Transcription-termination-mediated immunity and its prevention in bacteriophage SfV of Shigella flexneri

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The temperate phage SfV encodes the genes responsible for the serotype conversion of Shigella flexneri strains from serotype Y to 5a. Bacteriophages often encode proteins that prevent subsequent infection by homologous phages; the mechanism by which this is accomplished is referred to as superinfection immunity. The serotype conversion mediated following lysogenization of SfV is one such mechanism. Another mechanism is the putative λ-like CI protein within SfV. This study reports the characterization of a third superinfection mechanism, transcription termination, in SfV. The presence of a small immunity-mediating RNA molecule, called CI RNA, and its essential role in the establishment of immunity, is shown. The novel role of the gene orf77, located immediately downstream from the transcription termination region, in inhibiting the establishment of CI RNA-mediated immunity is also presented.

INTRODUCTION

Twelve of the 13 serotypes of Shigella flexneri are generated as the result of structural modifications to the O antigen, part of the lipopolysaccharide. These modifications to the O antigen are conferred by temperate serotype-converting bacteriophages present within S. flexneri. Phage-encoded enzymes, glucosyltransferases and/or O-acetyltransferases mediate the addition of glucosyl and/or O-acetyl residues to specific sugars of the basic tetrasaccharide repeat unit of the O antigen present in serotype Y strains of S. flexneri (Macpherson et al., 1991; Simmons & Romanowska, 1987). Lysogenization by the S. flexneri bacteriophage SfV results in the serotype conversion of serotype Y strains to serotype 5a. The genome of SfV has been sequenced (37 074 bp) and contains 53 predicted open reading frames (ORFs) that are organized in a manner similar to that of bacteriophage λ (Allison et al., 2002).

Bacteriophages exist within a cell as part of the chromosone, plasmid or linear DNA and have developed numerous mechanisms to maintain themselves within the bacterial cell. Often these mechanisms serve a dual function and act in trans to prevent subsequent infection of other, often homologous, bacteriophages into the same cell. Many temperate bacteriophages maintain their lysogenic state through the actions of a λ CI repressor-like protein. The CI protein binds to operator sequences to repress the early promoters from which the lytic genes are transcribed, establishing the lysogenic state (reviewed by Oppenheim et al., 2005). The CI repressor protein is expressed constitutively by the integrated phage and can function in trans to prevent the propagation of superinfecting phages. In this way, the repressor protein can establish what is referred to as immunity or superinfection immunity. Sequence analysis of SfV suggests that SfV contains a λ-like repressor protein system. Putative CI and Cro homologues have been identified as being encoded by orf34 and orf35 (Allison et al., 2002).

Temperate bacteriophages may also utilize superinfection exclusion mechanisms to prevent another bacteriophage from entering the bacterial cell. In P22, T4, P1 and the Lactococcus lactis phage Tuc2009, the superinfection exclusion mechanism is encoded by the sieA and sieB, imm, sim and sie2009 genes, respectively (Lu & Henning, 1989; Maillou & Dreiseikelmann, 1990; McGrath et al., 2002; Susskind et al., 1971). Generally, these proteins are membrane-associated and appear to function by blocking the injection of phage DNA into the host cell (Hofer et al., 1995; Lu et al., 1993; McGrath et al., 2002; Susskind et al., 1974). An exception to this is the sieB gene in P22, the expression of which has been hypothesized to cause abortive infection rather than blocking DNA injection (Ranade & Poteete, 1993). Modification of the receptor required for bacteriophage absorption into the host cell also results in the exclusion of other related phages that utilize the same receptor. A number of temperate serotype-converting bacteriophages, including SfV, encode a glucosyltransferase cassette that results in the modification of O antigen polysaccharide chains during lysogeny,
preventing superinfection through the newly modified receptor (Huan et al., 1997a; Markine-Goriaynoff et al., 2004; Mavris et al., 1997; Vander Byl & Kropinski, 2000).

