Chapter 5 – Effect of *virK* and *rmlD* mutations on IcsP and *S. flexneri* virulence

5.1 Introduction

S. flexneri strains carrying a transposon insertion in the *virK* gene (*virK*::Tn*10* mutant) have been reported to express increased levels of IcsP (Wing *et al.*, 2005). The *virK* gene was originally discovered to affect the intracellular spreading of *S. flexneri* (Nakata *et al.*, 1992) and is located in an operon with several genes such as *msbB2* (refer to Fig. 1.6), the product of which has been shown to modify the lipid A component of LPS in *Shigella* (D'Hauteville *et al.*, 2002). The effect of *virK* on LPS structure has not been investigated. The *S. flexneri virK* mutant used by Wing *et al*. (2005) was made by Nakata *et al*. (1992). This mutant was shown to have decreased intracellular ABM and was unable to form plaques on cell monolayers. Complementation of the *virK* mutation was seen to restore plaque formation. However, no other *S. flexneri* independent *virK* mutant has ever been constructed to confirm these data.

In addition to this, Omptin family members have been shown to be affected by the presence of LPS. OmpT, for example, has been shown to be active only in the presence of LPS (Kramer *et al.*, 2000) and LPS Oag has been shown to inactivate the ability of Pla and PtgE to activate plasminogen (Kukkonen *et al.*, 2004). Since IcsP possesses 56% to OmpT and ~38% homology to Pla and PtgE, it is resonable to hypothesise that IcsP activity may be affected by LPS structure.

This chapter describes the construction of a 2457T *virK* and *rmlD* single mutants, and a *virK*/*rmlD* double mutant using a slight modification of the λ Red recombinase system by

Datsenko and Wanner (2000). Since Oag is known to affect the activity of Omptins, the *rmlD* gene was mutated as *rmlD* is required for the synthesis of dTDP-rhamnose which is a precursor for Oag synthesis (refer to Section 1.2.1). Inactivation of *rmlD* results in rough LPS (Van den Bosch *et al.*, 1997; Van den Bosch & Morona, 2003). Derivatives with the *icsP* mutation (2457T *virK*/*icsP*::*kan*^{R}, *rmlD*/*icsP*::*kan*^{R} and *virK*/*rmlD*/*icsP*::*kan*^{R}) were also made. The mutants were compared to wild-type with respect to IcsA and IcsP production, and the ability to form plaques.

5.2 Construction of *S. flexneri* 2457T *virK* and *rmlD* mutants

5.2.1 Construction of 2457T *virK* mutant

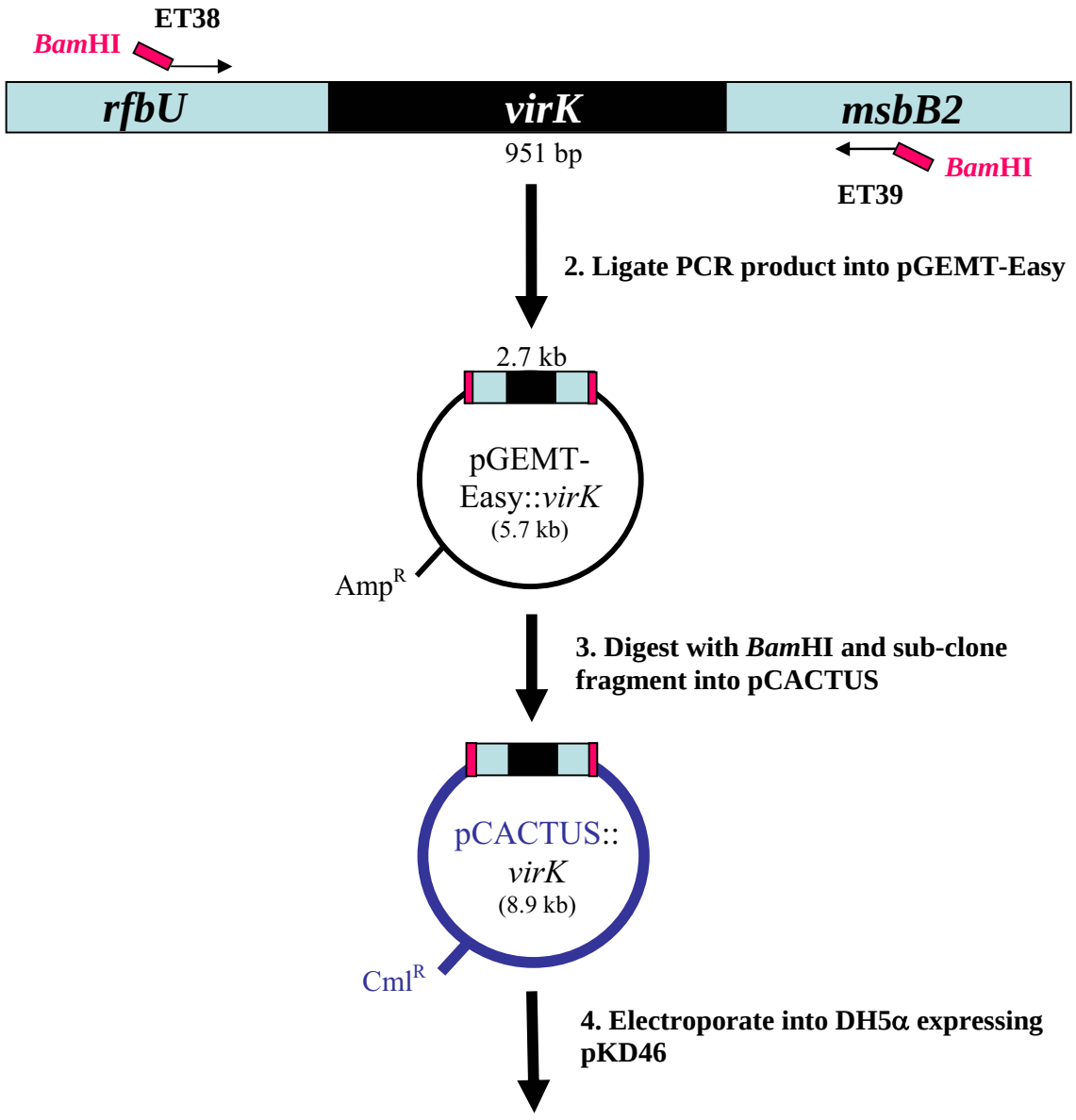
The *S. flexneri* 2457T *virK* mutant was constructed using a modified version of the λ Red recombinase system of Datsenko and Wanner (2000) (Section 2.8.2.2) and is summarised in Fig.ures 5.1 and 5.2. A deletion mutation was constructed to avoid any polarity effect from occurring on adjacent genes in the *virK* operon (refer to Fig. 1.7). Primers with *Bam*HI restriction enzyme sites and homologous to ~900 bp upstream and downstream of *virK* (ET38 and ET39) were used to PCR amplify the *virK* gene from *S. flexneri* 2457T chromosomal DNA (Table 2.2) (Fig. 5.1). The amplified 2.7 kb *virK* fragment (Fig. 5.3, lane 6) was cloned into pGEMT-Easy to give pGEMT-Easy::*virK*, and then digested with *Bam*HI to allow the 2.7 kb *Bam*HI-*Bam*HI fragment to be sub-cloned into likewise digested pCACTUS. The resulting plasmid pCACTUS::*virK* was electroporated into DH5α to give ETRM237 (DH5α [pCACTUS::*virK*]). ETRM237 was then electroporated with pKD46 (Table 2.5) to give ETRM287 (DH5α [pKD46][pCACTUS::*virK*]).

Primers with tag sequences homologous to regions upstream and downstream of *virK* (ET40 and ET41) were then designed to PCR amplify the *kan*^{R} gene from pKD4 (Table 2.5) (Fig.

