RfaH Enhances Elongation of *Escherichia coli* *hlyCABD* mRNA

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Escherichia coli *hlyCABD* operons encode the polypeptide component (HlyA) of an extracellular cytolytic toxin, as well as proteins required for its acylation (HlyC) and *sec*-independent secretion (HlyBD). Previous reports suggested that the *E. coli* protein RfaH is required for wild-type hemolysin expression, either by positively activating *hly* transcript initiation (M. J. A. Bailey, V. Koronakis, T. Schmoll, and C. Hughes, *Mol. Microbiol.* 6:1003–1012, 1992) or by promoting proper insertion of hemolysin export machinery in the *E. coli* outer membrane (C. Wandersman and S. Letoffe, *Mol. Microbiol.* 7:141–150, 1993). RfaH is also required for wild-type levels of mRNA transcribed from promoter-distal genes in the *rfaQ-K*, *traY-Z*, and *rplK-rpoC* gene clusters, suggesting that RfaH is a transcriptional antiterminator. We tested these models by analyzing the effects of *rfaH* mutations on *hlyCABD* mRNA synthesis and decay, HlyA protein levels, and hemolytic activity. The model system included a uropathogenic strain of *E. coli* harboring *hlyCABD* on the chromosome and *E. coli* K-12 transformed with the *hlyCABD* operon on a recombinant plasmid. Our results suggest that RfaH enhances *hlyCABD* transcript elongation, consistent with the model of RfaH involvement in transcriptional antitermination in *E. coli*. We also demonstrated that RfaH increases toxin efficacy. Modulation of hemolysin activity may be an indirect effect of RfaH-dependent *E. coli* outer membrane chemotype, which is consistent with the model of lipopolysaccharide involvement in hemolytic activity.

Synthesis of *Escherichia coli* hemolysin is directed from operons consisting of four contiguous genes, *hlyCABD*. Hemolysin determinants are found on large, transmissible plasmids in animal isolates of *E. coli* or within unique chromosomal inserts, absent in *E. coli* K-12, of human uropathogenic *E. coli* isolates (22, 35). The *hlyA* gene encodes the polypeptide component of hemolysin, which undergoes HlyC-dependent acylation (15, 23) and is then transported across both bacterial membranes without an amino-terminal cleavage or a periplasmic intermediate (12, 13). Hemolysin transport requires HlyB and HlyD (32), as well as the unlinked *tolC* gene product (33). The coding regions of individual hemolysin determinants display 97% sequence identity (16); however, untranslated regions and sequences 5' to the promoters are heterologous (9, 16, 19, 20, 31, 36). This suggests that effectors of hemolysin transcript initiation differ among individual *hlyCABD* determinants, whereas regulatory features common to hemolysin operons, such as the putative *rho*-independent *hlyA-B* intergenic transcriptional terminator, may exist within the conserved transcribed regions (13).

Recently, a *trans*-acting factor affecting the expression of several hemolysin determinants of chromosomal or plasmid origin was identified independently by two groups of investigators. The *rfaH* gene product was described by Bailey et al. (1) as a positive activator of *hlyA* transcription and by Wandersman and Letoffe (34) as a product required for expression of TolC, an HlyA secretion facilitator. The *rfaH* locus encodes an 18.3-kDa protein that enhances expression of promoter-distal genes in at least three operons in *E. coli*, putatively at the level of transcription antitermination. These include the *rfaQ-K* gene cluster (24, 26) and the homologous *rfa* operon in *Salmonella typhimurium* (5), the *traY-Z* operon (4), and the *rplK-*

rpoC gene cluster (25). In *rfaH* mutants, steady-state levels of mRNA from genes downstream of putative transcriptional terminators in the *traY-Z* operon are reduced to a significantly greater degree than those of mRNA from promoter-proximal genes (4). The model for RfaH-dependent transcript antitermination of the *traY-Z* operon is supported by a sevenfold reduction in expression of *lacZ* when it is fused to genes in distal portions of the operon (14). Within the *rplK-rpoC* gene cluster, about 23% of the transcripts are elongated through the *rplL-rpoB* intragenic terminator. Transcription through the terminator decreases to 14% in an *rfaH* mutant, similar to the decrease seen in a *nusA1* mutant (25). In the *rfaQ-K* gene cluster, the effect of mutations in *rfaH* on downstream gene expression is more dramatic than that on expression of genes near the promoter(s) (8, 24). Recently, RfaH was implicated in enhanced *E. coli* *kps*, group II capsular polysaccharide gene, transcription (30). The effect of RfaH on a wide array of secreted and outer-membrane-associated macromolecules is striking and suggests a common regulatory phenomenon.

To examine the models of RfaH-enhanced hemolysin expression, we first characterized the effects of nine independent *rfaH* alleles on hemolysin expression from a recombinant *hlyCABD* operon in *E. coli* K-12. The effect of a null *rfaH* allele on *hlyCABD* mRNA synthesis and decay was further investigated. In this report, we provide evidence to support the role of RfaH in transcript elongation. In addition, evidence is presented which suggests that *rfaH* mutations indirectly affect the activity of the toxin as a result of the deep rough lipopolysaccharide (LPS) phenotype. Previous work has shown that hemolysin activity is compromised in deep rough *rfaC* and *rfaP* mutants (2, 29) and that the LPS chemotype strongly correlates with levels of toxin efficacy (6). Our results complement these data.

