The VirF$_{21}$:VirF$_{30}$ protein ratio is affected by temperature and impacts Shigella flexneri host cell invasion

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Abstract.

Shigella spp, the etiological agents of bacillary dysentery in humans, have evolved an intricate regulatory strategy to ensure fine-tuned expression of virulence genes in response to environmental stimuli. A key component in this regulation is VirF, an AraC-like transcription factor, which at the host temperature (37°C) triggers, directly or indirectly, the expression of > 30 virulence genes important for invasion of the intestinal epithelium. Previous work identified two different forms of VirF with distinct functions: VirF$_{30}$ activates virulence gene expression, while VirF$_{21}$ appears to negatively regulate virF itself. Moreover, VirF$_{21}$ originates from either differential translation of the virF mRNA or from a shorter leaderless mRNA (llmRNA). Here we report that both expression of the virF$_{21}$ llmRNA and the VirF$_{21}$:VirF$_{30}$ protein ratio are higher at 30°C than at 37°C, suggesting a possible involvement of VirF$_{21}$ in minimizing virulence gene expression outside the host (30°C). Ectopic elevation of VirF$_{21}$ levels at 37°C indeed suppresses Shigella’s ability to infect epithelial cells. Finally, we find that the VirF$_{21}$ C-terminal portion, predicted to contain a Helix-Turn-Helix motif (HTH2), is required for the functionality of this negative virulence regulator.

Keywords: Shigella, virulence genes, regulation, infection, cell invasion, Shigellosis

Introduction

Enterobacterial pathogens coordinate the expression of virulence factors through complex regulatory networks in order to colonize and disseminate in the host gut epithelium. Shigella flexneri bacteria, facultative intracellular microbes causing bacillary dysentery in humans, have become paradigmatic for the study of virulence gene regulation. The expression and combined action of numerous virulence factors, mainly encoded on a large virulence plasmid (pINV), ultimately result in the invasion of colonic epithelial cells in the lower gut (Mattock and Blocker 2017). Subsequently, the bacteria multiply intracellularly and spread to adjacent cells, resulting in cell death and inflammatory destruction of the gut mucosa (Schroeder and Hilbi 2008, Arena et al. 2015). A crucial regulator of the Shigella infection process is VirF, an AraC-like transcription factor, responsible for the invasive phenotype (Di Martino et al. 2016a). The synthesis of VirF occurs when Shigella senses the transition from the external environment to the human host (Falconi et al. 1998, Prosseda et al. 2004). VirF then triggers a regulatory cascade involving the expression of virB and icsA genes (Tobe et al. 1993, Tran et al. 2011). VirB activates a second wave of virulence genes involved in the assembly of a type 3 secretion system (T3SS), its effectors (the ipa-spa operons), and a second AraC-like transcriptional activator, mxiE (Le Gall et al. 2005, Parsot 2005, Schroeder and Hilbi 2008). IcsA, on the other hand, promotes Shigella dissemination across adjacent cells through host actin polymerization (Bernardini et al. 1989, Lett et al. 1989). Finally, the master regulator VirF also activates some chromosomally located genes (e.g. the spermidine excretion complex MdU1; the chaperones IbpA, HtpG, DnaK and the protease Lon) whose expression may optimize Shigella’s intracellular life style (Barbagallo et al. 2011, Leuzzi et al. 2015).

The activation of virF is a key event for the successful invasion and dissemination of Shigella within the host epithelium, and is therefore stringently regulated. A multitude of environmental signals (e.g. temperature, pH, osmolarity) affect virF expression through several regulatory mechanisms. Some of these mechanisms have been described at the molecular level, as the temperature-dependent expression of the virF gene (Falconi et al. 1998, Prosseda et al. 2004). At temperatures below 32°C (non-permissive), the nucleoid-associated protein H-NS tightly binds two sites within the virF promoter. This prevents access of the RNA polymerase and therefore leads to virF transcriptional silencing. At the permissive host temperature (37°C), relaxation of an intrinsically-curved DNA region, located between the two H-NS binding sites, hampers H-NS binding and favors access of the nucleoid associated protein FIS to one of its binding sites (Falconi et al. 2001, Prosseda et al. 2004). This results in activation of virF transcription. VirF subsequently acts as an anti-silencer, countering H-NS-mediated repression on for example virB and icsA promoters. Besides direct binding to the icsA promoter, VirF also stimulates icsA expression by lowering the intracellular concen-
Figure 1. Low temperature (30°C) stimulates virF21 lliRNA expression and an increased VirF21:VirF30 protein ratio. (A) β-Galactosidase activity of the virF-lacZ transcriptional fusion pRS-F(+205) containing the internal promoter for the leaderless mRNA. The analysis was performed in E.coli DH10b. pRS-F(+305) was used as a negative control. The β-Galactosidase activity was determined after subculture at 30 or 37°C. The activity is reported in Miller Units and represents the mean and standard deviation of 7 (pRS-F(+305)) and 13 (pRS-F(+205)) biological replicates from 3 different experiments. Statistical significance, comparing the β-Galactosidase activity of the pRS-F(+205) fusion at 30 or 37°C, was determined by Mann-Whitney U test, *P < 0.05.

