Transcription of the Subtilase Cytotoxin Gene *subAB*₁ in Shiga Toxin-Producing *Escherichia coli* Is Dependent on *hfq* and *hns*

Laura Heinisch,^a Katharina Zoric,^a Maike Krause,^a Herbert Schmidt^a

^aInstitute of Food Science and Biotechnology, Department of Food Microbiology and Hygiene, University of Hohenheim, Stuttgart, Germany

ABSTRACT Certain foodborne Shiga toxin-producing *Escherichia coli* (STEC) strains carry genes encoding the subtilase cytotoxin (SubAB). Although the mode of action of SubAB is under intensive investigation, information about the regulation of *subAB* gene expression is currently not available. In this study, we investigated the regulation of the chromosomal *subAB*₁ gene in laboratory *E. coli* strain DH5 α and STEC O113:H21 strain TS18/08 using a luciferase reporter gene assay. Special emphasis was given to the role of the global regulatory protein genes *hfq* and *hns* in *subAB*₁ promoter activity. Subsequently, quantitative real-time PCR was performed to analyze the expression of Shiga toxin 2a (Stx_{2a}), SubAB₁, and cytolethal distending toxin V (Cdt-V) genes in STEC strain TS18/08 and its isogenic *hfq* and *hns* deletion mutants. The deletion of *hfq* led to a significant increase of up to 2-fold in *subAB*₁ expression, especially in the late growth phase, in both strains. However, deletion of *hns* showed different effects on the promoter activity during the early and late exponential growth phases in both strains. Furthermore, upregulation of *stx*_{2a} and *cdt-V* was demonstrated in *hfq* and *hns* deletion mutants in TS18/08. These data showed that the expression of *subAB*₁, *stx*_{2a}, and *cdt-V* is integrated in the regulatory network of global regulators Hfq and H-NS in *Escherichia coli*.

IMPORTANCE Shiga toxin-producing *Escherichia coli* (STEC) strains are responsible for outbreaks of foodborne diseases, such as hemorrhagic colitis and the hemolytic uremic syndrome. The pathogenicity of those strains can be attributed to, among other factors, the production of toxins. Recently, the subtilase cytotoxin was detected in locus of enterocyte effacement (LEE)-negative STEC, and it was confirmed that it contributes to the cytotoxicity of those STEC strains. Although the mode of action of SubAB₁ is under intensive investigation, the regulation of gene expression is currently not known. The global regulatory proteins H-NS and Hfq have impact on many cellular processes and have been described to regulate virulence factors as well. Here, we investigate the role of *hns* and *hfq* in expression of *subAB*₁ as well as *stx*_{2a} and *cdt-V* in an *E. coli* laboratory strain as well as in wild-type STEC strain TS18/08.

KEYWORDS STEC, Shiga toxin-producing *Escherichia coli*, gene expression, global regulator, *hfq*, *hns*, subtilase cytotoxin

Shiga toxin-producing *Escherichia coli* (STEC) strains are a major cause of serious foodborne diseases, leading to diarrhea, hemorrhagic colitis, and the hemolytic uremic syndrome (HUS) (1, 2). The main virulence factors described so far are the production of Shiga toxins (Stx) (3) and the components of the locus of enterocyte effacement (LEE) (4). Although the LEE is widely distributed in STEC, many LEE-negative STEC strains have been isolated and characterized (5, 6). Characterization of those strains led to identification of other putative virulence factors, such as the subtilase cytotoxin (SubAB). So far, SubAB was found to be produced only by LEE-negative STEC strains and was first identified in *E. coli* O113:H21 strain 98NK2, involved in an HUS outbreak (7). SubAB belongs to the family of AB₅ toxins and consists of a

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Address correspondence to Herbert Schmidt, herbert.schmidt@uni-hohenheim.de.

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catalytic A subunit and a homopentameric B subunit, the latter mediating binding to the terminal sialic acids of glycoproteins of the host cell (8). The catalytic activity of SubA is the cleavage of the endoplasmic chaperone BiP/GRP78, leading to an unfolded protein response and apoptosis (9, 10).

Recent studies demonstrated the contribution of SubAB to STEC cytotoxicity (11, 12). Until now, three genetic *subAB* variants have been described in STEC strains. Whereas *subAB*₁ is located on the virulence plasmid pO113, the variants *subAB*₂₋₁, *subAB*₂₋₂, and *subAB*₂₋₃ are located on the chromosome (13, 14). The regulatory pathways leading to increased expression of *subAB* are currently not known.

Global regulatory proteins regulate many cellular processes in bacteria. The host factor of bacteriophage Q β (Hfq) is an RNA chaperone and interacts with mRNA and small regulatory RNAs in *E. coli* (15). Hfq itself or the RNAs that are processed by Hfq are implicated in regulation of metabolism, quorum sensing, and regulation of adhesion and motility (16, 17). Moreover, Hfq regulates many stress responses, such as response to acids or oxidative stress (18, 19). In addition to Hfq, the histone nucleoid structuring protein H-NS is a pleiotropic regulator. It regulates transcription by binding directly to DNA or to the RNA polymerase (20). H-NS is known to alter DNA topology (20) and to regulate many processes during the bacterial life cycle. It regulates metabolic processes and is integrated in the regulatory pathway of genes involved in motility (21). Moreover, H-NS plays a role in stress response, such as cold shock response (22). Regulation of cellular processes by Hfq and H-NS have been studied extensively in recent years. Both Hfq and H-NS are integrated in the regulation of virulence factors of pathogenic bacteria as well. Kendall et al. identified the role of Hfq in regulation of virulence in enterohemorrhagic *E. coli* (EHEC) O157:H7. In this study, it was shown that deletion of the *hfq* gene led to an increase of *stx*_{2AB} expression (23). The nucleoid binding protein H-NS silences foreign DNA with high percentages of adenosine and thymine (A/T) content (24) and is therefore integrated in regulation of virulence genes. H-NS was also identified to regulate genes of the LEE. H-NS also regulates expression of genes of the hemolysin in EHEC (25).

First insights in gene expression of *subAB*₁ were given by Hauser et al. (12). In STEC strain TS18/08, isolated from minced meat, the expression of *subAB*₁ was analyzed using quantitative real-time PCR (qRT-PCR). It was shown that the expression of *subAB*₁ was highest in the exponential growth phase under standard batch culture conditions (12).