MATERIALS AND METHODS

Null *rfaH1.8* strain constructions. The *rfaH1.8::Tn5* allele from *E. coli* 5KC1.8 (*hsdR hsdS*) (1) was introduced into *E. coli* RZ4500 (*hsdR⁺ S⁺*) in two steps. First, *E. coli* LE392 (*hsdR hsdS⁺*) was transduced to kanamycin resistance (Kan^r) with a P1vir lysate of 5KC1.8. Next, RZ4500 was transduced to Kan^r with a P1vir lysate of LE392 *rfaH1.8* to create WAM1925. *E. coli* J96, a hemolytic human

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TABLE 1. Recombinant plasmids and *E. coli* strains used in this study

Strain or plasmid	Properties or genotype	Source or reference
Plasmids		
pKZ17	pBR322 <i>rfaH</i> ⁺	K. Sanderson; 26
pSF4000	J96 <i>hlyCABD</i> operon in pACYC184	This laboratory; 35
pWAM744	1,386-bp pSF4000 <i>HindIII-EcoRI</i> fragment, contains <i>hlyBD</i> in opposite orientation to T3 promoter in pGEMII, source of <i>hlyBD AccI-EcoRI</i> probe	This laboratory
pWAM750	pWAM04 (<i>hlyCABD</i> on pUC19) Δ <i>PstI</i> -H	36
pWAM764	2,151-bp pSF4000 <i>PstI</i> fragment, contains portion of <i>hlyA</i> in opposite orientation to T3 promoter in pGEMII, source of <i>hlyA DraI-PstI</i> probe	This laboratory
pWAM823	4.7-kb pSF4000 <i>TaqI</i> fragment, contains <i>hlyCA</i> under control of <i>hly</i> promoter in pACYC184	This laboratory
pWAM1452	997-bp pSF4000 <i>PstI</i> fragment, contains portion of <i>hlyCA</i> in opposite orientation to SP6 promoter in pBS ⁺ , source of <i>hlyCA RsaI-PstI</i> probe	This laboratory
pWAM2123	1,069-bp pWAM04 <i>BamHI</i> fragment, contains portion of <i>hlyC</i> in opposite orientation to T3 promoter in pBS ⁺ , source of <i>hlyC SmaI-BamHI</i> probe	This laboratory
<i>E. coli</i> strains		
5KC	<i>recAI hsdR hsdS</i>	C. Hughes; 1
5KC1.8	<i>recAI hsdR hsdS rfaH::Tn5 1.8</i>	C. Hughes; 1
5KC10.4	<i>recAI hsdR hsdS rfaH::Tn5 10.4</i>	C. Hughes; 1
RZ4500	<i>lacZΔ145</i>	P. Kiley; 7
WAM1925	<i>RZ4500 rfaH::Tn5 1.8</i>	This laboratory
M1174	<i>rfaH</i> ⁺	K. Sanderson
M1160	M1174 <i>rfaH1</i>	L. Beutin; 3
M1161	M1174 <i>rfaH2</i>	L. Beutin; 3
M1162	M1174 <i>rfaH3</i>	L. Beutin; 3
M1170	M1174 <i>rfaH11</i>	L. Beutin; 3
M1171	M1174 <i>rfaH12</i>	L. Beutin; 3
M1172	M1174 <i>rfaH13</i>	L. Beutin; 3
SAB2562	M1174 <i>rfaH14</i>	K. Sanderson; 26
J96	Human clinical uropathogenic strain	This laboratory; 35
WAM2096	<i>J96 metE3079::Tn10</i>	This laboratory
WAM2097	<i>J96 metE3079::Tn10 rfaH::Tn5 1.8</i>	This laboratory

urinary tract isolate, was transduced to *rfaH1.8* in two steps. J96 could not be transduced directly to Kan^r because of high frequencies of resistance associated with loss of the hemolytic phenotype (21), so a linked Tet^r marker was utilized. First, a *Plvir* lysate of *E. coli* CAG18491 (MG1655 *metE3079::Tn10*) (28) was used to transduce WAM1925 to Tet^r with coselection for Kan^r. A *Plvir* lysate of this strain was used to transduce *E. coli* J96 to Tet^r. The resulting colonies were screened for cotransduction to Kan^r to create WAM2097. The isogenic *rfaH*⁺ partner (WAM2096) was constructed by transducing *E. coli* J96 directly to Tet^r with a *Plvir* lysate of CAG18491. The acquisition of the deep rough *rfaH* phenotype in transductants was confirmed by resistance to phage U3 and sensitivity to phage C21 (27). The strains and recombinant plasmids used in this study are described in Table 1.

Media and reagents. Cultures were grown in Luria-Bertani medium at 37°C with aeration in the presence of the appropriate antibiotics (chloramphenicol, 12.5 µg/ml; kanamycin, 25 µg/ml; ampicillin, 50 µg/ml; tetracycline, 12.5 µg/ml). SP6 and T3 polymerases, rRNasin, RQ1 DNase, avian myeloblastosis virus reverse transcriptase, and nucleoside triphosphates were purchased from Promega (Madison, Wis.). Radionuclides were purchased from Amersham (Arlington Heights, Ill.). RPAII RNase protection reagents were from Ambion (Austin, Tex.).

Assay of hemolytic activity. *E. coli* cells were grown in Luria-Bertani broth plus appropriate antibiotics to an optical density at 600 nm (OD₆₀₀) of 0.80, the cells were pelleted, and supernatants were filtered through 0.2-µm-pore-size Acrodiscs (Gelman Sciences, Ann Arbor, Mich.). Hemolytic activities of cell-free media were assayed by addition of a 2% sheep erythrocyte suspension in 0.85% NaCl–20 mM CaCl₂ to an equal volume of filtered supernatant serially diluted in 0.85% NaCl–20 mM CaCl₂ for a final concentration of 1% sheep erythrocytes. The mixtures were incubated for 1 h at 37°C, the sheep erythrocytes were pelleted, and the A₅₇₀s of the media containing released hemoglobin were determined. Hemolytic activities of supernatants from individual cultures harboring hemolysin determinants were compared by graphing the supernatant dilution factors versus the A₅₇₀s of hemoglobin released by those dilutions of culture supernatants. The average initial slopes of these curves, generated from at least five replicates, were used to compare hemolytic activities.

