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Reciprocal Transcriptional and Posttranscriptional Growth-Phase-Dependent Expression of *sfh*, a Gene That Encodes a Paralogue of the Nucleoid-Associated Protein H-NS[▽]

Marie Doyle and Charles J. Dorman*

Department of Microbiology, Moyne Institute of Preventive Medicine, University of Dublin, Trinity College, Dublin 2, Ireland

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The IncHI1 self-transmissible plasmid pSf-R27 from *Shigella flexneri* 2a strain 2457T harbors *sfh*, a gene that codes for a protein with strong amino acid sequence homology to the global transcription regulator and nucleoid-associated protein H-NS and to its paralogue, StpA. Previously, we discovered that the expression of *sfh* mRNA is growth phase dependent such that in cultures growing in Lennox broth at 37°C, the transcript is readily detectable in the early stages of exponential growth but is not detectable at the onset of stationary phase. In contrast, the Sfh protein is poorly expressed in early-exponential growth when *sfh* mRNA is abundant whereas it is expressed to a high level in early stationary phase, when *sfh* transcript expression is low (P. Deighan, C. Beloin, and C. J. Dorman, Mol. Microbiol. 48:1401–1416, 2003). This unusual pattern of reciprocal mRNA and protein expression is not due to growth phase-dependent effects on either mRNA or protein stability, nor is it due to the known abilities of the Sfh, StpA, and H-NS proteins to influence *sfh* gene expression. Instead, our data point to a blockade of *sfh* mRNA translation in early-exponential growth that is relieved as the culture enters the stationary phase of growth. Replacing the 5′ end and translation initiation signals of the *sfh* mRNA with heterologous sequences did not alter the growth phase-dependent expression of the Sfh protein, suggesting that growth phase control of translation is intrinsic to another component of the message.

DNA supercoiling and the nucleoid-associated proteins collaborate to organize the structure of the bacterial nucleoid. These factors also influence transcription on a global level and modulate other major DNA transactions, such as replication, recombination (general and site specific), and transposition (17, 24, 47). H-NS is a prominent member of the nucleoid-associated protein family. It can constrain DNA supercoiling both in vitro and in vivo, and it influences transcription throughout the cell, usually by acting as a repressor (10, 11, 15, 41). H-NS has been identified as a repressor of virulence gene transcription in several gram-negative pathogens, including *Shigella flexneri* (4, 15, 16, 39). Here, it binds to the promoters of the major virulence gene operons located on the 230-kb virulence plasmid (4), where the transcription activator VirF or VirB counteracts its gene silencing activity (3, 6, 35, 39, 46). Virulence gene activation occurs under growth conditions that approximate those found in the lower human gut and involves a combination of environmental signals, such as temperature, osmolarity, and pH (16, 37). The molecular mechanism of gene activation operates through disruption of the H-NS-imposed silencing of the virulence gene promoters. The model that currently applies in the case of the *S. flexneri* virulence genes envisions an environmentally wrought change in DNA structure accompanied by a disruption of the H-NS-DNA complex leading to transcription derepression (16, 39).

S. flexneri 2a strain 2457T has been used extensively in studies of *Shigella* virulence gene regulation. It harbors an IncHI1 plasmid that is closely related to R27 but lacks genes for resistance to antibiotics or other antimicrobials (4, 50). This plasmid has not been found in other *S. flexneri* isolates, although the R27 prototype plasmid is widely distributed among epidemic strains of *Salmonella enterica* serovar Typhi from South and Southeast Asia (48, 49) and was discovered originally in *S. enterica* serovar Typhimurium (42).

The plasmid from strain 2457T, pSf-R27, encodes an H-NS-like protein called Sfh. This brings to three the number of H-NS-like proteins found in this bacterium: H-NS, StpA, and Sfh. Each protein can repress the transcription of its own gene and the genes coding for the other two paralogues (12). In addition, each protein can form a heteromeric complex with either of the other two, presumably owing to the strong amino acid sequence conservation seen in their oligomerization domains (12). There has been considerable speculation that the homomeric and heteromeric forms of H-NS-like proteins might have distinct biological activities (18, 27, 43, 51). Knockout mutants lacking Sfh do not have obvious phenotypes (12). This can be explained by the abilities of the H-NS, StpA, and Sfh proteins to substitute for one another functionally. For example, classic phenotypes of *hns* mutants, such as loss of *Escherichia coli* motility (44), derepression of the cryptic *bgl* locus (14, 26), low-osmolarity expression of the osmotic upshock-inducible *proU* locus (26, 40, 43), and an abnormal outer membrane porin protein expression profile (13), can all be complemented by genes coding for Sfh or StpA (5). Furthermore, combining *sfh* and *hns* knockout mutations in the same *S. flexneri* strain results in an enhancement of the already-derepressed virulence gene expression pattern normally seen in an *hns* single mutant (5). The three proteins can bind to the same DNA sequences and all share the well-documented preference of H-NS for binding to intrinsically curved DNA se-

* Corresponding author. Mailing address: Department of Microbiology, Moyne Institute of Preventive Medicine, University of Dublin, Trinity College, Dublin 2, Ireland. Phone: 353 1 896 2013. Fax: 353 1 679 9294. E-mail: cjdorman@tcd.ie.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
S. flexneri 2a 2457T		
BS184	*mxiC*::MudI*1734*; Km^r	33
BS185	BS184 *hns*::Tn*10*; Tc^r	34
CJD1199	BS184 Δ*stpA*::Tc^r	38
CJD1216	CJD1199 *hnrG*::*bla hns*205::Tn*10*	36
CJD1650	BS184 Δ*sfh*::Gen^r	5
E. coli K-12		
MC4100	F^- λ^- *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC ptsF25 rbsR*	9
MD11	MC4100/pSf-R27Gen^r	This work
Plasmids		
pBAD18	Arabinose-inducible P_{BAD} promoter; no RBS	23
pBAD18sfh	Arabinose-inducible *sfh* with native 5′-UTR	This work
pBAD2	Arabinose-inducible P_{BAD} promoter and RBS	23
pBAD24sfh	Arabinose-inducible *sfh* with foreign 5′-UTR	This work
pSf-R27Gen^r	pSf-R27 tagged with gentamicin; Gen^r	19
pBR322	Routine cloning vector; Ap^r	New England Biolabs
pZep08	*gfp* promoterless trap vector; Cm^r Km^r	25
psfh-gfp	*sfh* promoter region cloned upstream of the promoterless *gfp* gene in pZep08	This work
p18sfh	*sfh* gene in pSPT18	12
p18rrnA	*rrnA* internal region gene in pSPT18	12

[a] RBS, ribosome-binding site.

quences (5, 12). This DNA binding preference contributes to the abilities of the three proteins to auto-repress transcription of their own genes and to cross-regulate negatively one another's genes. Preliminary analysis has shown that the three proteins have distinct expression patterns: H-NS is present at an approximately constant level throughout growth, StpA is expressed principally in early exponential phase, and Sfh is seen predominantly in late-logarithmic growth (12, 20, 43). The expression pattern of the Sfh protein is unusual in being the reciprocal of that of the *sfh* mRNA. The protein is abundant at the onset of stationary phase, whereas the mRNA is plentiful in early exponential phase and becomes difficult to detect by the onset of stationary phase (12). Here, we investigated the expression pattern of the *sfh* gene in order to elucidate its molecular biology in more detail.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains were derivatives of *S. flexneri* 2a strain 2457T or *Escherichia coli* K-12 strain MC4100 and are listed in Table 1. *E. coli* strain MD11 (MC4100/pSf-R27Gen^r) was constructed by conjugating plasmid pSf-R27Gen^r (19) into strain MC4100 as previously described (45). Exconjugants were selected for and twice single-colony purified on L–$Str^{50}Gen^{15}$ plates, and the presence of pSf-R27Gen^r was confirmed by Southern blot and PCR analyses (data not shown). Bacteria were cultured routinely in Lennox (L) broth or on L agar plates at 37°C. Antibiotics were used at the following concentrations: carbenicillin, 50 μg ml^{-1}; streptomycin, 50 μg ml^{-1}; gentamicin, 15 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; tetracycline, 15 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; spectinomycin, 200 μg ml^{-1}; and rifampin, 250 μg ml^{-1}.

