Hfq Regulates Biofilm Gut Blockage That Facilitates Flea-Borne Transmission of *Yersinia pestis*

Katherine A. Rempe,* Angela K. Hinz, and Viveka Vadyvaloo

Paul G. Allen School for Global Animal Health and Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, USA

The plague bacillus *Yersinia pestis* can achieve transmission by biofilm blockage of the foregut proventriculus of its flea vector. Hfq is revealed to be essential for biofilm blockage formation and acquisition and fitness of *Y. pestis* during flea gut infection, consistent with posttranscriptional regulatory mechanisms in plague transmission.

*Y*ersinia pestis, the etiological agent of bubonic plague, is transmitted to humans by fleabite. Colonization and biofilm formation by *Y. pestis* in the flea gut are essential steps for proventricular foregut blockage and facilitate subsequent transmission of *Y. pestis* during "frustrated feeding" by an infected flea (16, 20). The known transmission factors *hmsHFRS*, *gmhA*, and *ymt* are required for flea-borne transmission but are dispensable for infection within the mammalian host (8, 16–19). Whole-genome comparative transcriptional profiling revealed, however, that these three genes are not differentially transcribed during *Y. pestis* biofilm formation and gut blockage of the flea relative to temperature-matched *in vitro* conditions (40). Clearly, the transcriptome provides only a first order of gene regulation, while a second order of posttranscriptional regulation may shape the unique flea-associated biofilm that allows transmission. This is in keeping with the emerging paradigm for extensive posttranscriptionally regulated virulence in numerous bacterial pathogens (6, 25, 29, 32–34) mediated by the ubiquitous RNA chaperone protein Hfq. The characterization of Hfq function by the use of rodent infections with several bacterial pathogens, including *Salmonella* spp. (34), *Francisella tularensis* (28), uropathogenic *Escherichia coli* (24), and *Y. pestis* (13), has shown significant virulence attenuation in *hfq* mutants.

The *hfq* gene is abundantly transcribed during *Y. pestis* biofilm proventricular blockage (40), predicting a second order of posttranscriptional regulation directed by Hfq during *Y. pestis* flea infection. To investigate the role of Hfq in mediating flea gut blockage, an *hfq* deletion mutant in the *Y. pestis* KIM6+ strain was constructed using the lambda red recombinase system (9). This strain contains the *hmsHFRS* gene locus required for the synthesis of extracellular matrix polysaccharide (EPS) essential for biofilm formation and flea foregut blockage (18, 31, 39). We hypothesized that the Δ*hfq* mutant growing *in vitro* at the 21°C insect host temperature or within the flea gut would be impaired in its growth and biofilm gut blockage capability essential for plague survival and transmission.

Growth of a *Y. pestis* KIM6+ *hfq* deletion mutant. Functional mutations of *hfq* frequently result in compromised bacterial growth fitness (6, 13), including in *Y. pestis* growing at the mammalian host temperature of 37°C versus laboratory growth at 28°C (3, 13). Here, at 21°C, the insect host temperature, the Δ*hfq* mutant, relative to its isogenic wild type, exhibited a significantly impaired specific growth rate and a decrease in stationary-phase cell density (Fig. 1A) similar to those seen with growth at 37°C (3, 13) and at 28°C (Fig. 1B) in brain heart infusion broth (BHI). The Δ*hfq* mutant, complemented with the native gene and promoter region of *hfq* on a high-copy-number plasmid, pCR4-TOPO (In-

vitrogen), sufficiently rescued this growth fitness defect in BHI broth. However, the Δ*hfq* mutant showed no significant growth fitness defect compared to the wild-type strain when grown in biofilm-supporting minimal TMH medium (supplemented with 0.2% galactose as the carbon source instead of gluconate and 1 μg/ml hemin) (37).