Assay of HlyA protein levels. Cultures were grown to an OD₆₀₀ of 0.80. For quantitative analyses of extracellular HlyA concentrations, supernatants were lyophilized and resuspended in 2× treatment buffer (0.125 M Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% 2-mercaptoethanol)–

0.85% saline (1:1). To assess total cellular HlyA, whole culture material was mixed 1:1 with 2× treatment buffer. Samples were boiled for 10 min, separated by SDS–10% polyacrylamide gel electrophoresis alongside β-galactosidase protein concentration standards, and stained with Coomassie brilliant blue. Signals were quantified by densitometric analysis with a Zeineh soft laser scanning densitometer. Two independent cultures of each test strain were used to determine concentrations of extracellular HlyA. For Western blot (immunoblot) analyses, proteins were electrophoretically transferred to nitrocellulose and then reacted with rabbit anti-HlyA serum, followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G. The HlyA protein was detected by development of the blots by the LumiGLO chemiluminescence method as recommended by the manufacturer (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.).

Preparation of total cellular RNA. Cellular RNA was prepared from 5 to 10 ml of *E. coli* grown to an OD₆₀₀ of 0.50 by the hot phenol extraction method of Emory and Belasco (11).

Preparation of antisense RNA probes. Recombinant plasmids pWAM744, pWAM764, pWAM1452, and pWAM2123 were linearized and used as templates for in vitro generation of full-length runoff transcripts (Table 1; see Fig. 3A). RNA probe syntheses were carried out in a final volume of 10 µl containing 2.2 µl of H₂O, 1 µl of template DNA (0.2 to 1.0 µg/ml), 2 µl of 5× transcription buffer, 0.5 µl of dithiothreitol (100 mM), 0.5 µl of RNasin, 1 µl of 2.5 mM ATP-GTP-CTP, 0.5 µl of 1 mM UTP, 1.8 µl of [α-³²P]UTP (800 Ci/mmol), and 0.5 µl of (T3 or SP6) RNA polymerase. The reactions were carried out for 1 h at 37°C (T3 polymerase) or 39°C (SP6 polymerase). The DNA templates were removed by addition of RNase-free DNase (1 U/µg of DNA; Promega), followed by incubation at 37°C for 15 min. The reaction mixtures were extracted once with phenol-chloroform-isoamyl alcohol (24:1), precipitated with 100% ethanol and 10 µg of carrier tRNA, and resuspended in RNase-free H₂O.

RNase protection of *hly* mRNA. Target RNA and excess probe RNA (~50,000 cpm) were mixed in 80% formamide hybridization solution {200 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid); pH 6.4], 2 M NaCl, 5 mM EDTA}, heated to 85°C for 5 min, and then allowed to hybridize for 6 to 12 h at 45°C. RNase T1 (0.05 U) digestion solution (Ambion) was added, and the mixtures were incubated at 37°C for 30 min. The reactions were stopped by addition of an RNase inactivation-RNA precipitation solution (Ambion). Following precipitation, the RNA-RNA hybrids were resuspended in formamide loading buffer and separated on a 6% acrylamide–7 M urea gel at 300 V (20 mA). The radiola-

belled, protected RNA fragments were detected by autoradiography following exposure of the gels to DuPont NEF-496 film (with an intensifying screen when necessary) at -80°C for 4 to 18 h. For RNA quantitation experiments, dried RNA gels were exposed to a Molecular Dynamics (Sunnyvale, Calif.) phosphor screen for 12 h and scanned by a PhosphorImager SI (Molecular Dynamics). Band intensities were analyzed by using the ImageQuaNT V4.2 program (Molecular Dynamics). RNAs from at least three independent extractions were used to quantify *hlyCABD* mRNA.

Determination of *hly* mRNA half-life. Overnight cultures of *E. coli* were diluted 1:50 in fresh Luria-Bertani medium plus antibiotics and incubated at 37°C with aeration for 2 h (OD_{600} , 0.50). Rifampin was added to a final concentration of 200 $\mu\text{g}/\text{ml}$, and 10-ml aliquots were shifted to 0°C at various times following treatment. Cells were pelleted, and total RNA was extracted as described above. Equal amounts of total RNA from each time point were subjected to RNase protection analyses as described above. The radiolabelled, protected RNA fragments were detected by autoradiography following exposure of the gels to DuPont NEF-496 film (with an intensifying screen when necessary) at -80°C for 4 to 18 h. Autoradiographic films were analyzed by scanning densitometry (Zeineh). Half-lives were calculated by least-squares analyses of plots of autoradiographic signal intensity as a function of time. Errors in the measurements of half-lives were estimated from the standard deviation of the slope of each plot. The error bars shown in Fig. 4 represent the standard error for each time point. Only signals within the linear range of the film were used in calculating half-lives.

Primer extension and determination of *hlyCABD* 5' ends. Synthetic oligonucleotide primer 5' TCAACCAGCACAACCATAG 3', complementary to pSF4000 bases 424 to 444 (*hly* mRNA leader), was end labelled with [γ - ^{32}P]ATP by using T4 polynucleotide kinase as described previously (18). RNA was hybridized to the labelled primer for 15 min at 45°C and extended for 30 min at 45°C by 2 U of avian myeloblastosis virus reverse transcriptase in the presence of MgCl_2 hybridization-extension buffer (36 mM MgCl_2 , 60 mM NaCl, 50 mM Tris [pH 8.3], 10 mM dithiothreitol). The products were denatured and analyzed on a 6% polyacrylamide-7 M urea sequencing gel along with a pSF4000 DNA sequence primed with the same oligonucleotide.