RNA isolation and analyses by RT-PCR and Northern blotting. Total RNA extracts were prepared by harvesting 6 optical density at 600 nm (OD_{600}) units of bacteria and RNA isolated using TRI reagent (Sigma-Aldrich Ltd.) according to the manufacturer's guidelines. Total RNA was then DNase I treated using a DNA-free kit (Ambion Inc.) to ensure no DNA contamination. Reverse transcription (RT)-PCR analyses were carried out using a OneStep RT-PCR kit (QIAGEN), 1.2 μg DNA-free RNA as a template, and gene-specific primer pairs to yield amplicons of ~200 to 250 bp in size (Table 2). The RT-PCR program was used according to the manufacturer's guidelines. Reaction mixtures were subject to one cycle at 50°C for 30 min and 95°C for 15 min, followed by 20 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. The RT-PCR products were electrophoresed through 2% (wt/vol) Tris-acetate-EDTA agarose gels and stained with ethidium bromide and then quantified by densitometry using Quantity-One software (Bio-Rad, Hercules, Calif.). For Northern blot analyses, samples of total RNA (5 μg) were denatured for 10 min at 70°C in 50% formamide, loaded onto 1.25% Reliant agarose gels (Flowgen), electrophoresed in MOPS (morpholinepropanesulfonic acid) buffer, and capillary transferred to Biodyne B nylon membrane (PALL). Digoxigenin-labeled riboprobes were produced from plasmids p18sfh and p18rrnA as described previously (5). Overnight hybridization was carried out at 68°C, and after stringency washes, the bound digoxigenin-labeled probes were detected using the chemiluminescence substrate CDP-Star (Roche). Transcript levels were quantified by densitometry using Quantity-One software (Bio-Rad, Hercules, Calif.). To correct for possible differences in RNA integrity and loading in each lane, the analysis of a reference transcript, the 16S *rrnA* rRNA, was included. All experiments were performed on at least three independent occasions.

Western immunoblotting. Whole-cell lysates were prepared by harvesting 2 OD_{600} units of bacteria and resuspending them in 50 μl B-PER reagent (Pierce) supplemented with lysozyme (500 μg ml^{-1}) and DNase I (100 U ml^{-1}). The

TABLE 2. Primers

Primer	Sequence[a]
pBAD18-F	5′-CTG GCT AGC GTA TTC TAT TGA TTT TAT TTA TTA-3′
pBAD24-F	5′-TCC GAA GCA CTC AAA TCA TT-3′
sfh+780	5′-GAT AAA GCT TAC TAC AAA GTA G-3′
sfh-FG	5′-CTG CCC GGG CAC TTT ATG AAC GGC TCG-3′
sfh-RG	5′-CTG TCT AGA CCA GCA GTT CTT CAA GG-3′
sfh-FRT	5′-GTA CTC TTC GTG CGC AG-3′
sfh-RRT	5′-CAG GGC GCG GTT CGC G-3′
sfh-BSF	5′-GCA GCA AAC GTT AAG AAC GC-3′
sfh-BSR	5′-CCA GCA GTT CTT CAA GGA TC-3′
hns-RTF	5′-CCG TAC TCT TCG TGC GC-3′
hns-RTR	5′-CCG GAC GCT GAG CAC G-3′

[a] Restriction enzyme cleavage sites are underlined.

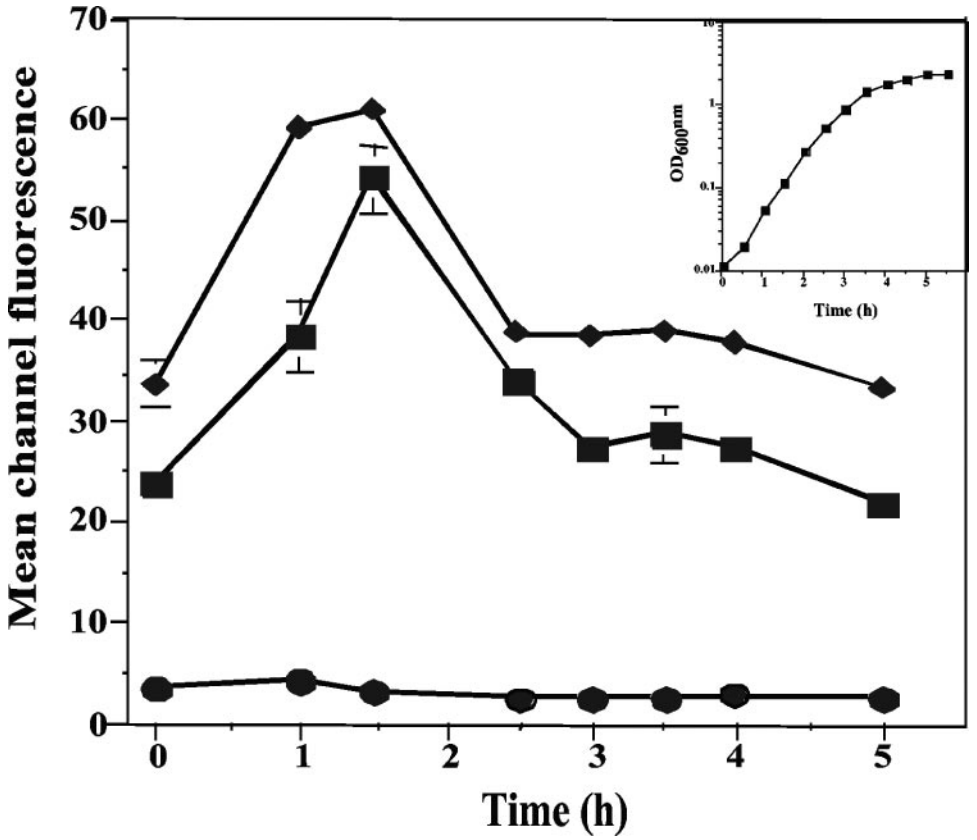

FIG. 1. Expression of the *sfh* gene as a function of growth phase. Expression of an *sfh-gfp* fusion in *S. flexneri* 2a strain 2457T (filled squares) and its *sfh* derivative (filled diamonds) in L broth at 37°C as a function of time is shown. Expression of the *gfp* plasmid vector without the *sfh* promoter insert (filled ovals) is also shown. The inset is the growth curve of the *S. flexneri* 2457T culture. The other two strains exhibited identical growth curves (not shown).

protein concentrations of the lysates were determined using the Bradford assay (8). Total protein extracts were resolved using 12% sodium dodecyl sulfate-polyacrylamide gels, and proteins were electroblotted to Protran nitrocellulose membranes (Schleicher and Schuell). Nitrocellulose membranes were stained with Ponceau (0.2% Ponceau dye, 3% trichloroacetic acid) to confirm consistent transfer before being blocked with 5% dried skim milk in phosphate-buffered saline. Membranes were probed with primary anti-Sfh antiserum (1:1,000) diluted in blocking solution. Membranes were then washed in phosphate-buffered saline and incubated with a secondary goat anti-rabbit horseradish peroxidase-conjugated antiserum. Membranes were developed using a chemiluminescent Pierce West Pico Super Signal kit. Protein levels were quantified by densitometry using Quantity-One software (Bio-Rad, Hercules, Calif.).