The aim of this study was to investigate whether *subAB*₁ expression is under the control of Hfq and H-NS. Therefore, deletion of *hfq* and *hns* genes was performed, and the expression of *subAB*₁ was measured with a luciferase reporter gene assay in laboratory *E. coli* strain DH5 α and the foodborne STEC strain TS18/08 (26). *E. coli* TS18/08 produces three toxins: the subtilase cytotoxin SubAB1, Shiga toxin 2a (Stx2a), and the cytolethal distending toxin V (Cdt-V) (11). Therefore, we were interested in whether the expression of the corresponding toxin genes is dependent on the global regulators Hfq and H-NS. Quantitative real-time PCR was applied to study *in vitro* transcription of those toxin genes in *E. coli* TS18/08.

RESULTS

Transcription of cloned *hfq* or *hns* genes present on complementation plasmids. Prior to the use of plasmids pLH01 and pLH02 for complementation studies, expression of cloned *hfq* and *hns* genes on these plasmids was investigated. Therefore, deletion mutants DH5 α Δ *hfq*/pHL01 and DH5 α Δ *hns*/pHL02 were grown under standard batch conditions and total RNA was isolated. Transcription analyses of *hfq* and *hns* were conducted as described previously. PCR analysis with *hfq*- and *hns*-specific primers and subsequent agarose gel electrophoresis of the resulting cDNA demonstrated PCR products with the molecular size of *hfq* (309 bp) and *hns* (414 bp). Laboratory strain *E. coli* DH5 α (positive control), as well as its *E. coli* DH5 α Δ *hfq* and DH5 α Δ *hns* (negative controls) isogenic deletion mutants, were also applied in the procedure. cDNAs of the *hfq* gene and *hns* gene were not amplified in either control, as representatively shown

FIG 1 Growth curves of *E. coli* DH5 α wild-type and mutant strains. Cultivation was conducted for 5 h in LB at 37°C with aeration. The OD₆₀₀ was determined over a period of 5 h for *E. coli* DH5 α /pKMD3, DH5 α Δ *hns*/pKMD3, and DH5 α Δ *hfq*/pKMD3 strains. Data represent mean values and standard deviations from three biological replicates.

for the *E. coli* DH5 α Δ *hfq* strain (see Fig. S1 in the supplemental material). For both the wild-type strain and the *E. coli* DH5 α Δ *hfq*/pLH01 and DH5 α Δ *hns*/pLH02 complemented strains, bands with an expected size of around 300 bp and 414 bp, respectively, were visible on the agarose gels.

Reverse transcriptase negative controls show that no cross-contamination with DNA occurred during the experimental procedure. This indicated that the plasmids pLH01 and pLH02 expressed the genes of interest and can be used for complementation of deletion mutants.

Deletion of *hfq* and *hns* leads to slower growth in STEC TS18/08. In order to investigate the effects of *hfq* and *hns* on *subAB* gene expression, growth curves of *E. coli* DH5 α (Fig. 1) and STEC strain *E. coli* TS18/08 (Fig. 2), as well as of their respective *hfq* and *hns* mutants, were initially compared. It could be shown that deletion of either *hfq* or *hns* led to a minor decrease in growth in DH5 α and both mutants, whereas the highest decreases in values of optical density at 600 nm (OD₆₀₀) were measured as 1.6-fold in the DH5 α Δ *hns* strain and 1.8-fold in the DH5 α Δ *hfq* strain in the late growth

FIG 2 Impact of deletion of genes *hfq* and *hns* on growth of STEC O113:H21 strain TS18/08. Optical density of wild-type strain (TS18/08), TS18/08/pKMD3 + pBR322 (TS18/08/pBR322) empty vector backbone control, TS18/08 Δ *hns*/pKMD3 (TS18/08 Δ *hns*) and TS18/08 Δ *hfq*/pKMD3 (TS18/08 Δ *hfq*) deletion mutants, and TS18/08 Δ *hns*/pKMD3 + pLH02 (TS18/08 Δ *hns* + *hns*) and TS18/08 Δ *hfq*/pKMD3 + pLH01 (TS18/08 Δ *hfq* + *hfq*) complemented strains is shown for measurements over 5 h. Data from three biological replicates are shown; error bars indicate standard deviations from mean values. Asterisks indicate statistical significance ($P < 0.05$) compared to values for TS18/08.

FIG 3 Comparison of *PsubAB₁* promoter activities in DH5α and TS18/08. Relative reporter gene activity (RLU/OD₆₀₀) is shown. Bars represent data for *E. coli* DH5α (dark blue) and an STEC strain (TS18/08, light blue). OD₆₀₀ is shown as squares. Cultivation was conducted for 5 h in LB at 37°C with aeration. Data from three biological replicates are shown; error bars indicate standard deviations from mean values.

phase. This effect was stronger in STEC TS18/08 than in DH5α. The deletion mutants showed significantly lower OD₆₀₀ values over the cultivation period. Whereas deletion of *hns* led to a decrease of up to 4.6-fold in the exponential growth phase, the decline in OD₆₀₀ values was 2.3-fold lower in the TS18/08 Δhfq strain than in the wild type. After 5 h of incubation, decrease rates diminished up to 1.5-fold and 1.3-fold in TS18/08 Δhns and TS18/08 Δhfq strains, respectively. The reduction in OD₆₀₀ values in the TS18/08 Δhns strain could be restored after complementation with the wild-type gene on plasmid pLH02 (Fig. 2). However, reduction in OD₆₀₀ values could not be restored in the TS18/08 Δhfq strain by complementation with plasmid pLH01. Although TS18/08 Δhfq and TS18/08 Δhns deletion mutants grew more slowly than wild-type TS18/08, all strains reached the exponential growth phase in a period of 5 h. Therefore, a growth period spanning 5 h was chosen for further investigations of *PsubAB₁* activity.