(B) Detection of VirF30 and VirF21 at 30°C and 37°C in protein extracts of the Shigella M90T strain carrying virF-3xFT grown in LB medium. A representative western blot with serial dilutions of the protein extracts is shown.

(C) Detection of VirF30 and VirF21 at 30°C in protein extracts of the Shigella M90T strain carrying virF-3xFT grown in M9 medium. A representative western blot in which protein extracts were concentrated to facilitate quantification is shown.

(D) Detection of VirF30 and VirF21 at 30°C and 37°C in protein extracts of the Shigella M90T strain carrying virF-3xFT grown in M9 medium. A representative western blot with serial dilutions of the protein extracts is shown.

(E) Detection of VirF30 and VirF21 at 30°C in protein extracts of the Shigella M90T strain carrying virF-3xFT grown in M9 medium. A representative western blot in which protein extracts were concentrated to facilitate quantification is shown.

(F) Detection of VirF30 and VirF21 at 30°C and 37°C in protein extracts of the Shigella M90T strain carrying virF-3xFT grown in LB medium. A representative western blot with serial dilutions of the protein extracts is shown.

(G) Detection of VirF30 and VirF21 at 30°C and 37°C in protein extracts of the Shigella M90T strain carrying virF-3xFT grown in M9 medium. A representative western blot with serial dilutions of the protein extracts is shown.

(H) Detection of VirF30 and VirF21 at 30°C and 37°C in protein extracts of the Shigella M90T strain carrying virF-3xFT grown in M9 medium. A representative western blot in which protein extracts were concentrated to facilitate quantification is shown.

(I) Detection of VirF30 and VirF21 at 30°C and 37°C in protein extracts of the Shigella M90T strain carrying virF-3xFT grown in M9 medium. A representative western blot in which protein extracts were concentrated to facilitate quantification is shown.
concentrations of IPTG (0, 0.1, 0.25, 1 mM) to induce VirF21 expression. GroEL protein was detected and used as internal loading control. A loading control using the Stain free protein: VirF30 (30 kDa) and VirF21 (21 kDa). VirF30 acts as primary activator of the virulence gene cascade, whereas VirF21 appears to negatively autoregulate virF expression through direct promoter binding (Di Martino et al. 2016b). In addition, VirF21 can originate also from a shorter, leaderless mRNA (llmRNA), transcribed from a gene-internal promoter (Di Martino et al. 2016b). While the molecular interactions of this regulatory loop were defined in the previous study, it had remained unknown which conditions affect VirF21 expression and how this can impact Shigella’s host cell invasive phenotype.

Here we characterized the conditions governing virF21 llmRNA expression and the overall VirF21:VirF30 protein ratio. We found that at 30°C, a common condition Shigella encounters during its extracellular (non-invasive) lifestyle, transcription of the virF21 llmRNA from the internal promoter is favored and the VirF21:VirF30 protein ratio is elevated, as compared to the permissive host temperature of 37°C. Moreover, ectopically elevating VirF21 levels at 37°C resulted in a marked and reversible block of the Shigella host cell invasive phenotype. The C-terminal part of VirF21 was found to be required for this suppression. We discuss the possible connections between environmental sensing, the fitness costs of virulence gene expression, and VirF21-dependent suppression of the host cell invasive program.

