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Pleiotropic Effects of a Mutation in *rfaC* on *Escherichia coli* Hemolysin

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Several genes involved in the lipopolysaccharide (LPS) biosynthetic pathway have been shown to affect the expression or activity of *Escherichia coli* hemolysin (Hly), a secreted cytotoxin that is the prototype of the RTX family of toxins. To further study this relationship, *E. coli* K-12 strains harboring mutations in the LPS biosynthetic genes *rfaS*, *rfaQ*, *rfaJ*, *rfaP*, and *rfaC* were transformed with a recombinant plasmid harboring the *hlyCABD* operon and examined for their effects on extracellular expression and hemolytic activity. A mutation in *rfaC* that affected both extracellular expression and activity of Hly was studied in greater detail. This mutation led to a growth-phase-dependent decrease up to 16-fold in the steady-state level of extracellular HlyA, although transcription and secretion of HlyA were decreased no more than 2-fold. Specific hemolytic activity in toxin produced from the *rfaC* mutant strain was significantly reduced, in a growth-phase-dependent manner. With the *rfaC* gene supplied in *trans*, both the decreased expression and activity of Hly were restored to wild-type levels. Hly from the *rfaC* mutant strain exhibited much slower kinetics of hemolysis, a more rapid rate of decay of activity, and greater formation of apparently inactive HlyA-containing aggregates in culture supernatants than was exhibited in the wild-type strain. A model is proposed for a physical interaction between LPS and Hly in which LPS with intact inner core participates in forming or maintaining an active conformation of Hly and helps to protect it from aggregation or degradation.

Escherichia coli hemolysin (Hly) is a well-characterized member of the RTX family of cytotoxins, which are associated with a wide variety of gram-negative human and animal pathogens (recently reviewed in reference 24). Hly is a virulence factor associated with urinary tract and other extraintestinal *E. coli* infections (25). The 110-kDa protein component of this toxin, HlyA, is expressed from the *hlyCABD* operon as pro-HlyA. Pro-HlyA is activated to HlyA by an acylation event requiring HlyC and is secreted through a *sec*-independent pathway involving both HlyB and HlyD as well as the genetically unlinked TolC outer membrane protein (24).

Several studies suggest a connection between Hly and another gram-negative toxin, lipopolysaccharide (LPS). The overall structure of *E. coli* LPS (recently reviewed in reference 18) consists of lipid A, a heptose-rich inner core, an outer core of hexoses, and O antigen. *E. coli* K-12 strains produce rough LPS, in which no O antigen is expressed. A number of LPS biosynthetic genes, including the *rfa* gene cluster that participates in core synthesis, have been identified. Mutations specifically affecting inner core assembly or modification yield a deep rough phenotype, which includes resistance to LPS-specific bacteriophages, decreased expression of some outer membrane proteins, including OmpF and OmpC, and susceptibility to hydrophobic compounds such as novobiocin (14).

Ostolaza et al. (13) and Bohach and Snyder (5) purified Hly by size exclusion and ion-exchange chromatography and demonstrated the presence of the LPS-associated 3-hydroxytetradecanoic acid and 2-keto-3-deoxyoctulonic acid (KDO) in active fractions of Hly. Further, Bohach and Snyder showed that hemolytically active fractions affinity purified from Hly-producing culture supernatants with an anti-HlyA monoclonal antibody (MAb) also reacted with an anti-LPS MAb, and they postulate that the two toxins may exist in a complex (4).

Genetic evidence for a connection between Hly and LPS is provided by studies in which chromosomal mutations in *E. coli* K-12 strains were found to affect expression and activity of Hly produced from a recombinant plasmid within the mutant strains. Three such mutations were localized to *rfaH*, *rfaP*, and the *galU* locus, all of which are involved in LPS biosynthesis and whose mutants yield a deep rough LPS phenotype (22, 23). A mutation in the *galU* locus impairs secretion of HlyA (23), while the *rfaP* mutant allele greatly decreases activity of Hly but has no effect on its expression or secretion (22). HlyA produced from the *rfaP* mutant migrates in sucrose density gradients as a larger species than the wild-type HlyA does, indicating formation of a molecular complex that reduces activity (22). Further, chaotropic agents increase the level of activity from the mutant strain to wild-type levels (22).

RfaH, a positive effector of the *rfa* operon as well as several other *E. coli* operons, also positively affects expression of HlyA (1, 11, 23) by enhancing elongation of *hlyCABD* mRNA, potentially via an antitermination process (11). Hemolytic activity is also reduced in various *rfaH* mutant strains (11).

In this study, we examined a panel of LPS biosynthetic mutants for their effects on the extracellular expression and activity of Hly. Mutants tested included *rfaS*, *rfaQ*, *rfaJ*, *rfaP*, and *rfaC*. *rfaS* and *rfaQ* function in modification of the intact outer core backbone (9, 10), whereas *rfaJ* encodes a sugar transferase that adds the terminal galactose residue of the outer core backbone (15). *rfaP* functions to modify the inner core heptoses, with a resulting charge difference in the LPS molecule (14). *rfaC* encodes a heptosyltransferase which adds the first heptose of the inner core to the lipid A-attached KDO