A less common superinfection immunity mechanism is transcription termination in which the mediator is not a protein but a small RNA molecule. Transcription-termination-mediated immunity has been characterized in bacteriophages P4, P1, P7 and N15 (Citron & Schuster, 1990; Deho et al., 1988, 1992; Ravin et al., 1999). In all cases, immunity is established through RNA–RNA interactions between the small RNA molecule and complementary regions located immediately upstream and downstream on the nascent transcript. This interaction with the nascent transcript results in the premature termination of transcription. In P4, this premature transcription termination directly prevents expression of genes in the lytic cycle, in contrast to P1, P7 and N15 where the expression of anti-repressors is prevented (Biere et al., 1992; Briani et al., 2000; Ravin et al., 1999). Much like the repressor protein, the small RNA molecule also targets superinfecting phages, preventing them from propagating within the cell, thus providing superinfection immunity.

Two superinfection mechanisms, O antigen modification and a putative λ-like repressor protein system, have been characterized in SfV as described above. Here, we report a third superinfection mechanism in bacteriophage SfV. In this work, the region encoding the putative transcription-termination mechanism in SfV – specifically the predicted small CI RNA-encoding region – was found to be sufficient to confer immunity. The small RNA molecule was expressed by all constructs conferring immunity and was shown to be essential for the establishment of immunity. Furthermore, we present the possible role of orf77, located immediately downstream of the cl coding region, in preventing the establishment of immunity by transcription termination.

### METHODS

**Bacteria, bacteriophages and plasmids.** Bacteriophage SfV was originally induced from S. flexneri EW595/52 (Huan et al., 1997b). Bacteriophage stocks of SfV were propagated on SFL124, and both phage purification and DNA extraction were performed as described previously for bacteriophage λ (Sambrook & Russell, 2001).

The plasmids and bacterial strains utilized in this study are listed in Table 1. Routine cloning steps were performed in *E. coli* JM109, whilst *S. flexneri* serotype Y strain SFL124 was used for determination of the efficiency of plaque formation (EOPF). Both *E. coli* and *S. flexneri* strains were routinely grown in Luria–Bertani (LB) broth or LB agar, supplemented with 100 μg ampicillin ml⁻¹.

**Recombinant DNA techniques.** Plasmid DNA was prepared by alkaline lysis (Sambrook & Russell, 2001). DNA was digested with restriction enzymes according to the manufacturer’s instructions (Fermentas) and purified from agarose gels using a QIAquick gel extraction kit (Qiagen). Site-directed mutagenesis was performed as specified by the QuikChange site-directed mutagenesis kit (Stratagene).

**RNA extraction and Northern blot hybridization.** Total RNA was extracted from *S. flexneri* cells as described previously (Sung et al., 2003). The RNA was fractionated by electrophoresis through 10 % polyacrylamide/urea denaturing gels and subsequently transferred to Hybond ™ nitrocellulose membrane (Amersham) (Brown et al., 2004). Hybridization was performed using a 32P-labeled seqB oligonucleotide probe (Proligo), spanning 24 nt (5'-GTAACCGGTGAT-CAAGGAAAAGCCG-3').

**Efficiency of plaque formation.** SFL124 recombinant strains were infected with SfV and the EOPF was determined by dividing the number of p.f.u. ml⁻¹ by that obtained for the control strain, SFL124 (pUC18). Antibiotic selection was maintained throughout phage infections and EOPF experiments were repeated in triplicate.

**One-step growth curves and burst size.** Overnight bacterial cultures were diluted 1:100 in 10 ml fresh LB broth and grown at 37 °C with shaking at 150 r.p.m. to approximately 2 × 10⁶ c.f.u. ml⁻¹. The bacteria were infected with SfV at an m.o.i. of 0.1 and the culture returned to 37 °C for 5 min, with shaking, to allow phage adsorption. The culture was diluted 10⁴-fold in a final volume of 100 ml LB broth. Samples were taken at 10 min intervals for 180 min. The samples were serially diluted and a plaque assay was carried out on the indicator strain, SFL124. The p.f.u. ml⁻¹ for the strains at each time point was then determined. One-step growth curves were repeated in triplicate and the average burst size was determined from the curves by calculating the p.f.u. ml⁻¹ after the burst divided by the p.f.u. ml⁻¹ initially present upon infection.