Generation of scanned images. Figures (see Fig. 1A, 2, 3B, 3C, 5, and 6) were generated by scanning DuPont NEF-496 film into Adobe Photoshop 3.0 (Adobe Systems, Inc., Mountain View, Calif.) at 600 dots per in. (2.54 cm) with a UMAX reflective scanner (UMAX Data Systems, Inc., Hsinchu, Taiwan). The images were arranged and cropped in Adobe Photoshop 3.0 and then copied into Canvas 3.5 (Denaba Software, Miami, Fla.) as "picture objects" for addition of text (10). No image manipulations were performed in Canvas.

RESULTS

Analysis of extracellular HlyA protein concentration and hemolytic activity from *E. coli rfaH*⁺ versus *rfaH* mutants. In 1979, Beutin et al. isolated a panel of *E. coli* M1174 *rfaH* mutants carrying the F plasmid that were defective for normal *tra* operon functions (3). These strains were well characterized phenotypically with respect to F plasmid transfer, F pilus production, and surface exclusion. The *rfaH* alleles that most severely affected *traY-Z* functions also strongly altered LPS (*rfa*)-associated phenotypes, including phage and antibiotic sensitivities. Those *rfaH* alleles that only moderately affected *tra* expression were also leakiest for LPS-associated phenotypes. To determine how hemolysin expression is affected by these and two newly isolated *rfaH::Tn5* alleles (1), nine independently derived *E. coli* K-12 *rfaH* mutant strains and the isogenic *rfaH*⁺ strains were transformed with the recombinant chromosomally derived *hlyCABD* operon in pSF4000 (Table 1). Table 2 summarizes the results of extracellular HlyA concentration and hemolytic activity determinations.

Our results demonstrate that the effects of *rfaH* mutations on hemolytic expression are allele specific. The *rfaH1.8* allele, a null mutation containing a Tn5 insertion in the eighth codon (1), conferred the greatest reduction in hemolytic expression. HlyA protein levels were reduced 15-fold, and supernatants from the *rfaH1.8* mutant displayed 1,000-fold less hemolytic activity than the parent *rfaH*⁺ strain RZ4500 harboring pSF4000. *rfaH11* and *rfaH13*, the *rfaH* mutations in strain M1174 most detrimental to *tra* and *rfa* expression, conferred the strongest reduction in HlyA protein from this group, about 5.5- and 4-fold respectively. Allele *rfaH13* reduced hemolytic activity 11-fold, whereas *rfaH11* was entirely nonhemolytic. Allele *rfaH1*, which had the least effect on *tra* and *rfa* expres-

TABLE 2. Effect of *rfaH* on extracellular hemolytic activity and HlyA protein levels

Strain ^a	<i>rfaH</i> allele	Hemolytic activity ^b , A_{570} units/supernatant dilution (fold difference)	Extracellular [HlyA] ^c , $\mu\text{g}/\text{ml}$ (fold difference)
RZ4500	Wild type	670 $\pm$ 79	2.32
WAM1925	Tn5 1.8	0.5 $\pm$ 0.2 (1,300 $\pm$ 540)	0.15 (15)
5KC	Wild type	250 $\pm$ 21	0.56
5KC10.4	Tn5 10.4	2.6 $\pm$ 0.3 (95 $\pm$ 14)	0.08 (7)
M1174	Wild type	500 $\pm$ 35	0.99
M1160	1	170 $\pm$ 16 (2.9 $\pm$ 0.3)	0.49 (2)
M1161	2	94 $\pm$ 6.4 (5.3 $\pm$ 0.5)	0.30 (3.3)
M1162	3	20 $\pm$ 3.1 (25 $\pm$ 4.3)	0.42 (2.3)
M1170	11	<0.1 (>5,000)	0.18 (5.5)
M1171	12	72 $\pm$ 1.5 (6.9 $\pm$ 0.5)	0.48 (2.1)
M1172	13	46 $\pm$ 5.5 (11 $\pm$ 1.5)	0.25 (4)
SAB2562	14	74 $\pm$ 7.0 (6.8 $\pm$ 0.8)	0.46 (2.2)

^a All strains tested were transformed with pSF4000.

^b Hemolytic activities were determined as described in Materials and Methods. The unit is defined as the slope of the A_{570} of hemoglobin released as a function of supernatant dilution. The values shown are average slopes of at least five independent assays $\pm$ the standard errors of those values.

^c Extracellular HlyA concentrations were determined as described in Materials and Methods. The values shown are averages of two independent assays.

sion, acted similarly on hemolysin expression, reducing HlyA protein levels and hemolytic activity only two- and threefold respectively.

To ensure that the *rfaH* mutations alone were responsible for the reduction in HlyA protein and hemolytic activity, we cotransformed the *rfaH* mutants harboring pSF4000 with a plasmid containing the wild-type *rfaH*⁺ gene (pKZ17). Figure 1A depicts the HlyA antigen content (Fig. 1A, lanes 1 to 5) and hemolytic activity (Fig. 1B) of null mutant WAM1925 (*rfaH1.8*) harboring pSF4000 with and without the *rfaH*⁺ complementing plasmid. *Trans* complementation of WAM1925 (*rfaH1.8*)/pSF4000 with pKZ17 (*rfaH*⁺) restored both hemolytic activity and HlyA protein content to wild-type levels.