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant phenotype	Reference or source
E. coli strains		
CL632	F$^-$ *thi-1 tfr-8 rfaC*	6
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CS2767	*his leu proA argT his thi galK* Δ*lac trpE non mtl yxl ara rpsL sup*$^+$	C. Schnaitman
CS2198	*rfaJ19*::Tn*lac* derivative of CS2767; Kan^r	C. Schnaitman
CS2209	*rfaG13*::Tn*lac eps-5*::Tn*10* derivative of CS2767; Kan^r Tet^r	C. Schnaitman
CS2210	*rfaP23B*::Tn*lac cps-5*::Tn*10* derivative of CS2767; Kan^r Tet^r	C. Schnaitman
CS2274	*rfaB7*::Tn*lac* derivative of CS2767; Kan^r	C. Schnaitman
CS2774	*rfaQ9*::Tn*lac* derivative of CS2767; Kan^r	C. Schnaitman
CS2775	*rfaS2007*::Tn*lac* derivative of CS2767; Kan^r	C. Schnaitman
Plasmids		
pACYC184	Cm^r	This laboratory
pCG26	*rfaC* gene on 1-kbp *Alu*I-*Dra*I fragment in pUC8; Amp^r	6
pSF4000	*hlyCABD* in pACYC184	25
Recombinant strains		
WAM1583	pSF4000 in CS2767 (*rfa*$^+$)	This study
WAM1584	pSF4000 in CS2198 (*rfaJ*)	This study
WAM1585	pSF4000 in CS2209 (*rfaG*)	This study
WAM1586	pSF4000 in CS2210 (*rfaP*)	This study
WAM1587	pSF4000 in CS2274 (*rfaB*)	This study
WAM1588	pSF4000 in CS2774 (*rfaQ*)	This study
WAM1589	pSF4000 in CS2775 (*rfaS*)	This study
WAM1821 (Hly$_{rfaC}$)	pSF4000 in CL632 (*rfaC*)	This study

sugar (6). In *rfaC* mutant strains, LPS is truncated at this point, and the resultant LPS moiety consists of lipid A and two KDO molecules (6). Mutations in some LPS biosynthetic genes, especially *rfaP*, demonstrate varied pleiotropic effects on the cell (18). Such effects have not been reported for *rfaC* (6). Hly expression and activity from a *rfaC* mutant which possesses the most severely truncated LPS moiety of the mutants was examined in detail; this analysis showed that there is a decrease in the steady-state level of extracellular HlyA and reduced activity that was less stable than that of the wild-type Hly. These data provide further evidence for a link between these two toxins and also suggest a specific role of LPS in stabilizing the toxin's active conformation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and materials. All chemicals were purchased from Sigma (St. Louis, Mo.) unless stated otherwise. Strains and plasmids used are described in Table 1. DNA manipulations were performed by standard methods (17). Plasmids were maintained in *E. coli* K-12 CL633 or CS2767. CL632, CL633, and pCG26 were provided by William Coleman, Jr. (6). All other *rfa* mutants and CS2767 were provided by Carl Schnaitman.

Culture growth and toxin preparations. Bacterial cultures were grown in Luria-Bertani (LB) broth containing the appropriate antibiotics (chloramphenicol, 10 μg per ml; ampicillin, 50 μg per ml; kanamycin, 25 μg per ml; and tetracycline, 10 μg per ml), with aeration, to an optical density at 600 nm (OD_{600}) of 0.8 unless indicated otherwise. Growth-phase-dependent assays were performed with each culture inoculated at an OD_{600} of 0.05 from overnight broth cultures, and samples were harvested at the indicated OD_{600}. Cells were pelleted by centrifugation, and toxin-containing supernatants were passed through a 0.45-μm-pore-size Acrodisc filter (Gelman Sciences, Ann Arbor, Mich.), kept on ice, and used within 1 h of harvest. WAM1821 was routinely checked after growth for sensitivity to 30 μg of novobiocin per ml, a phenotypic consequence of the truncated LPS, to control for reversion of the point mutation in *rfaC*.

Hemolysis assays. Sheep erythrocytes (RBC) were obtained fresh, defibrinated with glass beads, stored at 4°C, and used within 1 week of harvest. Hemolysis assays were performed as described previously (3). Briefly, serial dilutions of toxin-containing culture supernatants were incubated with 1% RBC suspensions in 0.85% NaCl–20 mM $CaCl_2$ at 37°C for 1 h. Hemolysis was measured spectrophotometrically at A_{570} and reported as HU_{50} (reciprocal of the titer of supernatant required for 50% lysis of RBC) or initial slope of lytic activity. Initial slope (A_{570} units per microliter of supernatant) was calculated as the slope of the line made by the first three points above background in dose-response curves of hemolysis. Specific activity was calculated by normalizing the initial slopes to nanograms of HlyA and reported as A_{570} units per nanogram of HlyA protein.

For kinetic studies, toxin-containing supernatants were diluted in saline and incubated with a 1% (final concentration) suspension of RBC for times ranging from 15 s to 70 min at 37°C, after which RBC were removed by centrifugation, and the level of hemolysis was read spectrophotometrically at A_{570}. Rates of hemolysis were determined from the slopes of curves made by the three earliest time points at which lysis was observed. Hemolytic rates were compared between strains at dilutions of supernatant such that the HlyA concentrations differed by less than twofold, as confirmed by immunoblot analysis.

To determine rates of decay of activity, samples of culture supernatants were incubated immediately after harvest at 0 to 4, 22, or 37°C and assayed for hemolytic activity at 1, 2, 4, and 8 h postharvest. Supernatants from the *rfaC*$^+$ strain were diluted 1:16 in saline to approximate the concentration of HlyA in the *rfaC* strain. Immunoblot analysis confirmed that the concentrations of HlyA in the diluted supernatants were less than twofold from those of the *rfaC* strain.