Figure 2. Elevated expression of VirF21 suppresses Shigella virulence at 37°C. (A) Western blot with VirF antibodies on extracts from a Shigella M90T ΔvirF mutant and M90T strains harbouring pControl (empty vector) or pVirF21, a plasmid carrying the virF21 coding sequence under an inducible pTaq promoter. The strains were grown in the presence of increasing concentration of IPTG (0, 0.1, 0.25, 1 mM) to induce VirF21 expression. GroEL protein was detected and used as internal loading control. A loading control using the Stain free method is also shown. (B) (upper panel) % CR- colonies upon spreading of the indicated strains on CR plates containing increasing concentrations of IPTG (0, 0.1, 0.25, 1 mM) to induce VirF21 expression. (bottom panel) % CR- colonies upon spreading and re-plating of the colonies obtained on the previous plates, onto new CR plates without IPTG selection. Data come from at least three replicates from two independent experiments. ~200-600 colonies/replicate were examined for the CR phenotype. (C) Invasion efficiency of the indicated Shigella M90T strains in sub-confluent Caco-2 cell layers. Cells were infected at MOI 100 for 1h, and analysed by selective plating of intracellular bacteria. Shown are CFU data expressed as the percentage of the inoculum retrieved in the intracellular population. Shown are CFU data for 7 (M90T pControl) and 9 (M90T ΔmixID, M90T pVirF21) biological replicates from 3 independent experiments. Bars represent mean and standard deviation. Statistical significance was determined by Mann Whitney U test, **P < 0.01.

Material and methods

Bacterial strains and general procedures

Strains and plasmids used in this study are listed in Table S1. M90T is a S. flexneri serotype 5 strain (Sansonetti et al. 1982). Strain M90T ΔvirF carries a deletion of the virF gene (Leuzzi et al. 2015). Strain M90T virF21-3xFt carries the 3xFLAG tag sequence at the C-terminus of the pINV-encoded virF gene (Leuzzi et al. 2015). Strain M90T ΔmixID carries a deletion of the mixID gene and has been constructed using the one-step gene inactivation method (Datsenko and Wanner 2000), transforming M90T pKD46 with the PCR product obtained using plasmid pKD4 as template and the oligo pairs mixID_delF/mixID_delR (Table S2). The plasmids pControl (previously named pGIP7), pVirF21 (previously named pAC-21), pRS-F(+205) and pRS-F(+305) were described previously (Di Martino et al. 2016b, Falconi et al. 2001).

The plasmids pVirF21_J97N, pVirF21_V108A, pVirF21_V145T and pVirF21_Y141stop were obtained by Gibson Assembly (Gibson et al. 2009), ligating in vitro synthesized PCR products (purchased from GeneWiz) into the BamHI restriction site in pControl using the Gib-
Figure 3. The C-terminal HTH2 motif is required for VirF21 function. (A) Schematic representation of the VirF21 protein sequence, with relevant mutagenized amino acid positions indicated. (B) virF mRNA expression levels (2−ΔΔCt) as a function of protein induction with 0.25 mM IPTG in Shigella M90T strains harbouring pControl, pVirF21, pVirF21_I97N, pVirF21_V108A, pVirF21_V145T, or pVirF21_Y141stop. Data come from 6–7 biological replicates from 3 independent experiment and were normalized to the virF expression in the M90T pControl strain. (C) VirF21 protein levels detected by western blot as a function of IPTG induction (increasing concentration: 0, 0.1, 0.25, 1 mM) in Shigella M90T strains harbouring pControl, pVirF21, pVirF21_I97N, pVirF21_V108A, pVirF21_V145T, or pVirF21_Y141stop. VirF21_Y141stop produces a smaller protein (∼15kDa), since the last 39 aa in the C-terminal part are deleted. (D) VirF21 protein levels detected by western blot in a Shigella M90T ΔmxiD mutant and in Shigella M90T strains harbouring pControl, pVirF21, or pVirF21_Y141stop plasmids in the presence of 0.25 mM IPTG. (E) Invasion efficiency of the indicated Shigella M90T strains in sub-confluent Caco-2 cell layers. Cells were infected at MOI 100 for 1h, and analysed by selective plating of intracellular bacteria. Shown are CFU data expressed as the percentage of the inoculum retrieved in the intracellular population. Shown are CFU data for 4 (ΔmxiD) and 6 (Shigella M90T strains harbouring pControl, pVirF21, or pVirF21_Y141stop plasmids) biological replicates from 2 independent experiments. Bars represent mean and standard deviation. Statistical significance was determined by Mann Whitney U test, ns = non significant, *P < 0.05, **P < 0.01.

