the *cam* resistance cassette was excised from pLR25::Cm as a *Xho*I-*Sma*I fragment and

ligated into pHM5 digested with *Sal*I and *Eco*RV to generate pLR27. pLR27 was mated from *E. coli* SM10λ*pir* to *S. flexneri* SM100, and single crossovers in the *impB* gene were selected by plating on TSB agar plus 0.1% Congo red containing chloramphenicol and streptomycin. Double crossover recombinants were then selected on L agar containing 5% sucrose, chloramphenicol, and streptomycin and screened for carbenicillin sensitivity. PCR analysis, using primers that flank the *cam* insertion in *impB*, confirmed that the wild-type *impB* allele was replaced with the *cam*-disrupted *impB* allele in the chloramphenicol-resistant, sucrose-resistant, carbenicillin-sensitive recombinants. One recombinant, designated SM162, was used for further study.

Tissue culture cell invasion and plaque assays. Henle cell monolayers were used in all experiments and were routinely maintained in Earle's minimal essential medium plus 2 mM glutamine plus 10% fetal calf serum (Life Technologies, Grand Island, N.Y.) in a 5% CO_2 atmosphere at 37°C. Plaque assays were done as described previously (34) with the following modifications. Confluent Henle cell monolayers in 35-mm plates were infected with 10^3 and 10^4 bacteria. After a 60-min incubation, the cells were washed four times with PBS and overlaid with fresh medium containing 0.45% (wt/vol) glucose, 0.5% agarose, and 20 μg of gentamicin/ml. Plaques were scored after 72 h.

Detection of GFP expression controlled by the *umuDC* promoter. The promoterless *gfp* vector pGTXN3 was constructed as follows. The promoterless *cat* vector pKK232-8 (Pharmacia, Piscataway, N.J.) was digested with *Nco*I and *Hin*dIII to generate a 4.5-kb DNA fragment which is missing the 5′ 576 bp of the *cat* gene. The 4.5-kb fragment was treated with the Klenow fragment of DNA polymerase I, isolated by electrophoresis, and religated with T4 DNA ligase to generate pKK232-8Δ. This plasmid was digested with *Sal*I, treated with the Klenow fragment of DNA polymerase I, and digested with *Bam*HI. The promoterless *gfp* gene was isolated from pGFP3 (6) by digestion with *Hin*dIII and treated with the Klenow fragment of DNA polymerase I, followed by digestion with *Bam*HI. The resulting 0.75-kb fragment was purified by electrophoresis and ligated to pKK232-8Δ isolated as described above. The resulting plasmid was designated pGTXN3.

A 479-bp PCR product containing the *umuDC* promoter was amplified from *E. coli* AB1157 by using PCR primers umuDCP1 and umuDCP2. This DNA fragment contained 420 bp 5′ of the transcriptional start site and 28 bp 3′ of the translational start codon and was cloned into the vector pGTXN3 cut with *Sma*I to generate pLR20. The same primers were used to amplify a 479-bp PCR product containing the *umuDC* promoter from SA100, which was cloned into the vector pKK232-8 cut with *Sma*I to generate pLR1R. The SA100 *umuDC* promoter was isolated from pLR1R by digestion with *Eco*RI and *Bam*HI and cloned into pBSK⁻ cut with *Eco*RI and *Bam*HI to generate pLR7. The SA100 *umuDC* promoter was isolated from pLR7 by digestion with *Eco*RV and *Bam*HI and cloned into pGTXN3 digested with *Xba*I, treated with the Klenow fragment of DNA polymerase, and digested with *Bam*HI to generate pLR13.

umuDC promoter activity was assessed by quantitating the amount of green fluorescent reporter protein with a Molecular Dynamics Fluorimager. Bacteria were grown in low-salt L broth containing the appropriate antibiotics to an OD_{650} of approximately 1.0, pelleted by centrifugation, and resuspended to a concentration of 10^9 bacteria per ml in PBS. One-half of each culture was exposed to 50 J/m² of UV radiation. Each sample was then pelleted by centrifugation and resuspended to a concentration of 10^9 bacteria per ml in L broth. After a 2-h incubation at 37°C, the samples were concentrated to 10^{11} bacteria per ml by centrifugation. The levels of green fluorescent protein (GFP) were measured at 530 ± 15 nm. The background fluorescence of the cells containing the vector was subtracted from each sample. Specific activity of GFP for each sample was expressed in fluorescence units, which were calculated by dividing the relative fluorescence by the OD_{650} and volume of the sample.

Nucleotide sequence accession number. The sequence data of a 2.4-kb DNA fragment containing the *imp* operon have been submitted to the GenBank database under the accession no. AF079316.