Electrophoresis and immunoblot analysis. The preparation and identification of HlyA by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Coomassie brilliant blue staining, and immunoblot analysis were done as described previously (3). HlyA was quantified by electrophoretic separation of toxin-containing supernatants alongside serial dilutions of known amounts of a 116-kDa β-galactosidase standard. Gels were stained with Coomassie brilliant blue, and densitometric tracings of the 110-kDa bands were used to estimate the toxin concentrations within the standard curve of β-galactosidase concentrations. Figures of immunoblots are scanned images of the lumnigraphs, as described elsewhere (11).

RNase protection assays. RNA manipulations and RNase protection assays were performed as described elsewhere (11), using RPAII RNase protection reagents from Ambion (Austin, Tex.). Briefly, bacterial RNA was prepared from 5 to 10 ml of broth culture at the indicated OD_{600} by hot phenol extraction (7). The α-^{32}P-labeled antisense RNA probe internal to *hlyA* was derived by runoff transcription from a DNA template covering bases 3790 through 4277 of *hlyCABD* (11). Denatured target RNA was hybridized with excess probe RNA overnight at 45°C, followed by RNase T_1 digestion. Reactions were terminated by precipitation of the RNA species, which were then resuspended in loading buffer (11) and electrophoretically separated on 6% acrylamide–7 M urea gels at 300 V. Protected *hlyA* mRNA fragments were detected by autoradiography. Relative levels of *hlyA* mRNA were estimated by densitometric tracings of autoradiographs. Data are represented by scanned images of the autoradiographs, as described elsewhere (11).

Pulse-chase assays. Bacterial cultures were grown in M9 minimal salts medium (12) supplemented with 0.6% glucose and 0.0005% thiamine. At the indicated OD_{600}, culture material was labeled with 50 μCi of [^{35}S]methionine per ml at 37°C for 20 s, followed by addition of a 1,000-fold molar excess of unlabeled methionine. Samples were removed at 10, 20, 60, 140, and 300 s after addition of unlabeled methionine, and 500-μl aliquots of supernatant from these samples were processed for electrophoresis as described above. Radiolabeled HlyA was detected by fluorography, and relative levels of secreted toxin were determined from densitometric tracings of fluorographs. Data are represented by scanned images of the fluorographs, as described elsewhere (11).

HlyA sedimentation analysis. Fresh, toxin-containing culture supernatants were sequentially passed through 0.45- and 0.2 μm-pore-size Acrodisc filters and subjected to ultracentrifugation at 150,000 × *g* for 3.5 h in a Beckman Ti50.2 rotor at 4°C under conditions that pellet membrane vesicles and other large (≥~500-kDa)-molecular-mass particles (8). The cleared supernatants were removed from pellets and kept on ice until use in hemolysis and immunoblot analyses.

RESULTS

Effects of *rfa* mutant alleles on extracellular expression and activity of Hly. *E. coli* CS2767, CL633, and several derivatives with mutations in *rfa* genes were transformed with pSF4000 (Table 1) to examine effects of these mutations on Hly. All *rfa* mutant transformants produced zones of hemolysis on blood agar plates that were similar in size to those of the isogenic *rfa*$^+$ transformants. As an initial survey of the effects of *rfa* mutants on the extracellular expression and activity of Hly,

TABLE 2. Hemolytic activity in culture supernatants from *rfa* mutant strains

HlyA-producing strain[a]	Mutant *rfa* allele	HU_{50}[b]	Fold difference vs wild type
WAM1583[c]		4,294 ± 927	
WAM1589	*rfaS*	5,084 ± 364	0.84
WAM1588	*rfaQ*	4,348 ± 710	0.99
WAM1584	*rfaJ*	1,361 ± 507	3.2

[a] Activities of untransformed mutants or mutants transformed with the vector pACYC184 were <2 HU_{50}.
[b] Reciprocal of the titer at which 50% of RBC are lysed. Each value represents the mean ± standard error of the mean from at least three independent assays.
[c] Isogenic parent of the *rfaS*, *rfaQ*, and *rfaJ* mutants.

culture supernatants of these transformants harvested at an OD_{600} of 0.8 (late logarithmic growth) were tested for hemolytic activity and HlyA antigen levels. As summarized in Table 2, lesions in *rfaS* and *rfaQ* had little effect on hemolytic activity of Hly, while the *rfaJ* mutant demonstrated nearly a fourfold decrease in activity. Immunoblot analysis showed no effect on the steady-state level of HlyA antigen secreted to the supernatant in these strains compared with the isogenic rfa^+ strain (data not shown). No hemolytic transformant was obtained from CS2209, which harbors a mutation in *rfaG*. Additionally, hemolytic transformants of strains CS2274 and CS2210, harboring mutations in *rfaB* and *rfaP*, respectively, grew very slowly and could not be stably maintained upon storage at −70°C in LB–50% glycerol.

To examine the effects of the *rfaP* allele, supernatants from three independent *rfaP* transformants were assayed upon the first passage after transformation. No effect was observed on the level of HlyA antigen expressed in the supernatant; however, the activity varied with each transformant, with either undetectable or wild-type levels of hemolytic activity (data not shown).