***E. coli* DH5α/pKMD3 is suitable for measuring *subAB₁* promoter activity.** Promoter activity of *subAB₁* was measured as described above in *E. coli* DH5α, *E. coli* TS18/08, and their respective Δhfq and Δhns mutants. As shown in Fig. 3, the relative promoter activity of *subAB₁* (*PsubAB₁*) of *E. coli* DH5α/pKMD3 significantly increased up to 11-fold compared to the starting value in the exponential growth phase, showing the highest activity of 2.29×10^5 relative light units (RLU)/OD₆₀₀ after 2 h of cultivation. This activity did not increase any further. Similar to the activity measured in *E. coli* DH5α/pKMD3, the *PsubAB₁* activity of TS18/08/pKMD3 increased significantly up to 7-fold compared to the starting value in the exponential growth phase and did not increase further. The highest *PsubAB₁* activity in STEC TS18/08 was detected with 3.3×10^5 RLU/OD₆₀₀ after 3 h of cultivation (Fig. 3). The results of these experiments demonstrated that the luciferase reporter system worked well for measuring *subAB₁* promoter activity.

Hfq represses *PsubAB₁* activity in *E. coli* DH5α and TS18/08 in the late exponential growth phase. In Fig. 4, the effect of the *hfq* deletion in *E. coli* DH5α (Fig. 4A) and *E. coli* TS18/08 (Fig. 4B) on *PsubAB₁* activity is shown. In the *E. coli* DH5α Δhfq /pKMD3 mutant, the relative reporter gene activity was significantly increased up to 1.8-fold compared to that of wild-type DH5α/pKMD3 in the exponential growth phase and reached its highest activity of 4.0×10^5 RLU/OD₆₀₀ after 5 h of growth. Moreover, the observed effects were reduced in the DH5α Δhfq /pKMD3 + pLH01 strain to a level similar to that of the wild type. TS18/08 Δhfq /pKMD3 deletion mutants showed behavior similar to that of the DH5α deletion mutants. The relative reporter gene activity was significantly increased up to 2-fold in the late growth phase, showing its highest activity of 5.6×10^5 RLU/OD₆₀₀ after 4 h of growth. The effects were reduced to the wild-type level after complementation of the TS18/08 Δhfq /pKMD3 strain with pLH01. Moreover, DH5α and TS18/08 did not show great differences in *PsubAB₁* activity

FIG 4 Impact of Hfq on *PsubAB*₁ activity in *E. coli* DH5α (A) and STEC *E. coli* TS18/08 (B). Relative reporter gene activity (RLU/OD₆₀₀) is shown. Bars represent data from the wild-type strain (DH5α/pKMD3 [A] or TS18/08/pKMD3 [B], solid blue) and an empty vector control (DH5α/pKMD3 + pBR322 [A] or TS18/08/pKMD3 + pBR322 [B], dashed blue). Additionally, data from *E. coli* DH5α *Δhfq*/pKMD3 (DH5α *Δhfq*) (A) and *E. coli* TS18/08 *Δhfq*/pKMD3 (TS18/08 *Δhfq*) (B) (solid green) deletion mutants and DH5α *Δhfq*/pKMD3 + pLH01 (DH5α *Δhfq* + *hfq*) (A) and TS18/08 *Δhfq*/pKMD3 + pLH01 (TS18/08 *Δhfq* + *hfq*) (B) (dashed green) complemented strains are depicted. Data are presented for measurements over 5 h of cultivation in LB at 37°C with aeration. Data from three biological replicates are shown; error bars indicate standard deviations from mean values. Statistical significance compared to the wild-type strain is indicated (*, $P < 0.05$; **, $P < 0.01$).

in either complemented strains or empty vector controls (pBR322-harboring strains). This result indicated the pBR322 backbone had no effect on the *PsubAB*₁ promoter activity.

The deletion of *hfq* led to a significant increase of *PsubAB*₁ activity in the late growth phase for both *E. coli* DH5α and STEC TS18/08. Moreover, observed effects were restored to the wild-type level if complementation was performed. These results indicate that Hfq represses *subAB*₁ promoter activity under wild-type conditions.

***PsubAB*₁ activity is dependent on H-NS in *E. coli* strains.** The impact of deletion of the *hns* gene on *PsubAB*₁ activity is depicted in Fig. 5. Relative reporter gene activity was significantly increased to a value of 1.9×10^5 RLU/OD₆₀₀ in the DH5α *Δhns*/pKMD3 laboratory strain compared to that of the control DH5α/pKMD3 after 1 h of cultivation. After 2-, 3-, and 4-h time points, the *PsubAB*₁ activity of the DH5α *Δhns*/pKMD3 strain was similar to that of the corresponding wild type. However, in the late exponential growth phase, the activity decreased up to a significant reduction of 1.5-fold relative reporter gene activity compared to that of the control. Nevertheless, the effects detected in the deletion mutant were restored in the complemented strain to levels comparable to that of *E. coli* DH5α.

FIG 5 Impact of H-NS on *PsubAB₁* activity in *E. coli* DH5α (A) and STEC TS18/08 (B). Relative (rel.) reporter gene activity (RLU/OD₆₀₀) is shown. Bars represent data from the wild-type strain (DH5α/pKMD3 [A] or TS18/08/pKMD3 [B], blue) and an empty vector control (DH5α/pKMD3 + pBR322 [A] or TS18/08/pKMD3 + pBR322 [B], dashed blue). Additionally, data from *E. coli* DH5α Δhns/pKMD3 (DH5α Δhns) (A) and *E. coli* TS18/08 Δhns/pKMD3 (TS18/08 Δhns) (B) (solid red) deletion mutants and DH5α Δhns/pKMD3 + pLH01 (DH5α Δhns + hns) (A) and TS18/08 Δhns/pKMD3 + pLH01 (TS18/08 Δhns + hns) (B) (dashed red) complemented strains are depicted. Data are presented for measurements over 5 h of cultivation in LB at 37°C with aeration. Data from three biological replicates are shown; error bars indicate standard deviations from mean values. Statistical significance compared to the wild-type strain is indicated (**, $P < 0.01$).

The increase of *PsubAB₁* activity after 1 h of cultivation in the *hns* deletion mutant was not detected in STEC strain TS18/08, but, comparable to the case in *E. coli* DH5α, the deletion of *hns* led to a decrease of *PsubAB₁* activity. In the TS18/08 Δhns/pKMD3 strain, the decrease in relative reporter gene activity was measured during the whole period of cultivation, reaching a decrease of up to 2.9-fold compared to that of the wild-type strain. As described above for DH5α, the plasmid-based complementation led to a reduction of those effects.