RESULTS

Sensitivity of *S. flexneri* isolates to UV radiation. The ability of *S. flexneri* isolates to survive UV irradiation was assessed by measuring the fraction of cells that survived exposure to increasing doses of UV radiation. We used *E. coli* AB1157, a strain previously shown to be relatively UV resistant, as a reference (42). *S. flexneri* SA100 and 2457 were as UV resistant as AB1157 (Fig. 1). However, SA102, a derivative of SA100 lacking the 220-kb virulence plasmid pVP, was significantly more UV sensitive than SA100 ($P < 0.05$). At doses of UV radiation of 20 and 40 J/m², the numbers of SA102 cells that survived exposure to UV radiation were 22- and 100-fold less than SA100, respectively (Fig. 1). *S. flexneri* M90T and 229272, both of which contain a virulence plasmid, were also significantly more UV sensitive than SA100 ($P < 0.05$). The numbers of M90T and 229272 cells that survived exposures to UV radia-
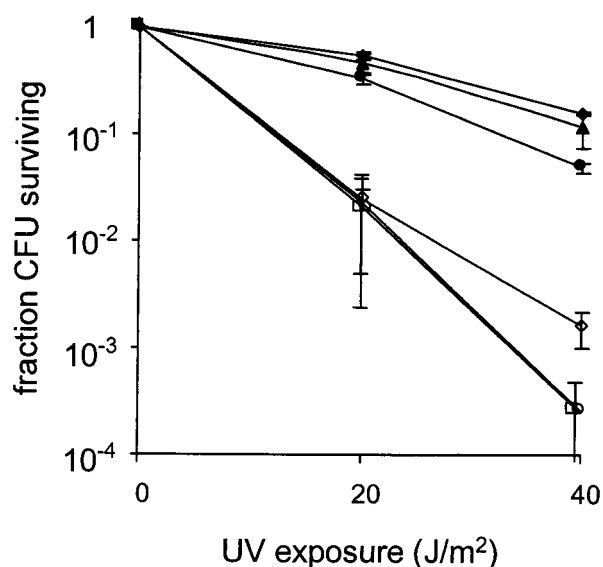

FIG. 1. Survival of *E. coli* AB1157 and *S. flexneri* isolates after UV irradiation. Cells were subjected to varying doses of UV light, and the CFU were determined by plating onto L agar. The data are the means of three experiments, and the standard deviations of the means are indicated. Symbols: ●, AB1157; ▲, 2457; ◆, SA100; ◇, SA102; ○, M90T; □, 229272.

tion of 20 and 40 J/m² were 25- and 600-fold less, respectively, than the number of surviving SA100 cells (Fig. 1).

***impB* is located on the virulence plasmid of *S. flexneri* SA100.** The different sensitivities to UV radiation of SA100 and SA102 suggested that survival following exposure to UV radiation was enhanced by the presence of pVP in SA100 (Fig. 1). Because a fragment of *S. flexneri* DNA homologous to the error-prone DNA repair gene *impB* had been identified in *S. flexneri* in our preliminary studies (15), it seemed likely that a pVP-encoded ImpB homologue contributes to resistance to UV radiation. To determine whether pVP encodes an ImpB homologue, we designed primers based on the plasmid TP110 *impB* sequence to amplify an 87-bp *impB* fragment from SA100 and SA102 by using PCR. A PCR product of this size was amplified from SA100 but not from SA102. Since the only known difference between SA100 and SA102 is that SA102 lacks pVP, it appeared that ImpB is encoded on pVP.

To provide further evidence that *impB* was located on the virulence plasmid in *S. flexneri*, the virulence plasmid was isolated from a derivative of *S. flexneri* SA100 (SM100/pVP::Cm) and transferred by electroporation into *E. coli* DH5α, which does not contain *impB*. An 87-bp PCR product corresponding to *impB* was amplified from these transformants but not from DH5α (data not shown); thus, we concluded that *impB* is located on pVP.

The presence of *impB* correlates with increased resistance to UV radiation. We examined isolates of other *Shigella* spp. for the presence of *impB* and for their ability to survive UV irradiation. Although *impB* was present in representatives of all four species of *Shigella*, not all isolates tested contained the gene (Table 2). Within each species, isolates that contained *impB* were significantly more resistant to UV radiation than strains that lacked *impB* ($P < 0.05$) (Table 2). Eight isolates of enteroinvasive *E. coli* were also tested for the presence of the *impB* gene because enteroinvasive *E. coli*, which causes a disease that is similar to shigellosis, has a virulence plasmid that is related to the *Shigella* virulence plasmids (40). None of the eight isolates tested contained the *impB* gene (Table 2 and

TABLE 2. Correlation between the presence of *impB* and
resistance to UV radiation in *Shigella* spp.
and enteroinvasive *E. coli*