The Δ*hfq* mutant displays a differential ability to form biofilm *in vitro*. Biofilm formation is a dynamic process that is initiated by bacterial adherence and development of multicellular aggregates on a favorable surface (22, 27). To examine biofilm formation on different surfaces, *Y. pestis* strains were grown in borosilicate glass tubes and on polystyrene plates at 21°C with shaking in biofilm-supporting minimal TMH medium (supplemented with 0.2% galactose and 1 μg/ml hemin), and the biofilm was quantified by safranin staining (7, 39). Relative to the wild-type strain, the Δ*hfq* mutant was unable to grow a comparable biofilm on polystyrene (Fig. 2A) but retained a wild-type ability to form biofilm on borosilicate glass (Fig. 2B) over time. The slightly lower growth rate of the Δ*hfq* mutant may account for the reduced ability to form biofilm up to 48 h. However, there was no difference in the stationary-phase cell density between the wild-type and Δ*hfq* mutant strains under these minimal culture conditions (Fig. 1C), indicating disparate abilities of these strains to form biofilm on different surfaces.

A *Y. pestis* strain with the *hmsHFRS* gene locus can be assessed for biofilm formation at <28°C by streaking bacteria on agar medium containing Congo red dye on which pigmented red colonies form when the bacterial cells bind Congo red, which correlates with the ability to form a biofilm that is largely dependent on production of EPS (39). The wild-type and complemented Δ*hfq* mutant strains formed large red pigmented colonies with rugose centers, while the Δ*hfq* mutant colonies appeared to have smooth centers (Fig. 2C) on Congo red agar. Rugose colony morphology represents enhanced EPS production and biofilm (2, 35, 38, 42).
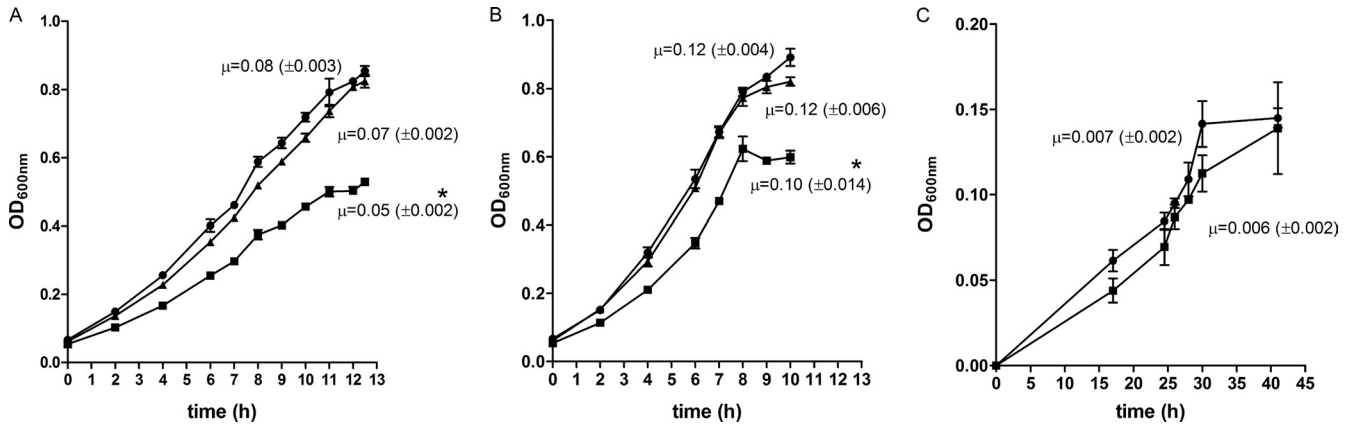
FIG 1 Growth fitness comparison of *Y. pestis* KIM6+ wild-type (●), Δ*hfq* mutant (■), and complemented mutant Δ*hfq* (pCR4::*hfq*) (▲) strains at 21°C (A) and 28°C (B) in brain heart infusion (BHI) broth and wild-type (●) and Δ*hfq* mutant (■) strains at 21°C in TMH medium (0.2% galactose, 1 μg/ml hemin) (C). The specific growth rate, μ, represents the linear regression slope calculated from exponential-phase time spans 4 to 10 h and 17 to 28 h for growth in BHI broth and TMH broth, respectively. Means and standard deviations of the results of 3 independent experiments are indicated. Repeated analysis of variance (ANOVA) measurements were used to determine significant differences in slopes (μ). * indicates $P < 0.001$.