### RESULTS

**Confirmation of the ability of transcription termination regions to confer immunity**

To determine whether other superinfection immunity mechanisms exist in SfV in addition to O antigen modification and the CI-like repressor, various phage fragments were cloned into pUC18 or pUC19 and introduced into the serotype Y *S. flexneri* strain SFL124. The EOPF on the resulting recombinant strains was then determined. Strain SFL1369 containing a 6.7 kb *BamHI* fragment located upstream of the serotype-conversion genes was found to confer a significant decrease in EOPF (Fig. 1a) and to cause an alteration in plaque morphology. Very small pinpoint plaques were formed by SfV on SFL1369 compared with the large, clear plaques formed on the control strain, SFL124 (pUC18). As this fragment contained the putative λ-like *cl* and *cro* genes, which could account for the immunity seen, smaller fragments not containing these genes were examined by subcloning. An 814 bp *Sphi–BamHI* fragment (pNV917, SFL1379) spanning the 3' end of *orf36* (encoding the C-terminal 36 aa) and the 5' end of *orf37* (encoding the N-terminal 237 aa) was found to confer significant immunity to SfV (EOPF 4.4 × 10⁻³). The Orf37 protein showed sequence identity to putative anti-repressors in *Salmonella typhimurium* phages ST64B (NCBI protein accession no. NP_700414; 58 % identity, E value 6e⁻⁵) and Gifsy-1 (NCBI protein accession no. AAX65104; 66 % identity, E value 2e⁻³⁰), and to Orf179 (NCBI protein accession no. CAA80648; 48 % identity, E value 2e⁻¹⁴), which is located adjacent to...
dicF-like sequences in *S. flexneri* (Faubladier & Bouche, 1994). These three proteins show similarity to the Eta protein of bacteriophage P4 (Forti et al., 1999). Nested within *eta* is a region encoding a small RNA molecule, called CI, that confers superinfection immunity to P4 (Deho et al., 1992; Ghisotti et al., 1992). To gain insight into the immunity-conferring agent in SfV, the 814 bp fragment was further subcloned and a 383 bp HindIII–BamHI fragment (pNV930, SFL1392) was found to be sufficient to confer immunity (EOPF 2.6 × 10^{-3}) (Fig. 1b).

The 383 bp HindIII–BamHI SfV fragment showed nucleotide identity with a hypothetical protein in bacteriophage P27 (GenBank accession no. AJ298298, 84% identity in a 293 nt overlap) and with a putative anti-repressor in phage ST64B (GenBank accession no. AY055382, 96% identity in a 59 nt overlap), which has similarity to the P4 Eta protein. Regulation of the early region in P4 by CI RNA relies on premature termination of transcription. The constitutive promoter PLE, located within *eta*, expresses full-length transcripts early during infection, resulting in the expression of early genes. Rapidly following infection, the immune state is established, with only short transcripts being transcribed from the PLE promoter (Briani et al., 1996, 2000; Deho et al., 1992). Consequently, transcripts from the PLE promoter do not proceed through to the genes required for the lytic cycle. This immunity is established and maintained in the lysogen by CI RNA (79 nt), encoded by the *cI* region and derived from longer transcripts by a process that involves RNase E and PNPase (Briani et al., 2002; Forti et al., 2002; Piazza et al., 1996). The CI RNA, specifically its internal seqB region, mediates immunity through RNA–RNA interactions with complementary regions seqA and seqC (seqC′ and seqC") located upstream and downstream of the CI RNA-coding region, respectively, on the nascent transcript (Deho et al., 1992; Forti et al., 1995; Ghisotti et al., 1992; Sabbattini et al., 1995). The CI RNA of P4 has a complex predicted

<table>
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*Swedish Institute for Infectious Disease Control.*
secondary structure composed of a double-stranded stalk, a minor and major stem–loop, and a single-stranded bulge (Forti et al., 1995).

Analysis of the Hinfl–BamHI fragment identified features of the P4 immunity mechanism: a PLE-like promoter and an RNA-coding region containing a seqB-like sequence complementary to both upstream and downstream regions, putatively called seqA, seqC and seqC* (Fig. 2). The ATG start codon at nt 27846 and its preceding strong ribosome-binding site (RBS) are located within the seqC* sequence of SfV, similar to the kil gene of phage P4 in which both its start codon and RBS are also located within the seqC* region. The ORF encoded from the start codon at nt 27846 in SfV was named orf77 as it encodes a protein of 77 aa. Both the size and location of orf77 make it similar to the Kil protein of P4. The predicted secondary structure of the 81 nt seqB-containing CI RNA-like region of SfV (Fig. 3) was the same as the CI RNA of P4, although the sequence between the two molecules was not completely conserved (Zuker et al., 1999). This secondary structure of the immunity-mediating RNA molecule appears to be important for transcription termination, as the bacteriophages N15, P1, P7 and φR73 share a remarkably similar CI RNA secondary structure without being homologous in sequence (Fig. 4) (Citron & Schuster, 1990, 1992; Ravin et al., 1999).