son Assembly Cloning kit from NEB (#E5510S). Amino acid substitutions were adjusted according to the Shigella codon usage. The resulting plasmids were transformed into the wild-type M90T strain. Sequences of the PCR products used are listed in Table S2. Bacteria were routinely grown in LB medium at 37°C, unless otherwise specified. When required, strains were grown in M9 complete medium (M9 minimal medium supplemented with 10 mg/ml thiamine, 0.2% glucose, 0.5% casamino acids and 10 mg/ml nico- tinic acid). When necessary, antibiotics were supplemented at the following concentrations: ampicillin, 50 μg/ml, chloramphenicol, 15 μg/ml, kanamycin, 50 μg/ml, streptomycin, 50 μg/ml. Plasmid DNA extraction, DNA transformation, electrophoresis, purification of DNA fragments and sequencing were performed as de-

cribed previously (Green and Sambrook 2012). PCR reactions were performed using DreamTaq DNA polymerase (Thermo Fisher Scientific, #EP0702) or Phusion DNA polymerase (Thermo Fisher Scientific, #F-530L). All oligonucleotide primers used in this study are listed in Table S2.

β-galactosidase assays

β-galactosidase assays were performed as previously described (Miller 1992) on sodium dodecyl sulphate-chloroform-permeabilized cells grown in LB or M9 medium (to OD600 0.5–0.6). β-galactosidase activity of the pRS-F (+205) and pRS-F (+305) transcriptional fusions was assessed under different conditions. For temperature shift, ON cultures grown at 30°C in LB were
subcultured 1:100 in LB at 30°C or 37°C. To mimic intestine-like conditions, ON cultures grown at 30°C were subcultured 1:100 at 37°C in M9 medium supplemented with the following compounds: Sodium deoxycholate (0, 2.5, 5 mg/ml), Bile salts (0, 6, 9 mg/ml), NaCl (0, 0.1, 0.2M), Hydrogen peroxide (0, 10, 50, 100 mM). To screen different pH conditions, bacteria were subcultured 1:100 in M9 medium at pH 5, 6 and 7.

Immunodetection of VirF proteins

VirF protein levels were detected by western blot through enhanced chemiluminescence. In brief, equal amount of proteins was extracted from strains grown at OD600 ~0.6, separated on Any kD™ Mini-PROTEAN® TGX Stain-Free™ Protein Gels (Biorad, #4568126) and transferred onto Trans-Blot Turbo Mini 0.2 μm PVDF Transfer Packs (Biorad, #1704156). The stain-free method was used to obtain the loading control (Coella et al. 2012). The method is based on the fluorescent detection of tryptophan residues in the protein sequence, as a result of the presence of a trihalo compound in the gel. After protein separation by electrophoresis, each gel was imaged upon exposure to UV-light for a trihalo compound in the gel. After protein separation by electrophoresis, each gel was imaged upon exposure to UV-light for 5 min and the same region was selected as loading control for all western blots. Immunodetection was performed as described in Di Martino et al. 2016b using polyclonal halon anti-VirF, anti-FLAG (Sigma, #F1804) and anti-GroEL (Sigma, #A8705) antibodies. Quantification by Western blots were obtained by serial dilution of protein extracts, with the relative amounts calculated from a standard curve. For the protein extracts derived from cultures grown at 30°C, concentrated samples were used to calculate the standard curves.

Congo red binding assay

Congo Red (CR) plates were prepared adding 0.108 mg/ml of Congo Red dye (Sigma, #C6277) to Trypticase Soy Agar (Sigma, #20167) and supplemented with 0, 0.1, 0.25, or 1 mM IPTG (Sigma, #H1758). The indicated Shigella flexneri M9OT strains were grown ON at 30°C with appropriate antibiotics, diluted 1:40 and subcultured at 37°C for ~2 h (OD600 ~0.7). Subcultures were serially diluted and plated on CR plates containing increasing concentrations of IPTG. The following day Congo Red positive (CR+) and negative (CR−) colonies were enumerated. Subsequently, colonies were scraped from each plate, serially diluted and plated on new CR plates without the IPTG selection.