These results suggested an impact of the global regulator H-NS on *PsubAB₁* activity. Repression of *subAB₁* by H-NS in early growth phase and activation in the exponential growth phase were measured for *E. coli* DH5α. In STEC strain TS18/08, only activation of the *subAB₁* promoter activity was detected.

The global regulators Hfq and H-NS control expression of *subAB₁*, *stx_{2a}*, and *cdt-V*. Since TS18/08 was previously shown to express the three toxin genes *subAB₁*, *stx_{2a}*, and *cdt-V*, transcription analysis of wild-type and mutant strains was carried out by qRT-PCR. The gene expression of the three toxins was analyzed and normalized to expression of the reference gene *rrsB* (ΔC_T value, where C_T is threshold cycle). Tran-

FIG 6 Expression of *subAB*₁, *stx*₂, and *cdt-V* in *E. coli* TS18/08 Δhfq (A) and TS18/08 Δhns (B) strains. Transcription is shown as fold change relative to the level of the control (*E. coli* TS18/08). Gene expression was measured under standard batch conditions (LB medium, 37°C, with aeration) using quantitative real-time PCR. Quantification was conducted using *rrsB* as a reference gene. Error bars indicate standard deviations from the mean values. Data from three biological replicates are shown. Asterisks indicate significance in fold change compared to the level for the control ($P < 0.05$).

scription of all investigated strains was normalized to transcription of TS18/08 ($\Delta\Delta C_T$ value) at each time point separately. The effect of the deletion of the *hfq* gene (Fig. 6A) and the *hns* gene (Fig. 6B) in *E. coli* TS18/08 is shown as fold change of mRNA levels in Fig. 6. The expression of *subAB*₁ was increased significantly up to 2-fold in the *E. coli* TS18/08 Δhfq strain in the late exponential growth phase. The transcription of *stx*_{2a} in this strain showed a similar pattern, whereas a 2.8-fold increase was measured after 5 h of cultivation compared to the level for TS18/08. The highest upregulation was detected for *cdt-V* expression. Similar to that of the other toxins, the 14-fold upregulation was measured in the late growth phase.

Upregulation of the expression of all three toxins was also found in the *E. coli* TS18/08 Δhns strain. *subAB*₁ transcription increased up to 6-fold after 1 h of cultivation and up to 3-fold in the late growth phase at 5 h of cultivation. The expression of *cdt-V*

increased up to 16-fold in the late growth phase and therefore was comparable to the one obtained in the *E. coli* TS18/08 Δhfq strain. The expression of *stx*_{2a} increased over the whole bacterial life cycle in the *E. coli* TS18/08 Δhns strain and was 10-fold upregulated after 2 h of cultivation.

The results obtained by qRT-PCR showed that both Hfq and H-NS have an impact on the expression of the toxins produced by STEC TS18/08. The strongest effects were detected in the late growth phase. In comparisons of the expression of the three toxins, the highest increase was detected in the *E. coli* TS18/08 Δhns for *stx*_{2a} transcription. Therefore, the results of these experiments let us conclude that Hfq and H-NS had a strong impact on toxin gene transcription in STEC TS18/08.

DISCUSSION

Regulation of transcription of virulence factors is of major importance for pathogens, since maximum transcription of such factors at the site of infection may be advantageous for the colonization and infection process. In recent years, STEC strains have been analyzed frequently for the presence and absence of genes encoding virulence factors, such as Shiga toxins, type III effectors, adhesins, and EHEC-hemolysin, or for the qualitative description of their expression (14, 27–30). In some cases, data on the regulation of virulence factors have been published. For example, Shiga toxin expression is included in the phage induction cycle and is *recA* dependent (31). Moreover, the complex regulation of the LEE operon is well elucidated, and there is some knowledge on regulation of EHEC-hemolysin.

During recent years, STEC strains that carry genes encoding a subtilase cytotoxin have been described increasingly, and it was demonstrated that this toxin contributes to virulence of STEC (11). In a recent study, we could show that the subtilase is expressed during the bacterial growth cycle in batch culture, but the dependence of regulators is currently not known (12). Therefore, we were interested in getting deeper insight into the transcription regulation of *subAB*₁.

In this study, we investigated the effects of *hfq* and *hns* deletions in laboratory strain *E. coli* DH5 α and wild-type STEC O113:H21 strain TS18/08. Both strains showed their highest promoter activity in the exponential growth phase. Hauser et al. (12) have demonstrated that transcription of *subAB*₁ was highest after 3 h of cultivation under standard batch conditions, showing a 7-fold increase in transcription compared to the initial value. This is similar to the results obtained in our study. Interestingly, the promoter activity varied only slightly between the two tested strains.

Moreover, we could show that Hfq acts as a repressor of *subAB*₁ promoter activity in the late growth phase. This finding was strain independent. Hfq is a pleiotropic regulator and has an impact on the expression of many virulence genes (23, 32–35). In former studies, the regulation of toxin gene expression by Hfq was shown. Kendall et al. studied EHEC O157:H7 strain 86-24 and its mutant with a *hfq* deletion and its respective Shiga toxin expression. They could show that transcription of *stx*_{2AB} was increased in this deletion mutant (23). This result could be reproduced in our study with the *E. coli* TS18/08 Δhfq strain. In addition, upregulation of *cdt-V* was detected in the same mutant strain, assuming that Hfq is integrated in the regulation of different toxins. Both SubAB and Shiga toxin belong to the family of AB₅ toxins (36, 37), whereas the Cdt-V toxin is an AB₂ toxin consisting of three subunits (Cdt-A, Cdt-B, and Cdt-C) (38). The regulation by Hfq seems not to be dependent on the type of toxin.

Hfq can directly interact with DNA and regulate transcription, but most processes described indicate regulation by Hfq-dependent small regulatory RNAs (sRNAs) (25, 34, 39). In recent studies, the prevalence of sRNAs in gene regulation were shown (40). Oogai et al. have shown that the regulation of expression of Cdt-V is dependent on sRNAs in *Aggregatibacter actinomycetemcomitans* (41). We suggest that this is the same regulatory pathway in STEC strain TS18/08. Regulation of toxins could be integrated in networks regulated by sRNAs due to the fast response to environmental changes. This cascade is controlled by the RNA chaperone Hfq.