The Δ*hfq* mutant is unable to form proventricular biofilm blockage of the flea gut. To determine whether the Δ*hfq* mutant infects, survives, and forms biofilm blockage of the proventriculus, 150 *Xenopsylla cheopis* fleas were infected with wild-type, Δ*hfq* mutant, or complemented mutant Δ*hfq* (pCR4::*hfq*) strains using a previously described artificial feeding system (18, 20) and a blood meal containing a mean of 9.61×10^9 ($\pm 1.52 \times 10^{10}$)

bacteria/ml. Fleas that took a blood meal were maintained at 21°C and 75% relative humidity, fed twice weekly on uninfected mice, and monitored for proventricular blockage (18). To quantify bacterial survival, 20 fleas were processed at days 0, 7, and 28 postinfection (18, 20, 39). In the flea gut, the Δ*hfq* mutant was able to grow at the same rate as the wild-type strain (Fig. 3A), achieving similar bacterial loads at 28 days postinfection. However, signifi-
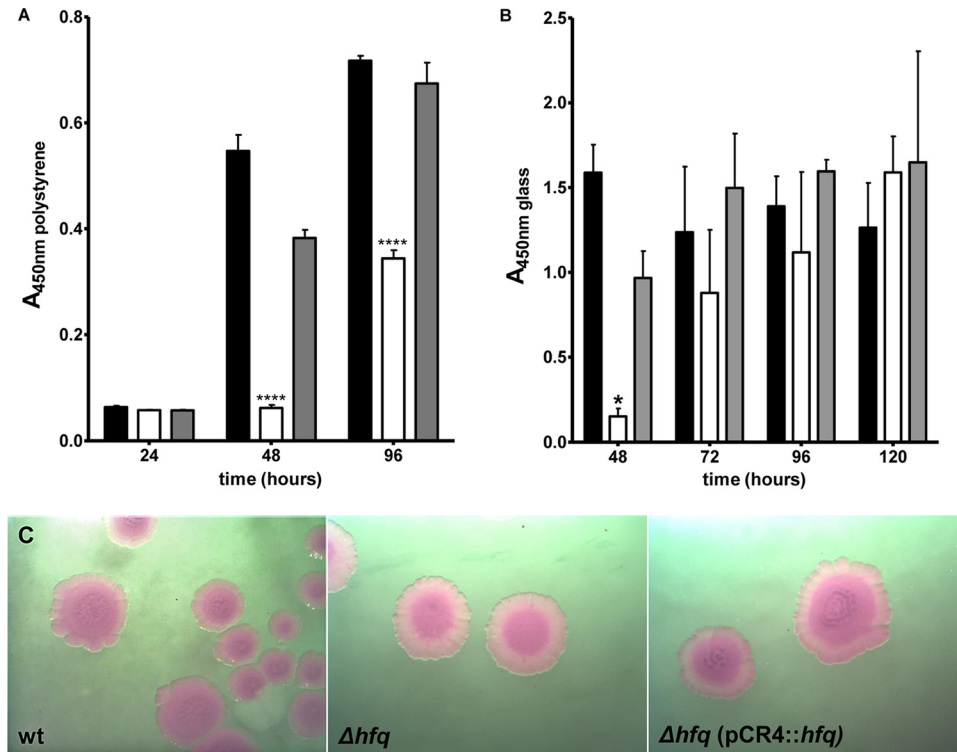

