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ORIGINAL PAPER

Two promoters and two translation start sites control the expression of the *Shigella flexneri* outer membrane protease IcsP

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Abstract The *Shigella flexneri* outer membrane protease IcsP proteolytically cleaves the actin-based motility protein IcsA from the bacterial surface. The *icsP* gene is monocistronic and lies downstream of an unusually large intergenic region on the *Shigella* virulence plasmid. *In silico* analysis of this region predicts a second transcription start site 84 bp upstream of the first. Primer extension analyses and beta-galactosidase assays demonstrate that both transcription start sites are used. Both promoters are regulated by the *Shigella* virulence gene regulator VirB and both respond similarly to conditions known to influence *Shigella* virulence gene expression (iron concentration, pH, osmotic pressure, and phase of growth). The newly identified promoter lies upstream of a Shine–Dalgarno sequence and second 5′-ATG-3′, which is in frame with the annotated *icsP* gene. The use of either translation start site leads to the production of IcsP capable of proteolytically cleaving IcsA. A bioinformatic scan of the *Shigella* genome reveals multiple occurrences of in-frame translation start sites associated with putative Shine–Dalgarno sequences, immediately upstream and downstream of annotated open reading frames. Taken together, our observations support the possibility that the use of in-frame translation start sites may generate different protein isoforms, thereby expanding the proteome encoded by bacterial genomes.

Keywords *Shigella* · IcsP · VirB · Gene regulation · Transcription

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Introduction

Shigella species are gram-negative intracellular pathogens that cause bacillary dysentery in humans by invading cells of the colonic epithelium (Labrec et al. 1964; Sansonetti 1998). Once inside host cells, *Shigella* move through the cytoplasm and into adjacent cells using actin-based motility. This process is mediated by the *Shigella* outer membrane protein IcsA (VirG), which polymerizes eukaryotic actin monomers into a tail of tightly bundled filaments on one pole of the bacterium (Bernardini et al. 1989; Goldberg et al. 1993). *Shigella flexneri* mutants lacking *icsA* are avirulent in animal models (Makino et al.

1986), demonstrating that actin-based motility is essential for *Shigella* pathogenicity.

The outer membrane protease IcsP modulates the amount and distribution of IcsA associated with *Shigella*. The activity of this protease was originally observed when growth medium was found to contain a 95-kDa polypeptide of IcsA after it had supported *Shigella* growth (Goldberg et al. 1993). Two groups identified IcsP (SopA) as the protease responsible for the cleavage of IcsA (Egile et al. 1997; Shere et al. 1997). Data collected by these two groups throughout five studies demonstrated that IcsP plays a role in the modulation of IcsA and the actin-based motility of *Shigella* (d'Hauteville et al. 1996; Egile et al. 1997; Shere et al. 1997; Steinhauer et al. 1999; Wing et al. 2005). Although IcsA is localized to the old pole of the bacterium in wild-type *Shigella* (Goldberg et al. 1993), expression of a non-cleavable form of IcsA in *Shigella* was found to lead to an increase in the circumferential localization of IcsA (d'Hauteville et al. 1996). Similar phenotypes were reported for *icsP* mutants in vitro (Egile et al. 1997; Shere et al. 1997; Steinhauer et al. 1999). When the intra- and intercellular phenotypes of *icsP* mutants were analyzed, abnormal actin-based motility and cell-to-cell spread were observed regardless of the serotype examined (Egile et al. 1997, Shere et al. 1997). Furthermore, *Shigella* cells expressing plasmid-borne *icsP* were found to lack detectable IcsA on their surfaces, and the effects of *icsP* mutation on intercellular movement and plaque formation were also serotype dependent (Steinhauer et al. 1999; Wing et al. 2005). Since dysregulation of the *icsP* gene generates *Shigella* phenotypes that are consistent with the attenuation of virulence, these studies strongly suggest that the *icsP* gene and its protein product will be tightly regulated.

Like many of the genes required for virulence of *S. flexneri*, *icsA* and *icsP* are encoded by the large ~230-kb virulence plasmid of *S. flexneri*. This virulence plasmid encodes the transcription factor VirB that positively regulates many genes on the plasmid including *icsP* (Dorman and Porter 1998; Wing et al. 2004). The VirB-dependent regulation of the *icsP* promoter requires two distal VirB sites located between positions −1,144 and −1,130 relative to the annotated transcription start site (TSS; Castellanos et al. 2009). These binding sites are located within an unusually large (~1.2 kb) intergenic region, which separates the *icsP* gene and the divergently transcribed *ospZ* gene.

In *Shigella flexneri* serotype 2a, coding sequences account for 76.24% of the virulence plasmid (Jin et al. 2002). Although the coding density of the *Shigella* virulence plasmid is lower than the *Escherichia coli* K12 chromosome (87.8%; Blattner et al. 1997), the intergenic region upstream of the *icsP* gene is still abnormally large when compared to the average size of an *E. coli* K-12 intergenic region (1.2 kb vs. 246 bp, respectively; Pupo et al. 2000). Furthermore, the remote location of the VirB-binding sites that influence *icsP* expression already implicates this large intergenic region in the transcriptional regulation of the *icsP* gene.

Based on the role that IcsP plays in maintaining the surface distribution of IcsA and how this ultimately regulates *Shigella* actin-based motility, we hypothesize that the *icsP* gene and/or its protein product will be tightly regulated. The aim of this study was to further characterize the regulation of IcsP at both the transcriptional and the translational level. To do this, we chose to examine the entire upstream intergenic region for sequence elements involved in the regulation of IcsP.

Results

In silico analyses of DNA sequences upstream of *icsP*

Due to the unusual length of the intergenic region and its involvement in the regulation of *icsP*, we wanted to further analyze this region for elements contributing to the transcriptional regulation of the *icsP* gene. To do this, our initial approach was to analyze the entire 1,232-bp sequence using *in silico* tools. To identify putative promoter sequences, the intergenic region upstream of *icsP* was entered into the BPROM program for prediction of promoters regulated by the σ^{70} subunit of RNA polymerase (http://www.linux1.softberry.com). The BPROM software identified four putative transcription start sites (TSSs) with associated −10 and −35 sequences at positions −84, −422, −769, and −1,106 relative to the previously annotated TSS (Fig. 1a). Interestingly, the BPROM program did not identify the originally annotated promoter. Analysis of the divergent strand predicted four additional promoters approximately 20 bp upstream of each TSS found on the complementary strand (Fig. 1a). This is not surprising considering the adenine and thymine composition of the −10 sequence.