The lesion in *rfaC* affected both the extracellular expression and activity of Hly. Immunoblot analysis demonstrated a clear reduction in the steady-state level of extracellular HlyA antigen present in supernatants containing toxin produced from the *rfaC* transformant (Hly_{rfaC}) compared with supernatants containing toxin produced from the transformed parent strain (Hly_{rfaC^+}) (Fig. 1). Quantitative electrophoretic analysis (described in Materials and Methods) showed that supernatants from the $rfaC^+$ transformant harvested at OD_{600} of 0.8 contained 3.5 ± 0.1 μg of HlyA per ml (n = 3), while supernatants from the *rfaC* transformant had 0.46 ± 0.01 μg of HlyA per ml (n = 3), an eightfold reduction. The steady-state level of extracellular HlyA antigen was fully restored to that of wild type when the mutant strain was complemented with the intact *rfaC* gene supplied in *trans* (Fig. 1).

As a further refinement, the hemolytic activity of Hly_{rfaC} was also less than that of Hly_{rfaC^+}, when the activities were calculated as the initial slope of hemolysis dose-response curves normalized to nanograms of HlyA protein. The specific hemolytic activity of Hly_{rfaC^+} was 344 ± 19 A_{570} units per ng of protein (n = 27), while Hly_{rfaC} demonstrated a broad range of specific activities, averaging 44 ± 8.7 A_{570} units per ng of protein (n = 66). The specific activity was restored to near wild-type levels in toxin produced from the complemented mutant ($Hly_{rfaC/prfaC^+}$), which exhibited 273 ± 18 A_{570} units of activity per ng of protein (n = 16).

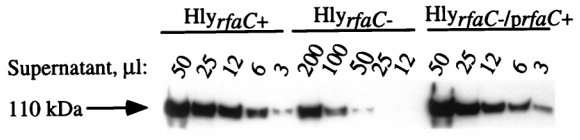

FIG. 1. Immunoblot of extracellular HlyA antigen from *E. coli* strains that are $rfaC^+$, *rfaC*, and $rfaC/rfaC^+$. Lanes: 1 to 5, serial twofold dilutions of Hly_{rfaC^+}; 6 to 10, serial twofold dilutions of Hly_{rfaC}; 11 to 15, serial twofold dilutions of $Hly_{rfaC/prfaC^+}$. Numbers above the lanes represent microliters of supernatant loaded. Proteins were detected with polyclonal anti-HlyA antiserum.

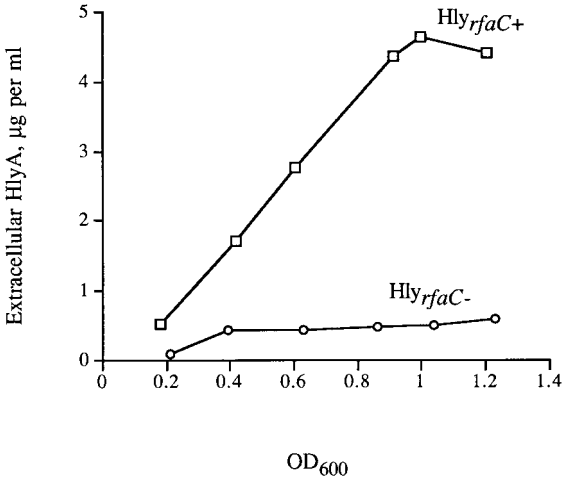

FIG. 2. Extracellular HlyA antigen production during growth of $rfaC^+$ and *rfaC* cultures. Squares indicate HlyA concentrations from the $rfaC^+$ strain, while circles depict HlyA concentrations from the *rfaC* strain. Data shown are representative of four independent assays.

Effect of *rfaC* lesion on extracellular expression of HlyA. The *rfaC* mutant, which produces the most severely truncated LPS moiety of the mutants tested, was examined further for its effects on Hly. Figure 1 demonstrates differences in the steady-state levels of extracellular HlyA from transformants that are $rfaC^+$, *rfaC*, or *rfaC* complemented in *trans* with the intact *rfaC* gene ($rfaC/prfaC^+$) when all cultures were harvested at an OD_{600} of 0.8. Immunoblot analysis of culture supernatants harvested at points throughout culture growth showed that differences in the steady-state levels of extracellular HlyA varied with the growth phase, as illustrated in Fig. 2. While HlyA accumulated in Hly_{rfaC^+} supernatants throughout log growth, there was little accumulation of HlyA in Hly_{rfaC} supernatants at any point during growth. Thus, in early to mid-logarithmic growth (OD_{600} of 0.2 to 0.5), no more than a 4-fold difference was observed in the production of extracellular HlyA antigen between the $rfaC^+$ and *rfaC* transformants, while the steady-state level of secreted HlyA antigen during late log growth (OD_{600} of 0.8 to 1.0) in the supernatant of the *rfaC* strain was approximately 16-fold less than that in the $rfaC^+$ strain. The steady-state cell-associated HlyA varied only two- to fourfold at the same OD_{600} (data not shown). The growth phase of each strain was also measured by determining the increase in CFU over time. These data support the observation that the pair of strains have similar growth rates (data not shown) but with the *rfaC* strain entering stationary phase sooner (OD_{600} of 0.7 to 0.9) than the $rfaC^+$ strain (OD_{600} of 0.9 to 1.1).