To assess the effect of RfaH on hemolytic expression from a native chromosomal *hlyCABD* operon, the null *rfaH1.8* allele was transduced into hemolytic, uropathogenic *E. coli* J96 (see Materials and Methods). As seen in the *E. coli* K-12 background, both HlyA antigen levels (Fig. 1A, lanes 6 and 7) and hemolytic activity (Fig. 1B) were severely reduced in supernatants of the *rfaH1.8* mutant clinical isolate. These results demonstrate that RfaH is required for wild-type levels of extracellular HlyA protein and hemolytic activity from both the native chromosomal and multicopy recombinant *hlyCABD* operons. Our data also indicate that the decrease in hemolytic activity from *rfaH* mutants did not always correlate with the decrease in HlyA protein levels. For example, allele *rfaH3* reduced HlyA protein levels 2-fold but conferred a 25-fold reduction in hemolytic activity. In addition, 1,000-fold reductions in hemolytic activity from *E. coli rfaH1.8* and *rfaH11* resulted from mutations reducing HlyA protein levels 5- to 15-fold. These observations are consistent with the proposal that hemolytic activity is sensitive to changes in LPS phenotype (2, 29) independent of the effect of RfaH on HlyA protein levels.

Assays of total cellular protein (Bio-Rad protein assay) and plasmid DNA that were normalized to total cell number demonstrated that the *rfaH* mutations do not have a general effect on protein expression or plasmid copy number (data not shown).

Analysis of intracellular HlyA protein levels in *E. coli rfaH*⁺ versus *rfaH1.8*. Wandersman and Letoffe (34) proposed that RfaH indirectly enhances hemolysin expression at the level of

FIG. 1. Analysis of extracellular HlyA antigen levels and hemolytic activity in *E. coli rfaH*⁺ versus *rfaH1.8*. (A) Immunoblot analysis of extracellular HlyA antigen from *E. coli rfaH*⁺ and *rfaH1.8*. Supernatants of RZ4500 carrying pSF4000 (*rfaH*⁺) (lanes 1 and 2) alone or with the complementing *rfaH*⁺ plasmid (pKZ17) (lane 4), WAM1925 carrying pSF4000 (*rfaH1.8*) (lane 3) alone or with the complementing *rfaH*⁺ plasmid (lane 5), WAM2096 (*rfaH*⁺) (lane 6), and WAM2097 (*rfaH1.8*) (lane 7) were subjected to immunoblot analysis. The amount of supernatant analyzed is indicated below each lane. (B) Hemolytic activities of supernatants were determined as described in Materials and Methods. The data shown are averages of at least three separate assays. Symbols: △, RZ4500 (*rfaH*⁺)/pSF4000; ▲, WAM1925 (*rfaH1.8*)/pSF4000; ○, WAM1925 (*rfaH1.8*)/pSF4000 + pKZ17 (*rfaH*⁺); ■, WAM2096 (*rfaH*⁺); □, WAM2097 (*rfaH1.8*).

HlyA secretion, rather than affecting HlyA synthesis. To determine if RfaH only affects secretion of HlyA, we transformed *E. coli* K-12 *rfaH*⁺ and the *rfaH1.8* null mutant with pWAM823, a subclone of the *hlyCABD* operon with the *hlyBD* secretion genes deleted, and assayed the levels of intracellular HlyA protein by Western analysis. The data in Fig. 2 demonstrate that *E. coli rfaH*⁺ harboring only *hlyCA* continued to produce greater than eightfold more intracellular HlyA than *E. coli rfaH1.8* harboring *hlyCA*. This observation indicates that the *rfaH1.8* mutation affects HlyA synthesis and contradicts the model which states that reduced HlyA protein expression in *E. coli rfaH* is a result of a malfunctioning HlyA transport system.

Analysis of steady-state levels of *hlyCABD* mRNA in *E. coli rfaH*⁺ versus *rfaH1.8*. To determine whether the reductions in HlyA protein levels in the *rfaH1.8* mutants were due to loss of *hly* mRNA, we compared the steady-state levels of *hlyCABD* mRNA in *E. coli* K-12 *rfaH*⁺ (RZ4500) and *rfaH1.8* (WAM1925) harboring pSF4000 and *E. coli* J96 *rfaH*⁺ (WAM2096)

FIG. 2. Immunoblot analysis of cell-associated HlyA antigen from *E. coli* K-12 *rfaH*⁺ versus *rfaH1.8*. Whole *E. coli* cultures were subjected to immunoblot analysis as described in Materials and Methods. The amount of culture analyzed is indicated below each lane. Lanes: 1, 2, and 5 to 7, RZ4500 (*rfaH*⁺); 3, 4, and 8 to 10, WAM1925 (*rfaH1.8*). Cultures in lanes 1 to 4 were transformed with pSF4000 (*hlyCABD*). Cultures in lanes 5 to 10 were transformed with pWAM823 (*hlyCA*). The data shown are representative of three independent assays.

and *rfaH1.8* (WAM2097). As a control for general transcription efficiency from the vector pACYC184, we assayed chloramphenicol acetyltransferase activity (FAST-CAT; Molecular Probes, Eugene, Oreg.) from WAM1925 and RZ4500 transformed with pSF4000. No significant difference in enzyme activity between the strains was detected (data not shown). The antisense RNA probes used and the results of representative RNase protection assays are shown in Fig. 3.