Strain	Sero-type	Presence of *impB*[a]	Resistance to UV[b] (% survival ± SD)
S. flexneri SA100	2a	+	+ (15.4% ± 9.3%)
S. flexneri 2457	2a	+	+ (11.8% ± 4.4%)
S. flexneri M90T	5	−	− (0.025% ± 0.023%)
S. flexneri 229272	5	−	− (0.024% ± 0.024%)
S. dysenteriae SD125	1	+	+ (2.6% ± 2.0%)
S. dysenteriae O-4576	1	+	+ (2.4% ± 2.0%)
S. sonnei PB66		+	+ (20.4% ± 2.4%)
S. sonnei 1-1245		+	+ (15.6% ± 1.4%)
S. boydii 224860	7	+	+ (0.20% ± 0.10%)
S. boydii O-1392	5	−	− (0.010% ± 0.003%)
Enteroinvasive *E. coli* 1107-81		−	− (0.002% ± 0.002%)
Enteroinvasive *E. coli* 550-3076		−	− (<0.001%)

[a] The presence of *impB* was detected by PCR using primers impB1 and impB2, which are complementary to plasmid TP110 *impB*.

[b] + and − indicate that ≥0.1% and ≤0.1% of the cells, respectively, survived UV irradiation at a fluence of 40 J/m².

data not shown). We examined the ability of two enteroinvasive *E. coli* isolates to survive UV irradiation and found that they were significantly more sensitive to UV radiation than SA100 ($P < 0.05$) (Table 2).

Sequencing the *imp* operon from SA100 pVP. We isolated the entire *imp* operon from *S. flexneri* SA100 pVP by PCR. The *imp* upstream primer was designed using the sequence upstream from the TP110-encoded *imp* operon. However, the DNA fragment containing the *imp* operon could not be amplified using the *imp* upstream primer and a primer with a nucleotide sequence based on the sequence downstream from the TP110 *imp* operon. Therefore, we used inverse PCR with primers corresponding to the coding sequence of *impB* to isolate a fragment downstream from the SA100 pVP *imp* operon. The nucleotide sequence of the inverse PCR product was determined, and a downstream primer was designed using this sequence. The *imp* upstream and downstream primers were used to amplify a 2.4-kb DNA fragment containing the *imp* operon, and the nucleotide sequence of this fragment was 96% identical to that of a DNA fragment containing the *impCAB* operon on the conjugative plasmid TP110.

The nucleotide sequence of the 2.4-kb PCR product from SA100 pVP contained three overlapping open reading frames (ORFs) encoding putative proteins of 9.5, 16.2, and 47.6 kDa, which were designated ImpC, ImpA, and ImpB, respectively, based on their amino acid homologies (see below). The predicted pIs of these proteins were 5.13, 4.98, and 9.52, respectively. Because the overlapping arrangement of the three *imp* genes suggested that they constituted an operon, the region upstream of *impC* was examined for promoter-like features (Fig. 2). We identified a putative σ⁷⁰ recognition sequence in which each of the −35 and −10 hexamers deviates in 1 of 6 nucleotides from the consensus binding sequence for σ⁷⁰ (14). A 20-bp SOS box, which may function as a binding site for the LexA repressor, overlapped the putative −10 sequence by 1

nucleotide. Nucleotides 12 and 19 deviated from the consensus SOS box; however, these 2 nucleotides are less critical for LexA binding (11). We were unable to locate a Rho-independent terminator downstream of the SA100 pVP *imp* operon. This suggests that transcriptional termination occurs via a Rho-dependent manner or that this operon contains an additional downstream gene.

impC, the first gene in the SA100 pVP *imp* operon, was preceded by a potential ribosome binding site (AGGGAGA) located 8 nucleotides 5′ to the translational start codon. The deduced amino acid sequence of *impC* was used to conduct a BLASTX search of the National Center for Biotechnology Information nonredundant database (1). The predicted protein was 100% identical to the ImpC protein encoded on the plasmid TP110. ImpC has been postulated to be involved in the regulation of ImpA and ImpB expression by an undefined mechanism (26). Additionally, *S. flexneri* pVP ImpC was homologous to ORFf, encoded by the retronphage ΦR67 in *E. coli* (8, 17); Tum, encoded by prophage 186 in *E. coli* (4); and *E. coli* DinI (59) (Table 3). The function of ORFf has not been determined; however, Tum is an antirepressor that causes induction of prophage 186 by directly interfering with the ability of the phage repressor to bind DNA (45). DinI has been shown to inhibit RecA*-mediated self-cleavage of LexA and UmuD (58). Thus, ImpC may have a similar role in the regulation of ImpA self-cleavage to ImpA′.