Fig. 5.1 Construction of pCACTUS::*virK*

The pCACTUS::*virK* plasmid was constructed by PCR amplification of the 951 bp *virK* gene from 2457T chromosomal DNA with upstream and downstream primers ET38 and ET39. The 2.7 kb PCR *virK* fragment was then ligated into pGEMT-Easy and the resulting pGEMT-Easy::*virK* construct (5.7 kb) was digested with *Bam*HI, and ligated into likewise digested pCACTUS at 30°C. pCACTUS::*virK* (8.9 kb) was then transformed into DH5α expressing the λ Red recombinase plasmid pKD46, to give strain ETRM287 (DH5α [pKD46][pCACTUS::*virK*]).

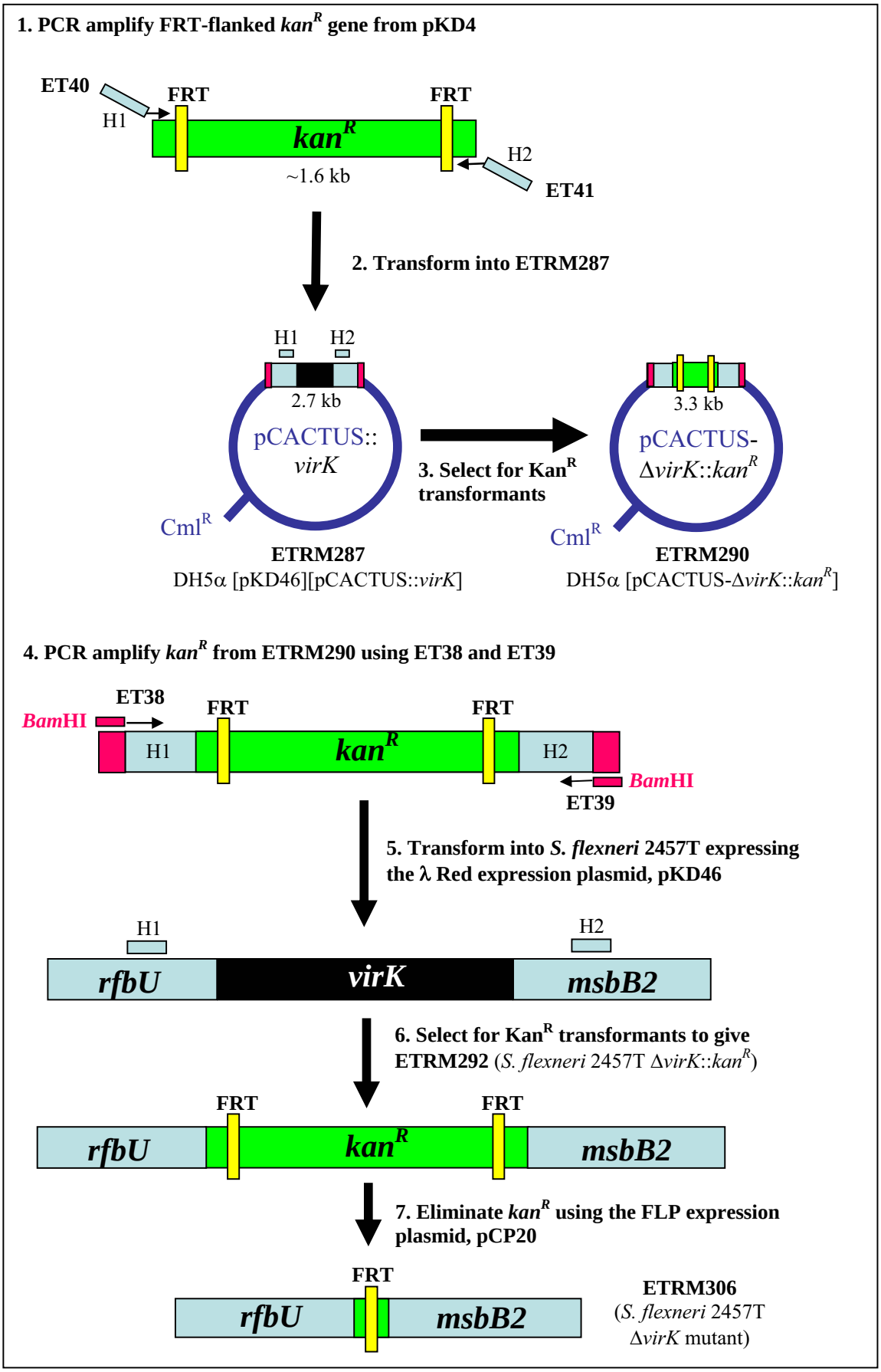
1. PCR amplify *virK* gene from 2457T chromosomal DNA



ETRM287
DH5α [pKD46][pCACTUS::*virK*]

Fig. 5.2 Construction of the 2457T *virK* mutant

The 2457T *virK* mutant was made by PCR amplifying the FRT-flanked kan^R gene from pKD4 with primers ET40 and ET41 containing homologous sequences tags to *virK*. H1 refers to the homologous sequence before the *virK* start codon, and H2 to the homologous sequence after the *virK* stop codon. The ~1.6 kb kan^R product was then electroporated into the DH5α strain ETRM287 carrying pCACTUS::*virK* and pKD46 (refer to Fig. 5.1). Kan^R transformants were selected to give ETRM290 (DH5α [pCACTUS-Δ*virK*::kan^R]), and *virK*-specific upstream and downstream primers ET38 and ET39 (Fig. 5.1) were then used to PCR amplify the kan^R gene from ETRM290. The resultant 3.3 kb product was transformed directly into *S. flexneri* 2457T carrying pKD46, and Kan^R transformants were selected to give the 2457T Δ*virK*::kan^R mutant ETRM292. Excision of the kan^R gene was achieved using pCP20 to give the final mutant ETRM306 (2457T Δ*virK* mutant).


1. PCR amplify FRT-flanked kanR gene from pKD4
ET40
FRT
FRT
H1
kanR
H2
~1.6 kb
ET41
2. Transform into ETRM287
H1
H2
2.7 kb
pCACTUS::
virK
CmlR
3. Select for KanR
transformants
3.3 kb
pCACTUS-
ΔvirK::kanR
CmlR
ETRM287
DH5α [pKD46][pCACTUS::virK]
ETRM290
DH5α [pCACTUS-ΔvirK::kanR]
4. PCR amplify kanR from ETRM290 using ET38 and ET39
ET38
BamHI
FRT
FRT
H1
kanR
H2
BamHI
ET39
5. Transform into S. flexneri 2457T expressing
the λ Red expression plasmid, pKD46
H1
H2
rfbU
virK
msbB2
6. Select for KanR transformants to give
ETRM292 (S. flexneri 2457T ΔvirK::kanR)
FRT
FRT
rfbU
kanR
msbB2
7. Eliminate kanR using the FLP expression
plasmid, pCP20
FRT
rfbU
msbB2
ETRM306
(S. flexneri 2457T
ΔvirK mutant)