**Immunity-conferring plasmids produce CI RNA**

If SfV encodes a CI RNA-mediated transcription-termination mechanism, then the ci sequence alone should suffice to confer immunity via a trans effect on the incoming phage. Consequently, the predicted P4-like immunity region in SfV was further subcloned into pUC18 to isolate the minimum region required to confer immunity. The constructs were transformed into SFL124 and the EOPF of the resulting recombinant strains was determined (Fig. 5). Fragments cloned into pUC18 lacking the native PLE promoter will be expressed by the pUC18-encoded P_{Lac} promoter. The following experiments were performed without IPTG induction, as this is not required for expression from the P_{Lac} promoter in _S. flexneri_ strains.

SFL1675 contained a 521 bp fragment spanning the entire predicted immunity region from the putative PLE-like promoter to the end of orf77. As expected this fragment was capable of conferring immunity, with an EOPF of $4.1 \times 10^{-3}$, and was accompanied by the production of pinpoint plaques. Both SFL1676 and SFL1677 contained fragments spanning the ci sequence, starting at the PLE promoter and ending within orf77 and seqC*, respectively. As expected for fragments containing the ci region, both of these strains had significant immunity to SfV, with EOPFs of $3.6 \times 10^{-3}$ and $3.9 \times 10^{-3}$, respectively. A 94 bp fragment spanning the predicted ci sequence of 81 bp was cloned to produce pNV1359, which was determined to be sufficient to confer immunity (EOPF $4.6 \times 10^{-3}$) and alter plaque morphology. However, when a 56 bp fragment internal to the ci sequence was introduced into SFL124 (SFL1681), no immunity was conferred on SfV and plaque morphology remained unchanged. **ORF FINDER** (NCBI) predicted orf77 but also predicted another upstream start codon at position 27708, three bases into the ci coding region. This predicted start codon shared the same stop codon as orf77 but was not preceded by a RBS. Lacking a RBS makes this ORF less likely to encode a protein than the predicted orf77. The 94 bp cloned fragment contained only the front portion of this putative ORF, such that even if this ORF did encode a protein, only a very truncated and presumably non-functional fragment would be transcribed. Given that the 94 bp fragment conferred immunity whilst plasmid pNV1357, which contained this entire larger predicted ORF, did not, it would appear that something...
other than a protein, most likely RNA, is exerting this immunity effect. It was subsequently shown that immunity was lost when a region smaller than the predicted CI RNA-coding sequence was cloned. Consequently, as there is not a complete protein encoded within the 94 bp fragment and immunity is lost when a region smaller than the predicted CI RNA coding sequenced is cloned, collectively these data indicated that an RNA molecule is involved in the establishment of immunity to SfV.

To elucidate further the nature of the immunity conferred by the cI-encoding plasmid-containing SFL124 strains, one-step growth curves were performed and the mean burst size was determined (Fig. 6b). One-step growth curves were repeated in triplicate for each strain using an m.o.i. of 0.1 and the mean burst size was determined as the mean of the results from these curves. Infection of SFL1682, the control strain, showed a latent period of 60 min before phage numbers started to increase, followed by a growth period of approximately 70 min (Fig. 6a). The resulting burst sizes correlated with the earlier EOPF data. The burst size of the non-immune strains SFL1678, SFL1679 and SFL181 did not significantly differ from the control strain, SFL1682 (P > 0.01). However, the burst sizes of the immune strains SFL1675, SFL1676, SFL1677 and SFL1680 were found to be significantly smaller than that of SFL1682 (P < 0.01).