Epithelial cell culture and infections

Caco-2 cells were grown in DMEM GlutaMAX (Gibco, #31966–021) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, #10270106) and 0.1 mM Non Essential Amino Acids (Gibco, #11140035) at 37°C with 10% CO2. Cultures were passaged two times/week in the presence of 100U/ml penicillin and 100 μg/ml streptomycin, but antibiotics were omitted during infection experiments. Caco-2 cells were seeded in 12-well plates 24–48 h before infection. The indicated S. flexneri strains were grown ON at 30°C in LB with appropriate antibiotics, diluted 1:50 in the presence of 0.25 mM IPTG to allow the induction of VirF21. The subcultures were further incubated for 2 h at 37°C or for 1 h at 30°C before shifting for 1.5 h to 37°C (OD600 ~0.7). Upon infection, bacteria were centrifuged on top of the cultured epithelial cells for 15 min at 700 g, followed by 45 min incubation at 37°C and 10% CO2. The culture medium was replaced with fresh medium containing 200 μg/ml Gentamicin (Sigma, #G1914) and the cells were further incubated for 2 h. At 3 h post-infection (p.i.) cells were washed and lysed adding 0.1% Sodium deoxycholate, the lysates were then diluted and plated on LB agar plates with appropriate antibiotics, followed by enumeration of the number of colony-forming units (CFUs).

qRT-PCR

Total RNA purification and cDNA synthesis were performed as previously described (Di Martino et al. 2016b). qRT-PCR was performed using Maxima SYBR green/ROX qPCR master mix (2X) (Thermo Fisher Scientific, #K0222) on a CFX384 Touch Real-Time PCR Detection System (Biorad). The levels of virF, virF30, virB, mxiE and nusA transcripts were analysed using the 2−ΔΔCT (cycle threshold [CT]) method and results are reported as the fold increase relative to the reference (Livak et al. 2001). The housekeeping gene nusA was used for normalization. The following oligonucleotide primers were used (see Table S2): nusAQF/nusAQR, virFQF/virFR, virF30QF/virF30QR, virBFQF/virBQR, mxiEQF/mxiEQR and nusAQL/nusAQR.

Results and discussion

Expression levels of the virF21 llmRNA and the VirF21:VirF30 protein ratio are both elevated at non-permissive temperature (30°C)

Previous work identified a virF21 translationally capable llmRNA, whose transcription is dependent on the presence of an internal promoter located two nucleotides upstream the VirF21 translational start site (Di Martino et al. 2016b). To determine how and when expression of the virF21 llmRNA occurs, E. coli strain harbouring a virF21(llmRNA)-lacZ transcriptional fusion construct (pRS-F (+205)), or a control fusion (pRS-F (+305)) (Di Martino et al. 2016b) were grown under different conditions, mimicking either the environment Shigella encounters during host cell invasion in the gut (i.e. exposure to sodium deoxycholate, bile salts, high osmolarity, oxidative stress, low pH) or the environment outside the host (non-permissive temperature: 30°C) (Marteyn et al. 2012). No difference in the pRS-F (+205) β-galactosidase activity was observed during osmotic, oxidative and pH-stress, as compared to the untreated control (Fig S1A-B-C). Increasing concentrations of either sodium deoxycholate alone or a bile salt mixture, hence mimicking the biliary secretions encountered in the human intestinal tract (Faherty et al. 2012, de Buy Wenniger et al. 2013, Di Claua et al. 2017, Nickerson et al. 2017, Chanin et al. 2019), resulted in ~6 and ~3-fold increase in β-galactosidase activity, respectively (Fig S1D-E). This suggests an increase in virF21 llmRNA transcription under these two conditions. However, the relative abundance of VirF21 protein, measured in a Shigella flexneri M9OT strain harbouring a 3xFlag-tagged version of the VirF proteins, did not increase accordingly in response to these stimuli (Fig S1F-G-H). This may imply that under the conditions tested here, the majority of VirF21 protein originates from alternative translation of the virF full length mRNA. The existence of some other unknown post-transcriptional regulatory mechanisms, hampering VirF21 translation, can also not be ruled out. In either case, typical conditions encountered by Shigella within the host may influence virF21 llmRNA transcription, but do not seem to significantly alter VirF21 protein levels.