The translational stop codon for ImpC overlapped the translational initiation codon for ImpA by 2 nucleotides. The deduced amino acid sequence of *S. flexneri* pVP *impA* was 100% identical to the TP110-encoded ImpA protein and was similar to other members of the UmuD family of error-prone DNA repair proteins (Table 3). Amino acid residues that are highly conserved among the UmuD family and are important in the RecA*-mediated self-cleavage reaction were conserved in *S. flexneri* pVP ImpA. These include the Ala-Gly or Cys-Gly residues (amino acids 24 and 25 in *E. coli* UmuD) where cleavage occurs; a serine residue (amino acid 60), which functions as a nucleophile; and a lysine residue (amino acid 97), which most likely functions as an activator (5, 32, 36, 46). Like other UmuD homologues, the N terminus of *S. flexneri* pVP ImpA was not as well conserved as the C terminus. Since the N terminus has been shown to be proteolytically removed in several UmuD homologues, it is probably not essential for ImpA function.

The translational stop site for ImpA overlapped the translational initiation site for ImpB by 1 nucleotide. The deduced amino acid sequence of *S. flexneri* pVP *impB* was 97% identical to the TP110-encoded ImpB protein and was similar to other members of the UmuC family of error-prone DNA repair proteins (Table 3). The nucleotide sequences of the 3′ ends and downstream regions of the TP110- and SA100 pVP-encoded *impB* genes were less conserved than the rest of the *imp* operon. The nucleotide sequences are highly conserved (98% identity) from 370 nucleotides 5′ to the *impC* translational start codon to 49 nucleotides 5′ to the *impB* translational stop codon. Beginning at this point and continuing downstream from *impB*, the nucleotide sequence is not as highly conserved between *S. flex-*
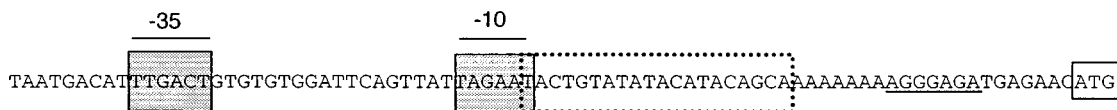

FIG. 2. Nucleotide sequence of the *impCAB* promoter from *S. flexneri*. The dotted box indicates the putative SOS box for LexA binding, and the shaded box indicates the putative −10 and −35 hexamers for σ⁷⁰. The proposed translational initiation codon is boxed, and the Shine-Delgarno sequence is underlined.

TABLE 3. Selected homologies of *S. flexneri* pVP-encoded ImpC, ImpA, and ImpB to other proteins[a]

Protein	Organism	Location	% Identity	% Similarity[b]	Reference
ImpC homologues					
ImpC	*Salmonella typhimurium*	Plasmid TP110	100	100	26
ORFf	*E. coli*	Retronphage Φ67	34	64	17
DinI	*E. coli*	Chromosome	30	55	59
Tum	*E. coli*	Coliphage 186	25	50	4
ImpA homologues					
ImpA	*Salmonella typhimurium*	Plasmid TP110	100	100	26
SamA	*Salmonella typhimurium*	60-MDa virulence plasmid	59	73	33
MucA	*Serratia marcescens*	Plasmid R471a	46	61	22
MucA	*Salmonella typhimurium*	Plasmid R46	43	56	GenBank accession no. X16596
MucA	*E. coli*	Plasmid pKM101	44	57	36
RumA	*Proteus rettgeri*	Plasmid R391	46	56	23
RulA	*Pseudomonas syringae*	Plasmid pPSR1	28	43	50
UmuD	*E. coli*	Chromosome	43	58	36
UmuD	*Salmonella typhimurium*	Chromosome	42	57	49, 52
ImpB homologues					
ImpB	*Salmonella typhimurium*	Plasmid TP110	97	98	26
SamB	*Salmonella typhimurium*	60-MDa virulence plasmid	70	83	33
MucB	*Serratia marcescens*	Plasmid R471a	51	67	22
MucB	*Salmonella typhimurium*	Plasmid R46	55	69	GenBank accession no. X16596
MucB	*E. coli*	Plasmid pKM101	55	69	36
RumB	*Proteus rettgeri*	Plasmid R391	57	74	23
RulB	*Pseudomonas syringae*	Plasmid pPSR1	42	61	50
UmuC	*E. coli*	Chromosome	57	72	36
UmuC	*Salmonella typhimurium*	Chromosome	58	71	49, 52

[a] Homologies were calculated using the CLUSTAL W algorithm (53) in MacVector 6.0 (Oxford Molecular Group).
[b] Includes conserved substitutions.

neri SA100 pVP and plasmid TP110 (45% identity). Additionally, the nucleotide sequences of the virulence plasmids from *S. flexneri* SA100 and 8-2031, another *S. flexneri* strain, were not as highly conserved in this region (47% identity). A sequence containing a direct repeat (<u>GGCAAATG</u>N$_4$<u>GGGAAATG</u> [the underlining indicates the repeated sequence]) precedes the point of nucleotide divergence. The nucleotide sequence 3′ of *impB* in 8-2031 contained an 8-bp sequence (TTGTTATT) that was tandemly repeated six times, beginning 22 bp from the stop codon. The significance of these direct repeats is unknown.