Regulation of toxin gene expression was reported not only for sRNAs and Hfq but

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant geno- and phenotype ^a	Reference or source
<i>E. coli</i>		
DH5α	<i>tonA lacZΔM15 endA1 recA1 thi-1 supE44 phoA gyrA96 hsdR17 Δ(lacZYA-argF)U169 relA1</i>	Invitrogen
DH5α <i>Δhns</i>	<i>tonA lacZΔM15 endA1 recA1 thi-1 supE44 phoA gyrA96 hsdR17 Δ(lacZYA-argF)U169 relA1 Δhns</i>	This study
DH5α <i>Δhfq</i>	<i>tonA lacZΔM15 endA1 recA1 thi-1 supE44 phoA gyrA96 hsdR17 Δ(lacZYA-argF)U169 relA1 Δhfq</i>	This study
HB101	<i>F⁻ mcrB mrr hsdS20(r_B⁻ m_B⁻) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20 (Sm^r) supE44 λ⁻</i>	52
TS18/08	O113:H21, <i>cdt-V</i> ⁺ <i>subAB</i> ₁ ⁺ <i>stx</i> ₂ ⁺	26
TS18/08 <i>Δhns</i>	O113:H21, <i>cdt-V</i> ⁺ <i>subAB</i> ₁ ⁺ <i>stx</i> ₂ ⁺ <i>Δhns</i>	This study
TS18/08 <i>Δhfq</i>	O113:H21, <i>cdt-V</i> ⁺ <i>subAB</i> ₁ ⁺ <i>stx</i> ₂ ⁺ <i>Δhfq</i>	This study
Plasmids		
pKD46	Contains genes of red recombinase system under control of <i>araB</i> promoter, Amp ^r	46
pKD4	Template plasmid for kanamycin resistance cassette, Amp ^r , Kan ^r	46
pCP20	Contains genes for FLP recombinase, Amp ^r	46
pKMD3	Luciferase reporter gene plasmid containing the <i>subAB</i> ₁ promoter region	This study
pBR322	Cloning vector, pMB1 origin of replication, Amp ^r , Tet ^r	53
p3121	Luciferase reporter gene (<i>luc</i>) template vector, Amp ^r , Kan ^r	54
pWSK29	Cloning vector (low copy number), Amp ^r	55
pLH01	Complementation plasmid, pBR322 backbone, with <i>hfq</i> gene under control of its own promoter, Tet ^r	This study
pLH02	Complementation plasmid, pBR322 backbone, with <i>hns</i> gene under control of its own promoter, Tet ^r	This study

^aAmp^r, ampicillin resistance gene; Kan^r, kanamycin resistance gene; Tet^r, tetracycline resistance gene.

also for H-NS. Wan et al. revealed, by RNA sequencing, the repressing role of H-NS on expression of the hemolysin and Shiga toxin in EHEC (42). In our study, we showed by quantitative real-time PCR that H-NS repressed the expression of not only *stx*_{2a} but also *cdt-V* and *subAB*₁. Upregulation of *subAB*₁ expression in the TS18/08 *Δhns* strain was detected in the early and late growth phases. For the *E. coli* DH5α *Δhns* strain, upregulation could be confirmed only in the early growth phase. Moreover, we measured a decreased promoter activity in the latter strain in the late growth phase. This result might be due to the different methods applied, and factors such as RNA decay could influence the results. Another possibility is that the toxin genes studied are not regulated directly by H-NS but are integrated in the regulatory pathway of H-NS. Wan et al. identified, by RNA sequencing, up to 983 genes which are regulated by H-NS in EHEC (42). H-NS is an important factor in xenogeneic gene silencing. Genes involved in response to environmental stimuli, such as the acidic environment, temperature, pH value, or host signal peptides, could have a direct impact on promoter activity and posttranscriptional processes. Moreover, H-NS interacts directly with other nucleoid binding proteins, such as the DNA binding protein StpA (24). Those proteins regulate gene expression if H-NS is not present (43, 44). Moreover, it has been shown that StpA in combination with Hfq could reduce effects of an *hns* deletion mutant. Thus, it was shown that phenotypic characteristics of a *Δhns* mutant were counteracted by *hfq* on a multicopy plasmid (45). This counteraction by Hfq and StpA might be the reason why different results were acquired for *subAB*₁ expression in the TS18/08 *Δhns* strain in this study by comparing the luciferase reporter gene assay and qRT-PCR.

Given the fact that both global regulators showed influence on gene expression of three important STEC toxins, the role of these proteins in virulence and pathogenicity of STEC should be further investigated.

Conclusion. In this study, we demonstrated that expression of *subAB*₁, *stx*_{2a}, and *cdt-V* is dependent on functional global regulatory proteins Hfq and H-NS in both *E. coli* DH5α and STEC strain TS18/08. The presented data reveal that the expression of the virulence factors is integrated in the circuit of global regulatory proteins. Further studies will be necessary to elucidate the complex regulatory network.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1. Strains were routinely grown in Luria-Bertani (LB) broth consisting of 1.0% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, and 1.0% (wt/vol) sodium chloride, adjusted to pH 7.0. For solid media, 1.5% (wt/vol) agar-agar was added. If necessary, ampicillin, kanamycin, and tetracycline were added to the

medium in final concentrations of 100 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, and 10 $\mu\text{g/ml}$, respectively. Overnight cultures were prepared by incubating two colonies in 10 ml LB medium in 100-ml Erlenmeyer flasks at 37°C on a rotary shaker at 180 rpm.

Plasmids were prepared using a QIAprep spin miniprep kit (Qiagen, Germany) by following the manufacturer's recommendations (QIAprep miniprep handbook from July 2006; Qiagen). Genomic DNA for cloning procedures was isolated using a DNeasy Blood and Tissue kit (Qiagen) according to the procedure described in the DNeasy Blood and Tissue handbook (July 2006). Concentration and purity of nucleic acids were determined using a NanoDrop 2000 spectrophotometer (ThermoScientific, USA) and by agarose gel electrophoresis.