Interestingly, we found that the β-galactosidase activity of the pRS-F (+205) fusion was significantly higher at 30°C than at the permissive host temperature of 37°C (Fig. 1A). Translation of both VirF forms in Shigella flexneri M9OT was observed at both temperatures (Fig. 1B-E). In agreement with the positive regulation of virF expression at 37°C, VirF30 protein abundance was ~8-10-
fold higher at 37°C than at 30°C both in LB (Fig. 1B-C-F) and in M9 medium (Fig. 1D-E-G). Notably, the VirF21:VirF30 protein ratio differed dramatically between the two temperatures. While VirF21 represented ~5–10% of the VirF30 protein content at 37°C (~5% in LB; ~10% in M9), it reached peaks of ~20–50% at 30°C (~20% in LB; ~50% in M9) (Fig. 1C-E-H-I).

Taken together these results suggest that while VirF21 lmmRNA transcription may be affected by several different stimuli, a high VirF21:VirF30 protein ratio is favored at environmental temperature, rather than under host-like conditions. In this context, VirF21 might function as a molecular brake to minimize fitness costs when the Shigella virulence program is not required or undesired.

The VirF genetic arrangement leading to the transcription and translation of two proteins from a single gene is not an isolated example. The E.coli copper chaperone CpaA and the Salmonella LysR-type regulator LtrR also display transcription of two mRNA molecules and the translation of two protein forms under specific environmental conditions (Drees et al. 2017, Rebollar-Flores et al. 2020). Furthermore, computational analysis aimed at discovering overlooked regulatory elements showed that gene internal promoters are often associated with horizontally transferred genes, both in E.coli and in some archaeal species (Ten-Caten, Vêncio et al. 2018). This is particularly relevant here, since virF was horizontally acquired on the pINV during Shigella’s evolution towards pathogenicity (Yang et al. 2005). Altogether, this suggests the existence of a widespread adaptation strategy in bacteria to expand and diversify the protein repertoire and thereby optimize the response to changing external conditions.

**Ectopic expression of VirF21 at the permissive temperature reversibly suppresses the Shigella host cell invasive program**

VirF30 activity governs the transition of Shigella between non-invasive and invasive states. When switching from 30°C to 37°C, already a modest increase in virF transcription is sufficient for full activation of the downstream virulence cascade and a host cell invasive phenotype (Le Gall et al. 2005). To test the hypothesis that VirF21 expression can prevent switching to the invasive phenotype, we explored the consequences of elevating VirF21 protein levels under the permissive temperature (37°C).

First, we investigated the effect of ectopic VirF21 expression on the ability of Shigella to bind Congo Red (CR), a phenotype linked to virulence and the presence of virF, resulting in red colonies (CR+) on solid medium (Sakai et al. 1986a). *Shigella flexneri* M90T was transformed with either a plasmid that allowed IPTG-inducible expression of pVirF21 (pVirF21; carries the Pptc promoter; reported as pAC-21 in Di Martino et al. 2016b), or the corresponding empty vector (pControl; previously named pGIP7 in Falconi et al. 2001). Ectopic expression of VirF21 was detected in the presence of graded concentrations of IPTG (0.1–0.25–1 mM), but not in the absence of IPTG (Fig. 2A). We plated dilutions of exponential cultures of M90T pControl, M90T pVirF21 and M90T ΔvirF on CR plates containing increasing concentrations of IPTG and incubated at 37°C. Fig. 2B and figure S2 show that IPTG-induced ectopic expression of VirF21 led to the appearance of a high percentage (~50–90%) of white (CR-) colonies, reaching comparable levels as the non-virulent M90T ΔvirF mutant, a strain known to exhibit a completely CR- phenotype (Sakai et al. 1986b). In particular, a robust CR- phenotype (>80% white colonies) was observed on CR plates containing 0.25 and 1 mM IPTG, while the percentage while colonies was somewhat variable in the presence of 0.1 mM IPTG. This observation suggests a borderline VirF21 expression at IPTG concentrations lower than 0.25 mM. Importantly, when the CR- M90T pVirF21 colonies were collected from the plates supplemented with IPTG and re-plated on new CR plates devoid of the IPTG inducer, the bacteria reverted back to virtually exclusively CR+ colonies (Fig. 2B; <3% CR-). These results suggest that CR binding is subjected to a VirF21-driven reversible switch, linked also to a decrease in virulence gene expression (Fig S3A-B; and Di Martino et al. 2016b).