Upstream from and in the opposite orientation of the *imp* operons on SA100 pVP and plasmid TP110, we identified a partial ORF with a deduced amino acid sequence that was 92% identical to that of an 8-kDa putative protein of unknown function encoded downstream of the *sop* locus. The *sop* locus controls partitioning of the F plasmid, but the 8-kDa putative protein is not required for plasmid partioning (30). On pVP, there was a stop codon in the ORF located 40 nucleotides downstream of the translational start codon. Additionally, on both pVP and TP110, there was a 1-bp insertion 76 nucleotides 5′ to the ORF translational stop codon that shifted the reading frame. Thus, it appears that this ORF does not encode a functional protein in strains carrying pVP or TP110.

Characterization of an ImpB mutant. To determine whether any of the genes in the pVP *imp* operon was required for survival or induced mutagenesis in *S. flexneri* following UV irradiation, we constructed an *impB* mutation in which the wild-type allele was replaced with one containing a *cam* gene insertion. The mutation was constructed in SM100, a streptomycin-resistant derivative of SA100, and the resulting ImpB mutant was designated SM162. The ability of SM162 to survive UV irradiation was examined as described above. SM162 was significantly more sensitive to UV radiation than the parental

strain SM100 ($P < 0.05$) (Fig. 3); however, SM162 was not as sensitive as SA102, which does not contain pVP ($P < 0.05$) (Fig. 3).

A 2.4-kb PCR fragment containing the entire *imp* operon
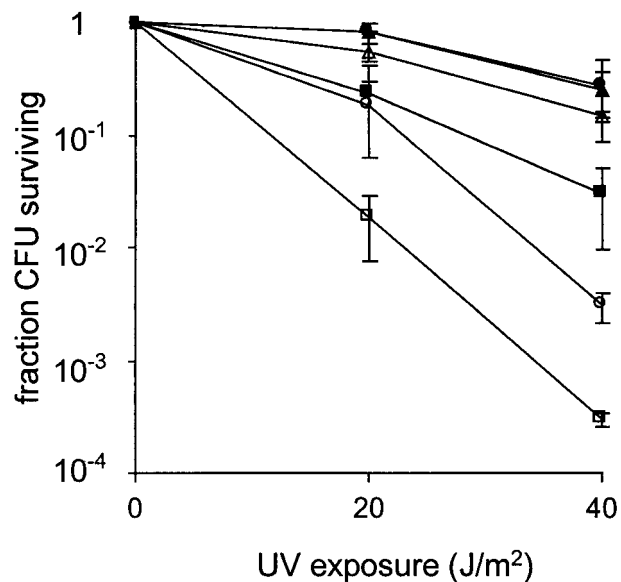

FIG. 3. Effect of *impB* on survival after UV irradiation. Cells were subjected to varying doses of UV light, and the CFU were determined by plating on L agar. The data are the means of three experiments, and the standard deviations of the means are indicated. Symbols: △, SM100; ▲, SM100/pIMP; □, SA102; ■, SA102/pIMP; ○, SM162; and ●, SM162/pIMP.
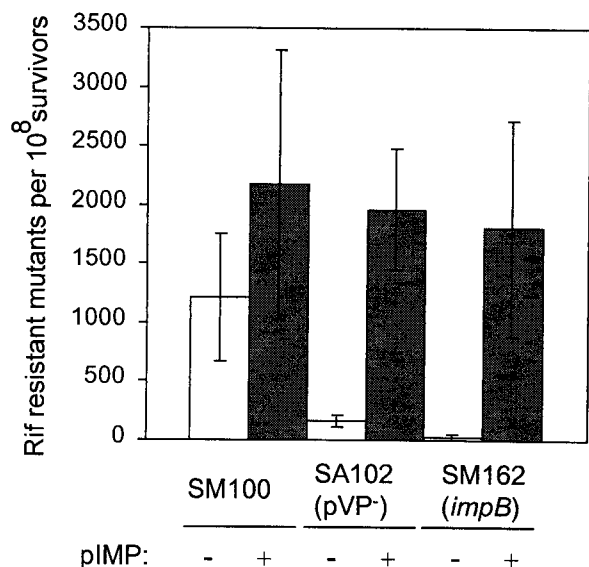
FIG. 4. Effect of *impB* on UV-induced mutagenesis. Rifampin-sensitive strains were grown to mid-logarithmic phase and irradiated with 10 J/m² of UV light, and the number of rifampin-resistant colonies was determined. The data are graphed as rifampin-resistant mutants per 10⁸ survivors, and the number of spontaneous mutations conferring rifampin resistance in the absence of UV irradiation has been subtracted. The data are the means of three experiments, and the standard deviations of the means are indicated.