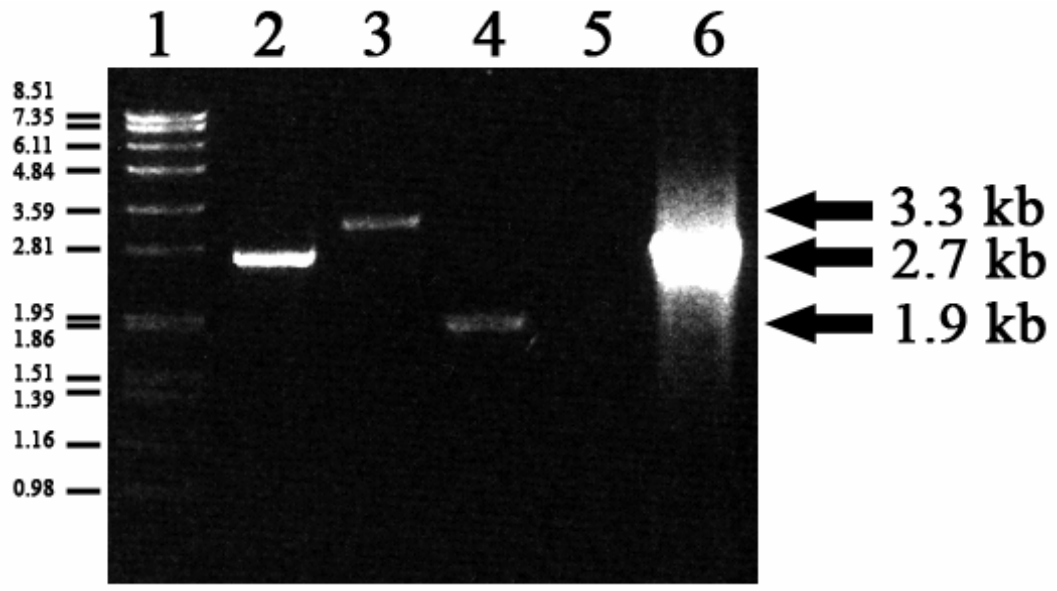


Fig. 5.3 PCR analysis on 2457T *virK* mutant

PCR was performed on strains (indicated below) with *virK* upstream and downstream primers ET38 and ET39 (Table 2.2). The size of the undisrupted (2.7 kb), *kan*^{R} disrupted (3.3 kb) and deleted (1.9 kb) *virK* gene PCR products are indicated on the right. The migration positions of the SPP1 DNA marker is indicated on the left in kb. *S. flexneri* 2a 2457T genomic DNA (diluted 1/100) was used as a positive control and MQ water was used as a template for the negative control. The lane order is as follows:

1. SPP1 DNA marker
2. *S. flexneri* 2a 2457T
3. ETRM292 (2457T *virK*::*kan*^{R})
4. ETRM306 (2457T *virK*)
5. MQ control
6. *S. flexneri* genomic DNA control

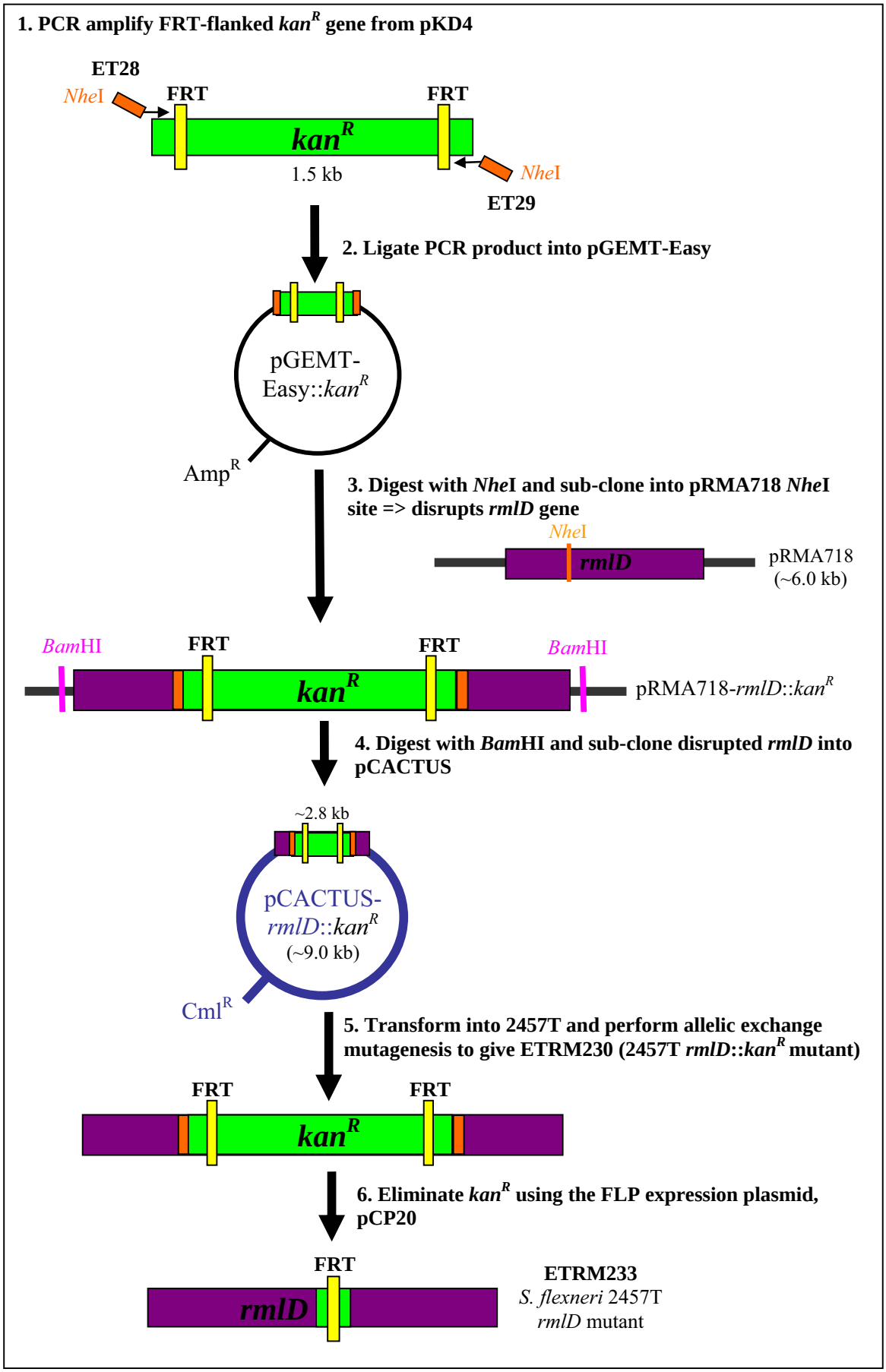
5.2) and the 1.6 kb fragment transformed via electroporation into ETRM287 at 37°C. Kan^R transformants were selected to give ETRM290 (DH5α [pCACTUS-Δ*virK*::*kan*^{R}]). Primers ET38 and ET39 were then used to PCR amplify the *kan*^{R} gene from ETRM290 and the resultant 3.3 kb product was transformed into 2457T carrying pKD46 at 37°C, followed by Kan^R selection, resulting in ETRM292 (Table 2.4). ETRM292 (2457T Δ*virK*::*kan*^{R}) was confirmed by PCR (with ET38 and ET39) showing insertion of *kan*^{R} in place of *virK* (~3.3 kb) (Fig. 5.3, lane 3). ETRM292 was then transformed with the temperature sensitive plasmid pCP20 (Table 2.5) at 30ºC to flip out the FRT flanked *kan*^{R} gene. Amp resistant colonies were then re-purified non-selectively at 43ºC for loss of all antibiotic resistances. The resultant 2457T Δ*virK* mutant strain ETRM306 (denoted as 2457T Δ*virK*::scar^{FRT} in Tables 2.4, and as 2457T *virK* in the following text) was again confirmed by PCR for loss of the *kan*^{R} gene and deletion of *virK* (by the presence of a 1.9 kb fragment) with primers ET38 and ET39 (Fig. 5.3, lane 4).