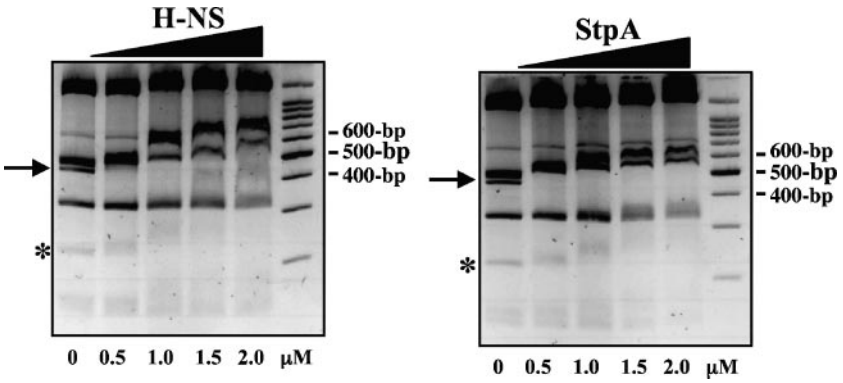

FIG. 2. Binding of the H-NS and StpA proteins to the *sfh* promoter. Results for competitive electrophoretic mobility shift assays showing binding of the H-NS (left) and StpA (right) proteins to the *sfh* promoter are shown. Plasmid pBR322 DNA was digested with TaqI and SspI, and the resulting DNA fragments were mixed with a 438-bp PCR amplimer of the *sfh* promoter region. The DNA mixture was incubated with H-NS or StpA in the 0 to 2 μM range. An arrow and an asterisk indicate the positions of the *sfh* and the pBR322 *bla* promoter fragments, respectively. The curved DNA *bla* fragment is a positive control that is known to bind H-NS-like proteins (4). The positions of the 400-, 500-, and 600-bp molecular size markers are also indicated.

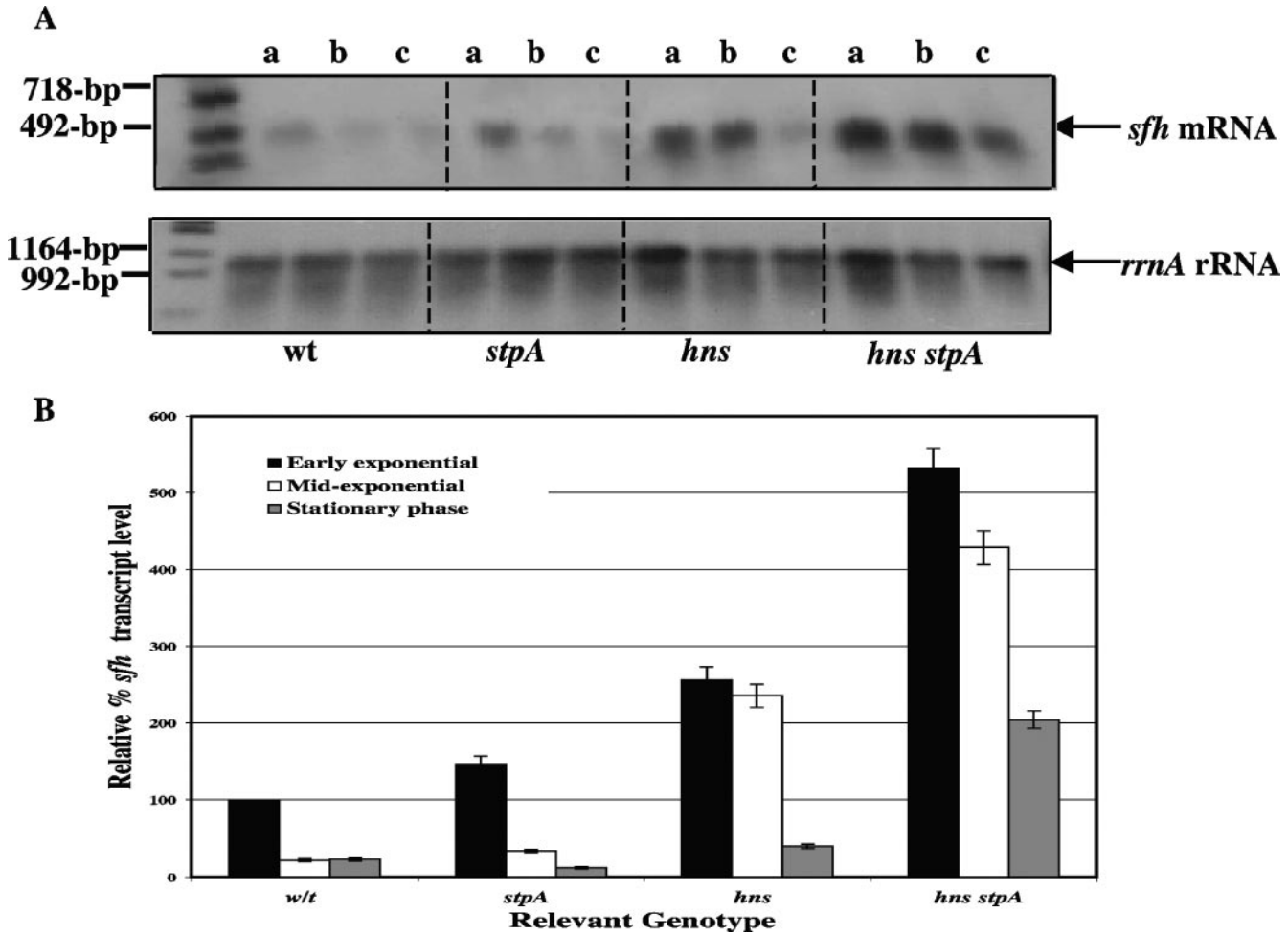

FIG. 3. Expression of *sfh* mRNA as a function of growth phase in the wild type (wt) and mutants deficient in H-NS, StpA, or both. (A) Northern blotting with a specific *sfh* riboprobe was used to monitor *sfh* mRNA levels at the early, mid-, and late exponential phases of growth in the wild type and its *stpA*, *hns*, and *hns stpA* derivatives. The data indicate that the *sfh* transcript is approximately 500 nucleotides in length, which is believed to be a more accurate estimate than our previous one of 650 nucleotides (12). As a control for RNA integrity and loading, levels of *rrnA* rRNA were measured in the same samples with an *rrnA*-specific riboprobe. Samples were isolated at OD_{600}s of 0.1 (early exponential phase) (lanes a), 0.6 (mid-exponential phase) (lanes b), and 1.5 (early stationary phase) (lanes c). (B) The Northern blots were scanned and the data used to generate histograms. The transcript levels are expressed as percentages of the *sfh* mRNA content of wild-type BS184 cells at an OD_{600} of 0.1 (early exponential phase), the value of which was set at 100%. The data are averages for three independent experiments, and a representative blot is shown.