from *S. flexneri* SA100 pVP was cloned into pWKS30, a low-copy-number vector, to generate pIMP. SM100, SM162, and SA102 were transformed with pIMP, and the ability of the strains to survive UV irradiation was assessed (Fig. 3). Strains carrying the vector pWKS30 showed a response to UV irradiation that was identical to that of the same strain without the vector. The addition of pIMP to SM162 restored survival following UV irradiation to levels similar to that of the parental strain SM100 carrying pIMP. SA102 carrying pIMP showed an intermediate level of UV sensitivity that was between the sensitivity of SA102 and that of SM100 carrying pIMP.

We examined the UV-induced mutagenesis phenotype of SM162 by measuring the frequency at which mutations that conferred rifampin resistance arose. SM100, SM162, and SA102, all of which are sensitive to rifampin, were exposed to 10 J/m² of UV light and then tested for rifampin resistance. The UV-induced mutagenesis frequency was 40-fold lower in SM162 than in the parental strain SM100 ($P < 0.05$) (Fig. 4). Surprisingly, even though SA102 was more sensitive to UV exposure than SM162 (Fig. 3), the UV-induced mutagenesis frequency in SA102 was significantly higher than in SM162 ($P < 0.05$), although it was still sevenfold lower than that in the parental strain SM100 ($P < 0.05$) (Fig. 4). The addition

of pIMP to SM162 and SA102 restored the UV-induced mutagenesis frequency to the levels of SM100/pIMP (Fig. 4).

Examination of the ability of SM162 to invade and spread in cultured epithelial cells. Many of the genes on the *S. flexneri* virulence plasmid are important for invasion of colonic epithelial cells, for intracellular survival and proliferation, and for spread to adjacent cells (28, 40). To determine whether ImpB was essential for any of these processes, we examined the ability of the ImpB mutant SM162 to form plaques on a Henle cell monolayer. SM162 formed plaques that were similar in size and number to the plaques formed by the parental strain SM100, suggesting that *impB* was not required for the invasion of Henle cells or for cell-to-cell spread in a plaque assay (data not shown).

Identification of the *umuDC* operon in *S. flexneri* SA100 and analysis of its induction by UV radiation. The *E. coli* error-prone DNA repair system UmuDC is chromosomally encoded (20, 36). Although *Shigella* spp. are closely related to *E. coli*, *S. flexneri* appears to require the virulence plasmid-encoded *impB* gene for induced mutagenesis. This suggested that *S. flexneri* does not contain a functional, chromosomally encoded UmuDC system. To investigate this possibility, we designed the primers umuDCP1 and umuDC3 based on the nucleotide sequence of the *E. coli* *umuDC* operon to amplify a 2.1-kb fragment containing the *umuDC* operon from *S. flexneri*. A PCR product of this size was amplified from SA100 and SA102, which lacks the virulence plasmid. These data demonstrate that *S. flexneri* contains a *umuDC* operon and suggests that the operon is chromosomally encoded.

To begin characterization of the contribution of the SA100 *umuDC* operon to survival and induced mutagenesis following UV irradiation, we measured the level of induction of a *umuDC* promoter-*gfp* transcriptional fusion after exposure to UV radiation. pLR13, which contained the *S. flexneri* SA100 *umuDC* promoter-*gfp* transcriptional fusion, was transformed into *E. coli* AB1157 and *S. flexneri* SA100. Expression of *gfp* controlled by the *S. flexneri* *umuDC* promoter on pLR13 in either AB1157 or SA100 was not induced by UV irradiation. In contrast, expression of *gfp* controlled by the *E. coli* AB1157 *umuDC* promoter on pLR20 was induced 12-fold by UV irradiation. These data suggest that although *S. flexneri* SA100 contains the *umuDC* operon, it is not expressed in response to UV irradiation.

Sequence analysis of the *S. flexneri* *umuDC* promoter. To verify that the lack of UV-induced expression from the *S. flexneri* SA100 *umuDC* promoter was not a result of a PCR-derived mutation in the promoter, we sequenced two independently isolated *umuDC* promoter clones and a *umuDC* promoter PCR product. Sequence analysis of these DNA fragments showed that all three sequences were identical, demonstrating that there was not a PCR-derived mutation in the *umuDC* promoter amplified from SA100.