To examine whether any of the predicted promoters were aligned with potential open reading frames (ORFs), the entire intergenic region was analyzed with the microbial gene-finding system Glimmer (NCBI). Only one ORF, identified on the *icsP* coding strand, lay within 150 bp of a putative TSS found by BPROM. This was the TSS at the −84 position (Fig. 1b). This ORF begins 33 bp upstream of the annotated *icsP* gene (Fig. 1b). This would allow for the production of a polypeptide exactly 11 amino acids longer than and yet still in frame with the previously described *icsP* gene. A common problem with ORF prediction software is that they identify the longest ORFs and frequently miss internally coded start codons (Delcher et al. 1999). This may explain why the Glimmer program did not identify the beginning of the originally annotated *icsP* gene (Egile et al. 1997).

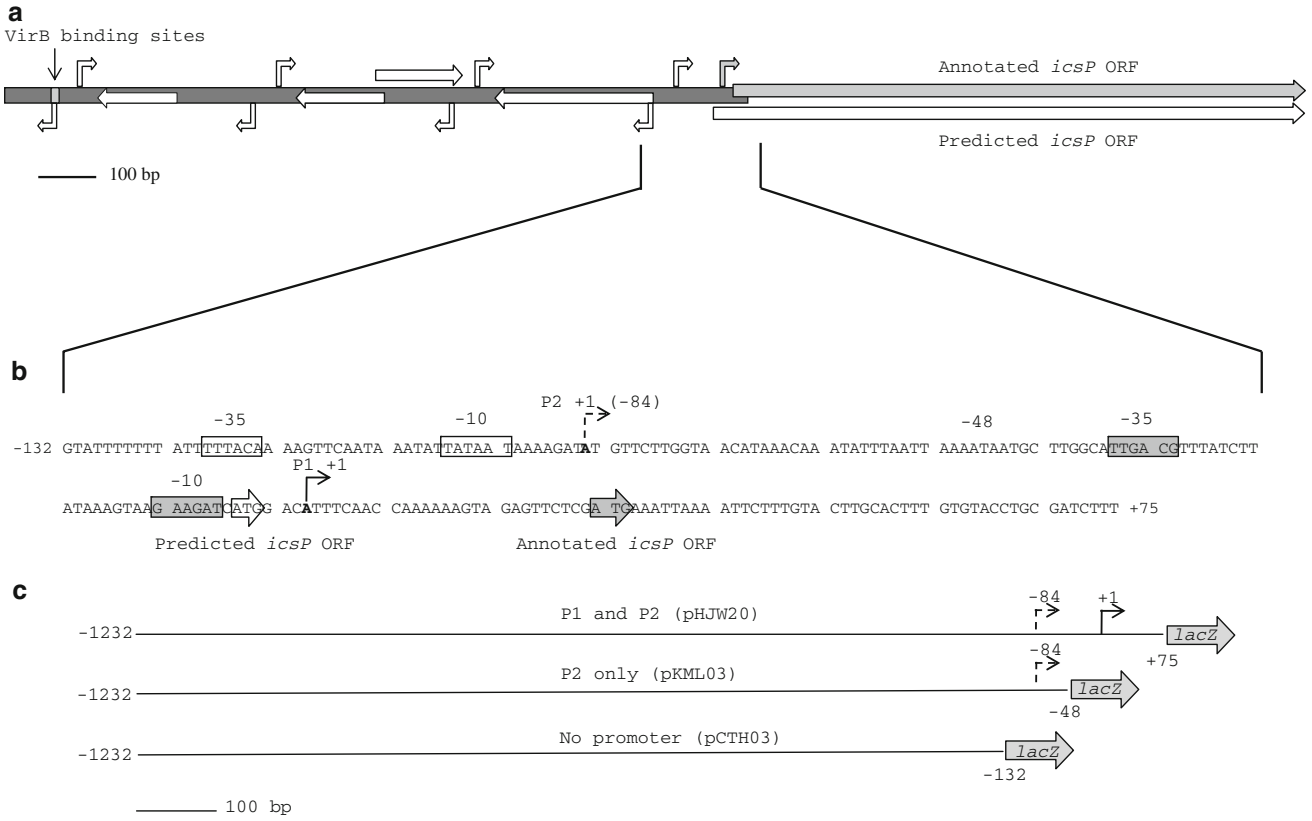

Fig. 1 Schematic of the entire *icsP* intergenic region, *icsP* promoter elements and *PicsP-lacZ* fusions used in this study. Overview of the entire *icsP* intergenic region (**a**). Locations of the annotated TSS, ORF, upstream VirB-binding sites (*gray arrows and box*) and predicted TSSs and ORFs (*white arrows*). Promoter elements of the *icsP* P1 and P2 promoters (**b**). P1 *solid angled arrow*; P2 *dashed angled arrow*; −10 and −35 sites are *boxed and labeled*. The P1 and P2 translation start sites are enclosed by *arrows* and *labeled*. Diagram of the *PicsP-lacZ* fusions in pHJW20, pKML03, and pCTH03, respectively (**c**). The inserts are drawn to scale, and numbers are relative to the P1 TSS

Two promoters control the expression of *icsP* gene

Given the discrepancy between predicted and annotated TSSs, we wanted to experimentally determine the position of all TSSs using primer extension analysis (Fig. 2). Primer extension analyses were performed on mRNA isolated from the wild-type *S. flexneri* 2a strain (2457T), the isogenic *virB* mutant (AWY3) and from wild-type *Shigella* carrying either the *icsP* promoter and gene (pHJW6) or a P*icsP-lacZ* promoter fusion (pHJW20). Regardless of whether the *icsP* promoter was carried by the virulence plasmid or the low copy cloning vectors, two products were observed, indicating that the *icsP* gene is transcribed from two promoters. The sizes of these two products indicate that TSSs occur at +1 and −84 relative to the previously annotated *icsP* TSS (Fig. 2). These data are in agreement with our *in silico* analyses (Fig. 1b; Egile et al. 1997). We therefore designated the +1 and −84 TSSs and their accompanying promoter elements P1 and P2, respectively. Quantification of the primer extension products by densitometry indicate that the P2 signal is approximately half that of the P1 signal in all wild-type strains. The increased signal intensity of products generated from strains carrying plasmids of the pHJW series was attributed to the copy number of these plasmids. In summary, these analyses identify two promoters involved in the transcriptional regulation of the *icsP* gene. We next wanted to examine how these two promoters are regulated.