5.2.2 Construction of 2457T *rmlD* mutant

The 2457T *rmlD* mutant was constructed as summarised in Figure 5.4. Primers ET28 and ET29 (Table 2.2) with *Nhe*I restriction enzyme sites were used to PCR amplify the *kan*^{R} gene from pKD4. The amplified 1.5 kb PCR fragment was cloned into pGEMT-Easy, resulting in pGEMT-Easy::*kan*^{R}, and the construct was then digested with *Nhe*I to sub-clone the 1.5 kb *Nhe*I-*Nhe*I fragment into likewise digested (and SAP treated) pRMA718 (Table 2.5). pRMA718 contains the *S. flexneri rfb* region which includes the *rmlD* gene. The *Nhe*I site is located within *rmlD* and hence, insertion of *kan*^{R} into this site allowed disruption of *rmlD* to give pRMA718-*rmlD*::*kan*^{R}. pRMA718-*rmlD*::*kan*^{R} was then digested with *Bam*HI (there are two *BamH*I sites located just before and after the *rmlD* gene) and the ~2.8 kb *rmlD*::*kan*^{R} fragment was gel purified and sub-cloned into likewise digested pCACTUS, resulting in pCACTUS-*rmlD*::*kan*^{R}. *S. flexneri* 2457T was then electroporated with pCACTUS-

Fig. 5.4 Construction of the 2457T *rmlD* mutant

The 2457T *rmlD* mutant was constructed by PCR amplifying the FRT-flanked *kan*^{R} gene from pKD4 with *Nhe*I sites using primers ET28 and ET29. The amplified 1.5 kb *kan*^{R} product was then ligated into pGEMT-Easy, and pGEMT-Easy::*kan*^{R} was digested with *Nhe*I. The *Nhe*I-*Nhe*I *kan*^{R} fragment was then sub-cloned into the *Nhe*I site in the *rmlD* gene expressed on pRMA718 (hence disrupting the *rmlD* gene) to give pRMA718-*rmlD*::*kan*^{R}. This plasmid was then digested with *Bam*HI and the *rmlD*::*kan*^{R} fragment was cloned into the *Bam*HI site of pCACTUS. The pCACTUS-*rmlD*::*kan*^{R} construct was then electroporated into 2457T. Allelic exchange mutagenesis was induced to give the 2457T *rmlD*::*kan*^{R} mutant ETRM230. Excision of the *kan*^{R} gene was achieved using pCP20 to give the final mutant ETRM233 (2457T *rmlD* mutant).


1. PCR amplify FRT-flanked kanR gene from pKD4
ET28
NheI
FRT
FRT
kanR
1.5 kb
NheI
ET29
2. Ligate PCR product into pGEMT-Easy
pGEMT-Easy::kanR
AmpR
3. Digest with NheI and sub-clone into pRMA718 NheI site => disrupts rmlD gene
NheI
rmlD
pRMA718 (~6.0 kb)
BamHI
FRT
FRT
BamHI
kanR
pRMA718-rmlD::kanR
4. Digest with BamHI and sub-clone disrupted rmlD into pCACTUS
~2.8 kb
pCACTUS-rmlD::kanR (~9.0 kb)
CmlR
5. Transform into 2457T and perform allelic exchange mutagenesis to give ETRM230 (2457T rmlD::kanR mutant)
FRT
FRT
kanR
6. Eliminate kanR using the FLP expression plasmid, pCP20
FRT
rmlD
ETRM233
S. flexneri 2457T
rmlD mutant

rmlD::*kan*^{R} and allelic exchange mutagenesis was induced as described in Section 2.8.1. The resultant strain ETRM230 (2457T *rmlD*::*kan*^{R}) was transformed with pCP20 (Table 2.5) at 30ºC to flip out the FRT flanked *kan*^{R} gene to give the final 2457T *rmlD* mutant ETRM233 (denoted as 2457T *rmlD*::scar^{FRT} in Table 2.4 and 2457T *rmlD* in the following text). A 2457T *rmlD*/*virK* double mutant (ETRM320) was also constructed for this study and made by mutating the *virK* gene in ETRM233 as described in Section 5.2.1.

5.2.3 Construction of *icsP* derivatives

The *icsP* mutant strains ETRM318 (2457T *virK*/*icsP*::*kan*^{R}), ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}) and ETRM309 (2457T *rmlD*/*virK*/*icsP*::*kan*^{R}), were made by subjecting ETRM306 (2457T *virK*), ETRM233 (2457T *rmlD*), and ETRM320 (2457T *rmlD*/*virK*) to allelic exchange mutagenesis with pCACTUS-*icsP*::*kan*^{R} as described in Section 3.2.

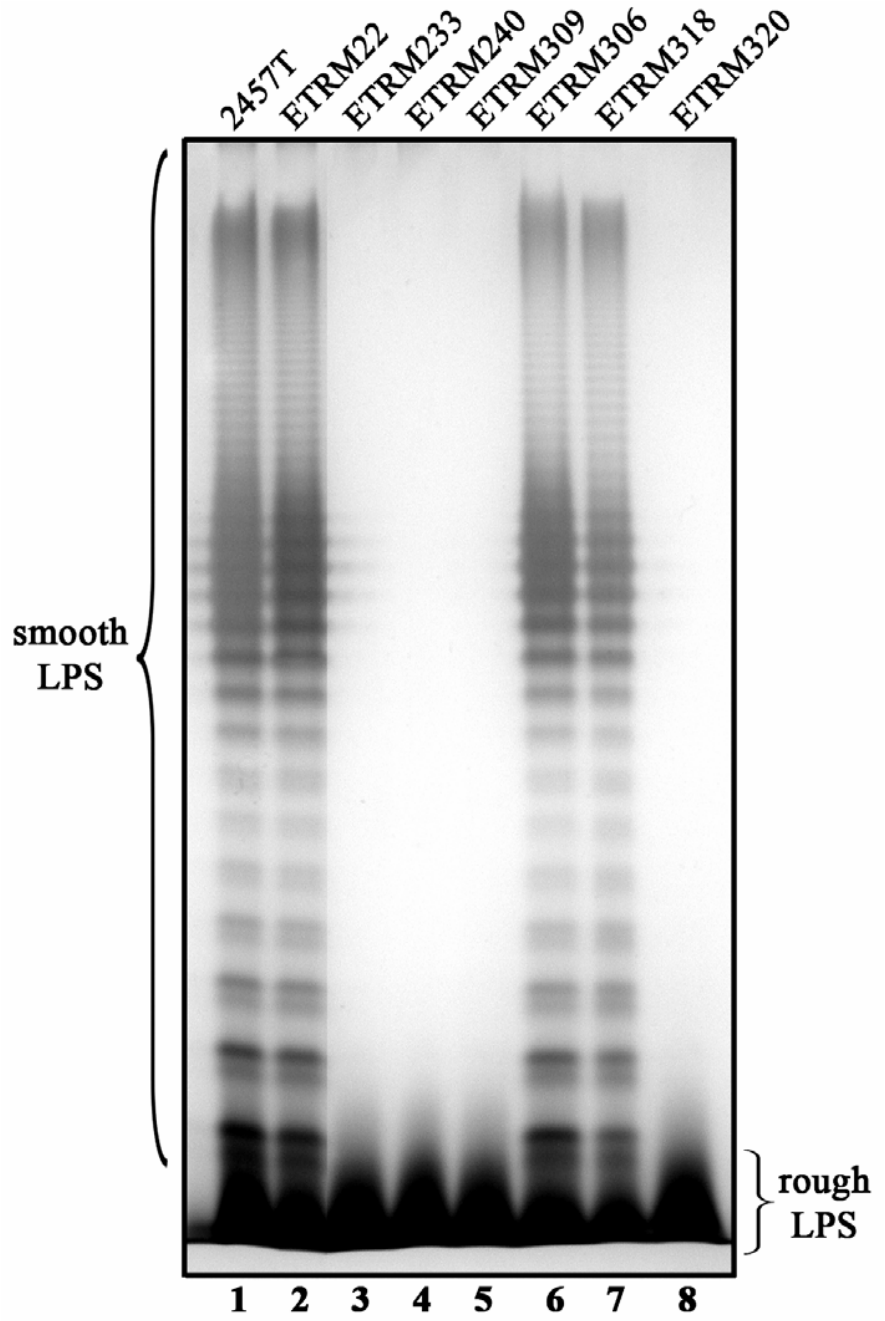
5.3 LPS profile of *virK* and *rmlD* mutants

To investigate the LPS profile of the 2457T *virK* mutant ETRM306 and confirm the expression of rough LPS in the *rmlD* mutants constructed, LPS samples were prepared as described in Section 2.10.2 and analysed by SDS-PAGE silver staining. The presence of Oag was observed in the LPS of 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), ETRM306 (2457T *virK*) and ETRM318 (2457T *virK*/*icsP*::*kan*^{R}) (Fig. 5.5, lanes 1, 2, 6 and 7 respectively). The absence of Oag repeating units was confirmed in the LPS of ETRM233 (2457T *rmlD*), ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}), ETRM309 (2457T *rmlD*/*virK*/*icsP*::*kan*^{R}) and ETRM320 (2457T *rmlD*/*virK*) (Fig. 5.5, lanes 3, 4, 5 and 8 respectively). The absence of an effect on the LPS banding profile of ETRM306 (2457T *virK*) compared to wild-type 2457T (Fig. 5.5, lanes 1 and 6) suggested that *virK* has little or no detectable effect on the structure of LPS.