The steady-state levels of *hlyCABD* mRNA in both *rfaH1.8* mutants, WAM2097 and WAM1925, were significantly lower than the levels of *hlyCABD* mRNA in isogenic *rfaH*⁺ strains WAM2096 and RZ4500, respectively. Ratios of RNA protected in WAM2096 versus WAM2097 (Fig. 3B) were 7.5-fold for the *hlyC* *SmaI-BamHI* probe, 17-fold for the *hlyCA* *RsaI-PstI* probe, 15-fold for the *hlyA* *DraI-PstI* probe, and 18-fold for the *hlyBD* *AccI-EcoRI* probe. The ratios of RNA protected in RZ4500 versus WAM1925 (Fig. 3C) were less than 2-fold for the *hlyC* *SmaI-BamHI* probe, 6-fold for the *hlyCA* *RsaI-PstI* probe, 7-fold for the *hlyA* *DraI-PstI* probe, and 11-fold for the *hlyBD* *AccI-EcoRI* probe. The sets of shorter *hlyC* *RsaI-PstI* signals protected by *E. coli* J96 and K-12 RNAs were due to RNase E cleavage in the *RsaI-PstI* region of *hlyC* (21). This cleavage event was not affected by the *rfaH1.8* mutation (21). These data indicate that diminished HlyA protein levels in the *rfaH1.8* mutants are a result of decreased levels of *hly* mRNA.

Analysis of *hlyCABD* mRNA decay in *E. coli rfaH*⁺ versus *rfaH1.8*. The decrease in steady-state levels of *hly* mRNA from *E. coli rfaH1.8* versus *rfaH*⁺ could be due to a decrease in mRNA synthesis or to an increase in mRNA decay. To distinguish between these possibilities, we determined the half-lives of mRNA within the *hlyA* and *hlyBD* regions of the operon from RZ4500 (*rfaH*⁺) and WAM1925 (*rfaH1.8*) harboring pSF4000 (Fig. 4). The half-lives of 19 ± 0.01 and 18 ± 2.1 min for the *hlyA* gene mRNAs from RZ4500 and WAM1925, respectively, were not significantly different (Fig. 4A). The half-lives of 2.9 ± 0.4 and 3.2 ± 0.6 min for the *hlyBD* gene mRNAs from RZ4500 and WAM1925, respectively, were also not significantly different (Fig. 4B). These data demonstrate that the loss of steady-state *hly* mRNA from *E. coli rfaH1.8* is not a result of increased *hly* mRNA decay.

The pSF4000 *hlyCABD* promoter is not associated with RfaH-enhanced hemolysin expression. Our results indicate that RfaH affects *hlyCABD* mRNA synthesis rather than decay. Decreased mRNA synthesis may be due to decreased transcript initiation, elongation, or both. Primer extension analyses were performed to determine the steady-state levels and loca-

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FIG. 3. RNase protection analysis of steady-state *hly* mRNA levels in *E. coli rfaH*⁺ versus *rfaH1.8*. Cultures were grown to an OD₆₀₀ of 0.50. Extraction of total RNA, antisense probe construction, and RNase protection analysis are described in Materials and Methods. The data shown are representative of three independent RNA isolations. (A) Map of *hlyCABD* operon antisense RNA probes used in this study. (B) Autoradiographs of radiolabelled antisense RNA probes protected from RNase T1 digestion following hybridization to serial dilutions of total RNAs extracted from WAM2096 (*rfaH*⁺) and WAM2097 (*rfaH1.8*). (C) Autoradiographs of radiolabelled antisense RNA probes protected from RNase T1 digestion following hybridization to serial dilutions of total RNAs extracted from RZ4500 (*rfaH*⁺) and WAM1925 (*rfaH1.8*) harboring pSF4000. Amounts of total RNA probed are indicated below the lanes.

tions of *hly* mRNA 5' ends from *E. coli rfaH*⁺ and *rfaH1.8*. By using a primer complementary to bases 424 to 444 of the pSF4000 *hlyCABD* operon, we detected five products that extended 132 to 119 bases on RNAs from RZ4500 (*rfaH*⁺) and WAM1925 (*rfaH1.8*) harboring pSF4000 (Fig. 5). No primer was extended in the absence of pSF4000 (data not shown). The steady-state level of *hly* 5' ends from WAM1925 harboring pSF4000 was twofold lower than that in RZ4500 harboring the same hemolysin determinant. Attempts to reverse transcribe *hlyCABD* leader RNA from WAM2096 and WAM2097 by using three nonoverlapping primers complementary to that

region failed to produce any detectable signals (data not shown).

Although the 5' end of *hlyCABD* mRNA from the native chromosomal operon remains to be identified, results of primer extension analyses of mRNA transcribed from the recombinant *hlyCABD* operon suggest that the decrease in *hlyCABD* mRNA from *E. coli rfaH1.8* compared with *rfaH*⁺ (Fig. 3) was not at the level of *hly* transcript initiation. The results of these experiments also necessitate modification of the location of the previously published pSF4000 *hlyCABD* 5' end from base 334 to base 312. Earlier mapping of the

FIG. 4. Determination of *hly* mRNA half-lives ($t_{1/2}$). RNA extraction, anti-sense RNA probe construction, and analysis of *hly* mRNA decay by RNase protection were done as described in Materials and Methods. The plots of mRNA concentration as a function of time are average results of three independent RNA isolations. Standard deviations of half-lives were calculated from the slopes of individual curves. Error bars represent standard errors of individual time points. (A) *hlyA* mRNA decay in RZ4500 (*rfaH*⁺)/pSF4000 (dark symbol) and WAM1925 (*rfaH1.8*)/pSF4000 (open symbol). (B) *hlyB* mRNA decay in RZ4500 (*rfaH*⁺)/pSF4000 (dark symbol) and WAM1925 (*rfaH1.8*)/pSF4000 (open symbol).

pSF4000 *hlyCABD* promoter employed a primer that produced 266- and 268-base products (36). Experiments in this study utilized a primer closer to the start of *hlyCABD* transcription, resulting in more precise mapping of the pSF4000 *hlyCABD* 5' end.