Both *icsP* promoters are regulated by VirB

Previous work demonstrated that *icsP* is regulated by the *Shigella* transcriptional regulator VirB (Wing et al. 2004). Two VirB-binding sites located over 1 kb upstream of the originally annotated TSS are required for this VirB-dependent regulation (Castellanos et al. 2009). To determine the effect of VirB-dependent regulation on P1 and P2, β-galactosidase assays were conducted with a series of P*icsP-lacZ* transcriptional reporter plasmids containing P1 and P2 (pHJW20), P2 only (pKML03), or no promoter (pCTH03; Fig. 1c) in wild-type *Shigella* or a *virB* mutant background.

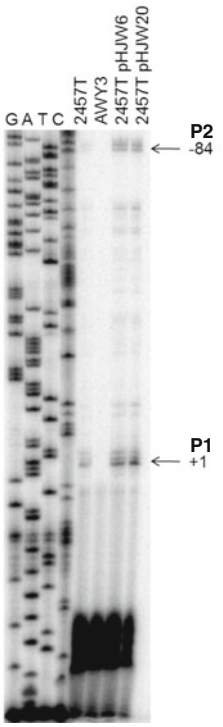

Fig. 2 Primer extension analysis of *icsP* transcripts generated by wild-type (2457T), the *virB* mutant (AWY3), or wild-type cells carrying the plasmid pHJW6 or pHJW20. The new TSS, P2, is identified with its relative location to the annotated TSS, P1. A sequencing reaction (*first four lanes*) was used to calibrate the gel. The experiment was repeated three times and representative data are shown

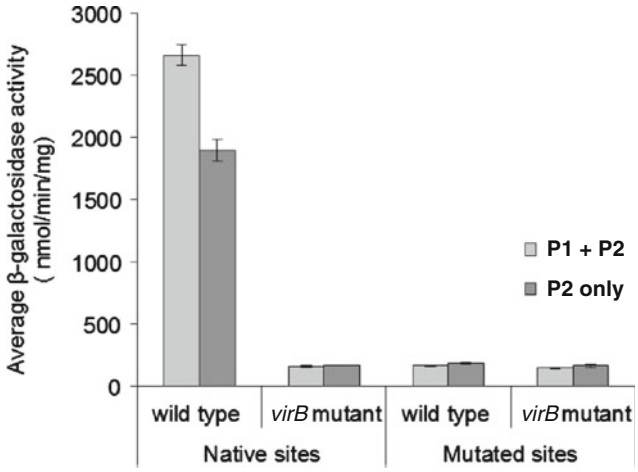

Fig. 3 Activity of *PicsP-lacZ* fusions carrying wild-type or mutated VirB-binding sites (centered at −1,137, with respect to P1) in wild-type *S. flexneri* (2457T) and the *virB* mutant (AWY3). Assays were run in triplicate, and the means and standard deviations are shown

Our data show that P2 alone contributes approximately 70% of the total promoter activity in wild-type cells. In contrast, in the *virB* mutant background, the activity of both promoters is significantly decreased (Fig. 3), indicating the activity of both promoters is dependent upon VirB. To further test the role of the distal VirB-binding sites on P1 and P2 regulation, base pair substitutions that completely abolish the previously annotated VirB-binding sites (Castellanos et al. 2009) were introduced into each of the reporter plasmids and promoter activity was measured in a wild-type and *virB* mutant background. In wild-type cells, both constructs carrying the mutated binding sites exhibited a significant decrease in *icsP* promoter activity, and this activity was similar to that observed in the *virB* mutant background (Fig. 3). Furthermore, our primer extension analysis revealed no detectable primer extension products in the *Shigella virB* mutant lane (Fig. 2, AWY3). Taken together, these data indicate that VirB acts as a transcriptional regulator for both *icsP* promoters and that both promoters require the presence of the two distal VirB-binding sites to mediate this effect.

Both *icsP* promoters respond similarly to changes in growth phase, iron concentration, pH, and osmotic pressure

Multiple promoters often allow for the differential regulation of a single gene product in response to bacterial growth or environmental stimuli (Erickson et al. 1987; Raina et al. 1995). In *Shigella*, many virulence genes are regulated at the level of transcription by changes in temperature, pH, osmolarity, and iron concentration (Mitobe et al. 2009; Murphy and Payne 2007; Porter and Dorman 1997). The presence of two promoters upstream of the *icsP* gene raises the possibility that these promoters respond differently to growth phase and/or environmental cues. Although expression of VirB is known to be regulated by these environmental signals and VirB is required for transcription from both promoters as demonstrated here, we wanted to investigate whether changes in environmental conditions experienced by *Shigella* during colonization would allow for more refined control of *icsP* transcription through capitalization of the two-promoter architecture. Therefore, we examined the change in activities of the two promoters following a decrease in pH, osmolarity, or iron concentration using the pHJW20 and pKML03 constructs. A decrease in iron concentration, pH, or osmotic pressure all seemed to affect each promoter similarly, with P2 contributing between approximately 60–75% of the combined promoter activity (Table 1). Since *icsA* expression is regulated in a growth phase-dependent manner (Goldberg et al. 1994), we also conducted a time course assay to determine whether the relative activity of the two promoters varies with phase of growth. Our data show that both *icsP* promoters are maximally active during stationary phase and that P2, represented by pKML03, contributes approximately 59% (2 h) to 76% (10 h) of the combined promoter activity, represented by pHJW20 (Fig. 4). These data indicate that the relative contribution of P1 and P2 to overall activity of the *icsP* promoters remains constant under a variety of conditions, suggesting that the two *icsP* promoters do not appear to be differentially regulated, at least under the conditions tested here.