Fig. 5.5 LPS analysis of *S. flexneri virK* and *rmlD* mutants

LPS was prepared by protease K treatment using cultures standardised to an OD_{600} of 2 and electrophoresed on a SDS 15% polyacrylamide gel prior to silver staining (refer to Section 2.10.3). The position of rough LPS (LPS lacking Oag) and smooth LPS (containing Oag repeat units) is indicated on the right and left, respectively. Samples represent approximately $2x10^8$ bacterial cells. The strains in each lane are as follows:

1. 2457T
2. ETRM22 (2457T *icsP*::*kan*^{R})
3. ETRM233 (2457T *rmlD*)
4. ETRM240 (2457T *rmlD/icsP*::*kan*^{R})
5. ETRM309 (2457T *rmlD*/*virK/icsP*::*kan*^{R})
6. ETRM306 (2457T *virK*)
7. ETRM318 (2457T *virK/icsP*::*kan*^{R})
8. ETRM320 (2457T *rmlD*/*virK*)


2457T
ETRM22
ETRM233
ETRM240
ETRM309
ETRM306
ETRM318
ETRM320
smooth LPS
rough LPS
1
2
3
4
5
6
7
8

5.4 Analysis of plaque and F-actin comet tail formation by *virK* and *rmlD* mutants

An important virulence characteristic of the *virK*::Tn*10* mutant phenotype described by Nakata *et al*. (1992) is the formation of smaller sized plaques on cell monolayers compared to wild-type. To investigate the intracellular phenotype of the *virK* mutant constructed in this study, HeLa and CV-1 cell monolayers were infected with 2457T and ETRM306 (2457T *virK*). ETRM318 (2457T *virK*/*icsP*::*kan*^{R}) was also included in the above plaque assay to determine whether a *virK*/*icsP*::*kan*^{R} double mutant had an effect on intercellular spread. Note that in Section 3.4.1, ETRM22 (2457T *icsP*::*kan*^{R}) formed significantly larger sized plaques than wild-type strain 2457T on CV-1 cell monolayers, but not on HeLa cell monolayers. In addition to this, previous studies have shown that *rmlD* mutants do not form plaques on cell monolayers and display deformed F-actin comet tails inside cells (Van den Bosch *et al.*, 1997; Van den Bosch & Morona, 2003). Hence, this aspect was also investigated for the strains carrying the *rmlD* mutation (ETRM233 (2457T *rmlD*), ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}), ETRM320 (2457T *rmlD*/*virK*) and ETRM309 (2457T *rmlD*/*virK*/*icsP*::*kan*^{R})).

5.4.1 Plaque formation on CV-1 cell monolayers

Plaque assays with CV-1 cells showed that ETRM306 (2457T *virK*) formed plaques of similar size to 2457T (Fig. 5.6 A and C), while ETRM318 (2457T *virK*/*icsP*::*kan*^{R}) formed larger sized plaques comparable to the plaques formed by ETRM22 (2457T *icsP*::*kan*^{R}) (Fig. 5.6 B and D). Statistical analysis using a two-tailed unpaired *t*-test on the average size plaques for each strain showed that the differences in plaque sizes for ETRM306 compared to wild-type, and ETRM318 compared to ETRM22, were statistically insignificant (Fig. 5.7). Strains carrying the *rmlD* mutation (ETRM233 (2457T *rmlD*), ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}),

Fig. 5.6 CV-1 cell plaque assays with *S. flexneri virK* and *rmlD* mutants

Confluent CV-1 cell monolayers were infected with LB grown *S. flexneri* strains (as indicated below) for 2 h, overlaid with gentamycin-containing agarose and incubated at 37°C with 5% CO_2 for 20 h, then overlaid with a second agaroseose layer containing Neutral Red and incubated for a further 8 h prior to taking pictures (refer to Section 2.12.4). The experiment was repeated four times with consistent results. The average size of 20 plaques ± the standard error mean for each strain is shown in the top corner of each image, and the genotype of each strain is indicated in the bottom right corner. The images are as follows:

A. CV-1 infected with 2457T

B. CV-1 infected with ETRM22 (2457T *icsP*::*kan*^{R})

C. CV-1 infected with ETRM306 (2457T *virK*)

D. CV-1 infected with ETRM318 (2457T *virK*/*icsP*::*kan*^{R})

E. CV-1 infected with ETRM233 (2457T *rmlD*)

F. CV-1 infected with ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R})

G. CV-1 infected with ETRM320 (2457T *rmlD*/*virK*)

H. CV-1 infected with ETRM309 (2457T *rmlD*/*virK*/*icsP*::*kan*^{R})

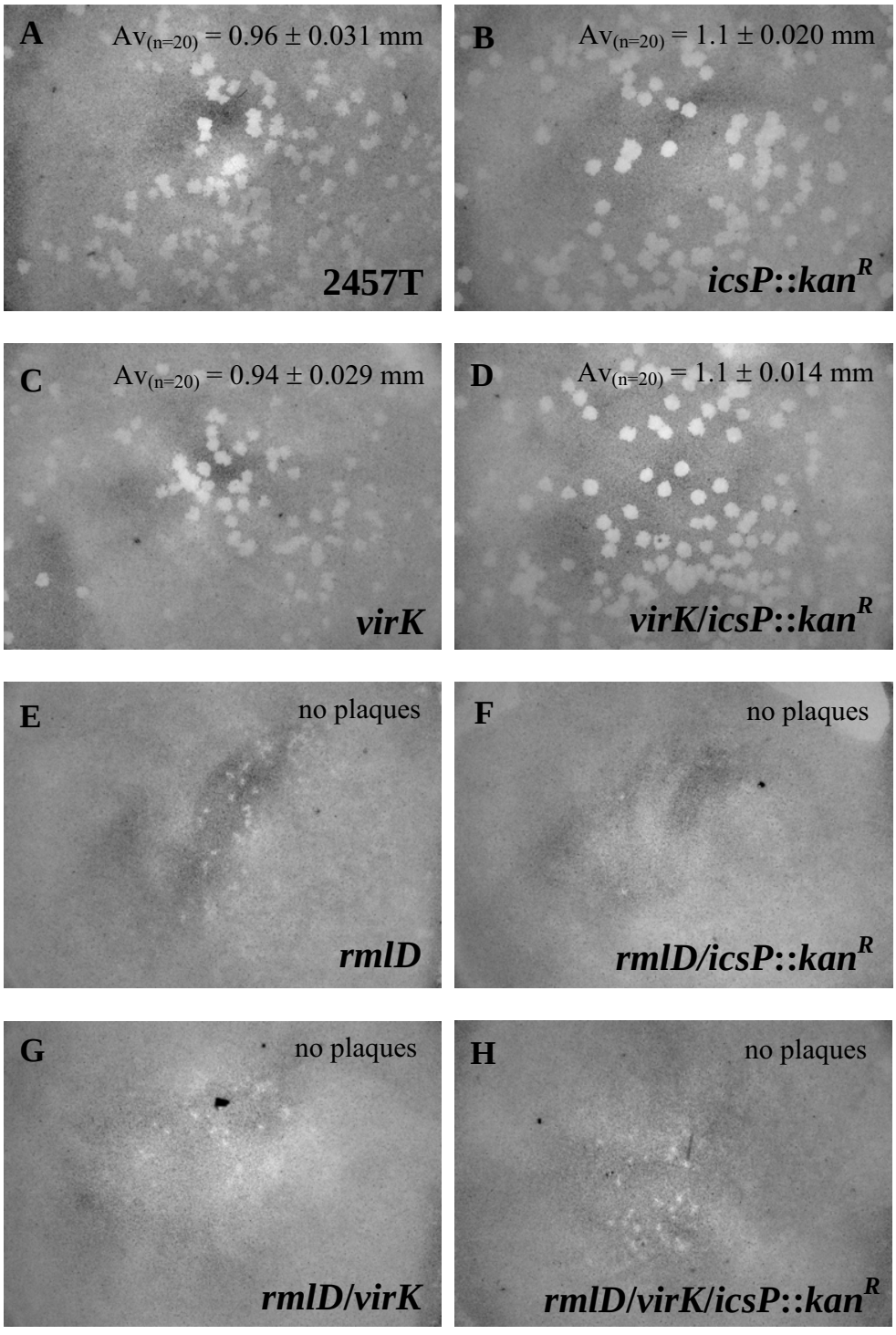
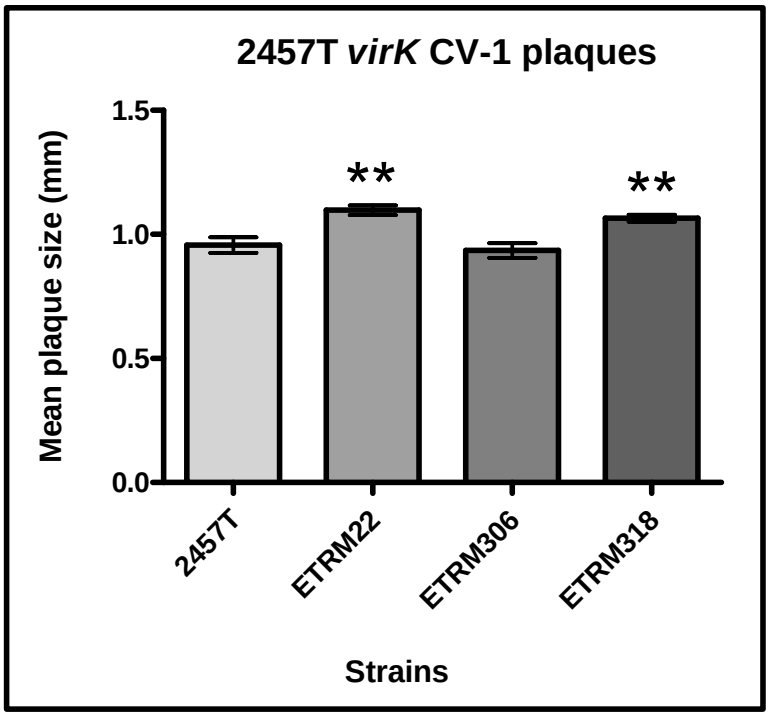