Determination of protein stability. The stability of Sfh protein was monitored using a method described previously (21, 27, 28). Bacterial cells were cultured to exponential phase (OD_{600} = 0.3) or early stationary phase (OD_{600} = ~1.5) and treated with spectinomycin (200 μg ml^{-1}) to inhibit translation. Protein samples were then isolated in a time course experiment, and determination of Sfh protein stability was followed by Western blotting. Experiments were performed on at least three independent occasions.

Determination of mRNA stability. The stability of *sfh* mRNA was monitored using a method described previously (13). Bacterial cells were cultured to exponential phase (OD_{600} = 0.2) or early stationary phase (OD_{600} = ~1.5) and treated with rifampin (250 μg ml^{-1}) to inhibit transcription. Total RNA samples were then isolated in a time course experiment, and determination of *sfh* mRNA stability was followed by RT-PCR using the primer pair sfh-FRT and sfh-RRT (Table 2). As a control, *hns* mRNA stability was monitored using the primer pair hns-RTF and hns-RTR (Table 2), as its mRNA stability does not change significantly with growth phase. Experiments were performed on at least three independent occasions.

Plasmid construction. To assay *sfh* promoter activity, the *sfh* regulatory region was cloned upstream of the promoterless *gfp* reporter gene of plasmid pZep08 (Table 1). Primers sfh-FG and sfh-RG (Table 2) were used to amplify by PCR a 587-bp product encompassing the *sfh* regulatory region (nucleotides [nt] −497 to +90 with respect to the translation start site of *sfh*). The amplimer and vector pZep08 were then both digested with XbaI and SmaI so that following ligation, the *sfh* promoter read into the *gfp* gene. The structure of the new plasmid was verified by DNA sequencing and designated psfh-gfp (Table 1).

For controlled expression of the Sfh protein, the *sfh* gene was cloned without its own promoter downstream of the arabinose-inducible P_{BAD} promoter in plasmids pBAD18 and pBAD24 (Table 1). To construct plasmid pBAD18sfh, PCR primers pBAD18-F and sfh+780 (Table 2) were used to amplify an 820-bp product encompassing the 5′ untranslated region (UTR) and open reading frame (ORF) of *sfh* (nt −40 to +780 with respect to the translation start site of *sfh*). The amplimer and vector pBAD18 were then both digested with NheI and HindIII so that following ligation, the P_{BAD} promoter read into the *sfh* gene. The structure of the plasmid was verified by DNA sequencing and designated pBAD18sfh (Table 1).

Plasmid pBAD24sfh was constructed in a manner similar to that for pBAD18sfh except that PCR primers pBAD24-F and sfh+780 (Table 2) amplified only the *sfh* ORF (nt +1 to +780 with respect to the translation start site of *sfh*) and not its 5′ UTR and translation initiation signals. The *sfh* ORF was cloned behind the pBAD24 ribosome binding site. The amplified *sfh* ORF DNA

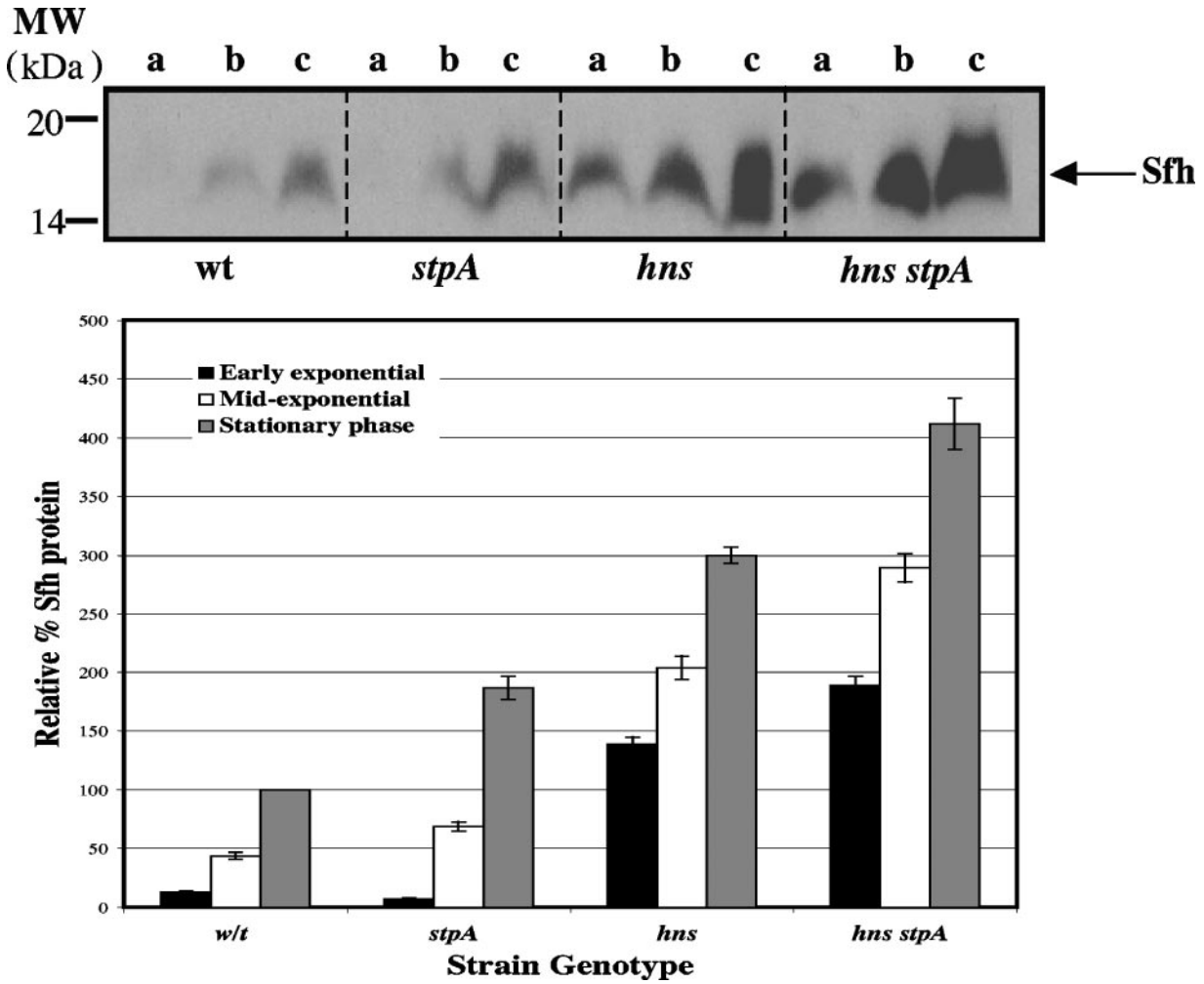

FIG. 4. Expression of Sfh protein as a function of growth phase in the wild type (wt) and mutants deficient in H-NS, StpA, or both. (A) Western blot analysis of Sfh protein levels in the BS184 wild type and its *hns*, *stpA*, and *hns stpA* mutant derivatives at fixed OD_{600} values during growth in L broth at 37°C. Samples were isolated at OD_{600}s of 0.1 (early exponential phase) (lanes a), 0.6 (mid-exponential phase) (lanes b), and 1.5 (early stationary phase) (lanes c). An arrow indicates the Sfh protein band. (B) The Western blots were scanned and the data used to generate histograms. The protein levels are expressed as percentages of the Sfh protein content of wild-type BS184 cells at an OD_{600} of 1.5 (early stationary phase), the value of which was set at 100%. The data are averages for three independent experiments, and a representative blot is shown.