Northern blot hybridization was performed with a seqB-spanning oligonucleotide probe on total RNA extracted from the strains that showed immunity to SIV and from SFL1681, which contained the internal cI fragment (Fig. 7a). All strains containing the cI-coding region were found to express the approximately 81 bp CI RNA. SFL1681, which does not confer immunity, was shown not to express this RNA molecule.
However, SFL1678 containing the cl-coding sequence and the entire orf77 in plasmid pNV1357 did not confer immunity (EOPF 0.28). SFL1678 differed from SFL1675 in that it lacked the PLE promoter and seqA. This fragment was cloned downstream of the PLac promoter in pUC18. As plasmid pNV1359, containing the cl sequence alone downstream of the P Lac promoter, was capable of conferring immunity, it is unlikely that the absence of immunity in SFL1678 was due to a lack of CI RNA expression. This was confirmed by Northern blot hybridization, which showed that SFL1678 expressed the CI RNA (Fig. 7b). As orf77 was encoded on the SfV fragment in pNV1357, it is possible that this protein is expressed and involved in inhibiting the establishment of immunity by the CI RNA in SFL1678.

Mutations within the seqB region of CI RNA abolish immunity

Northern blot hybridization confirmed the presence of a seqB-spanning RNA molecule, CI RNA, in the strains that showed immunity to SfV. If transcription termination is utilized by SfV, which the data presented in this work support, then the complementarity between seqB and seqA and seqC/C* located upstream and downstream of the cl-coding region, respectively, is essential for transcription termination to occur and for immunity to be established (Deho et al., 1992; Ghisotti et al., 1992; Sabbattini et al., 1995).

Site-directed mutagenesis was used to introduce a double mutation within the main loop of the CI RNA (Figs 2 and 3). This double mutation was not predicted to alter the secondary structure of the CI RNA, as determined by MFOLD, but was positioned such that it should affect both seqA and seqC interactions. Plasmid pNV1359 containing the 94 bp cl-spanning fragment shown previously to confer immunity was mutated such that the CT nucleotides within the seqB region in the main loop (nt 27749 and 27750) was altered to AG, producing plasmid pNV1378. pNV1378 was transformed into SFL124 producing the recombinant strain SFL1703. Subsequent testing of SFL1703 confirmed that immunity had been abolished, with an EOPF of 0.87 and large-sized plaques similar in size to the control strain. Expression of the mutant CI RNA in SFL1703 was confirmed by Northern blot hybridization (Fig. 7b). The burst size of SFL1703 (Fig. 6) differed significantly \( (P < 0.01) \) from SFL1680, which was immune and contained the identical cl-coding fragment without the seqB mutations. However, whilst immunity was lost in SFL1703, it was not restored to the equivalent level seen in the control strain SFL1682, as the burst size of SFL1703 was significantly smaller than that of SFL1682 \( (P < 0.01) \). This probably reflects that fact that the mutated CI RNA present

![Fig. 3. Secondary structure of the CI RNA as predicted by the MFOLD program (Zuker et al., 1999). The double mutation introduced into the CI RNA is indicated next to the wild-type structure by A* and G*.

![Fig. 4. Multiple sequence alignment of immunity-mediating RNAs from bacteriophages, adapted from Ravin et al. (1999) with the addition of the SfV CI RNA sequence. The immunity-mediating RNAs of SfV (CI), N15 (CA), P4 (CI), P73 (CI), P1 (C4) and P7 (C4) are aligned from the +1 base of the mature RNA. The different elements of the secondary structure are divided by a space in the sequence and are indicated above the sequence as st1 and st2 (stem 1 and stem 2), and lp1 and lp2 (loop 1 and loop 2), with the corresponding sequences indicated by a prime. Upper- and lower-case letters indicate double-stranded and single-stranded regions, respectively. Conserved regions within the alignment are indicated in bold.](image-url)
in SFL1703 was still able to interact with the seqA and seqC regions to a small extent, resulting in a slight inhibition in burst size. As the mutations within the main loop were not predicted to alter secondary structure, the loss of immunity implicated the essential role that complementarity between seqB and seqA/seqC plays in the establishment of immunity to SfV.