The primer extension data failed to correlate the level of the *hlyCABD* mRNA decrease with a significant decrease in transcription from the *hlyCABD* promoter in WAM1925 harboring pSF4000. As an alternative test of *hlyCABD* promoter involvement in RfaH-enhanced hemolysin expression, we transformed RZ4500 (*rfaH*⁺) and WAM1925 (*rfaH1.8*) with pWAM750, a subclone of *hlyCABD* with a 168-bp *Pst*I deletion that removes the pSF4000 *hly* promoter. Figure 6 shows that in the absence

FIG. 5. Analysis of *hlyCABD* 5' ends in *E. coli* K-12 *rfaH*⁺ versus *rfaH1.8*. Samples (20 μ g) of total RNA from RZ4500 (*rfaH*⁺)/pSF4000 and WAM1925 (*rfaH1.8*)/pSF4000 were used as templates for extension of a primer hybridizing to bases 424 to 444 of pSF4000 as described in Materials and Methods. Non-coding strand bases corresponding to 3' ends of primer extension products are depicted by capital letters next to the image, and the complementary coding strand bases are indicated by boldface capital letters in the operon sequence below. -10 and -35 sequences, as well as the *hlyC* translational start, are indicated in capital letters in the operon sequence. The numbers in parentheses indicate base pairs between highlighted sequences, and the numbers below the sequence indicate the location, in base pair numbering, of those features in the pSF4000 DNA sequence.

of the pSF4000 *hly* promoter, transcript initiation from a downstream *hlyCABD* sequence that was not utilized in the intact construct (36) still required RfaH for synthesis of wild-type levels of HlyA.

DISCUSSION

E. coli protein RfaH was shown by Bailey et al. (1) and Wandersman and Letoffe (34) to be required for expression of *E. coli* hemolysin. Bailey et al. proposed that RfaH is required for increased initiation of *hlyCABD* transcription (1). Wandersman and Letoffe concluded that RfaH indirectly affects *hlyCABD* expression by enhancing synthesis of LPS which, they propose, is required for correct membrane insertion of TolC, an outer membrane protein required for hemolysin transport (34). To address these conflicting models, the effects of nine independent alleles of *rfaH* on expression of a hemolysin determinant of chromosomal origin were investigated. RfaH enhances expression of promoter-distal genes in several *E. coli* gene clusters, including *rfaQ-K* (24, 26), the homologous *rfa* locus in *S. typhimurium* (5), *traY-Z* (4), and *rplK-rpoC* (25). Recently, RfaH was shown to be required for wild-type *E. coli* group II capsular polysaccharide expression. The data suggested that RfaH enhances type II capsular expression directly at the level of *kps* transcription rather than indirectly as a function of the LPS chemotype (30). The potential role for RfaH in antitermination in these operons prompted a test of the hypothesis that RfaH is involved in elongation of transcription in the *hlyCABD* operon, a model not examined in previous

FIG. 6. Analysis of HlyA antigen from a promoter deletion *hlyCABD* construct in *E. coli* K-12 *rfaH*⁺ versus *rfaH1.8*. Cultures were grown to an OD₆₀₀ of 0.80. Immunoblot analysis was performed as described in Materials and Methods. The operon schematic depicts the location of the *Pst*I deletion removing the pSF4000 *hlyCABD* promoter. Lanes: 1 to 3, 6 to 8, and 12, RZ4500 (*rfaH*⁺); 4, 5, and 9 to 11, WAM1925 (*rfaH1.8*). Cultures in lanes 1 to 5 were transformed with pSF4000. Cultures in lanes 6 to 11 were transformed with pWAM750. The amount of whole *E. coli* culture assayed is indicated below each lane. The data shown are representative of three independent assays.

studies. In this communication, evidence is provided that, contrary to both previous models, RfaH enhances elongation of *hlyCABD* transcription and indirectly enhances hemolytic activity at the level of LPS-dependent HlyA toxicity.

The initial characterization of extracellular HlyA protein concentrations and hemolytic activities from a panel of *rfaH* alleles demonstrated that the effects of *rfaH* mutations on hemolysin expression are allele specific. The null *rfaH1.8* allele (1) was most detrimental, overall, to hemolysin expression. Alleles *rfaH11* and *rfaH13*, which conferred strong reductions in *tra* and *rfa* expression (4, 14), greatly reduced HlyA expression. Beutin et al. showed that the effects of the *rfaH11* and *rfaH13* mutations on *tra* gene expression were suppressed by the *rho-15* allele (4), supporting a role for RfaH in transcript elongation. The effect of *rho* on hemolysin expression in *rfaH* mutants remains to be determined.

The *rfaH1* allele, which had little effect on *hly* expression, also had minimal effect on *tra* and *rfa* expression (3). Allele *rfaH10.4* contains a Tn5 insertion that, on the basis of Southern (1) and PCR (21) analyses, may be located between the *rfaH* transcriptional control region and the *rfaH* open reading frame. This allele may confer reduced levels of wild-type RfaH protein. The *rfaH3* allele reduced hemolytic activity to a much greater extent than HlyA protein levels. Creeger et al. (8) showed that the *rfaH3* allele reduced levels of *rfa*-encoded glycosyltransferases only 5- to 10-fold, as opposed to the 50-fold reduction conferred by *rfaH11*. *rfaH3* was also not as detrimental to F transfer efficiency as allele *rfaH11* or *rfaH13* (3). This allele may not strongly affect transcription of *hly*, *tra*, or *rfa*. However, even a mild reduction in synthesis of LPS biosynthetic enzymes dramatically reduced hemolytic activity. All of the non-null *rfaH* mutations retain some function, and all are recessive to the wild-type *rfaH*⁺ allele (3). Compilation of these data, in conjunction with sequence analysis of these mutations, should provide insight into the structure and function of RfaH. The null *rfaH1.8* allele was the focus of further analyses in this study.