FIG 2 Differential ability of the Δ*hfq* mutant to form biofilm *in vitro*. (A and B) Growth of *Y. pestis* KIM6+ wild-type (black bars), Δ*hfq* mutant (unfilled bars), and complemented mutant Δ*hfq* (pCR4::*hfq*) (dark gray bars) strains in TMH medium (0.2% galactose, 1 μg/ml hemin) seeded at 10^3 bacteria/ml on polystyrene microtiter plates (A) and in borosilicate glass tubes (B). (C) The Δ*hfq* mutant does not form rugose (wrinkled) colonies. Means and standard deviations of the results of 3 independent experiments are indicated for panels A and B. Data were analyzed using two-way ANOVA and a Bonferroni posttest. * indicates $P < 0.05$; **** indicates $P < 0.001$.
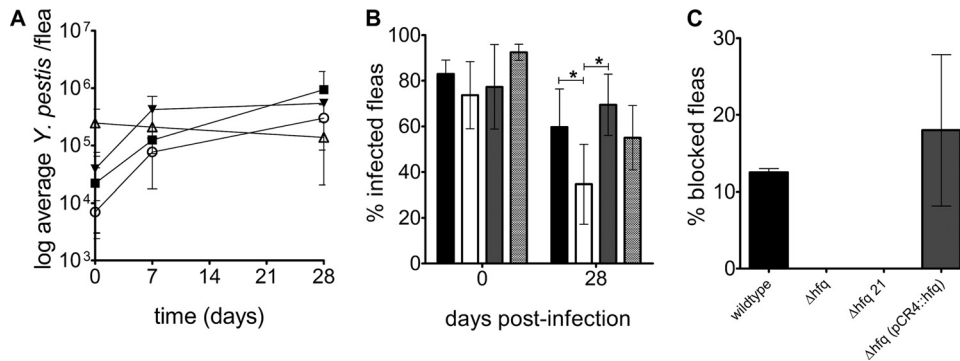
FIG 3 The Δ*hfq* mutant is deficient in its ability to form biofilm blockage of the flea proventriculus. (A) The Δ*hfq* mutant grows at a rate similar to that of the wild-type strain during flea infection. ■, wild-type strain; ○, Δ*hfq* mutant strain; ▲, complemented mutant Δ*hfq* (pCR4::*hfq*) strain; △, Δ*hfq* mutant strain pregrown at 21°C. (B) The Δ*hfq* mutant strain had reduced infection rates in fleas 28 days postinfection. Black bars, wild-type strain; unfilled bars, Δ*hfq* mutant strain; dark gray bars, complemented mutant Δ*hfq* (pCR4::*hfq*) strain; hatched bars, Δ*hfq* mutant strain pregrown at 21°C. (C) The Δ*hfq* mutant has a reduced ability to form biofilm blockage of the flea proventriculus. The means and standard deviations of the results of 3 independent experiments are represented for all infections except for the infections of the Δ*hfq* mutant strain pregrown at 21°C, for which the data represent 2 independent experiments. A one-way ANOVA and a Newman-Keuls posttest (* indicates $P < 0.05$) were used to statistically analyze wild-type, Δ*hfq* mutant, and Δ*hfq* (pCR4::*hfq*) strains grown at 37°C.

cantly fewer fleas remaining infected with the Δ*hfq* mutant by 28 days (Fig. 3B). Notably, the Δ*hfq* mutant was unable to form a biofilm blockage of the flea gut (Fig. 3C).

The ability to form biofilm blockage may be limited by infectious dose (10, 20) or defective acquisition of the pathogen; fleas infected with the Δ*hfq* mutant showed lower infectious doses ($\leq$50% wild-type CFU/flea at $T = 0$) immediately following feeding compared to the wild type (Fig. 3A). The lower infectious dose may be correlated with the poor growth, physiological effects, and elongated cell morphology of the Δ*hfq* mutant grown at 37°C in BHI broth (3). Therefore, the Δ*hfq* mutant was grown at 21°C, to adapt cells to the flea growth temperature, following which fleas were infected as described above using 1.95×10^8 ($\pm 7.07 \times 10^6$) bacteria/ml. The fleas acquired a higher infectious dose of the Δ*hfq* mutant pregrown at 21°C; however, there was no biofilm blockage. This confirmed that the Δ*hfq* mutant was defective in forming biofilm blockage in a manner independent of the infectious dose.

To further evaluate the liability of the *hfq* mutation, fleas were coinfected with the wild type carrying the green fluorescent protein (GFP)-expressing pACGFP1 plasmid (Clontech) and the Δ*hfq* mutant carrying plasmid pmCherry (Clontech) expressing red fluorescent protein by the use of a 1:1 ratio of 3.5×10^8 ($\pm 1.2 \times 10^8$) bacteria/ml from each strain cultured at 37°C. The competitive index (CI) was calculated as previously reported (26) 28 days postinfection to determine the fitness of the Δ*hfq* mutant versus that of the wild type following flea infection. The CI was determined by calculating the ratio of mean wild-type *Y. pestis* CFU/flea (X) and the mean Δ*hfq* mutant CFU/flea (Y) at 28 days postinfection divided by this same ratio at 0 days postinfection $[(X/Y)_{t = 28 \text{ days}}/(X/Y)_{t = 0 \text{ days}}]$. A CI of <1 would denote the wild type as the dominant strain, whereas a CI of >1 would denote dominance of the Δ*hfq* mutant. In two independent coinfection experiments, the CI was 0.22 ($\pm$0.06), which indicated a lower mean bacterial load per flea and reduced fitness of the Δ*hfq* mutant relative to the wild type in competition. Fluorescence microscopy of the coinfected flea guts demonstrated that the Δ*hfq* mutant formed smaller aggregates that developed separately from the wild-type strain (Fig. 4).