**Expression of the Orf77 protein prevents the establishment of immunity**

Nested orf77 located downstream of the cl region in SfV shares a common location and size with the kil gene of P4 and the icd gene of both N15 and P1. These small genes encoding proteins of 65, 54 and 73 aa in P4, N15 and P1/P7, respectively, are all located immediately downstream of the immunity-conferring RNA-coding region (Biere et al., 1992; Forti et al., 1999; Riedel et al., 1993b). In addition to location and size similarities, the Kil protein of P4 and the Icd proteins of N15, P1 and P7 share a common function of inhibiting cell division (Forti et al., 1999; Ravin et al., 1999; Riedel et al., 1993b). Sequence analysis at both the nucleotide and amino acid level showed no significant similarity between orf77 and the kil or icd gene (data not shown). However, the Icd protein of N15, which shares 54% identity in a 54 aa overlap with Kil of P4, has no significant sequence identity to Icd of P1, suggesting that the sequence may not be highly conserved among these genes (Ravin et al., 1999).

Plasmid pNV1357, containing orf77 and the cl-coding region without the PLE promoter, was shown above not to confer immunity, even though CI RNA was expressed (Fig. 7a). Whilst this plasmid construct lacked the seqA sequence, which could be involved in the lack of immunity, pNV1359 also lacked this sequence but was capable of conferring immunity. If Orf77 was expressed from pNV1357, it could be involved in the absence of immunity observed in SFL1678. To test this hypothesis, a frame-shift mutation was introduced by the removal of a nucleotide within orf77 in pNV1357 (Fig. 5). The resulting mutated

<table>
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<td>pNV1369</td>
<td>seqA &lt;----- cl &lt;----- orf77</td>
<td>3.8×10⁻³</td>
<td>Pinpoint</td>
</tr>
<tr>
<td>SFL1703</td>
<td>pNV1378</td>
<td>seqA &lt;----- cl &lt;----- orf77</td>
<td>0.87</td>
<td>Large</td>
</tr>
</tbody>
</table>

**Fig. 5.** Identification of the minimum SfV region required for establishment of immunity. Schematic representation of the SfV fragments containing the putative P4-like immunity region cloned into pUC18 and respective EOPF and plaque morphology results. Plasmids pNV1369 and pNV1378 are identical to plasmids pNV1357 and pNV1359, respectively, except that they have undergone site-directed mutagenesis, indicated by black arrows. A frame-shift deletion was introduced in pNV1369 within orf77, whilst a two-base substitution (CT→AG) was introduced into the seqB region of the cl-coding region in pNV1378.
Orf77 protein expressed from pNV1369 in SFL1695 was truncated after 9 aa (MATVPPSLV) and was therefore non-functional. The frame-shift deletion within orf77 did not inhibit the expression of CI RNA in SFL1695 (Fig. 7b) and resulted in the restoration of immunity (EOPF 3.8$^{+6}_{-10}$) to SfV. The burst size observed on SFL1695 was significantly smaller than the non-immune strains SFL1678 and SFL1682 ($P < 0.01$). Therefore, it appears that the expression of Orf77 is involved in the absence of immunity in SFL1678 in some manner.

Orf77 was cloned downstream of the P$_{Lac}$ promoter of pUC18, producing pNV1358, and transformed into JM109. Expression of Orf77 induced by the addition of IPTG had no effect on either optical density measurements or a live cell count of the culture over a 3 h time period (data not shown). Transformation of pNV1358 into SFL124, allowing constitutive Orf77 expression, also had no apparent effect on cell growth, as determined by optical density measurements and a live cell count (data not shown). Thus, unlike the kil and icd genes, orf77 in SfV is not involved in the inhibition of cell division.

DISCUSSION

In this work, we have demonstrated that a third superinfection mechanism operates in SfV. This mechanism is similar to transcription termination, which relies on a small RNA molecule to mediate immunity. All plasmids constructed in this work containing the predicted RNA-coding region of SfV and shown to express the CI RNA conferred immunity to SFL124 from subsequent SfV infection. In fact, a cloned fragment of 94 bp (only enough to span the cl region) was sufficient for the establishment of immunity. Northern blot hybridization confirmed the expression of the seqB-containing RNA molecule, predicted to span 81 bp, in all immune strains containing such plasmids.