Construction of deletion mutants. Deletion of genes was conducted as described by Datsenko and Wanner (46), with minor modifications. Briefly, the genes of interest were exchanged with a kanamycin resistance cassette by homologous recombination. Primers were designed with Serial Cloner, version 2.6.1 (SerialBasics; Franck Perez, Paris, France). Whole-genome sequences of *E. coli* DH5 α (NCBI accession number [NZ_JRYM01000001](#)), *E. coli* K-12 MG1655 (GenBank accession number [NC_000913.3](#)), and *E. coli* TS18/08 (NCBI accession number [ASM199093v1](#)) were used to design primers targeting *hfq* and *hns*, including their surrounding regions. Moreover, the genomic backgrounds of *hfq* and *hns* were compared in both strains by DNA sequencing. Thus, surrounding regions, meaning 86-bp upstream and 76-bp downstream sequences for the gene *hfq* and 144-bp upstream and 300-bp downstream sequences for the gene *hns*, were amplified using the primers stated in Table 1. Substitution of the kanamycin cassette was carried out using the helper plasmid pKD46, encoding the recombinase genes γ , β , and *exo*. Using a second helper plasmid, pCP20, which encodes FLP recombinase, the resistance cassettes were excised. Transformation of plasmids and linear fragments was performed as described below. Primers used for mutagenesis are shown in Table 2. Deletion mutants were verified by PCR with subsequent agarose gel electrophoresis and DNA sequencing. The *E. coli* DH5 α Δhns , *E. coli* DH5 α Δhfq , *E. coli* TS18/08 Δhns , and *E. coli* TS18/08 Δhfq deletion mutants were analyzed for a presumable 2nd copy of these genes by PCR using primers *hfq_for*, *hfq_rev*, *hns_for*, and *hns_rev* (Table 2).

Electroporation. Transformation of PCR products and plasmids was conducted by electroporation (GenePulser Xcell electroporation system; Bio-Rad, USA), and competent cells were prepared as described previously (47). To each cell aliquot, 300 ng of PCR products or 30 ng of plasmid DNA was added. Transformation was performed in electroporation cuvettes (2 mm; Bio-Rad) at 25 μF , 200 Ω , 2.5 kV, and 5 ± 0.2 ms. Residual steps were carried out as described previously (47).

Construction of reporter gene plasmid pKMD3. The luciferase reporter gene plasmid pKMD3 was constructed using the isothermal Gibson Assembly (NEB, USA) strategy. Therefore, a 405-bp region upstream of *subAB*₁, including the putative *subAB*₁ promoter region, was cloned from *E. coli* O113:H21 strain TS18/08. The plasmid map is shown in Fig. 7. The vector backbone consisted of plasmid pWSK29, which was linearized by restriction with PvuII and subsequent purification by agarose gel extraction using a QIAex II gel extraction kit (Qiagen, Germany) according to the manufacturer's instructions (QIAex II handbook, March 2015; Qiagen). The luciferase gene (*luc*) insert (1,653 bp) was amplified using plasmid p3121 as the target using primers *luc-gibson_for* and *luc-gibson_rev* (Table 2). The promoter region of the *subAB*₁ gene was amplified with primers *subAB-gibson_for* and *subAB-gibson_rev* (Table 2) using genomic DNA of *E. coli* TS18/08. The isothermal assembly was performed at 60°C for 50 min with an insert-to-vector ratio of 2:1. The assembly mixture was directly transformed by electroporation in competent *E. coli* DH5 α cells as described previously. Cloning of the reporter gene plasmid was verified by PCR and DNA sequencing.

Luciferase assay. Analysis of subtilase cytotoxin promoter activity (*PsubAB*₁) was conducted using a luciferase reporter system (Promega, Madison, WI) by following the recommendations of the manufacturer (luciferase assay system, technical bulletin from 2015; Promega Corporation). Strains harboring the reporter gene plasmid pKMD3 (Table 1) were grown in LB medium at 37°C, 180 rpm, for 16 h. Twenty-four milliliters of LB medium in a 100-ml Erlenmeyer flask was inoculated with an overnight culture to an initial OD₆₀₀ of 0.1 (unless stated otherwise) and further incubated at 37°C, 180 rpm, for 5 h. During cultivation, hourly sample taking and measurement of the OD₆₀₀ was conducted in duplicates for each culture. In addition, samples were collected to measure luciferase activity. Thus, 400- μl samples were taken in duplicates for each strain and centrifuged at $6,000 \times g$, 4°C, for 5 min. Supernatants were discarded and pellets were resuspended in 90 μl of $1 \times$ cell culture lysis reagent (CCLR; Promega, USA) containing 10 mg/ml bovine serum albumin (albumin fraction V; Carl Roth, Germany). Subsequent adding of 90 μl $1 \times$ CCLR containing 10 mg/ml lysozyme (Sigma-Aldrich, Germany) was performed, and samples were frozen immediately at -70°C . Thawing of samples was conducted by incubation at 23°C with 300 rpm for 20 min in a heating block.

Measurement of luciferase activity was performed as described here and prepared in duplicates for each sample. Fifty-microliter aliquots of thawed samples were transferred to a white microtiter plate (LUMITRAC 600; Greiner). As a substrate, 50 μl of luciferase assay reagent (Promega) was added to samples, and luminescence was measured immediately at 562 nm using a microplate reader (Infinite M200; Tecan). To compare the results during cultivation, relative reporter gene activity was defined as the ratio of relative light units (RLU) to the respective OD₆₀₀. All experiments were performed three times, each on a different day.

Cloning of vectors for complementation experiments. For complementation experiments, plasmids were constructed containing *hfq* and *hns*, including their putative promoter regions, as shown in Fig. 8. The medium-copy-number plasmid pBR322 (Table 1) was chosen as a cloning vector due to its compatibility with the luciferase reporter gene plasmid pKMD3. The plasmid was double digested with restriction enzymes PvuI (NEB, USA) and AseI (NEB, USA). The reaction was carried out using 1 μg of DNA