Next, we infected human colonic epithelial Caco-2 cells with the M90T pControl and M90T pVirF21 strains, to test how ectopic VirF21 expression impacts Shigella host cell invasion. M90T ΔmxiD (lacking the outer membrane ring MxiD protein, resulting in a nonfunctional T3SS) was used as a non-invasive control strain. To ensure robust VirF21 expression with minimal side effects, we induced VirF21 expression with the intermediate concentration of IPTG (0.25 mM, as informed by the CR binding assay; Fig. 2A and B). As expected, the M90T ΔmxiD strain failed at infecting Caco-2 cells (Fig. 2C). Notably, ectopic expression of VirF21 (M90T pVirF21) led to a ~4–5-fold decrease in Shigella’s ability to infect Caco-2 cells, as compared to the M90T pControl strain (Fig. 2C).

Taken together, these results show that elevated VirF21 expression at 37°C can suppress the Shigella invasive program, signified by a CR-phenotype on plates, reduced virulence gene expression, and hampered capacity to infect epithelial cells. Considering that VirF21 makes up a larger fraction of the total VirF protein pool at 30°C than at 37°C (Fig. 1), the above results reinforce the hypothesis that VirF21 may negatively tune Shigella virulence gene expression, when this is undesirable. It is known that the pINV virulence plasmid is subjected to high counter-selective pressure at 37°C. With increasing number of generations, mutations, insertions of IS sequences and/or complete loss of the virulence cascade top regulators virF or virB occur at 37°C (Sasakawa et al. 1986, Schuch and Maurelli 1997, Pilla et al. 2017). Occasionally, the selective pressure can escalate leading even to the loss, or integral silencing, of the entire pINV (Zagaglia et al. 1991, Pilla et al. 2017). At 30°C, virulence gene expression is silenced and therefore the selective pressure on the pINV is relieved, resulting in minimal loss or mutations (Schuch and Maurelli 1997). In this context, it is tempting to speculate that VirF21 expression represents an additional regulatory layer to minimize virulence gene expression leakage and therefore promote overall pINV stability under certain environmental conditions.

**The C-terminal region of VirF21 is required for negative regulation of Shigella host cell invasion**

The experimental setting based on ectopic VirF21 expression precludes assessment of the impact of endogenous VirF30 and VirF21 levels expressed from their native genetic context. Despite significant efforts, we have however been unsuccessful at generating a *Shigella flexneri* scarless mutant expressing VirF30 protein only from the endogenous locus. This may suggest that the native virF locus sequence is unusually intolerant to perturbation, although we cannot formally rule out other technical explanations.

To better understand the relationship between VirF21 sequence and function, and to verify the specificity of the above results, we therefore opted for a site directed mutagenesis approach, targeting the untagged virF21 gene cloned into the pVirF21 plasmid. VirF30 and VirF21 belong to the family of AraC-like transcriptional regulators (Cortés-Avalos et al. 2021). This group comprises both positive and negative transcriptional regulators, which often control virulence systems across different gram-negative bacterial species (i.e. Salmonella, Yersinia, Vibrio cholera, (Gallegos et al. 1997, Cortés-
Avalos et al. 2021). The mechanisms governing AraC-like protein expression and regulation have been successfully studied in many cases. However, their biochemical and structural properties have been less well characterized, since AraC-like proteins are often difficult to purify (Cortés-Avalos et al. 2021). The DNA sequences targeted by VirF21 have nevertheless been identified in some cases (i.e. within icsA, RNAG, and virB promoters) (Tobe et al. 1993, Giangrossi et al. 2010, Tran et al. 2011), and the VirF21 binding site within the virF promoter was also previously mapped (Di Martino et al. 2016b). VirF21 and VirF30 share the C-terminal portion, which contains the two typical AraC-like Helix-Turn-Helix (HTH) DNA binding motifs, separated by an alpha helix linker. VirF21 lacks the N-terminal domain of VirF30, which is believed to have oligomerization properties. Some of the amino acids likely involved in the interaction between VirF30 and its DNA targets have been identified by a combined random and site directed mutagenesis approach (Porter and Dorman 2002).