fragment was extended with a HindIII site at the 3′ end. After digestion with HindIII, the amplimer was cloned into pBAD24 that had been linearized with HindIII and NcoI. Following NcoI digestion and prior to HindIII digestion, the Klenow fragment of DNA polymerase was employed to create a blunt end at the NcoI-cut sites. The relative locations of the blunt end and HindIII-cut end of pBAD24 and the amplimer ensured that following ligation, the P_{BAD} promoter read into the *sfh* gene. The structure of the plasmid was verified by DNA sequencing and designated pBAD24sfh (Table 1). All PCRs were performed using *Pfu* polymerase (Promega) and *S. flexneri* BS184 genomic DNA as the template. GATC Biotech performed custom automated DNA sequencing, and MWG Biotech supplied all the oligonucleotides.

Controlled expression of Sfh. The plasmids pBAD18sfh and pBAD24sfh (Table 1) were transformed separately into the *E. coli* K-12 strain MC4100 (Table 1). Bacterial cells were cultured to early exponential phase (OD_{600} = 0.2), and arabinose was added at time zero to the cultures at a final concentration of 0.2% to induce expression of Sfh from the P_{BAD} promoter. Protein samples were then isolated in a time course experiment, and Sfh expression was monitored by Western blotting.

Bioinformatic analysis. The secondary structure of *sfh* mRNA was predicted by the folding program Mfold (32, 52) (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi).

Electrophoretic mobility shift assay. The association of recombinant StpA and H-NS proteins (5) with the *sfh* promoter was investigated using a gel retardation assay (7). A 438-bp PCR amplimer of the *sfh* promoter region (corresponding to nt −327 to +111 with respect to the *sfh* transcription start site) was amplified using *Pfu* polymerase (Promega) and the primer pair sfh-BSF and sfh-BSR (Table 2). The PCR probe was incubated with pBR322 digested with TaqI and SspI restriction enzymes and purified protein in a reaction buffer containing 40 mM HEPES (pH 8), 100 mM potassium glutamate, 10 mM magnesium aspartate, 0.022% NP-40, 0.1 μg ml^{-1} bovine serum albumin, and 10% glycerol. The reactions were incubated at room temperature for 15 min and then electrophoresed through 3% molecular screening agarose (Roche). After migration, the gels were stained with ethidium bromide. Experiments were performed on at least two independent occasions.

RESULTS

The *sfh* promoter is growth phase regulated. A *gfp* transcriptional fusion to the *sfh* promoter was monitored in *S. flexneri* 2a strain BS184 and its *sfh* knockout mutant derivative in cultures

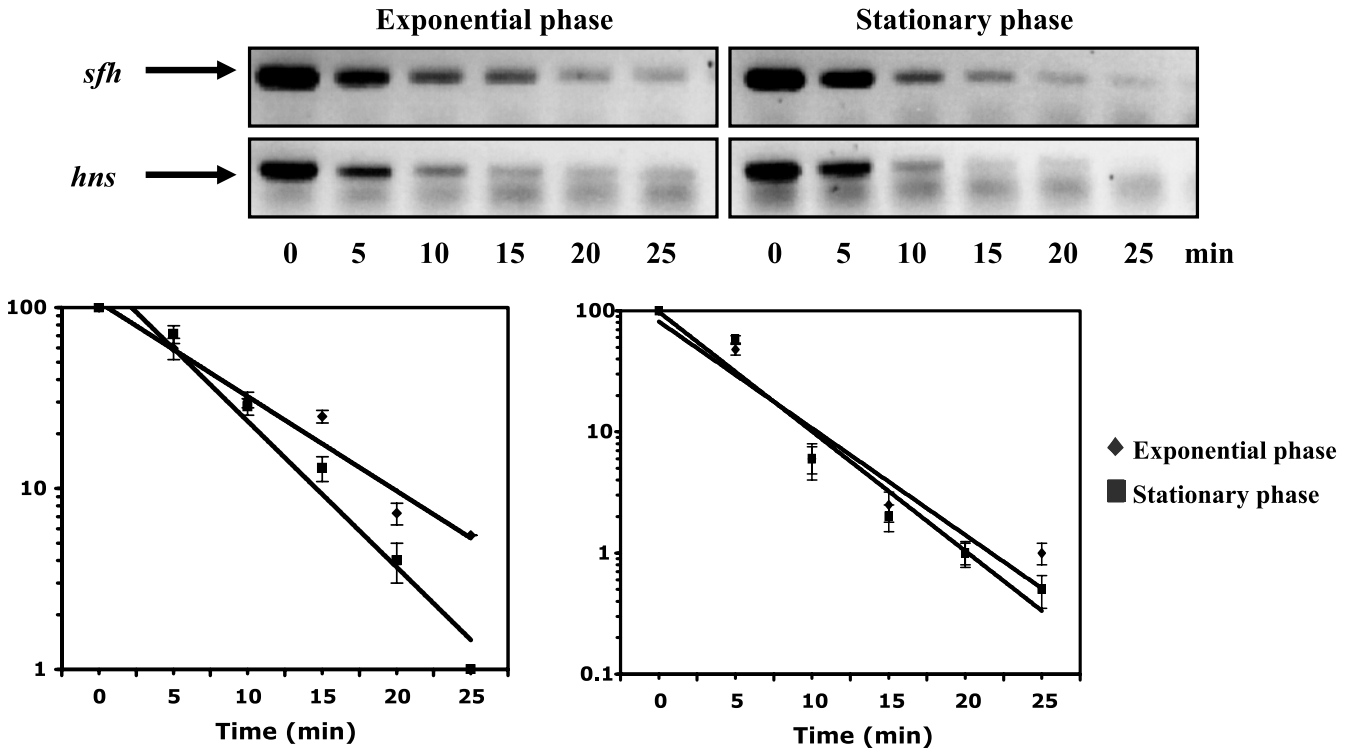

FIG. 5. Stability of *sfh* mRNA in exponential and stationary phases. Rifampin treatment was used to arrest transcription in wild-type BS184 cells growing in L broth at 37°C in exponential phase (OD_{600} = 0.2) or stationary phase (OD_{600} = 1.5). Total RNA was extracted at 5-min intervals over a 25-min period. The 0-min sample was taken just prior to addition of rifampin. RT-PCR analysis was used to assess mRNA stability by using primers specific for *sfh* and for a control transcript, *hns*. The data for *sfh* mRNA were scanned densitometrically and plotted as percentages of *sfh* mRNA remaining as a function of time. Similar decay rates were found for exponential-phase (filled diamonds) and stationary-phase (filled squares) cultures. The experiment was performed on three occasions, and representative blots are shown.

grown in L broth at 37°C. The patterns of expression were similar in both strains, although the level of *gfp* expression was higher in the absence of a functional *sfh* gene (Fig. 1). This was consistent with the known ability of Sfh to repress the *sfh* promoter (12). In both bacterial strains, *sfh-gfp* expression showed a marked growth phase dependency, with transcription being maximal in early exponential phase and declining steadily thereafter. Both *S. flexneri* strains contained the pSf-R27 plasmid. To investigate the possibility that growth phase-dependent expression was imposed by a factor or factors encoded by this plasmid, the *sfh-gfp* fusion was moved to *E. coli* K-12 strain MC4100. Here, a growth phase-dependent expression pattern similar to that observed in *S. flexneri* was seen, with maximal transcription occurring in the early exponential phase of growth (data not shown). Adding the pSf-R27 plasmid did not alter this expression pattern (data not shown). This ruled out a role for other pSf-R27 genes in the growth phase regulation of *sfh* transcription while showing that the pattern seen in *S. flexneri* was conserved when the *sfh* gene was transferred to *E. coli* K-12.