Table 1 Contribution of P2 promoter activity to total *icsP* promoter activity

Environmental parameter	Specific condition in Luria–Bertani medium	Percent contribution[a]
pH	7.4	75.54 ± 6.96
	5.5	61.88 ± 19.55
Iron concentration	Normal (0 μg ml^{-1} EDDA)	69.86 ± 1.12
	Decreased (15 μg ml^{-1} EDDA)	74.81 ± 5.48
Osmotic pressure	Normal NaCl concentration (LB)	72.32 ± 0.07
	Half NaCl concentration (LO)	67.20 ± 3.96

[a] Average percent contributions are based upon the promoter activity of P2 (pKML03) compared to the total promoter activity (pHJW20). Assays were run in triplicate, and the means and standard deviations are shown

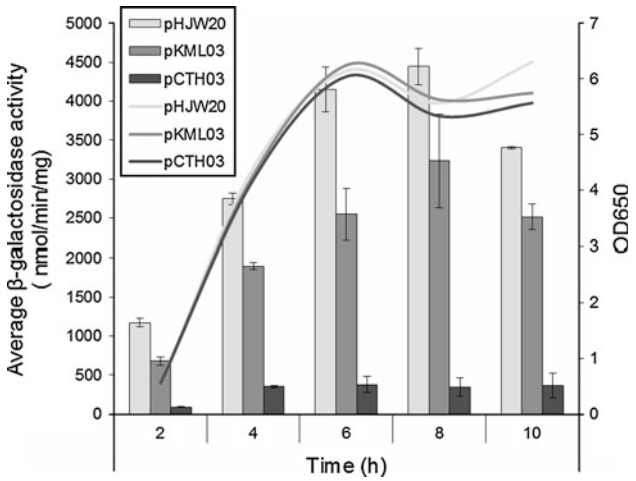

Fig. 4 Activity of the *icsP* promoter constructs in response to growth phase. pHJW20 is a construct carrying both promoters, pKLM03 carries the P2 promoter alone, pCTH03 carries neither *icsP* promoter. Promoter activities were measured throughout growth (0–10 h), and optical densities of the cultures carrying each promoter construct are shown. The data are normalized using pMIC21, the promoterless *lacZ* gene. Assays were run in triplicate, and the means and standard deviations are shown

Transcription from the two *icsP* promoters allows translation of IcsP from two different sites

Our *in silico* analysis revealed the potential for an additional translation start site upstream and yet still in frame with the annotated translation start site (Fig. 1b). This second translation start site lies downstream of P2, raising the possibility that it is unique to P2 regulated transcripts. To examine whether this translation start site is used in the production of the mature, secreted IcsP protein, IcsP levels were measured in an *icsP* mutant carrying a low copy plasmid containing both translation start sites (pHJW6), a single translation start site (pCTH16/downstream only or pCTH17/upstream only) or no translation start sites (pCTH18). Western blot analysis of whole-cell protein

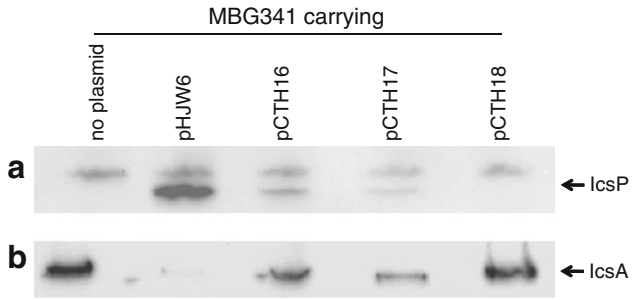

Fig. 5 Amount and activity of IcsP produced from either the upstream or the downstream translation start site. IcsP levels measured in the *icsP* mutant (MBG341) or the *icsP* mutant carrying pHJW6, pCTH16, pCTH17, or pCTH18 (**a**). Proteolytic activity of IcsP generated from either the upstream or the downstream translation start site, as judged by loss of IcsA. IcsA levels measured in the *icsP* mutant (MBG341) or the *icsP* mutant carrying pHJW6, pCTH16, pCTH17, or pCTH18 (**b**). The same whole-cell protein extracts were used for both IcsP and IcsA blots. The experiment was repeated three times and representative data are shown

preparations harvested from cells carrying each of these constructs shows that levels of the mature form of IcsP decrease when either the upstream or the downstream translation start sites are eliminated (pHJW6 compared to pCTH16 and pCTH17, respectively) and are undetectable when translation from both sites is prevented (pCTH18). These data indicate that both translation start sites can be used to produce the mature form of IcsP (Fig. 5a). Furthermore, since IcsP is an outer membrane protease, which is secreted across the inner membrane via the general secretion pathway, our detection of mature IcsP in cells that lack either one translation start site or the other strongly suggests that two isoforms of nascent IcsP are made and that each form is rapidly processed to a single mature form during secretion. This idea is supported by the signal peptide prediction program SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) that predicts a single signal peptide cleavage site for each of the predicted, nascent IcsP isoforms (data not shown).

IcsP produced from either translation start site is capable of cleaving IcsA

Having established that two translation start sites are used in the production of IcsP, we next wanted to test whether the resulting IcsP proteins differed in their proteolytic activity, as judged by their cleavage of IcsA. To do this, we probed the same protein cell extracts utilized for the IcsP analysis with an IcsA antibody. Our data show a reduction in the IcsA signal in all lanes except those containing cell extracts from the *icsP* mutant (MBG341) and 2457T pCTH18, a construct that lacks both translation start sites (Fig. 5b). This observed decrease in full-length IcsA protein levels is consistent with cleavage of IcsA by IcsP.

These data therefore suggest that regardless of which translation start site is used to make the IcsP protein, the resulting protease is capable of cleaving IcsA.

In *S. flexneri* start codons of annotated open reading frames are commonly associated with additional putative translation start sites

Our studies of the *icsP* promoter and gene had revealed that two in-frame translation start sites can each lead to the production of a mature IcsP protein. To address how frequently additional translation start sites are found associated with *S. flexneri* ORFs, we conducted a genome-wide scan of the *S. flexneri* genome. For this analysis, we assumed that translation start sites consist of two main elements: the start codon and Shine–Dalgarno sequence or ribosome-binding site (RBS).

First, a position-specific scoring matrix (PSSM) for the RBS associated with annotated ORFs on the *Shigella flexneri* genome (both plasmid and chromosome) was generated. We decided to use a PSSM instead of a consensus, because these matrices capture commonly observed variations within the motif. The reverse complement to the consensus of the generated PSSM is 100% identical to the 3′ end of the *Shigella flexneri* 16S rRNA 5-′CTCCTT-3′ and similar to the consensus SD sequence of *E. coli*, although offset by 1 nt: *E. coli* 5′-AGGAGG-3′; *Shigella flexneri* 5′-AAGGAG-3′. Consequently, the generated logo (Online Resource 1) is likely to be an accurate representation of the consensus RBS used in *Shigella flexneri*.