In an attempt to obtain a non-functional VirF21, we transplanted an assortment of mutations shown to affect VirF30 function in the prior study (Porter and Dorman 2002). The following mutations were introduced onto the pVirF21 plasmid: 197N, 208A, 145T and 141stop, here reported considering VirF30-Met84 (Porter and Dorman 2002) = VirF21-Met1 (Fig. 3A; Di Martino et al. 2016b; previously reported in Porter and Dorman 2002 as 1180N, 191A, 228T, and 224Och respectively). The substitutions 197N and 208A target the HTH1 DNA binding motif, while the 145T substitution is located within the HTH2 DNA binding motif. Finally, the deletion of the HTH2 motif was achieved by introducing a stop codon at position 141 (141stop, deletion of 39 aa in the C-terminus). Upon induction with IPTG in Shigella flexneri M90T, the wild type (wt) and the mutated versions of virF21 showed broadly similar transcriptional levels (Fig. 3B). However, VirF21 protein levels (monitored by a halon anti-VirF antibody) were markedly lower for the VirF21-197N, VirF21-208A and VirF21-145T mutant constructs than in the VirF21-wt carrying strain (Fig. 3C), suggesting a possible impact of these amino acid substitutions on protein stability. Only the truncated VirF21-141stop construct generated protein levels comparable to the strain harbouring the pVirF21-wt plasmid (Fig. 3C-D). Next, we infected Caco-2 cells with the strains ectopically expressing either VirF21-wt (pVirF21) or VirF21-stop (pVirF21-V141stop). As evident from Fig. 3E, the Shigella pVirF21-wt strain retained the ability to infect Caco-2 cells at similar levels as the control strain (Shigella pControl), while the Shigella pVirF21-V141stop strain again showed a ~3 fold lower invasion capacity (Fig. 3E; compare also with Fig. 2C). These results show that the VirF21-stop protein, lacking the predicted DNA binding HTH2 motif, can be expressed to similar levels as full-length VirF21, but is non-functional. This validates the specificity of the VirF21 suppressive effects observed in the above experiments (Fig. 2), and reveals a key role of the C-terminal portion for VirF21 functionality.

**Conclusions**

Pathogenic bacteria are masters at adapting to fast-changing environmental cues. Shigella encounters many different environmental conditions and switches flexibly between extracellular and intracellular lifestyles. While the activation of the virF regulatory cascade is a crucial event for Shigella expression of the invasive program (Schroeder and Hilbi 2008), it also constitutes a significant fitness cost for the bacterial population (Schuch and Maurelli 1997). Thus, it is not surprising that Shigella employs a multi-layered regulatory arsenal to ensure expression of the virulence genes only when these are needed. VirF21 has been identified as a possible negative autoregulator of VirF30, but the impact on the invasive Shigella phenotype had not been addressed (Di Martino et al. 2016b). Our results expand on these previous findings, by illustrating that virF21 lincRNA expression and the VirF21:VirF30 protein ratio is enhanced at 30°C, a common condition Shigella encounters outside of the host. In this context, it seems plausible that VirF21 serves to suppress virulence gene expression when not desired. Indeed, when ectopically expressed, VirF21 is capable of suppressing the Shigella virulence program at the permissive temperature 37°C, resulting in a CR- phenotype on plates, lowered levels of virulence gene transcripts, and an impaired ability to infect host cells. This suppressive activity requires the HTH2-motif-containing C-terminus of the VirF21 protein.

While the physiological impact of VirF21 remains to be completely explored under the multitude of possible environmental conditions, the findings presented here highlight the interconnected mechanisms that ensure fine-tuned regulation of virulence properties across the Shigella life cycle.

**Supplementary data**

Supplementary data are available at FEMSLE online.

**Author contribution**

Conceptualization: M.E.S., M.L.D.M. Methodology: E.S., M.E.S., M.L.D.M. Investigation: E.S., M.L.D.M. Formal analysis: E.S., M.L.D.M. Resources: B.C., G.P., M.E.S., M.L.D.M. Supervision: M.E.S., M.L.D.M. Project administration: M.E.S., M.L.D.M. Funding acquisition: M.E.S., M.L.D.M. Visualization: E.S., M.L.D.M. Writing—Original Draft: M.L.D.M. Writing—Reviewing & Editing: E.S., M.E.S., M.L.D.M. All authors read, commented on, and approved the final manuscript.

**Acknowledgments**

We thank members of the Sellin laboratory for helpful discussion.

**Funding**

This work was funded by the SciLifeLab Fellows program (to MES) and by the Carl Trygger Foundation CTS18:80 (to MLDM).

**Conflicts of interests statement**

None declared.

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