Cross-regulation as a function of growth phase. Previously, genetic evidence established roles for the H-NS and StpA proteins as repressors of *sfh* transcription (12). Electrophoretic mobility shift assays showed that H-NS and StpA bind to the *sfh* promoter region (Fig. 2). This was consistent with the previously demonstrated binding of Sfh to the same DNA fragment (12). We wished to investigate the possibility that the H-NS or StpA proteins might influence the growth phase regulation of *sfh*. Therefore, we examined *sfh* transcription by Northern blotting in wild-type, *hns*, *stpA*, and *hns stpA* strains at different stages of growth. In all four strains, the same growth phase-dependent expression pattern of *sfh* was detected (Fig. 3). However, the loss of the paralogous proteins affected the level of *sfh* expression at each stage of growth. Inactivation of *stpA* resulted in increased *sfh* transcription at early exponential phase but had no effect thereafter. Loss of the *hns* gene expression caused *sfh* to be transcribed at a higher level at all three stages of growth. This effect was enhanced when the *stpA* and *hns* mutations were combined in the same strain (Fig. 3). These data showed that while StpA and H-NS repress transcription of *sfh* at all stages of growth, they are not responsible for the growth phase-dependent pattern of *sfh* transcription.

We used Western blotting to monitor Sfh protein expression in the wild type and the *hns*, *stpA*, and *hns stpA* mutants. The results showed that each mutant expressed Sfh protein to a higher level than the wild type, in the following order: *hns stpA* > *hns* > *stpA* > the wild type (Fig. 4). These data supported those from the Northern blot analysis (Fig. 3), where H-NS and StpA were confirmed as negative influences on *sfh* gene expression but were found not to be determinants of growth phase-dependent expression.

Stability of the *sfh* mRNA as a function of growth. The previously described finding that *sfh* mRNA and Sfh protein

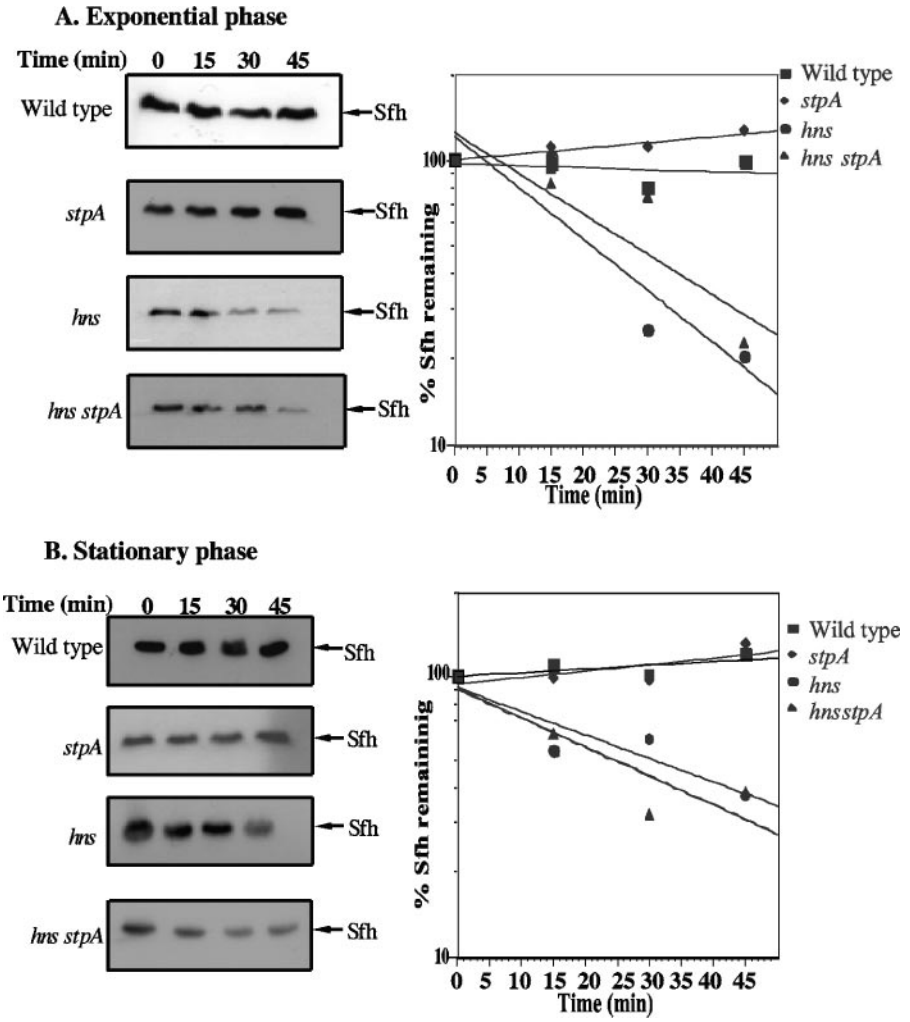

FIG. 6. Sfh protein stability. Treatment with spectinomycin was used to arrest translation in exponential (OD_{600} = ~0.3) (A)- and stationary (OD_{600} = ~1.5) (B)-phase cultures of wild-type BS184 cells and the *hns*, *stpA*, and *hns stpA* mutant derivatives growing at 37°C in L broth. Total protein was isolated for 45 min at 15-min intervals following translation inhibition, and Sfh protein was detected by Western blotting using a specific anti-Sfh antibody. The data from the Western blots were scanned densitometrically, and percentages of Sfh protein remaining were plotted as a function of time. The experiment was performed on three occasions, and representative blots are shown.

each reach their maxima at distinct phases of growth (12) suggested that *sfh* gene expression was regulated posttranscriptionally, at the level of either the *sfh* mRNA or the Sfh protein. Therefore, we decided to measure the stability of each of these molecules. Cultures of wild-type strain BS184, grown at 37°C in L broth to early exponential and early stationary phases, were treated with rifampin to stop all de novo transcription. The *sfh* transcript was then detected by RT-PCR at 5-min intervals over a 25-min period (Fig. 5). The experiment was also performed with *hns* mRNA. No significant difference in *hns* mRNA stability was detectable when results for early exponential and stationary phases of growth were compared. The rates of decay for the *sfh* mRNA, while similar, did suggest that the message was less stable in stationary-phase than in exponential-phase cultures. These results showed that if anything, the *sfh* mRNA was less stable at the later stage of growth, a period when the level of the Sfh protein was maximal. These results showed that the differential expression of Sfh protein at exponential and stationary phases of growth was not due to enhanced *sfh* mRNA stability in stationary-phase cultures.