The Δ*hfq* mutant's inability to form a biofilm blockage of the

foregut while surviving at high numbers in the flea is reminiscent of the phenotype of the *Y. pestis* KIM6 *pgm* mutant strain, which lacks the *hmsHFRS* EPS locus (18). However, 28 days after infection, 50 of 50 colonies grown from fleas ($n = 16$) infected with the Δ*hfq* mutant were pigmented on Congo red agar and were PCR positive for the *hmsF* gene. This ruled out spontaneous loss of the *pgm* locus (12), a known phenomenon which could have been enhanced in the Δ*hfq* mutant background.

We accept our hypothesis that Hfq is required for biofilm-mediated gut blockage in the flea. Development and assembly of the biofilm are affected by the coordinated synthesis of components, including surface appendages, signaling molecules promoting intercellular communication, and molecules essential for nutrient acquisition, stress adaptation, and biofilm regulation (1, 22, 27). In *Y. pestis*, the *hmsHFRS*-encoded EPS is a key component of the biofilm structure and its composition and is posttranslationally regulated by the chemical second messenger molecule, cyclic-di-GMP (5, 39). Although other outer surface factors impacting biofilm formation have not yet been defined, the observed auto-aggregative phenotype of a *Y. pestis* mutant impaired in EPS synthesis (39) may imply a role for such factors.

It is advantageous for *Y. pestis* to form large, dense multicellular aggregates to establish infection in the flea gut lumen, as these cannot be easily expelled in the feces (20). Importantly, biofilm blockage of the foregut proventriculus of the flea vector is the classical biological mechanism for transmission of the plague. Fewer fleas may remain infected with the Δ*hfq* mutant because it forms smaller bacterial aggregates, and altered intercellular signaling may contribute to the separated development of its bacterial aggregates. The lower numbers of the mutant during coinfection, however, may also reflect its inefficiency at acquiring available nutrients essential for survival and growth when in competition with the wild type. This could be influenced by an altered spatial structure of the Δ*hfq* mutant biofilm (1, 27). Stress adaptation signatures associated with nutrient starvation and membrane stresses are common in bacterial biofilms (1), including that of *Y. pestis* (40), and may be reduced in the Δ*hfq* mutant. This deficiency of the Δ*hfq* mutant may prove a competitive fitness disadvantage in sylvatic plague transmission, when the flea gut may be
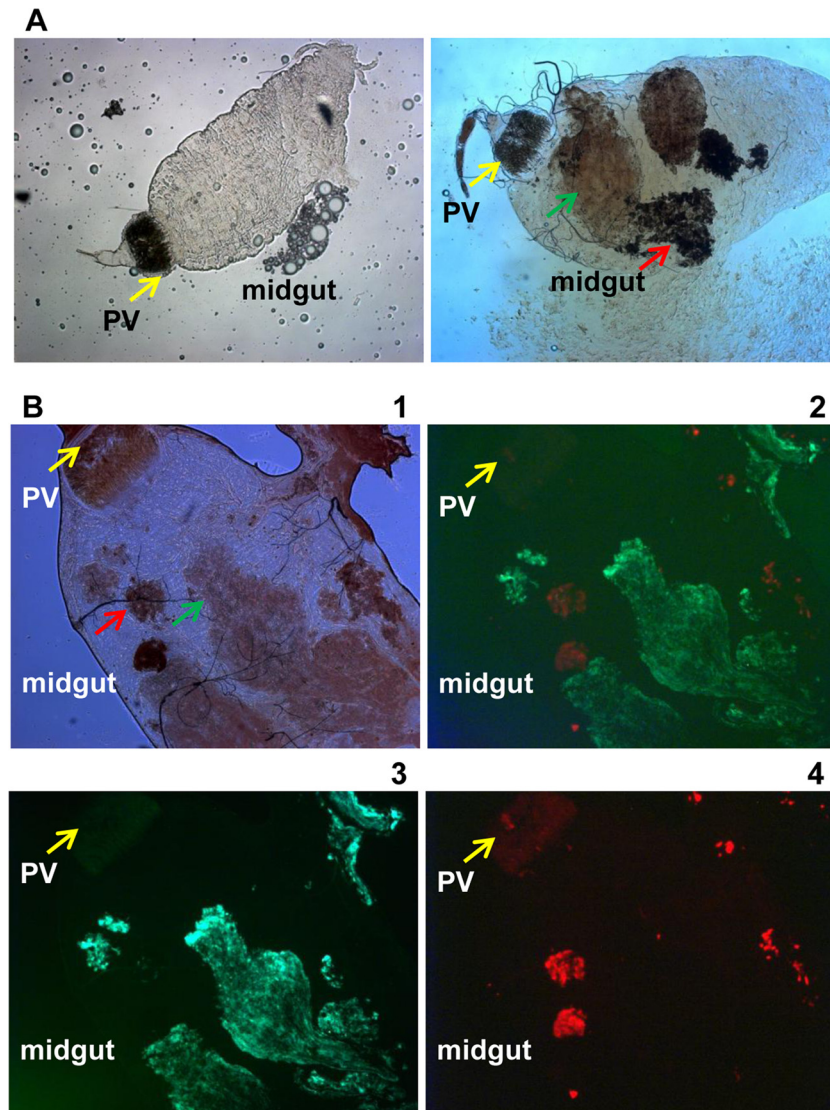
FIG 4 Coinfection of fleas with green fluorescent protein (GFP)-expressing wild-type (light-colored aggregates—green arrow) and mCherry red fluorescent protein-expressing Δ*hfq* mutant (dark-colored aggregates—red arrow) strains exhibited that the Δ*hfq* mutant strains form smaller aggregations that develop separately from the wild-type strains during flea infection, as visualized by fluorescence microscopy. (A) The left panel illustrates a whole uninfected flea midgut, and the right panel illustrates a coinfected flea gut containing GFP-expressing wild-type (green arrow) and mCherry-expressing Δ*hfq* mutant (red arrow) strains. (B) Coinfected flea gut: panel 1, bright-field image showing GFP-expressing wild-type (green arrow) and mCherry-expressing Δ*hfq* mutant (red arrow) strains; panel 2, separately developing aggregations of GFP-expressing wild-type and mCherry-expressing Δ*hfq* mutant strains; panel 3, GFP-expressing wild-type bacteria only; panel 4, mCherry-expressing Δ*hfq* mutant aggregations only. Images represent 8 fleas per experiment dissected between 21 and 28 days after infection. PV, proventriculus (yellow arrows).