The model that states that RfaH enhances hemolysin expression indirectly at the level of HlyA transport (34) was tested by expressing a subclone of the *hlyCABD* operon that was deleted in secretion genes *hlyBD* in *E. coli* *rfaH*⁺ and *rfaH1.8* (Fig. 2). One would predict that if mutations in *rfaH* reduced HlyA expression by interfering with secretion of HlyA, then removal of the hemolysin transport genes should reduce the expression of HlyA in an *rfaH*⁺ cell to the same level as

HlyA expression in an *rfaH1.8* cell. However, results of Western analyses of intracellular HlyA expression from the *hlyCA* construct versus the *hlyCABD* construct in *E. coli* K-12 *rfaH*⁺ and *rfaH1.8* indicated that HlyA expression was sensitive to RfaH independent of hemolysin transport. These data support a direct role for RfaH in HlyA synthesis rather than an indirect role in HlyA secretion.

A previous study demonstrated that steady-state levels of *hlyA* mRNA were reduced in the null *rfaH1.8* mutant (1). In this study, RNase protection analyses were performed to address the role of RfaH in expression of steady-state transcript levels across the entire *hlyCABD* operon. The results show that a null *rfaH1.8* mutant conferred a less-than-2-fold reduction in promoter-proximal RNA and an up-to-11-fold reduction in the promoter-distal *hly* message from a recombinant *hlyCABD* operon in *E. coli* K-12. In *E. coli* J96, the *rfaH1.8* mutation conferred a 7.5- to 18-fold reduction in the promoter-proximal-to-distal *hly* message from a native chromosomal *hlyCABD* operon. The null *rfaH1.8* allele did not decrease *hlyCABD* mRNA half-lives, indicating that RfaH affects transcript synthesis rather than decay. These results support the model for RfaH involvement in transcription in *E. coli*.

Analysis of *hlyCABD* mRNA 5' ends by primer extension indicated that the null *rfaH* mutation did not reduce transcript initiation of the recombinant *hlyCABD* operon from pSF4000 greater than twofold compared with the *rfaH*⁺ allele. For reasons that remain unexplained, primer extension and RNase protection assays of *hlyCABD* leader RNA performed with RNA extracted from either *E. coli* J96 strain failed to produce any detectable signal. The effect of RfaH on transcript initiation from the native chromosomal *hlyCABD* construct remains to be shown. However, the hypothesis that *hlyCABD* transcript initiation is not sensitive to RfaH is supported by Western analyses of HlyA protein levels from a pSF4000 promoter deletion construct in *E. coli* K-12 *rfaH*⁺ and *rfaH1.8* (Fig. 6). In the *Pst*I promoter deletion mutant, transcription from an alternate start site closer to *hlyC* did not relieve the dependence of hemolysin expression upon RfaH. These results are consistent with the model for RfaH involvement in transcript elongation.

Our model proposes that RfaH enhances transcription within the *hly* leader and into *hlyC* mRNA. This model is consistent with data from both the native chromosomal and recombinant plasmid *hlyCABD* operons. The differences in steady-state transcript levels between the *rfaH*⁺ and *rfaH1.8* strains of *E. coli* J96 versus *E. coli* K-12 may be an effect of *hlyCABD* copy number. Identification of the native *hlyCABD* mRNA 5' end will allow further development of our model.

What is the mechanism by which RfaH enhances transcript elongation in *hly* and other RfaH-sensitive operons, and what are the signals necessary for this activity? Hobbs and Reeves (17) recognized a 39-bp conserved sequence (JUMPstart) in the noncoding region upstream of several gene clusters whose expression is enhanced by RfaH, including *rfaQ-K* and *tra*. Stevens et al. (30) identified these sequences upstream of the *kps* operon and 5' to the *hlyC* gene from a native plasmid-borne *hlyCABD* operon. This provided the first example of sequence conservation among RfaH-regulated operons; however, the significance of this sequence is unknown. In our analysis, we recognized a portion of the JUMPstart sequence, including one of a pair of highly conserved direct repeats, GGCGGTAG, at bases 463 to 470 within the pSF4000 *hly* leader region, 150 bases downstream of the transcription initiation site mapped in our primer extension analysis. This sequence was not removed in the *hlyCABD* promoter deletion construct described in Fig. 6, which still retained RfaH sensi-

tivity. We transformed a subclone of pSF4000 with deletion of all bases 5' to the *Bam*HI site in *hlyC* (including the *hly* promoter and leader sequence and two-thirds of the first open reading frame) into *E. coli* *rfaH*⁺ and *rfaH*1.8. The results indicated that synthesis of wild-type levels of intra- and extracellular HlyA from the *tet* promoter in pACYC184 did not require RfaH (21). Although this is a crude test of the effect of JUMPstart on *hlyCABD* expression, the preliminary data do support a model in which RfaH enhances transcript elongation within operons containing the JUMPstart sequences.

In summary, this study demonstrated the effects of *rfaH* mutations on *hlyCABD* expression at the level of transcript initiation, transcript elongation, message stability, intra- and extracellular HlyA protein levels, and hemolytic activity. Our data contradict the previous models of RfaH-enhanced *hly* transcript initiation and RfaH-enhanced HlyA secretion. Instead, our results provide evidence that RfaH is necessary for transcript elongation within the *hly* operon. In addition, introduction of the *rfaH*1.8 allele into a wild clinical isolate of *E. coli* and analyses of *hly* operon expression under this condition constitute the first reported attempt to address the effect of a putative regulatory protein on the expression of the *hlyCABD* operon from a native chromosomal locale. The present study provides a preliminary understanding of RfaH-enhanced hemolysin expression. Further work is needed to define the mechanism of elongation and the precise locations of RfaH targets within *hlyCABD* and other RfaH-affected operons in *E. coli*.

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