The secondary structure of the immunity RNA molecules CI, C4, CA and CI of P4, P1/P7, N15 and SfV, respectively, are very similar. In all cases, the RNA molecule contains an internal sequence that has complementary target sequences on the nascent transcript, the interaction between which mediates the termination of transcription. Forti et al. (2002) analysed several CI mutants of P4 that fell in both single- and double-stranded regions of the RNA molecule. This research confirmed that bases in the bulge and main loop of the CI RNA were involved in the interaction between seqB and the targets, seqA and seqC. Furthermore, mutations within seqA or seqC in P4 that alter their complementarity to seqB have also been shown to either abolish or reduce immunity in P4 (Sabbattini et al., 1995).

The double base substitution (CU$\rightarrow$AG, nt 27749/27750) introduced into the seqB region of the CI RNA of SfV resulted in the loss of immunity, as demonstrated by EOPF, plaque morphology and burst size, whilst the secondary structure was predicted to remain unchanged. Northern blot hybridization confirmed CI RNA expression from plasmid pNV1378 containing the altered cl region. As such, the loss of immunity seen following the introduction of mutations within the seqB region of the CI RNA in SfV resulted in the loss of immunity, as demonstrated by EOPF, plaque morphology and burst size, whilst the secondary structure was predicted to remain unchanged. Northern blot hybridization confirmed CI RNA expression from plasmid pNV1378 containing the altered cl region. As such, the loss of immunity seen following the introduction of mutations within the seqB region of the CI RNA in SfV is analogous to that seen in P4 CI RNA mutants. Parallels can also be drawn between the two phages based on sequence and mutational analysis. Thus, the interaction between seqB and its target sequences in SfV must also be essential for immunity to be established and this interaction is reliant on the complementarity between seqB and seqA/C, in a manner similar to that of P4. Burst size data showed the impact of this interaction in SfV, as mutations within seqB abolish immunity but did not restore the burst size to
that of the control strain, suggesting that some minimal seqB–SeqA/C interaction still occurred in the mutant strain.

The major difference between the transcription-termination mechanism of P4 and that of N15, P1 and P7 is that, in P4, transcription termination prevents the expression of genes directly involved in the lytic cycle, as opposed to N15, P1 and P7 in which premature termination prevents the transcription of two anti-repressor genes (Deho et al., 1992; Heinrich et al., 1995a; Ravin et al., 1999). In SfV, however, the downstream genes are not predicted to be anti-repressors. PHD-BLAST analysis of the genes immediately downstream, orf38 and orf39, showed that they encode proteins of unknown and replication functions, respectively. The absence of immunity seen in SFL1678 containing orf77 suggested that these two downstream genes may not be involved in the transcription-termination-mediated immunity in SfV. Regardless, all bacteriophages so far characterized contain either an icd or kil gene downstream of the RNA coding region, which is believed to be involved in inhibiting cell division. Induction of plasmid-based expression of these genes has been shown to result in cell death, and microscopic observation has revealed filament formation (Forti et al., 1999; Heinrich et al., 1994; Ravin et al., 1999). Unexpectedly, the nested orf77 downstream of the cl region in SfV, initially thought to perform a function similar to Kil/Icd, had no affect on cell growth when expression was induced following cloning downstream of the P<sub>Lac</sub> promoter. This held true regardless of whether orf77 was expressed in an E. coli JM109 or an S. flexneri SFL124 background. Interestingly, the putative kil genes in the <i>S. flexneri</i> prophage-related sequences SFS and SFW containing transcription-termination features were also found to have no effect on cell growth (Faubladier & Bouche, 1994). Blastn analysis of these two sequences revealed their presence within both of the sequenced S. flexneri serotype 2a strains 301 and 2457T (data not shown). The presence of transcription-termination features in prophage-related sequences within the genome of two <i>S. flexneri</i> strains may imply that this type of superinfection immunity is present in other temperate phages of <i>Shigella</i>.