Sfh protein stability as a function of growth. The high level of *sfh* mRNA and the correspondingly very low level of Sfh protein at the same stage in growth were consistent with Sfh protein instability, possibly due to the rapid turnover of the protein. The wild-type strain BS184 was grown in L broth at 37°C to early exponential or early stationary phase. In each case, all protein synthesis was blocked by the addition of 200 μg ml^{-1} spectinomycin to the cultures. Protein samples were removed at the time of spectinomycin addition (time zero) and at intervals of 15, 30, and 45 min thereafter. The Sfh protein was then detected by Western blotting. The results showed that the Sfh protein was equally stable during early exponential growth (Fig. 6A) and at entry into stationary phase (Fig. 6B). These data did not support a model in which Sfh protein is differentially unstable at different stages of culture growth. Instead, they pointed to a regulatory regime in which abundant *sfh*

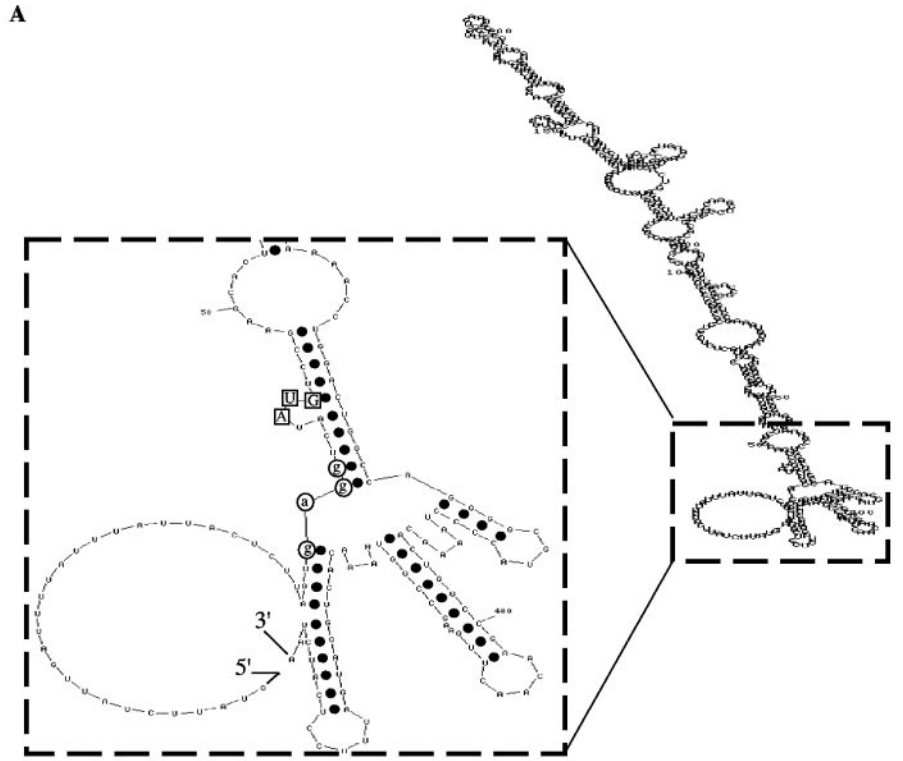

FIG. 7. Proposed secondary structure of *sfh* mRNA. (A) The Mfold RNA folding program (52) was used to predict the secondary structure of *sfh* mRNA (GenBank accession number NP_457198). The 5′ region of the transcript is magnified to show that the Shine-Dalgarno sequence (5′-gagg, with the bases within ovals) and translation initiation codon (5′-AUG, with the bases within squares) are sequestered within regions of base pairing. (B) The complete ribonucleotide sequence of *sfh* mRNA. The regions within the dashed boxes correspond to the magnified portion of the *sfh* mRNA secondary structure shown in panel A. The Shine-Dalgarno sequence is within the oval and the translation initiation codon within a rectangle. The bases in lowercase lettering form the 5′ untranslated part of the message.

mRNA in early-exponential-phase cultures was poorly translated while low levels of *sfh* transcript in early-stationary-phase cultures were translated very efficiently.

Sfh protein stability is dependent on H-NS. We had established that although the H-NS and StpA proteins were repressors of *sfh* transcription, growth phase-dependent expression of the *sfh* mRNA and Sfh protein was independent of these proteins (Fig. 3 and 4). We next examined the influence of knockout mutations in the *hns* and *stpA* genes, separately and in combination, on the stability of the Sfh protein. The results showed that the absence of H-NS (but not StpA) resulted in the reduced stability of the Sfh protein (Fig. 6). However, the same effect was seen in both exponential-phase (Fig. 6A) and stationary-phase (Fig. 6B) cultures. This ruled out a role for H-NS or StpA as a factor involved in growth phase-dependent control of Sfh protein stability.

Growth phase control is imposed after translation initiation. Our present and previous (12) data pointed to a scenario in which *sfh* mRNA was expressed in abundance in early exponential phase but not translated until the onset of stationary phase. The most straightforward explanation for these observations was that a translational blockade was imposed until an appropriate point in the growth of the culture was reached. Translational control might involve interference with the translational signals of the *sfh* gene, especially the ribosome binding site and the translation initiation codon. The Mfold mRNA secondary structure prediction algorithm (52) suggested that the native *sfh* mRNA could indeed adopt a secondary structure

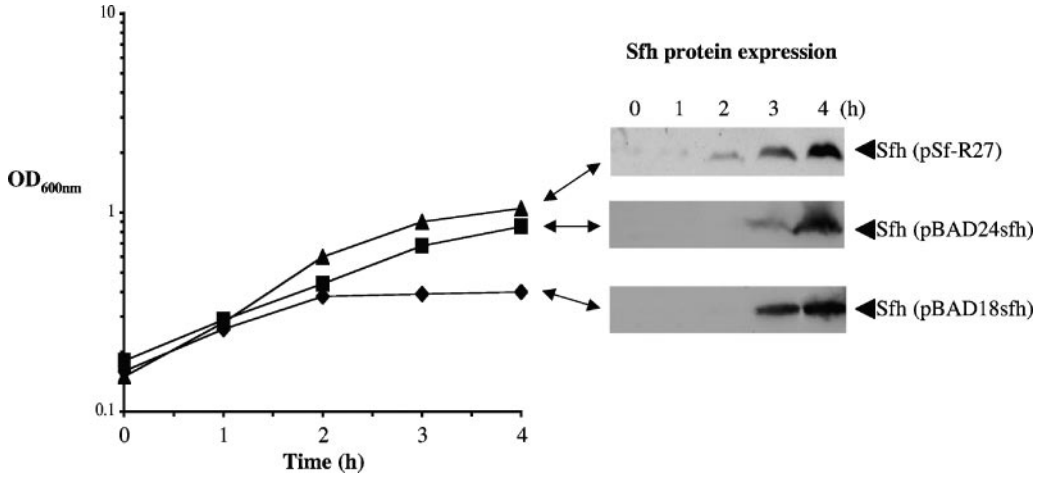

FIG. 8. Effect of altering translation signals on Sfh protein expression. Bacterial strains containing the native *sfh* gene in pSf-R27 (filled triangles), the *sfh* gene with heterologous translation initiation signals in plasmid pBAD24sfh (filled squares), or the *sfh* gene with its own translation initiation signals in plasmid pBAD18sfh (filled diamonds) were grown to early exponential phase (OD_{600}s of ~0.15 to 0.18) in LB at 37°C. The P_{BAD} promoter in plasmids pBAD18sfh and pBAD24sfh was induced by the addition of arabinose to a final concentration of 0.2% (wt/vol) at time zero. Samples were removed at the indicated time points, and Western blotting was used to monitor the expression of the Sfh protein. In each case, the maximum expression of the Sfh protein correlated with the onset of stationary phase.