We compared the nucleotide sequence of the *S. flexneri* *umuDC* promoter with those of the *E. coli* and *Salmonella*
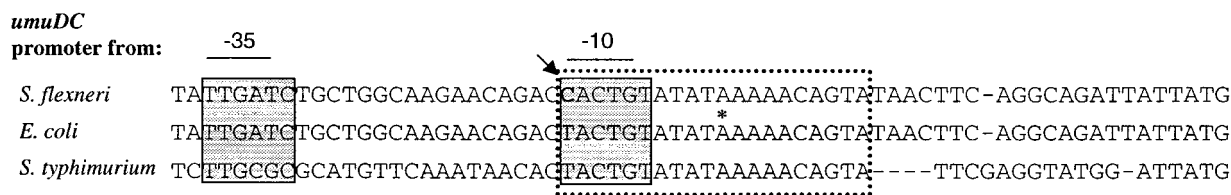

FIG. 5. Nucleotide sequence of the *umuDC* promoters from *S. flexneri*, *E. coli*, and *Salmonella typhimurium*. The dotted box indicates sequences corresponding to the SOS box for LexA binding, and the shaded boxes indicate sequences corresponding to the −10 and −35 hexamers for σ⁷⁰ binding. An asterisk indicates the transcriptional initiation site in *E. coli*. The arrow points to the nucleotide that is altered in the *S. flexneri* *umuDC* promoter.

typhimurium umuDC promoters. Over a 113-bp overlap, the *S. flexneri umuDC* promoter was 98% identical to the *E. coli umuDC* promoter and 56% identical to the *Salmonella typhimurium umuDC* promoter. In all three promoters, the SOS box and the $-10 \sigma^{70}$ recognition sequence overlapped (Fig. 5). The first nucleotide of the SOS box corresponds to the first nucleotide of the $-10 \sigma^{70}$ recognition sequence; however, this nucleotide was a T in *E. coli* and *Salmonella typhimurium* and a C in *S. flexneri* (Fig. 5). Although the T at position 1 in the SOS box is not essential for binding of the LexA repressor (11), it is a highly conserved nucleotide in *E. coli* $-10 \sigma^{70}$ hexamers (14). Thus, the presence of a C at this position in the *S. flexneri umuDC* promoter may weaken the binding of RNA polymerase and contribute to the lack of UV-induced expression of the promoter.

DISCUSSION

In the course of analyzing the response of *S. flexneri* to potentially stressful environments, including the intracellular environment, we discovered that *S. flexneri* contains a homologue of the *impB* gene, first identified on the plasmid TP110 in *Salmonella typhimurium* (9, 26). Our data suggest that, like the TP110-encoded *impB* gene, the *S. flexneri impB* gene is located on a large plasmid, the 220-kb virulence plasmid pVP. SA102, a derivative of *S. flexneri* SA100 that lacks pVP, does not contain the *impB* gene. Furthermore, the *impB* gene could be transferred to *E. coli* on pVP. We have not examined the location of the *impB* genes in other *Shigella* strains that contain *impB*; however, it is likely that *impB* is virulence plasmid encoded in these isolates since *Shigella* virulence plasmids have a high degree of similarity (40).

Based on amino acid sequence comparisons, it appears that two of the proteins encoded by the *S. flexneri* pVP *imp* operon (ImpA and ImpB) are members of the error-prone DNA repair family of proteins that contribute to survival after UV irradiation. Consistent with the sequence homology, *S. flexneri* SM162 and SA102, which contain an *impB* mutation and a deletion of the entire virulence plasmid, respectively, showed a reduced ability to survive UV irradiation. SA102 had a lower frequency of survival after UV irradiation than SM162, suggesting that pVP encodes another gene product that is important either specifically for resistance to UV irradiation or generally for survival under stressful conditions.

There is a precedent for other genes that contribute to resistance to UV irradiation. For instance, the *uvr* genes are important for surviving UV irradiation, but they are chromosomally encoded in *E. coli* (2, 3, 48). The plasmid pKM101, which encodes the MucAB error-prone DNA repair system, contains an additional uncharacterized gene which contributes to survival following UV irradiation (24). *Salmonella typhimurium* strains carrying a deletion derivative of pKM101, in which the *mucAB* genes were still present but the uncharacterized gene was absent, showed decreased survival following UV irradiation. Langer et al. (24) proposed that either this deletion in pKM101 resulted in overexpression of *mucAB*, leading to decreased survival following UV irradiation because of excessive levels of mutagenesis, or that the deletion removed a suppressor of a UV sensitization gene. Additionally, even among isolates of *Shigella* spp., there is a wide range of sensitivities to UV radiation (Table 2). For example, *Shigella boydii* 224860 is significantly less UV resistant than *S. flexneri* SA100 ($P < 0.05$), even though both contain *impB*, but is significantly more UV resistant than *S. boydii* O-1392 ($P < 0.05$), which does not contain *impB*. In general, among *Shigella* spp. isolates that contain *impB*, there is the following correlation between species type and UV resistance: *S. flexneri* = *Shigella sonnei* > *Shigella dysenteriae* > *S. boydii*. These observations emphasize the complexity of the UV sensitivity phenotype and suggest that although *impB* clearly contributes to UV resistance, *Shigella* spp. contain other genes that are also important for surviving UV irradiation.