A
Av(n=20) = 0.96 ± 0.031 mm
2457T
B
Av(n=20) = 1.1 ± 0.020 mm
icsP::kanR
C
Av(n=20) = 0.94 ± 0.029 mm
virK
D
Av(n=20) = 1.1 ± 0.014 mm
virK/icsP::kanR
E
no plaques
rmlD
F
no plaques
rmlD/icsP::kanR
G
no plaques
rmlD/virK
H
no plaques
rmlD/virK/icsP::kanR

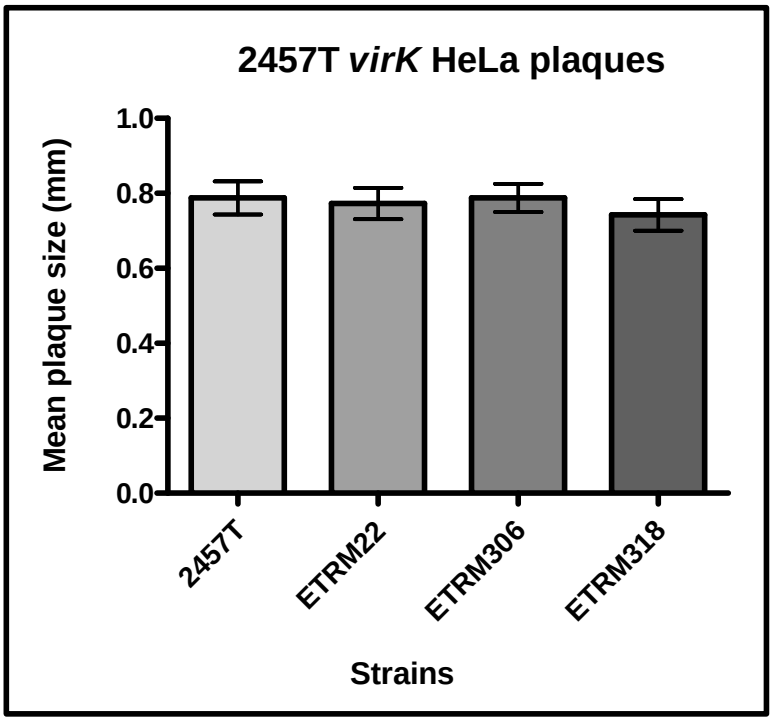
Fig. 5.7 Statistical analysis of *S. flexneri virK* mutant plaque sizes on CV-1 and HeLa cell monolayers

Confluent CV-1 and HeLa cell monolayers were infected with the following LB grown *S. flexneri* strains: 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), ETRM306 (2457T *virK*) and ETRM318 (2457T *virK*/*icsP*::*kan*^{R}). After 2 h, infected monolayers were overlaid with agarose containing gentamycin and incubated at 37°C with 5% CO_2 for either 20 h (for CV-1 cells) or 28 h (for HeLa cells), then overlaid with a second agaroseose layer containing Neutral Red and incubated for a further 8 h (for CV-1 cells) and 16 h (for HeLa cells), prior to calculating plaque sizes (refer to Section 2.12.4). The graphs represent (**A**) CV-1 cell mean plaque sizes and (**B**) HeLa cell mean plaque sizes relative to 2457T $\pm$ standard error; error bars are shown for each column. Strains which are statistically significant from 2457T are summarised above each column (whereby ** represents $P<0.01$) as determined by a two-tailed unpaired *t*-test.

A


2457T virK CV-1 plaques
Mean plaque size (mm)
1.5
1.0
0.5
0.0
**
**
2457T
ETRM22
ETRM306
ETRM318
Strains

B


2457T virK HeLa plaques
Mean plaque size (mm)
1.0
0.8
0.6
0.4
0.2
0.0
2457T
ETRM22
ETRM306
ETRM318
Strains

ETRM320 (2457T *rmlD*/*virK*) and ETRM309 (2457T *rmlD*/*virK*/*icsP*::*kan*^{R})), showed no plaque formation on CV-1 cell monolayers (Fig. 5.6 E, F, G and H). The experiment was repeated four times with consistent results.

5.4.2 Plaque formation on HeLa cell monolayers

In plaque assays with HeLa cells, ETRM22 (2457T *icsP*::*kan*^{R}), ETRM306 (2457T *virK*) and ETRM318 (2457T *virK*/*icsP*::*kan*^{R}) all formed plaques of similar size to 2457T (Fig. 5.8A, B, C and D). Further statistical analysis showed that the plaque sizes were statistically insignificant from 2457T (Fig. 5.7). Similar to Section 5.4.1, strains carrying an *rmlD* mutation (ETRM233, ETRM240, ETRM320 and ETRM309) showed no plaque formation on HeLa cell monolayers (Fig. 5.8 E, F, G and H). The experiment was repeated four times with consistent results. The results overall suggest that *virK* does not affect *S. flexneri* intercellular spread.