in which the translation initiation signals were sequestered in a region of base-paired RNA (Fig. 7). It was reasoned that if signal sequestration contributed to growth phase control of *sfh* mRNA translation, then the replacement of the native translation initiation signals with those from an unrelated gene would alter the pattern of growth phase regulation. To test this hypothesis, the *sfh* ORF was cloned with or without its native translation initiation signals into pBAD18 or pBAD24, respectively (see Materials and Methods). In each case, production of *sfh* mRNA was controlled by inducing the P_{BAD} promoter in each plasmid with arabinose. Following induction with arabinose at time zero, the expression of Sfh protein was monitored in the culture by Western blotting and compared with that of Sfh expressed from the native pSf-R27 plasmid (Fig. 8). The expression patterns of the Sfh protein were remarkably similar in all three cases, reaching a maximum 4 hours after induction of transcription. In each case, there was a strong correlation between the appearance of high levels of Sfh protein and the entry of the culture into the stationary phase of growth. These data showed that placing the *sfh* open reading frame under the control of foreign translation initiation signals did not disrupt its characteristic growth phase-dependent pattern of expression. This suggests that the point at which growth phase control is exerted lies at a later stage in the process of translation.

DISCUSSION

The discovery of the Sfh protein brings to three the number of H-NS-like proteins expressed in *S. flexneri* 2a strain 2457T. Because H-NS has such wide-ranging effects on the global gene expression patterns of the bacterium (7, 15, 40), it is important to understand the potential for Sfh to influence these patterns in bacteria that acquire the *sfh* gene following the horizontal transfer of the plasmid that harbors it. As a first step in increasing our understanding of *sfh* biology, we investigated the expression of this gene. The *sfh* promoter was found to be at its most active in early-exponential growth, declining to a low level of activity as the culture approached late exponential phase (Fig. 1). In addition, clear evidence of growth phase dependency was found for expression of both *sfh* mRNA and Sfh protein. However, we previously noted a marked and surprising mismatch between the expression patterns of the *sfh* gene at the levels of mRNA and protein: the expression patterns at the transcriptional and posttranscriptional levels were reciprocal, with *sfh* mRNA being abundant in early-exponential growth but scarce later in growth, while the Sfh protein had the opposite pattern of expression (12).

Previous work has shown that the H-NS, StpA, and Sfh proteins each repress the *sfh* promoter (12). In the present study, we show that H-NS and StpA bind to the same *sfh* promoter DNA fragment to which Sfh had been shown previously to bind (Fig. 2). However, we could find no evidence that the growth phase-dependent expression pattern of *sfh* mRNA was influenced by these proteins. The removal of H-NS resulted simply in an overall increase in transcription, while the removal of StpA had little effect (Fig. 3).

The Sfh protein is expressed poorly in early-exponential growth (Fig. 4). This was not due to the enhanced turnover of the protein compared with that in stationary-phase cultures. Sfh was found to be equally stable in exponential- and stationary-phase cultures following inhibition of de novo protein synthesis (Fig. 6). We could find no evidence that the presence or absence of the H-NS or StpA proteins was responsible for the differences in Sfh protein stability in exponential- and stationary-phase cultures. Removal of StpA had no effect on Sfh stability at either phase of growth, while removal of H-NS resulted in the enhanced turnover of the protein in both exponential and stationary phases of growth (Fig. 6). The enhanced instability of Sfh that was seen in the *hns* mutant was reminiscent of the previously reported destabilization of StpA that is seen in the absence of H-NS (26, 27). This is due to the protection of StpA from protease turnover through the forma-

tion of heteromeric H-NS-StpA complexes. It is likely that Sfh enjoys a similar protection through interaction with H-NS, and this is consistent with previous data showing that Sfh and H-NS can form heteromers (12). Presumably, in the absence of H-NS, the complex that the StpA protein is known to form with Sfh (12) is not protective because Sfh-StpA heteromers are vulnerable to proteolytic cleavage or because there is insufficient intact StpA to act protectively due to rapid StpA turnover.

Abundant *sfh* mRNA in exponentially growing bacteria is not accompanied by a high level of Sfh protein, whereas small amounts of *sfh* mRNA in stationary phase correlate with enhanced expression of Sfh protein (12). We have ruled out differential growth phase-dependent mRNA stability or Sfh protein stability as a cause of the observed reciprocal transcript and protein expression patterns. Our data are consistent with a blockade of translation that is active in early-exponential growth and relieved at the onset of stationary phase. Negative regulation of translation can be imposed in *cis*, in *trans*, or through a combination of *cis*- and *trans*-acting processes. Mechanisms acting in *cis* usually involve the formation of a secondary structure in the message that precludes translation (1, 2, 29). The *sfh* mRNA is predicted by Mfold analysis (52) to form a secondary structure in which the likely translation initiation signals are sequestered within a base-paired region (Fig. 7). An attractive hypothesis envisions that this secondary structure is modified by a *trans*-acting factor at the onset of stationary phase, allowing the message to be translated. Alternatively, the translational blockade may be imposed by a *trans*-acting factor that sequesters the translation initiation signals by directly binding to them in early exponential phase. In the first case, the *trans*-acting factor is a positive regulator, and in the second, it is a negative regulator of translation. The putative *trans*-acting factor could be a protein or another RNA molecule, such as one of the many small RNA molecules that are known to influence translation in gram-negative bacteria (13, 22, 28, 30, 31). When this hypothesis was tested by replacing the native *sfh* translation initiation signals and 5′ UTR with foreign sequences from the *E. coli araB* gene, it was discovered that the characteristic growth phase-dependent expression of the Sfh protein was retained (Fig. 8). Thus, it seems likely that growth phase control is exerted at a stage in the translation process later than initiation. This could still involve a role for mRNA secondary structure and a *trans*-acting regulatory factor. The identity of this *trans*-acting regulator remains elusive at the present time, but our data indicate that it is encoded by the chromosomes of both *S. flexneri* 2a strain 2457T and *E. coli* K-12 and not the pSf-R27 plasmid that harbors the *sfh* gene.

Why does the Sfh protein exhibit its particular pattern of expression? These bacteria already express two H-NS-like proteins (H-NS itself and StpA) from genes on the chromosome, and it is possible that the presence of a third protein may not be tolerated well under all physiological conditions. Consistent with this proposal is the observation that the bacterial culture harboring the pBAD18sfh plasmid in which Sfh was expressed from its natural translation signals entered stationary phase at a low optical density (OD_{600} = ~0.39) following the induction of the P_{BAD} promoter (Fig. 8). The culture carrying the pBAD24sfh plasmid in which *sfh* utilized foreign translation initiation signals also ceased growing earlier than the control following transcriptional induction. Control cultures harboring just the pBAD18 and pBAD24 plasmids do not exhibit this behavior following arabinose induction (data not shown). By confining Sfh protein expression to stationary phase, the *sfh* gene may ensure that the conjugative plasmid that harbors it imposes minimal disruption on the physiology of the bacteria that acquire it by horizontal transfer.

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