S. flexneri SM162 and SA102, which contain an *impB* mutation and a deletion of the entire virulence plasmid, respectively, also showed decreased levels of UV-induced mutagenesis. In contrast to the defect in survival following UV irradiation, which was greater in SA102, the defect in UV-induced mutagenesis was greater in SM162. One explanation for this result is that that expression of ImpA without ImpB in SM162 interferes with other systems that are mutagenic. This would not occur in SA102 because both ImpA and ImpB are absent. Although *S. flexneri* contains the UmuDC error-prone DNA repair system, the data presented in this article suggest that this system is not UV induced in *S. flexneri*. Furthermore, the *S. flexneri umuDC* operon does not appear to complement the defect in UV-induced mutagenesis in an *E. coli umuDC* mutant (12). However, *S. flexneri* may possess another mutagenesis system that is weakly induced by UV irradiation and has yet to be identified. Another possibility for the less severe defect in UV-induced mutagenesis in SA102 relative to that in SM162 is that pVP may encode a suppressor of another unidentified mutagenesis system which is activated upon deletion of pVP.

The analysis of the error-prone DNA repair *imp* operon presented here focused on the role of the operon in survival and induced mutagenesis after exposure to UV radiation. The UV inducibility of error-prone DNA repair operons is well conserved. To our knowledge the promoters of all error-prone DNA repair operons examined to date contain binding sites for the LexA repressor. Exposure to UV radiation induces LexA-repressed operons. Although we did not test directly whether expression of the pVP-encoded *imp* operon is UV inducible, the fact that a well-conserved LexA binding site overlaps the putative -10 sequence by 1 nucleotide in the *imp* promoter suggests that the *imp* operon is UV inducible. Additionally, the fact that UV exposure was required for ImpB-mediated induced mutagenesis supports this hypothesis. It is also possible that a signal other than or in addition to UV radiation may induce expression of the pVP-encoded *imp* operon in *S. flexneri*. Some of these signals may be encountered in the variety of stressful environments that *Shigella* encounters during its journey through the external environment and human host.

There are several possible molecular mechanisms by which *Shigella* spp. may have acquired the *imp* operon. The *imp* operon may have been acquired by fusion of the virulence plasmid or virulence plasmid progenitor with another plasmid, such as TP110, that contained the *imp* operon. Alternatively, the *imp* operon may have been acquired as part of a transposon or other mobile genetic element. There is evidence that error-prone DNA repair operons may have been located on transposable elements. Langer et al. (25) found that inverted repeats flank a 6-kb region that contains the *mucAB* genes on pKM101, suggesting that these genes were part of a transposon at one time. Additionally, direct repeats similar to the termini of the Tn*3* group of transposases flank a 12- to 14-kb region that contains the *umuDC* genes in a variety of *Escherichia* spp. (44). Kulaeva et al. (22) found a retroelement encoding a putative reverse transcriptase located upstream of and an insertion sequence located downstream from the *mucAB* genes on plasmid R471a. Finally, the large conjugative transposon Tn*5252* found in many clinical streptococci contains an error-prone DNA repair system (31). The fact that not all isolates of *Shi-*

gella contain the *imp* operon suggests that the *imp* operon may have been acquired by some but not all phylogenetic lines of the virulence plasmid early in the evolution of the plasmid. Alternatively, the *imp* operon may have been part of the progenitor virulence plasmid and this operon was subsequently lost from the virulence plasmids in some strains.

Although it is clear that the *impB* gene is not an essential gene in *S. flexneri*, it is possible that the acquisition of the *imp* operon by some *Shigella* species gives those strains a selective advantage. The presence of the *imp* operon on pVP most likely compensates for the inefficient UV induction of the chromosomal *umuDC* genes in *S. flexneri*. In environments in which UV radiation or other DNA-damaging agents are encountered, such as the external environment, strains containing the *imp* operon may survive better than strains that do not have the *imp* operon. If the *imp* operon provides a selective advantage for maintaining the virulence plasmid in the external environment, strains containing the *imp* operon may be more likely to have the plasmid when *Shigella* reencounters a human host, where the organism needs other virulence plasmid-encoded genes for survival. Additionally, it is possible that the *imp* operon may be protective in other stressful environments, some of which may be encountered when *Shigella* is inside the human host.

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