Second, to identify alternative translation start codons, fifty in-frame codons upstream and downstream of start codons of all annotated ORFs (both plasmid and chromosome) of *S. flexneri* 2a strain 301 were examined for the presence of potential start codons (Jin et al. 2002; Wei et al. 2006). The position-specific scoring matrix (PSSM), specific for the RBS in *S. flexneri* 2a strain 301 (Online Resource 1), was then applied to 20-nucleotide stretches of sequence upstream of every potential start codon to determine whether the identified potential alternative start codons were associated with RBS sequences.

Our analysis revealed that out of the 4,705 analyzed ORFs, 4165 have at least one additional putative start codon within a fifty codon range, upstream and downstream of the annotated start codon (raw data available upon request). In each case, the alternative translation start site lies in frame with the originally annotated start site and is not interrupted by a stop codon. For 2,070 of the ORFs, at least one of these alternative start codons is associated with a putative RBS (with a P value <0.04). Figure 6a and b shows the distribution of alternative start codons and alternative translation start sites within the examined range of genomic DNA. The trend within the distribution is better defined among ORFs on the chromosome, because of the larger number of ORFs examined. As expected, the number of detected start codons quickly declines within intergenic spaces compared to those within the ORFs, due to the presence of in-frame stop codons; however, local maximums of alternative translation start sites are located at codon 10 and between 35 and 40 codons upstream of the annotated translation start site. Within the coding regions, local maximums are found between codons 18 and 20 and at codon 35. The distribution of P values for RBSs associated with annotated and alternative start codons is shown in Online Resource 2. It is clear from these values that some alternative translation start sites are associated with much stronger RBSs than the annotated translation starts. In summary, although our data do not demonstrate use of these alternative translation sites, the frequency with which they occur and their association with sometimes strong RBS sequences have interesting implications when considering the evolution and potential expansion of bacterial proteomes. Further experiments will be required to establish which, if any, of these alternative translation start sites are currently active in the *S. flexneri* genome.

Discussion

This work has identified a second TSS for the *Shigella icsP* gene. Transcription from this site ultimately allows translation to proceed from a newly identified translation start site located 33 bp upstream of the originally annotated translation start site. Our work demonstrates that regardless of which translation start site is used, a mature form of IcsP is made that is capable of proteolytically cleaving the *Shigella* actin-based motility protein IcsA. It remains unclear whether the complex organization of the *icsP* regulatory region simply allows for transcriptional and translational redundancy or whether this organization allows for the exquisite control over *icsP* transcription and subsequent protein production in response to cellular and/or environmental cues.

Regulation from multiple promoters has been well documented in other bacterial systems and is usually found to contribute to the differential regulation of a single gene. While our experiments did not allow us to identify conditions that lead to the differential regulation of the two *icsP* promoters, we can now eliminate decreases in pH, osmolarity, and iron concentration from other environmental conditions encountered in the human host, which have the potential to differentially regulate the two *icsP* promoters. Our data show that both *icsP* promoters are most active during stationary-phase cultures. This pattern of expression is in agreement with the clearance of IcsA from the bacterial cell surface in stationary-phase cultures (Goldberg

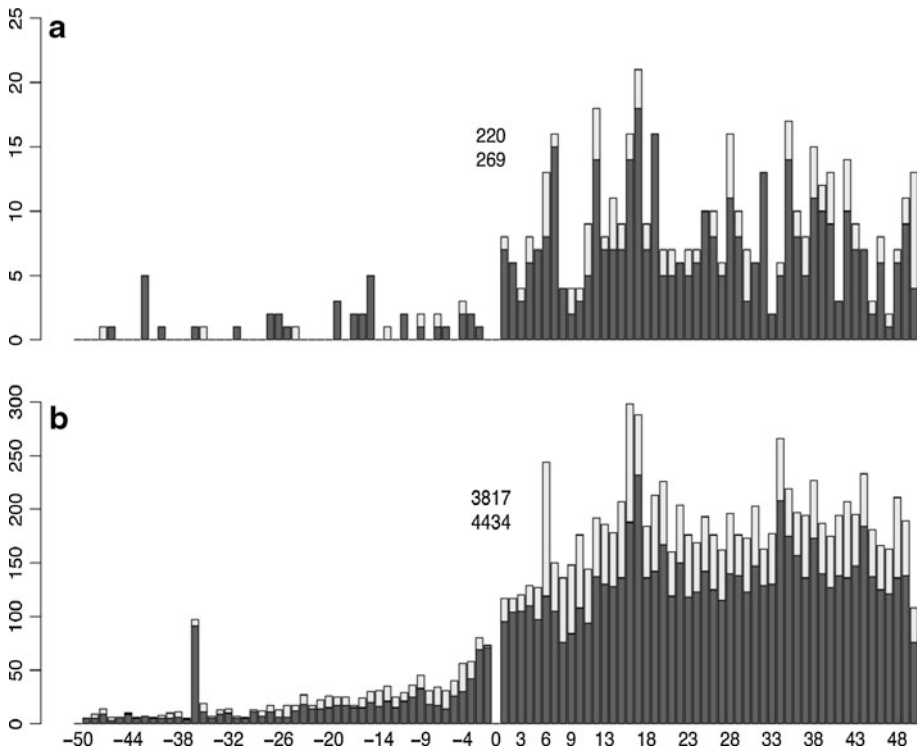

Fig. 6 Distribution of putative translation start codons positioned upstream and downstream of the annotated start codon (0) in the *S. flexneri* (**a**) and chromosome (**b**). The total number of predicted translation start codons are shown for each coordinate (*light gray*), the total number of predicted start codons associated with predicted Shine–Dalgarno sequences (*dark gray*). The inset values indicate the total number of annotated translation start sites (*bottom*) and annotated translation start sites associated with a predicted Shine–Dalgarno sequence (*top*)

et al. 1994) and the model for IcsP activity during pathogenesis (Steinhauer et al. 1999), which proposes a build-up of IcsA on one pole of the bacterial outer membrane, removal of IcsA that diffuses away from the pole through the activity of IcsP, and finally clearance of IcsA from the bacterial surface by IcsP.