TABLE 2 Oligonucleotide primers used in this study

Primer and/or category	Use	Nucleotide sequence ^a (5' to 3')	Source
Hfq_for	Detection of <i>hfq</i> gene	ATGCTAAGGGGCAATCTTTACAAG	This study
Hfq_rev	Detection of <i>hfq</i> gene	TTATTCGGTTCCTCGCTGTCCTG	This study
Hns_for	Detection of <i>hns</i> gene	TTATTGCTTGATCAGGAAATCGTCG	This study
Hns_rev	Detection of <i>hns</i> gene	ATAGCGAAGCACTTAAATTCCTG	This study
Mutagenesis			
Del_hfq_for	Deletion of <i>hfq</i> gene, creation of resistance cassette	AATGTGACAAATTGAGACGTATCTGTGCGCATTTTTTCAGAATC <u>CGGATTGTAGGCTGGAGC</u>	This study
Del_hfq_rev	Deletion of <i>hfq</i> gene, creation of resistance cassette	AGCGTATAACCTCTAAATAGATCAGCGGGGAACGCAGGATCCATGGTCCATATGAATATCCTCC	This study
Hfq + 186up_for	Verification of deletion of <i>hfq</i>	CCTGGCTGCGTGGTTGGGAAG	This study
Hfq-176down_rev	Verification of deletion of <i>hfq</i>	ACCAGAGATTCAAACTCTCGGAGTCC	This study
Del_hns_for	Deletion of <i>hns</i> gene, creation of resistance cassette	ACATCCGTATCGGTGTACACGAAACGGGTTGAGCAATCCGATTGTGTAGGCTGGAGC	This study
Del_hns_rev	Deletion of <i>hns</i> gene, creation of resistance cassette	ATAGGGAAATTCGTAAACACAACTAATACAGAAGACTGAAAGCATGGTCCATATGAATATCCTCC	This study
Hns + 244up_for	Verification of deletion of <i>hns</i>	TAGGTTACATGACAGGCCTTCGTTG	This study
Hns-705down_rev	Verification of deletion of <i>hns</i>	ACAGTGCATGCCCGGTTCC	This study
Construction of complementation plasmids			
Hfq + 86up_AseI_for	Complementation studies	GGGGGGGGGgattaatAATGTGTACAATTGAGACGTATCGTCGCAATTTTTTTCAGAATC	This study
Hfq-76down_PvuI_rev	Complementation studies	GGGGGgattcgAGCGTATACCTCTAAATAGATCAGCGGGGAACGCAGGATC	This study
Hns + 144up_AseI_for	Complementation studies	CCCCCgattaatACATCCGTATCGGTGTATCCACGAAACGGCGTTGAGCAATC	This study
Hns-300down_PvuI_rev	Complementation studies	CCCCCCgattcgATAGGGAAATTCCTCGTAAACACAACCTAATACAGAAGACTGAAAGG	This study
Construction of luciferase reporter gene plasmid pKMD3			
subAB-gibson_for	Cloning of <i>subAB</i> ₁ promoter region for pKMD3	<u>TTCGTATTACGCCAGCTGAACGGTATCGATCCG</u>	This study
subAB-gibson_rev	Cloning of <i>subAB</i> ₁ promoter region for pKMD3	<u>GTCTTCATAAGCTCTCCAGTTAGTAC</u>	This study
luc-gibson_for	Cloning of <i>luc</i> for pKMD3	AGGAGCTTATGGAAGACGCCAAAAAC	This study
luc-gibson_rev	Cloning of <i>luc</i> for pKMD3	<u>CCGATTCAATATGCAGTCACAATTTGGACTTTCCG</u>	This study
Gibson-1_for	Verification of pKMD3	ACACCCGCCGCTTAATGc	This study
Seq 2-gibson_rev	Verification of pKMD3	GATACCGCTCGCCGAGCCG	This study
Seq 3-gibson_for	Verification of pKMD3	GGTTGTGATCTGGATCCCG	This study
Seq 4-gibson_rev	Verification of pKMD3	AGGCGTATCTCTCATTAGC	This study

^aUnderlined bases depict homologous regions in mutagenesis, and underlined lowercase letters depict restriction sites for cloning strategy.

FIG 7 Plasmid map of the luciferase reporter gene plasmid pKMD3. The putative promoter region of *subAB*₁ (405 bp; *PsubAB*₁) was cloned upstream of the luciferase reporter gene *luc* (1,652 bp) in the pWSK29 backbone restricted with PvuII (pWSK29_PvuII).

template, 10 U of each enzyme, and NEB buffer 3.1. Restriction was performed at 37°C for 60 min. Inserts of the sequences were cloned, 86-bp upstream and 76-bp downstream sequences for the gene *hfq* and 144-bp upstream and 300-bp downstream sequences for the gene *hns*, and were amplified using primers with restriction sites at their 3' ends as shown in Table 2. Amplification was conducted using genomic DNA of *E. coli* strain DH5 α (DNeasy Blood and Tissue kit; Qiagen, Germany) as the template. Oligonucleotides (Eurofins MWG Operon, Germany) were designed using Serial Cloner, version 2.6.1 (SerialBasics; Franck Perez, Paris, France). Purification of linearized vector DNA was conducted by excising the respective band on an agarose gel (1%, wt/vol) and subsequently isolated using a Wizard SV gel and PCR clean-up system (Promega, USA). The amplified inserts were digested with PvuI and AseI as stated above and purified using a PCR purification kit (Qiagen, Germany) by following the manufacturer's recommendations (QIAquick spin handbook, April 2015; Qiagen). Ligation of vector and the respective insert was performed with T4 DNA ligase (ThermoFisher Scientific) at 22°C for 1 h using a vector-to-insert ratio of 1:5. After ligation, the resulting plasmids pLH01 and pLH02 were transformed in competent *E. coli* DH5 α cells as described previously, and transformants were selected on agar plates containing tetracycline (10 μ g/ml). Recombinant plasmids were then verified by DNA sequencing using the respective primers listed in Table 2 and finally transformed in reporter strains and deletion mutants using electroporation as described previously.

Analysis of gene transcription on complementation plasmids. The expression of the cloned genes on the recombinant plasmids used for complementation was verified by transcription analysis as described earlier (48). Briefly, RNA was isolated using the RNeasy minikit (Qiagen, Germany). Strains harboring complementation plasmids were grown in LB medium at 37°C with agitation to an OD₆₀₀ of 1.0. Five hundred microliters of the culture was added to 1 ml of RNeasyprotect bacterial reagent (Qiagen, Germany). Isolation of RNA was conducted by following the manufacturer's recommendation, and DNA

FIG 8 Cloning strategy of complementation vectors pLH01 (A) and pLH02 (B). Positions of *hns* and *hfq* genes refer to the genome sequence of *E. coli* DH5 α (NCBI accession number [NZ_JRYM01000001](https://www.ncbi.nlm.nih.gov/nuccore/NZ_JRYM01000001)).