To examine whether the extracellular production of HlyA was affected at the level of transcription in the *rfaC* strain, RNA was isolated from $rfaC^+$ and *rfaC* transformants at several points during culture growth and analyzed by RNase protection for the relative levels of *hlyA* mRNA present in each sample. As shown in Fig. 3A, the steady-state level of *hlyA*

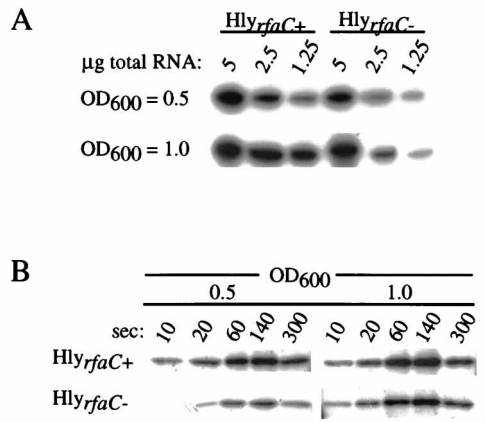

FIG. 3. Effects of *rfaC* on expression of HlyA. (A) Autoradiographs of radiolabeled antisense RNA probes protected from RNase digestion by hybridization to total RNA extracted from the indicated cultures harvested at an OD_{600} of 0.5 or 1.0. Numbers above the lanes indicate micrograms of total RNA probed in each reaction. Data shown are representative of three independent assays. (B) Autoradiographs of HlyA secreted from the indicated strains following a 20-s pulse with [^{35}S]methionine. Cultures were labeled at an OD_{600} of 0.5 or 1.0. Numbers above the lanes indicate the time in seconds after the label was chased with unlabeled methionine at which each sample was taken. Data shown are representative of two independent assays.

mRNA in the *rfaC* strain was twofold below that of the *rfaC*$^+$ strain at mid-log and stationary phases (OD_{600} of 0.5 and 1.0, respectively). Identical results were obtained with samples isolated at early and late log growth stages (OD_{600} of 0.2 and 0.8, respectively) (data not shown). As an internal control of plasmid-specific effects, the levels of transcription from pSF4000 in these cultures were assessed by a chloramphenicol acetyltransferase assay (FAST-CAT; Molecular Probes, Eugene, Oreg.). No differences in chloramphenicol acetyltransferase activity were observed between the *rfaC*$^+$ and *rfaC* strains at an OD_{600} of 0.8 (data not shown).

To assess if the *rfaC* mutation affects the efficiency of secretion of HlyA, *rfaC*$^+$ and *rfaC* cultures were radiolabeled in vivo at several points during culture growth with [^{35}S]methionine for 20 s and then chased with excess unlabeled methionine for 10 to 300 s. Supernatants from mid-log-and stationary-phase culture samples (OD_{600} of 0.5 and 1.0, respectively) taken after the addition of unlabeled methionine showed maximal accumulation of extracellular, labeled HlyA at 140 s postchase (Fig. 3B), indicating that the lesion in *rfaC* had no effect on the rate of secretion of ^{35}S-labeled HlyA. Identical results were obtained with samples isolated at early and late log growth stages (OD_{600} of 0.2 and 0.8, respectively) (data not shown). The signals of radiolabeled HlyA were twofold less in supernatants from the *rfaC* strain than from the *rfaC*$^+$ strain at all optical densities tested.

Effect of *rfaC* lesion on activity of HlyA. To examine the activity phenotype resulting from the *rfaC* lesion, the hemolytic activities of Hly$_{rfaC}$ and Hly$_{rfaC^+}$ were tested over a range of points throughout culture growth. As shown in Fig. 4, the hemolytic activities of Hly$_{rfaC}$ and Hly$_{rfa^+}$ varied during growth. The two strains showed similar levels of activity in early-log-phase growth (OD_{600} to 0.2 to 0.4). However, Hly$_{rfaC^+}$ activity increased throughout log growth, peaking at late log to early stationary phase (OD_{600} of 0.8 to 1.0) (Fig. 4A). In contrast, the activity of Hly$_{rfaC}$ peaked during mid-log phase and was rapidly and dramatically reduced at late log phase. Little activity was detectable in culture supernatants harvested at an OD_{600} of $\geq$1.0 (Fig. 4B).

Immunoblots of HlyA from *rfaC*$^+$ and *rfaC* transformants were probed with MAb D12, which recognizes only the acylated form of HlyA. These blots showed the same difference between extracellular HlyA levels in the two strains that was observed when the blots were probed with polyclonal anti-HlyA antiserum (data not shown), indicating that acylation at $HlyA_{K689}$ is not significantly affected in the *rfaC* transformant. Activity of Hly$_{rfaC}$ was recoverable to near wild-type levels when the toxin was precipitated in 60% ammonium sulfate and resuspended in 6 M urea buffer (data not shown), similar to results reported for the *rfaP* mutant (22).