Another way multiple promoters can be differentially regulated is by the use of different transcription factors. Many virulence genes in *Shigella* are commonly regulated by the transcription regulator, VirB. Previous work, by us and others, has demonstrated that transcription of *icsP* is positively regulated by VirB (Castellanos et al. 2009; Le Gall et al. 2005; Wing et al. 2004). The work presented here reveals that both *icsP* promoters are positively regulated by VirB and that this regulation is mediated by a VirB-binding site located over 1 kb upstream of the originally annotated TSS, because site-directed mutagenesis of these binding sites reduce promoter activity of both P1 and P2 to level observed in *virB* mutant. VirB functions at *Shigella* promoters, including the *icsP* promoter, by alleviating transcriptional repression mediated by the nucleoid structuring protein H-NS, rather than by activating the promoter per se. Since the activity of P1 and P2 increases in the presence of VirB, both promoters are likely to be repressed by H-NS. Whether or not other DNA binding proteins interact with the long intergenic region upstream of the *icsP* promoter, and what role this has in the regulation of the two *icsP* promoters, remains unclear at this stage, but this is an avenue of research investigation in our laboratory.

In bacterial genomes, alternative sigma factors sometimes allow the differential regulation of multiple promoters associated with a single gene. In our study, the BPROM software failed to identify the previously annotated TSS in our analysis. This might indicate the possible use of an alternative sigma factor, even though both *icsP* promoters contain −35 sequences closely resembling the consensus for σ^{70}-dependent regulation. Since the activity of both *icsP* promoters is maximal in stationary-phase cultures, the most likely alternative sigma factor to be used to control *icsP* expression is the stationary-phase sigma factor, σ^{S}. Despite maximal activity of the two *icsP* promoters in stationary-phase cultures, neither P1 nor P2 contain the 5′-TGTGC-3′ consensus sequence immediately upstream of the −10 element, which is a feature of promoters capable of binding σ^{S} (Gaal et al. 2001).

In all of our β-galactosidase assays, the relative contribution of each *icsP* promoter to total *icsP* promoter activity remained similar, regardless of the conditions used; P2 promoter activity contributed approximately 60–75% of the total promoter activity. Nevertheless, in our primer extension analysis, the P2-generated transcripts contribute to approximately 33% of the total signal intensity from both P1 and P2. Interestingly, the reduced level of P2 versus P1 transcripts in our primer extension analyses is consistent with the decreased amount of IcsP protein when the upstream translation start is used versus the downstream translation start. Therefore, it seems likely that the apparent discrepancy between the primer extension

analysis and the β-galactosidase assay results is most likely caused by differences in the mRNA leader of the *lacZ* reporter constructs, although this has yet to be confirmed.

The organization of the *icsP* promoter region means that if transcription occurs from P1, translation can only occur from the downstream translation start site, whereas if transcription occurs from P2, then translation has the potential to start from either the upstream or the downstream translation start site. This raises the question whether both translation start sites are used in a P2 transcript. Recent studies on polysome organization (Brandt et al. 2009) indicate that the distance between the two translation start sites in a P2 transcript (11 codons) is unlikely to allow simultaneous ribosome binding. This, along with the fact that our bioinformatics studies reveal no good match to the consensus Shine–Dalgarno sequence associated with the downstream translation start site, strongly suggests that the upstream translation start site would be favored in P2 transcripts, at least in the absence of accessory translation initiation factors.

Our work demonstrates that two translation start sites can be used to generate the mature and active form of IcsP. Since IcsP is secreted using the general secretion pathway, the two nascent isoforms must include an amino terminal signal sequence consisting of positively charged amino acids involved in protein targeting to the inner membrane for secretion (Fekkes and Driessen 1999). Interestingly, analyses of the two nascent isoforms of IcsP using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) predict that each isoform is likely to be cleaved at the same position to release two different signal peptides, but the same mature IcsP protein. The extended positively charged amino terminus of the longer nascent IcsP protein could allow for enhanced protein processing and translocation similar to the enhanced secretion of outer membrane proteins observed in *E. coli* (Akita et al. 1990). More efficient processing and translocation of the longer IcsP product may explain why the shorter IcsP protein product is expressed at a higher level but contributes to less IcsA cleavage as indicated by densitometry analyses of our IcsP and IcsA western blots.

While this study adds to our understanding of the *icsP* intergenic region and the role this region plays in the regulation of the *Shigella* outer membrane protease, further investigation is needed to understand whether additional regulatory elements exist within this intergenic region and how these elements affect the production of IcsP and ultimately *Shigella* actin-based motility. Although the purpose of the second promoter and second translation start site remains unclear, our results suggest that the production of the outer membrane protease IcsP may be more intricately regulated than previously thought.

The genome-wide screen for alternative translation start sites conducted within the present study, along with our observations made at the *icsP* promoter, provides the first evidence that functional alternative in-frame translation start sites in the genome of *S. flexneri* 2a strain 301 may be a general phenomenon rather than something specific for *icsP* gene. Whether our observation is restricted to the genome of *S. flexneri* 2a strain 301 or is the general property of bacterial genomes will require additional studies. It should be noted that recent work by Tucker and Escalante-Semerena (2010) demonstrates that two translation start sites allow the production of two isoforms of CobB to be made from a single gene in *Salmonella enterica* and that these two isoforms have different biological activities. Our findings, in conjunction with these studies, imply that the use of alternative translation start sites may increase the size of the proteome and, in some instances, lead to a larger range of physiological functions being encoded by the bacterial genome than was previously acknowledged.

Materials and methods

Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in the present study are listed in Table 2. *E. coli* strains were grown at 37°C in Luria–Bertani (LB) broth with aeration or on LB agar (LB broth containing 1.5% [wt/vol] agar). *S. flexneri* were grown at 37°C in trypticase soy broth (TSB) with aeration or in trypticase soy agar (TSA; TSB containing 1.5% [wt/vol] agar). Where appropriate, chloramphenicol was added at a final concentration of 25 μg ml^{-1}. To ensure that *Shigella* strains had maintained the large virulence plasmid during manipulation, Congo red binding was tested on TSA plates containing 0.01% (wt/vol) Congo red (Sigma Chemical Co., St. Louis, Mo.).

Plasmid construction

The starting point for this work was the P*icsP-lacZ* reporter plasmids pHJW20 and pMIC18 (described in Castellanos et al. 2009; Table 2). pHJW20 carries 1,232 bps upstream of the TSS of the *icsP* promoter annotated by Egile et al. (1997), the first 48 bp of the *icsP* coding region cloned upstream of a translation stop site, and a unique *Xba*I site upstream of a promoterless *lacZ* gene, so the expression of *lacZ* is directly regulated by the *icsP* promoter. pMIC18 is identical to pHJW20, but carries a 14-bp substitution that destroys the two upstream VirB-binding sites that are required for the VirB-dependent regulation of *icsP* (Castellanos et al. 2009).