TABLE 3 Oligonucleotide primers used for transcription analysis by qRT-PCR

Primer	Nucleotide sequence (5' to 3' direction)	PCR product size and conditions	Reference
rrsB-for	GCATAACGTCGCAAGACCAAA	91 bp; 95°C, 10 s; 60.0°C, 30 s; 72°C, 10 s	51
rrsB-rev	GCCGTTACCCACCTACTAGCT		
RTsubAB-2-for	GCAGGTATTATGGGATGTCT	177 bp; 95°C, 15 s; 60.3°C, 20 s; 72°C, 10 s	12
RTsubABrev	GCAGGCACTTATGGAGGAG		
Upper-stx2	TCATATCTGGCGTTAATGGAGTTC	99 bp; 95°C, 10 s; 52.4°C, 30 s; 72°C, 10 s	51
Lower-stx2	GCGTAAGGCTTCTGCTGTG		
RTCDTA-for	CCGATGGTAACACGCAATG	184 bp; 95°C, 10 s; 53.0°C, 30 s; 72°C, 10 s	12
RTCDTA-rev	GCCTTAATGGTTCGCTTATGG		

was digested using a DNA-free kit (Thermo Fisher Scientific, USA) according to the manual (user guide, October 2012; Life Technologies Corporation). RNA concentration and purity were determined spectrophotometrically using a NanoDrop 2000 device (Thermo Scientific, USA), followed by visual inspection on denaturing agarose gel electrophoresis (data not shown). Transcription to cDNA was performed using 1 µg RNA, a SuperScript II reverse transcriptase kit (Bio-Rad, USA), and primer for detection of the *hfq* (*hfq*_{for} and *hfq*_{rev}) or *hns* (*hns*_{for} and *hns*_{rev}) gene (Table 2) by following the manufacturer's recommendations (manual of SuperScript II from 2010; Life Technologies Corporation). As a control, cDNA synthesis was performed using random primers as given in the SuperScript II reverse transcriptase kit (random hexamer oligonucleotides). For each approach, the reverse transcriptase negative control was applied to avoid false-positive results due to residual DNA present in samples. Synthesized cDNA was analyzed by PCR using primers specific for genes *hfq* and *hns* as stated above. Subsequently, amplification was investigated by agarose gel electrophoresis. Expression control was performed in three biological replicates independently, each on different days.

RNA isolation. Erlenmeyer flasks containing 24 ml LB medium were inoculated with overnight culture to an OD₆₀₀ of 0.1. *E. coli* TS18/08 was cultivated at 37°C, 180 rpm, for 5 h, and samples were taken hourly for RNA isolation. Therefore, 1.0×10^9 cells were transferred to a 2-fold volume of RNeasy lysis buffer (Qiagen, Germany). The suspensions were mixed immediately for 5 s, followed by an incubation at room temperature for 5 min. Centrifugation was then performed at room temperature at $5,000 \times g$ for 10 min, and the supernatants were discarded. Pellets were stored at -70°C until further processing. RNA isolation was performed as described in the manual of the RNeasy lysis buffer (Qiagen, Germany) and RNeasy minikit (Qiagen, Germany) by following the protocol for enzymatic lysis and proteinase K digestion (protocol 4) and subsequent purification of total RNA (protocol 7). RNA purity and concentration were determined using a spectrophotometer (NanoDrop 2000; Thermo Scientific, USA), and integrity of RNA was examined using formaldehyde-based denaturing agarose gel electrophoresis.

Transcription analysis. Prior to cDNA synthesis, residual DNA was degraded in the samples using a DNA-free kit (ThermoScientific, USA) by following the recommendations stated in the manual (user guide, October 2012; Life Technologies Corporation). Reverse transcription was performed using an iScript cDNA synthesis kit (Bio-Rad, USA) with the following procedure: 500 ng of the RNA samples was used for reverse transcription and incubated according to the protocol stated by the manufacturer (manual from 2000; Bio-Rad Laboratories). For each sample, a reverse transcription-negative approach was conducted as a control to detect residual DNA. Synthesized cDNA was used for quantitative real-time PCR in a CFX96 system (Bio-Rad, USA) by following the experimental procedure described previously (12). Therefore, 10 µl of SsoAdvanced universal SYBR green supermix (Bio-Rad, USA), 2.8 ng of cDNA, 0.75 µl of each primer (10 pmol/µl), and nuclease-free water to the end volume of 20 µl were used for each qRT-PCR approach. Primers and respective PCR conditions are shown in Table 3. Analysis was performed in duplicates and with three biological replicates. Analysis of data was performed using the $2^{-\Delta\Delta C_T}$ method described by Pfaffl et al. (49, 50). Thus, expression data for genes *subAB*₁, *stx*₂, and *cdt-V* were normalized to expression of reference gene *rrsB* (16S rRNA gene) (ΔC_T). Subsequently, ΔC_T values were normalized to the values of *E. coli* TS18/08 ($\Delta\Delta C_T$). The data were analyzed for each point of time independently. For all targets, standard curves of recombinant plasmids were used to identify target efficiency as described previously (12, 51). No-template controls and reverse transcription-negative controls were applied in each approach and showed C_T values after 35 to 40 cycles of PCR (data not shown). Each sample was analyzed in at least two technical replicates, and experiments were conducted in three independent experiments on different days (biological replicates).

Statistical analysis. Means from biological replicates were used for statistical analysis. Data were analyzed on normal distribution using the Grubb's test. If data sets of two different time points measured were compared for one strain, paired Student's *t* test was used, and if data were not normally distributed, Mann-Whitney test was applied. Data sets of different strains at identical time points measured were analyzed by application of analysis of variance (ANOVA). The variance homogeneity was identified by residual plots (Q-Q-plots). If requirements were given, one-way ANOVA was performed using a *post hoc* Tukey test ($\alpha = 0.05$). If requirements were not met, data were analyzed using Welch's ANOVA and *post hoc* Games-Howell test. Statistical analysis was performed using the software SPSS Statistics 25 (IBM, USA), and statistical significance was given if the *P* value was $< \alpha$.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01281-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We have no conflicts of interest to declare.

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