To further characterize the less active hemolysin produced by the *rfaC* strain, the rates of hemolysis were measured for Hly$_{rfaC^+}$- and Hly$_{rfaC}$-containing supernatants from late log cultures. Figure 5 shows the results of a representative kinetic assay performed with supernatants at comparable concentrations of HlyA. Hly$_{rfaC^+}$ activity was detected within 90 to 120 s of the addition of RBC and led to complete lysis of the RBC

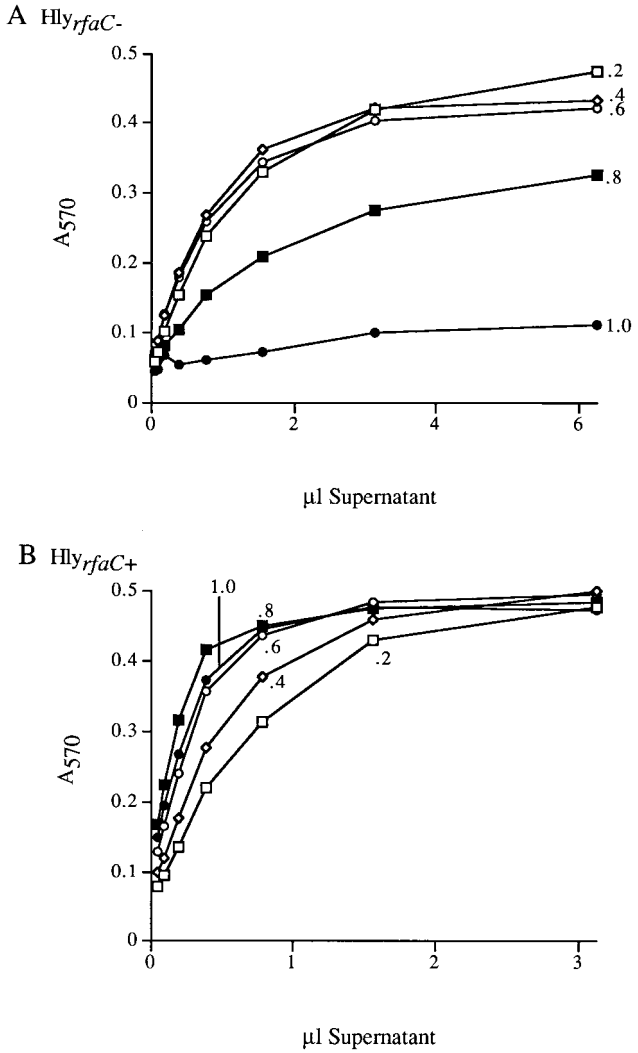

FIG. 4. HlyA activity during growth of *rfaC*$^+$ and *rfaC* cultures. (A) Hemolytic activity in Hly$_{rfaC}$-producing culture supernatants during growth. Samples were harvested and assayed at OD_{600} of 0.2 (open squares), 0.4 (diamonds), 0.6 (open circles), 0.8 (closed squares), and 1.0 (closed circles). Numbers indicate the OD_{600} represented by each curve. (B) Hemolytic activity in Hly$_{rfaC^+}$-producing culture supernatants during growth. Samples are depicted and labeled as in panel A. Data shown are representative of at least four independent assays.

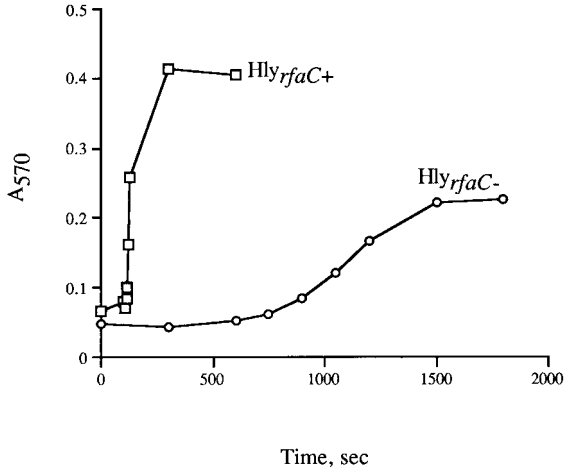

FIG. 5. Time course of hemolysis by Hly_{rfaC} and Hly_{rfaC^+}. Squares depict lysis by Hly_{rfaC^+}-containing supernatant; circles depict lysis by Hly_{rfaC}-containing supernatant. Supernatants were harvested at an OD_{600} of 0.8. The concentration of HlyA in supernatant from the $rfaC^+$ strain was diluted to be within twofold of the concentration of HlyA in the *rfaC* strain's supernatant. Data shown are representative of four independent assays.

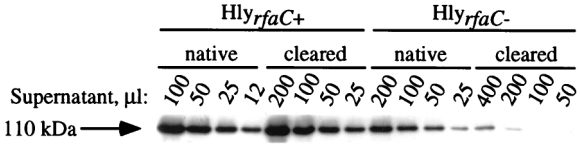

FIG. 6. Immunoblot of Hly_{rfaC^+}- or Hly_{rfaC}-containing supernatant prior to (native) or after (cleared) ultracentrifugation. Supernatants were harvested at an OD_{600} of 0.8. Numbers above each lane indicate the amount of supernatant represented in that lane. Proteins were detected with polyclonal anti-HlyA antiserum.

within 5 min. In contrast, no activity was detected in the first 10 min of assays with Hly_{rfaC}, and activity peaked and leveled off at 60 to 70 min without approaching 100% lysis. The rate of hemolysis by Hly_{rfaC^+} was 6.1 ($\pm$2.7) $\times$ 10^{-3} A_{570} units/s (n = 4), while the rate of hemolysis by Hly_{rfaC} was 1.2 ($\pm$0.4) $\times$ 10^{-4} A_{570} units/s (n = 4), a 50-fold decrease.