Table 2 Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
S. flexneri		
2457T	*S. flexneri* serotype 2a	Labrec et al. (1964)
AWY3	2457T *virB*::Tn5; Kn^r	Wing et al. (2004)
MBG341	2457T *icsP*::Amp^r	Shere et al. (1997)
Plasmids		
P*icsP-lacZ* reporters		
pHJW20	*icsP* promoter region transcriptionally fused to *lacZ* in pACYC184 Cm^r; carries 1,232 bp of wild-type sequence upstream of the *icsP* transcription start site and unique *XbaI* site upstream of *lac*Z gene	Castellanos et al. (2009)
pHJW36	pHJW20 lacking P2 promoter elements	Castellanos et al. (2009)
pMIC18	pHJW20 carrying 14-bp substitutions in the two upstream VirB-binding sites	Castellanos et al. (2009)
pMIC21	pHJW20 lacking all *icsP* promoter sequences	Castellanos et al. (2009)
pKML03	pHJW20 lacking previously annotated promoter elements	This work
pCTH02	pKML03 carrying 14-bp substitutions in the two upstream VirB-binding sites	This work
pCTH03	pHJW20 lacking P1 and P2 sequences	This work
P*icsP-icsP* reporters		
pHJW6	*icsP* promoter and gene cloned into pACYC184	Wing et al. (2004)
pCTH16	pHJW6 lacking P2 specific promoter elements	This work
pCTH17	pHJW6 with 4-bp substitutions in the downstream translation start site	This work
pCTH18	pCTH17 lacking P2-specific promoter elements	This work

Amp^r ampicillin resistance, *Cm^r* chloramphenicol resistance, *Kn^r* kanamycin resistance

To create pKML03, a truncated *icsP* promoter fragment was amplified from pHJW20 using oligonucleotides W93 (5′-TGGGTTGAAGGCTCTCAAGGGC-3′) and W123 (5′-TATTTTGCTCTAGATTTTAATTAAATATTTGTTTATGTTACC-3′). The PCR fragment was digested with *Pst*I and *Xba*I, and the resulting DNA fragment was ligated into pHJW20 previously digested with *Pst*I and *Xba*I. The resulting construct lacked the P1 TSS, and its −10 and −35 promoter elements, due to a 48-bp truncation at the 3′ end of the *icsP* promoter region. To create pCTH02, mutated VirB-binding sites from pMIC18 were isolated on a *Pst*I and *Pac*I restriction fragment and introduced into pKML03 previously digested with *Pst*I and *Pac*I. The resulting construct therefore carried mutated, instead of wild-type, VirB-binding sites. To create pCTH03, a truncated *icsP* promoter fragment was amplified from pHJW20 using oligonucleotides W93 and W167 (5′-TATTTTGCTCTAGACCTCATTGTGCGAATAAAGTAACGG-3′). The PCR fragment was digested with *Bgl*II and *Xba*I, and the resulting DNA fragment was ligated into pHJW20 previously digested with *Bgl*II and *Xba*I. The resulting construct therefore lacked both P1 and P2, due to a 132-bp truncation at the 3′ end of the *icsP* promoter region.

To measure IcsP production and IcsP protease activity, the plasmid pHJW6 and its derivatives were used (described in Wing et al. 2004; Table 2). pHJW6 is identical to pHJW20, but instead of carrying a P*icsP-lacZ* fusion, this plasmid carries the full *icsP* coding region downstream of the *icsP* promoter region.

To create pCTH16, the sequence encoding the *icsP* gene was isolated from pHJW6 using *Pac*I and *Bam*HI restriction enzymes and used to replace the *lacZ* gene in pHJW36. pHJW36 lacks the −35 and part of the −10 promoter elements for P2, and this has been demonstrated to result in an inactive P2, as evidenced by (1) primer extension analysis (unpublished data) and (2) the drop in total *icsP* promoter activity (Castellanos et al. 2009), consequently the newly formed construct pCTH16 could be used to measure IcsP protein production generated from P1 specific transcripts and hence the downstream translation start site. To create pCTH17, regions encoding a portion of the downstream Shine–Dalgarno sequence and the downstream methionine were mutated by introducing base pair substitutions in both sites using a Quick Change Lightning site-directed mutagenesis kit from Agilent Technologies and oligonucleotides W259 (5′-GTGCAAGTACAAAGAATTTTAATTTGAGCGAGAACTCGACTTTTTTGGTTGAAATGTCCATGA-3′) and W260 (5′-TCATGGACATTTCAACCAAAAAAGTCGAGTTCTCGCTCAAATTAAAATTCTTTGTACTTGCAC-3′). The substitutions used to disrupt the downstream translation start site were chosen to minimize the effect on the upstream translated protein

product. Specifically, the Shine–Dalgarno sequence was mutated from AAGTAG to AAGTCG, and this resulted in the substitution of a valine codon for another valine codon, and the methionine codon ATG was mutated to a leucine codon CTC. The resulting amino acids have similar biochemical properties. To create pCTH18, pCTH17 was digested with *Pac*I and *Bam*HI to obtain the mutated sequence eliminating the downstream translation start site, and the resulting DNA fragment was ligated into pCTH16 previously digested with *Pac*I and *Bam*HI. The resulting construct consequently lacked the upstream translation start site and carried a mutated downstream Shine–Dalgarno and translation start site.

Quantification of *icsP* promoter activity using P*icsP-lacZ* reporters

Activity of the *icsP* promoters was determined by measuring β-galactosidase activity [as described previously (Castellanos et al. 2009) using the Miller protocol (Miller 1972)] in strains carrying pHJW20 or derivatives. Routinely, transcription was analyzed in three independent transformants in early stationary-phase cultures. Cells were routinely back-diluted 1:100 and grown for 5 h in TSB, to ensure *icsP* expression. To measure the effects of growth phase on promoter activity, cells were grown for 2–10 h in 2-h intervals. To measure the effects of pH on promoter activity, cells were grown in LB with a pH of 5.5 buffered with a final concentration of 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) or a pH of 7.4 buffered with 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS). To measure the effects of osmotic pressure on promoter activity, cells were grown in either LB or LO (Porter and Dorman 1994). To measure the effects of iron concentration on promoter activity, cells were grown in either LB or LB supplemented with 15 μg ml^{-1} EDDA to chelate iron. Optical densities were measured using a DU 520 general purpose UV/Vis spectrophotometer (Beckman Coulter). Promoter activity was normalized using pMIC21, the promoterless *lacZ* reporter construct.