prone to increased colonization by other bacteria (11, 21). The difference in biofilm production between the wild type and the Δ*hfq* mutant may therefore be a consequence of alterations in several factors required for biofilm formation, development, and structure. This suggests a role for Hfq in modulating the dynamics of biofilm formation, optimal acquisition, and fitness during *Y. pestis* flea infection.

How Hfq functions in biofilm formation in the flea is currently unknown. However, a known function of Hfq is to stabilize and facilitate interaction of small regulatory RNA (sRNAs) with their cognate mRNA targets. The Hfq-regulated sRNAs function by base pairing to their cognate mRNAs and hindering their translation and/or increasing their degradation or sometimes enhancing

translation (33, 36, 41). Hfq and its associated sRNAs are implicated in biofilm development in uropathogenic *E. coli* (24) and *Vibrio cholera* (4) and modulation of the outer surface composition and envelope stress responses in other Gram-negative enterobacteria (14, 15, 30).

A *Y. pestis* Δ*hfq* mutant has been shown to be attenuated for virulence in a mouse infection model (13) and was observed here to be deficient in transmission by biofilm blockage of the flea proventriculus. This implicates Hfq-mediated mechanisms in the complete infective life cycle of the plague. The influence of a single regulatory protein on *Y. pestis* infection in two physiologically distinct host environments with discrete gene expression profiles (40) is interesting and not a previously described pheno-

type for Hfq. This suggests that Hfq may have separate targets in the two different host environments. Hfq-dependent sRNAs have recently been reported to contribute to virulence of *Y. pestis* (23). Therefore, uncovering the mechanisms and targets by which Hfq mediates transmission is the next step in better understanding of plague and in improving disease control.

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