To examine whether the activity of Hly_{rfaC} may be more labile than that of Hly_{rfaC^+}, cell-free Hly_{rfaC^+}- and Hly_{rfaC}-containing supernatants from late log cultures were incubated at 0 to 4, 22, or 37°C and assayed for hemolytic activity at 1, 2, 4, and 8 h postharvest. The initial slope of activity in each supernatant at each temperature was plotted against time to measure half-lives of hemolytic activity. In these experiments, supernatant from the $rfaC^+$ strain was diluted to the concentration of *rfaC* HlyA. As shown in Table 3, the half-life of each strain was affected by the temperature of incubation. Regardless of temperature, however, the half-life of Hly_{rfaC} was threefold less than that of Hly_{rfaC^+}.

Relative sizes of native *rfaC*$^+$ and *rfaC* HlyA. Estimates of the size of active Hly reveal a range of heterogeneously sized species (5, 16, 21). Hly_{rfaC^+}- and Hly_{rfaC}-containing culture supernatants from late log cultures were subjected to ultracentrifugation, and the levels of HlyA antigen that remained soluble or were pelleted from each strain were compared to roughly assess the relative levels of large and small HlyA-associated particles in each supernatant. Figure 6 shows that about 50% of the HlyA in $rfaC^+$ supernatant ($HlyA_{rfaC^+}$) remained soluble under conditions in which only 6% of HlyA from *rfaC* supernatant ($HlyA_{rfaC}$) remained soluble, indicating that a much greater proportion of $HlyA_{rfaC}$ than of $HlyA_{rfaC^+}$ is associated with particles large enough to be pelleted under these conditions. The hemolytic activity as measured by HU_{50} that remained in the soluble fraction was reduced about twofold in each strain, indicating that the bulk of active Hly may not be associated with the large, pelleted material (data not shown). When Hly_{rfaC^+}- and Hly_{rfaC}-containing culture supernatants were diluted 1:1 in 8 M urea–saline and subjected to ultracentrifugation, no detectable $HlyA_{rfaC^+}$ antigen was pelleted, and the cleared Hly_{rfaC}-containing supernatant retained about 25% of the HlyA, fourfold more than without urea (data not shown).

TABLE 3. Stability of hemolytic activity

Temp (°C)	Half-life[a] (min)		Fold difference vs $HlyA_{rfaC^+}$
	$HlyA_{rfaC^+}$	$HlyA_{rfaC}$	
0–4	314 ± 51	104 ± 40	3.0
22	194 ± 17	73 ± 38	2.6
37	87 ± 8	28 ± 12	3.1

[a] Mean ± standard error of the mean from three independent assays.

Conformational differences between $HlyA_{rfaC^+}$ and $HlyA_{rfaC}$ present in culture supernatants were assessed by reactivity of native $HlyA_{rfaC^+}$, $HlyA_{rfaC}$, and $HlyA_{rfaC^+/prfaC}$ with a panel of 15 anti-HlyA MAbs and 5 MAbs raised against the RTX adenylate cyclase/hemolysin of *Bordetella pertussis* that are cross-reactive with HlyA (3). Dilutions of fresh, toxin-containing supernatants were applied to nitrocellulose via vacum suction through a Minifold II slot blot apparatus (Schleicher & Schuell) before blocking and probing with antibodies as described for immunoblot analysis. The MAb reactivity profiles did not vary significantly among Hly_{rfaC^+}, Hly_{rfaC}, and $HlyA_{rfaC^+/prfaC}$ samples (data not shown).

DISCUSSION

In this study, a subset of mutations in LPS biosynthesis genes were examined for their effects on the extracellular levels and activity of Hly. In addition to providing a further link between these two bacterial toxins, these data support the hypothesis of Bohach and Snyder (5) that LPS and HlyA may form a toxin complex and suggest a role for LPS in Hly activity, specifically that of stabilizing the active moiety of the exotoxin.

The LPS mutants reported previously to affect Hly expression or activity also produce deep rough LPS. In this study, we tested several mutant strains for their effects on Hly, and the results are consistent with the trend that deep rough mutations affect Hly activity. Neither *rfaS* nor *rfaQ* showed a significant effect on Hly expression or activity, whereas *rfaJ* demonstrated a slight decrease in hemolytic activity of Hly, indicating that intact outer core may aid the efficiency of Hly, but not to the extent that inner core LPS does. Unfortunately, additional mutants with LPS truncations between those encoded by *rfaJ* and *rfaC* did not stably maintain the *hlyCABD* operon on a recombinant plasmid, and so their effects on Hly could not be adequately measured. This phenotype also included an *rfaP* mutant where independent hemolysin transformants yielded different levels of hemolytic activity. Such variation was not reported by Stanley et al. (22), which could be due to allelic differences in the two *rfaP* mutants.

Both expression and activity of Hly were affected by the lesion in *rfaC*, which encodes a heptosyltransferase that adds the first heptose of the inner core to KDO. These effects were growth phase dependent, with differences becoming more pro-

nounced at later stages in culture growth. Interestingly, the *rfaC* mutation appears to affect the steady-state level of HlyA accumulated in the culture milieu in a growth-phase-dependent manner, although the level of *hlyA* mRNA (Fig. 3A) and the amount of HlyA produced in a 20-s period of time (Fig. 3B) are both reduced twofold in the mutant regardless of the growth phase. Further, the rate of HlyA secretion is unchanged in this mutant. These data suggest that HlyA is degraded more rapidly in the liquid culture medium of the mutant than in that of the wild-type strain. Therefore, LPS may play a role in protecting the secreted HlyA protein from degradation.