Transcription start site mapping of the *icsP* gene

Transcription start sites of the *icsP* gene were identified through RNA extraction and primer extension analysis procedures as described previously (Wing et al. 1995) using a protocol adapted from Aiba et al. (1981; Wing et al. 1995). Total cellular RNA was extracted using the hot-phenol method from 10^9 cells harvested from early stationary-phase cultures (Aiba et al. 1981). Residual DNA within samples was digested with DNase I (Qiagen) at 37°C for 1 h in DNase I buffer according to Ambion instructions (Ambion 2001). Integrity of total RNA was checked by formaldehyde gel electrophoresis and ethidium bromide staining as described by Sambrook (2001). The oligonucleotide primer W183 (5′-AAAGTGCAAGTACAAAG-3′) was 5′ end labeled with [γ-^{32}P] ATP by using T4 polynucleotide kinase (Promega). One picomole of ^{32}P-labeled primer and 5 μg of total RNA were lyophilized and redissolved in 30 μl of hybridization buffer (Aiba et al. 1981). The reaction was incubated at 75°C for 15 min followed by a cooling and incubation at 37°C for a total of 3 h. Following an ethanol precipitation, reverse transcription was completed using Superscript II reverse transcriptase (Invitrogen) according to manufacturer's instructions. Remaining RNA was degraded with 10 mg/ml RNase A (Sigma) for 30 min at 37°C and the reaction terminated by ethanol precipitation. The precipitate was dissolved in 5 μl of loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and electrophoresed on a 6% glycerol-tolerant polyacrylamide gel containing 7 M urea. Following electrophoresis, the gel was transferred to Whatman paper and then vacuum-dried before overnight exposure to a phosphorescent screen. The screen was visualized the following morning using a Typhoon 9410 variable mode imager (Amersham). The sequencing ladder generated from pBluescript KSII+ (Stratagene) and a M13 reverse primer (5′-GAGCGGATAACAATTTCACACAGG-3′) with the Sequenase 2.0 kit (usb) according to manufacturer's instructions was used to size the primer extension products. Densitometry analysis was conducted using VisionWorksLS image acquisition and analysis software (UVP).

Quantification of IcsP production and IcsA cleavage in *Shigella*

IcsP production and activity (IcsA cleavage) was measured by western blot analysis. Cells from early stationary-phase cultures were harvested and whole-cell protein extracts were prepared as described previously (Steinhauer et al. 1999). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in a 12.5% SDS–PAGE gel. Equivalent amounts of protein were loaded by normalizing the volume to cell density. Western blot analyses were performed with an affinity-purified IcsP or IcsA rabbit antiserum. The IcsP and IcsA antisera were raised against peptide sequences predicted to fall in surface-exposed regions of the two proteins, the L3 loop of IcsP (based on the model of OmpT; (Vandeputte-Rutten et al. 2001) and the α-domain of IcsA, which is proteolytically cleaved from the surface of *Shigella* by IcsP. Each antibody was ultimately detected by chemiluminescence using a UVP BioSpectrum imaging system and accompanying software. Densitometry analysis was conducted as previously described.

In silico analyses of the *icsP* gene, its protein product and the position of translation start sites in *Shigella* open reading frames

Throughout our work, sequences files were accessed and analyzed using the software program "Clone Manager 9 Basic Edition" (Scientific and Educational Software). Transcription start site predictions were performed using the BPROM program (http://www.linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). This algorithm predicts potential transcription start positions regulated by σ^{70} promoters (major *E. coli* promoter class). The linear discriminant function combines characteristics describing functional motifs and oligonucleotide composition of these sites. BPROM has 80% accuracy for *E. coli* σ^{70}-dependent promoter recognition. Open reading frame predictions were performed using Glimmer (NCBI), which is a system for finding genes in microbial DNA using interpolated Markov models (IMMs) to identify the coding regions and distinguish them from noncoding DNA (Delcher et al. 1999). To predict the presence and location of proteolytic cleavage sites within nascent IcsP, the SignalP 3.0 Server program (http://www.cbs.dtu.dk/services/SignalP/) was used (Emanuelsson et al. 2007). This algorithm predicts potential signal peptides and cleavage sites based on a combination of several artificial neural networks and hidden Markov models.

Bioinformatics screen for alternative translation start sites and associated Shine–Dalgarno sequences in *S. flexneri* 2a str. 301

Translation start sites were assumed to consist of two main components: a start codon and a Shine–Dalgarno sequence (ribosome-binding site, RBS). The full-genome sequence of *S. flexneri* 2a str. 301 (chromosome and plasmid) as well as the most recent annotation was downloaded from GenBank FTP site (ftp://ftp.ncbi.nih.gov/genbank/genomes/Bacteria/Shigella_flexneri_2a/).

To identify position-specific scoring matrix (PSSM) for Shine–Dalgarno sequence specific for *S. flexneri* 2a str. 301, 20 nucleotides of sequence data upstream of the start codon of each annotated open reading frame (ORF) were extracted from the genome (chromosome and plasmid). Then, retrieved regions of DNA were searched for over-represented motifs using locally installed MEME program (Multiple EM for Motif Elicitation). The identified RBS motif was truncated to include only highly conserved positions (Online Resource 1). A motif sequence logo was created using an online program (http://www.weblogo.berkeley.edu/logo.cgi).

To identify the presence of alternative translation start sites in genome of *S. flexneri* 2a str. 301, fifty codons upstream and downstream of start codon of every annotated ORF were tested for the presence of possible in-frame start codon (ATGs or GTGs only), and if an in-frame stop codon (TAG, TAA or TGA) was detected between annotated and alternative start codons, further searches around this particular annotated start codon was terminated. Twenty nucleotide sequences upstream every possible alternative start codon were collected and then searched for the presence of RBS using the program MAST (motif alignment and search tool) and determined before PSSM for RBS. Distance form annotated start site, *P* value for motif presence (if RBS was found), and exact sequence of start codon were extracted. Data were manipulated, managed, and graphically represented using custom R and Perl scripts.

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