Under normal conditions, kil/icd expression is not lethal and expression is confined to the early phase of infection, before immunity is established. It has been postulated that the role of Kil/Icd in phage biology is to delay cell division early during infection, allowing replication of the phage genome before the cell divides (Forti et al., 1999; Ravin et al., 1999). This could increase the chance of the phage genome being inherited either as an integrated prophage or as a plasmid/linear DNA molecule. The absence of a gene with this function under the control of transcription-termination-mediated immunity in SfV suggests that the Kil/Icd function is not essential to the life cycle of SfV. In fact, it has been questioned whether Icd is in fact essential to the P1 phage life cycle (Riedel et al., 1993b). As the putative kil gene in the SFS and SFW sequences of <i>S. flexneri</i> also fail to inhibit cell division, the lack of a gene serving this function may be a common characteristic of phages of <i>Shigella</i>. With the absence of a kil-like gene between the cl region and the downstream genes, transcription termination as it occurs in SfV appears to differ from the mechanisms characterized so far. As Orf77 was not involved in inhibiting cell division, we undertook further investigation of its role in transcription termination in SfV.

Plasmid pNV1357 containing the cl region and orf77 downstream of the P<sub>Lac</sub> promoter was shown not to confer immunity to SFL124. To explore this, we used site-directed mutagenesis to introduce a frame-shift mutation in orf77 resulting in a truncated non-functional protein. The resulting plasmid, pNV1369, was able to confer immunity, suggesting that orf77 was expressed by pNV1357, preventing the establishment of immunity.

The two anti-repressor genes, ant1 and ant2, located downstream of the immunity RNA-coding region in N15, P1 and P7, are prevented from being transcribed by transcription termination. Similar to the mechanism that occurs in other phages, it has been proposed that the anti-repressors interact with the primary repressor, CB and CI in N15 and P1/P7, respectively, preventing the repressor from binding to operator sequences (Heinrich et al., 1995b; Ravin et al., 1999). Riedel et al. (1993a) found that, under appropriate conditions, the CI repressor of both P1 and P7 co-precipitated with Ant1/Ant2 complexes, but not Ant2 alone. This implies that the anti-repressors of P1 and P7 may execute their CI repressor-inactivating function directly through a protein–protein interaction. Consequently, it is possible that orf77 may encode an anti-repressor in SfV, explaining why its expression prevents immunity being established. If this is the case, SfV differs from other phages that utilize transcription termination to
prevent anti-repressor expression in that it appears to encode only one anti-repressor rather than two. The obvious target of such an anti-repressor in SfV would be the λ-like CI repressor protein predicted by sequence analysis of the genome. The anti-repressor and CI repressor of SfV, based on the data in P1, may interact through a direct protein–protein interaction (Riedel et al., 1993a). If orf77 functions as an anti-repressor, its expression could be expected to favour the lytic cycle, resulting in an increase in the number of plaques formed, as seen with induction of anTA expression in N15 (Ravin et al., 1999). However, orf77 cloned into pUC18 (pNV1358) had no effect on EOPF (Fig. 5), suggesting that Orf77 may not be an anti-repressor or at least not to the extent seen in N15, which would result in an EOPF of >1.

Small non-coding RNAs have begun to gain the interest of researchers of both prokaryotic and eukaryotic organisms. The extent in terms of both numbers and the roles played by these RNAs has only started to be elucidated in the last few years (Storz et al., 2005). Initially, the few known small non-coding RNAs were encoded by plasmids, bacteriophages and transposons; now more than 80 have been identified in E. coli and dozens within other bacteria (Huttenhofer & Vogel, 2006). Trans-acting small RNAs appear to play an important role in gene regulation, including genes involved in bacterial pathogenesis (Majdalani et al., 2005). This regulation can occur at a post-transcriptional level through modulating mRNA stability or translation through base pairing with the 5′ regions of the target mRNA (Storz et al., 2006). A much smaller subset of small non-coding RNAs acts by regulating transcription. As such, the further characterization of transcription termination and the variations present in SfV are of importance to the growing field of research addressing what appears to be a widely used mechanism regulating gene expression in a wide range of organisms.

In summary, we have identified an RNA-based immunity mechanism in SfV similar to transcription termination, capable of preventing subsequent SfV infection. We have shown that the predicted CI RNA is essential for the establishment of immunity. Transcriptional termination in SfV differs from the mechanisms characterized thus far in that it appears that the gene immediately downstream of cl does not function to inhibit cell division. In fact, initial results presented in this paper including complementation and mutagenesis data indicate that expression of orf77 inhibits the establishment of immunity in SfV. This newly characterized immunity mechanism brings the number of superinfection mechanisms in SfV to at least three. It is likely that these three mechanisms work concurrently to provide immunity in SfV.

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