The activity produced by cultures in early log growth was not significantly different than wild-type activity. However, levels of hemolytic activity dropped off dramatically as growth continued. The specific stage in growth at which the activity fell off varied across a wide range, from roughly an OD_{600} of 0.4 to 0.9. This variation in the OD_{600} at which activity dropped off may account for the range of specific activities observed at an OD_{600} of 0.8.

The changes in Hly_{rfaC} extracellular expression and activity during growth allowed us to examine effects of the truncated LPS on Hly in greater detail than with the *rfaP* mutant reported by Stanley et al., in which extracellular activity was completely abrogated while the steady-state level of secreted antigen was unaffected (22).

What causes the reduction in activity? One possibility is that the activity of the toxin in *rfaC* cultures is more labile than in $rfaC^+$ cultures. Hly_{rfaC} in cell-free culture supernatants demonstrated a threefold reduction in the half-life of activity compared with Hly_{rfaC^+}. The half-life measured at 37°C is short enough to affect the activity seen during the several hours of culture growth required to achieve an OD_{600} of 0.8. However, the half-life of Hly in culture may be affected by continuous production of fresh toxin or by environmental conditions other than temperature. Therefore, the half-lives of toxins in cell-free supernatants incubated without aeration may not be directly applicable to Hly in culture.

Another possible cause of the reduced activity in *rfaC* cultures is that Hly_{rfaC} may form inactive aggregates more readily than Hly_{rfaC^+}. Size estimates of native toxin indicate a heterodispersed population ranging from 300 to >1,000 kDa (5, 16, 21). Ostolaza et al. demonstrated that Hly aggregates can be dispersed by urea or other chaotropic agents, with a concomitant increase in activity (13). Ultracentrifugation of toxin-containing supernatants demonstrated that the bulk of Hly_{rfaC} (>90%) is found in species that would be roughly ≥500 kDa in size based on the method used here (8), while only ~50% of $HlyA_{rfaC^+}$ exists in forms this large. Further evidence for formation of inactive aggregates of HlyA comes from the precipitation of HlyA and resuspension in 6 M urea buffer that restores activity to Hly_{rfaC}. Taken together with the ultracentrifugation experiments and the activity at early log growth, these data suggest that Hly_{rfaC} is not intrinsically inactive as it is produced but, rather, that the toxin loses activity in the supernatant over time by formation of inactive aggregates. This process apparently occurs much more readily in *rfaC* than in $rfaC^+$ cultures.

What role does the mutant LPS play in increased aggregate formation of Hly? The differences in secreted Hly from $rfaC^+$ and *rfaC* strains, including a reduced rate of reaction and half-life of activity as well as increased aggregate formation, provide evidence that Hly_{rfaC^+} and Hly_{rfaC} are physically different. As the protein portion of Hly is wild type in both transformants, and the mutation lies in LPS, the data indicate that LPS may be physically associated with Hly in culture supernatants. Both Hly and LPS have large regions of hydrophobicity, including attached fatty acids, which may allow complex formation between the two molecules. Potentially, LPS core sugars, particularly those of the inner core, may be involved in maintaining a more stable, active conformation of Hly in such a complex. A physical interaction could also explain the apparent role of LPS in maintaining the integrity of the HlyA protein and preventing breakdown, as discussed above. Precedence for LPS involvement with specific protein structure comes from studies of OmpF porin formation. LPS is required for efficient trimerization of OmpF (19); further, deep rough LPS is insufficient for this function (20). Alternatively, LPS may affect the mature form of HlyA either indirectly or by transient complex formation that leads to an alteration of the final conformation of HlyA. There is also the possibility that LPS participates with an unidentified cofactor which aids Hly stability and reduces HlyA aggregation. In any of these cases, it is curious that the HlyA-LPS interaction does not affect reactivity of the epitopes recognized by the panel of 20 MAbs tested against the native forms of Hly_{rfaC^+} and Hly_{rfaC}.

It is unclear at which stage in production a putative HlyA-LPS complex may form. The active Hly conformation may be more poorly maintained by deep rough LPS, and the two toxins may interact to a greater extent via their fatty acids. This could cause micelle formation and lead to a rapid increase in aggregate formation and a consequent drop in activity. Several efforts to reconstitute *rfaC* HlyA with smooth or rough LPS in vitro were unsuccessful (data not shown), suggesting that the interaction may occur before or during secretion. Alternatively, it may simply be that the proper means of reconstitution have not yet been found.

In summary, the work presented here supports the model of deep rough LPS mutants affecting Hly. More specifically, these data demonstrate that removing the core sugars from LPS affects the kinetics and stability of secreted hemolytic activity. The LPS truncation also affects the degree of aggregation, as was observed with the *rfaP* mutation by Stanley et al. (22). Further, the *rfaC* mutation leads to a twofold reduction in the steady-state level of *hlyA* mRNA and affects the extracellular accumulation of full-length HlyA antigen without affecting the rate of secretion of the toxin polypeptide.

These data suggest a model for the role of LPS in Hly activity. According to this model, Hly exists as a complex including both HlyA and LPS, as well as potentially other unknown components. HlyA and LPS combine via hydrophobic and charge interactions to form an active toxin whose stability is aided by the LPS inner core. When the inner core is incomplete or improperly modified, LPS and Hly form large, inactive aggregates, possibly via fatty acid interactions, and render the protein component, HlyA, more susceptible to decay. Further research is required to test the hypotheses of this model, including whether HlyA and LPS interact in a complex, as proposed, or whether LPS exerts the observed effects by a more indirect mechanism.

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