5.4.3 Analysis of F-actin comet tail formation

The effect of the *virK* and *rmlD* mutation on *S. flexneri* F-actin comet tail formation inside CV-1 cells was investigated. The results obtained showed that ETRM306 (2457T *virK*), ETRM318 (2457T *virK*/*icsP*::*kan*^{R}) and ETRM22 (2457T *icsP*::*kan*^{R}) formed normal F-actin comet tails with no obvious difference to those formed by 2457T (Fig. 5.9 A, B, C and D). Strains carrying an *rmlD* mutation (ETRM233, ETRM240, ETRM320 and ETRM309) formed infrequent, shorter, and distorted F-actin comet tails (Fig. 5.9 E, F, G and H), as previously reported (Van den Bosch *et al.*, 1997; Van den Bosch & Morona, 2003).

Fig. 5.8 HeLa cell plaque assays with *S. flexneri virK* and *rmlD* mutants

Confluent HeLa cell monolayers were infected with LB grown *S. flexneri* strains (as indicated below) for 2 h, overlaid with agarose containing gentamycin and incubated at 37°C with 5% CO_2 for 28 h, then overlaid with a second agaroseose layer containing Neutral Red and incubated for a further 16 h prior to taking pictures (refer to Section 2.12.4). The experiment was repeated four times with consistent results. The average size of 20 plaques ± the standard error mean for each strain is shown in the top corner of each image, and the genotype of each strain is indicated in the bottom right corner. The images are as follows:

A. HeLa infected with 2457T

B. HeLa infected with ETRM22 (2457T *icsP*::*kan*^{R})

C. HeLa infected with ETRM306 (2457T *virK*)

D. HeLa infected with ETRM318 (2457T *virK*/*icsP*::*kan*^{R})

E. HeLa infected with ETRM233 (2457T *rmlD*)

F. HeLa infected with ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R})

G. HeLa infected with ETRM320 (2457T *rmlD*/*virK*)

H. HeLa infected with ETRM309 (2457T *rmlD*/*virK*/*icsP*::*kan*^{R})

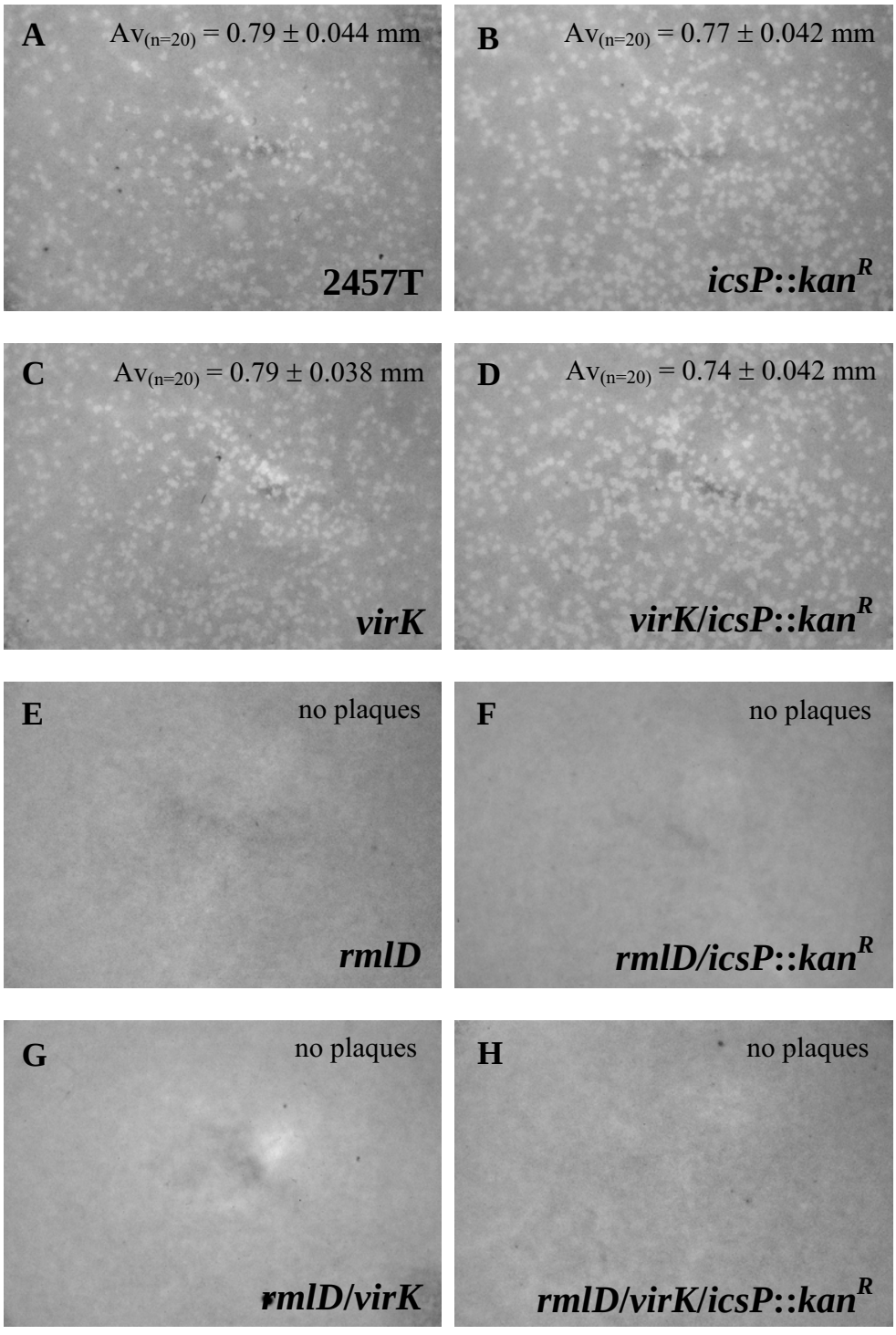

A
Av(n=20) = 0.79 ± 0.044 mm
2457T
B
Av(n=20) = 0.77 ± 0.042 mm
icsP::kanR
C
Av(n=20) = 0.79 ± 0.038 mm
virK
D
Av(n=20) = 0.74 ± 0.042 mm
virK/icsP::kanR
E
no plaques
rmlD
F
no plaques
rmlD/icsP::kanR
G
no plaques
rmlD/virK
H
no plaques
rmlD/virK/icsP::kanR

Fig. 5.9 F-actin tail formation inside CV-1 cells

Semi-confluent CV-1 cell monolayers were infected with LB grown *S. flexneri* strains (as indicated below) for 1 h, followed by centrifugation and 90 min incubation in medium containing 40 μg/ml gentamycin. Monolayers were then washed, fixed in 3.7% formaldehyde solution, incubated in DPBS + 50mM NH_4Cl, and then in PBS + 0.1% Triton X-100 prior to blocking in 10% FCS (refer to Section 2.12.5). Coverslips containing *S. flexneri* infected cells were then labelled with a rabbit group 3,4 anti-LPS antibody and a donkey anti-rabbit Alexa Fluor 594 IgG secondary antibody to stain the bacteria (red); FITC-phalloidin was used to stain F-actin (green). A typical F-actin comet tail is indicated by a white arrow for images A-D. The infrequent, shorter and distorted F-actin tails are indicated by a white arrow for images E-H if present. Scale bars represent 4 μm in size. Images are as follows:

A. CV-1 infected with 2457T

B. CV-1 infected with ETRM22 (2457T *icsP*::*kan*^{R})

C. CV-1 infected with ETRM306 (2457T *virK*)

D. CV-1 infected with ETRM318 (2457T *virK*/*icsP*::*kan*^{R})

E. CV-1 infected with ETRM233 (2457T *rmlD*)

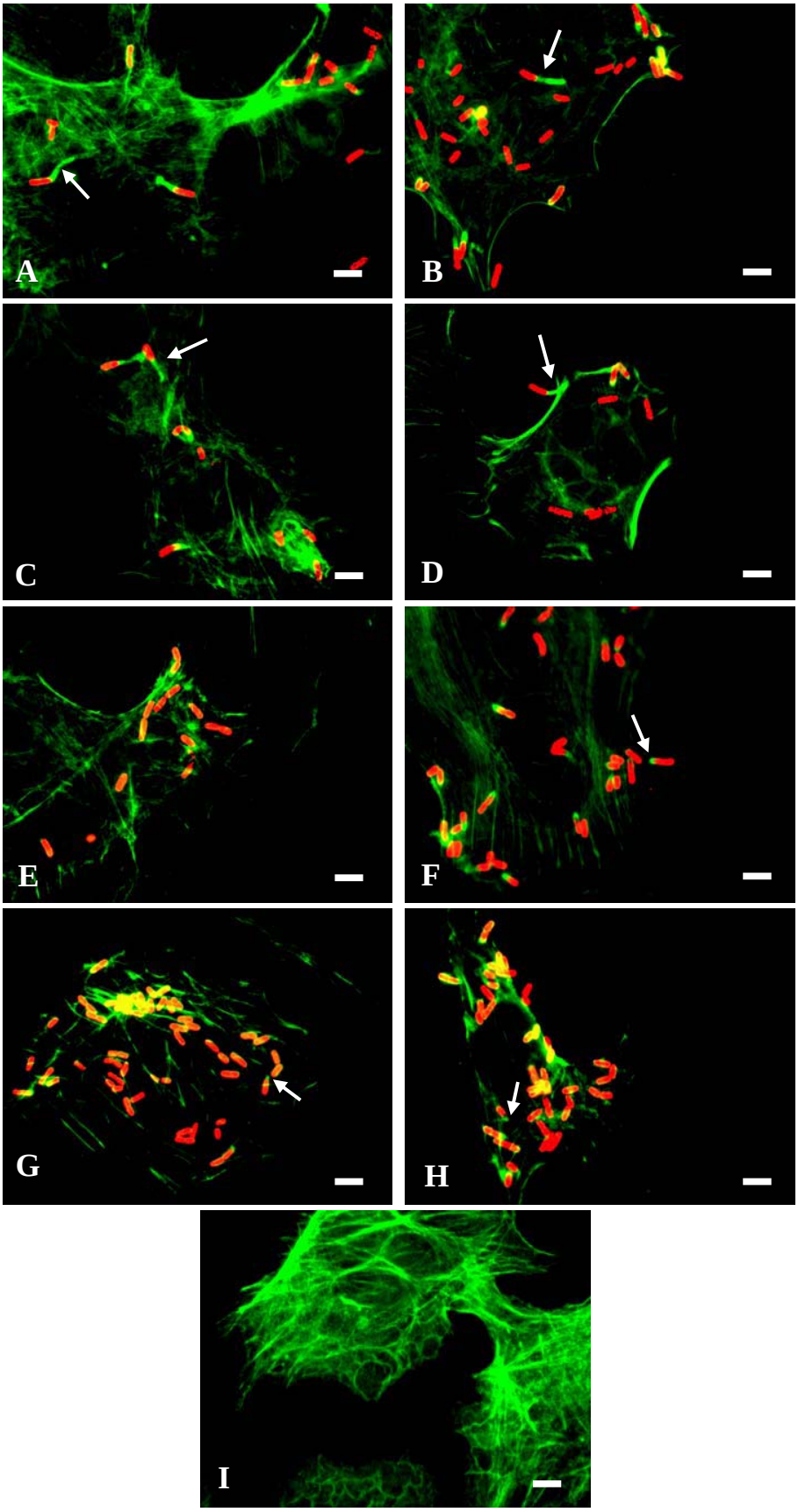
F. CV-1 infected with ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R})

G. CV-1 infected with ETRM320 (2457T *rmlD*/*virK*)

H. CV-1 infected with ETRM309 (2457T *rmlD*/*virK*/*icsP*::*kan*^{R})

I. CV-1 uninfected by RMA2519 (2457T VP^{-ve})

The control strain RMA2519 showed no invasion of bacteria into CV-1 cells as expected.


A
B
C
D
E
F
G
H
I

5.5 IcsA expression in *S. flexneri virK* and *rmlD* mutants

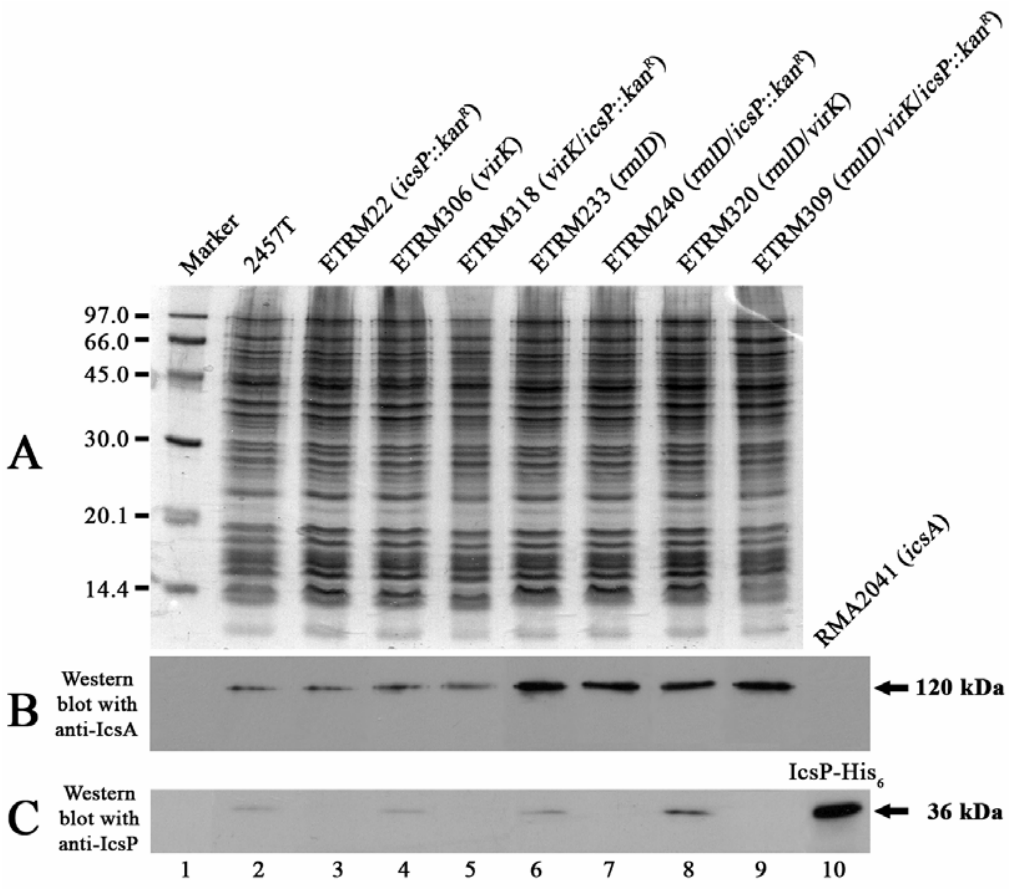
Nakata *et al*. (1992) showed by Western immunoblotting that their *Shigella virK*::Tn*10* mutant expressed decreased IcsA protein compared to the wild-type strain and that the decrease was not attributed to an effect on the levels of *icsA* transcription, as both the *virK*::Tn*10* mutant and wild-type strain expressed identical levels of *icsA* mRNA by Northern dot-blotting. As no effect in plaque formation was observed, the production of IcsA by the 2457T *virK* mutant constructed in this study was investigated. Whole cell samples of 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), ETRM306 (2457T *virK*) and ETRM318 (2457T *virK*/*icsP*::*kan*^{R}) were standardised as described in Section 2.9.3 and subjected to SDS 15% PAGE, followed by Coomassie blue staining (Fig. 5.10 A) and Western immunoblotting with anti-IcsA (Fig. 5.10 B and C). Additionally, whole cell samples for strains carrying an *rmlD* mutation (ETRM233 (2457T *rmlD*), ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}), ETRM320 (2457T *rmlD*/*virK*) and ETRM309 (2457T *rmlD*/*virK*/*icsP*::*kan*^{R})) were also standardised and prepared for the above analysis. RMA2041 (2457T Δ*icsA*::*tet*^{R}) and purified IcsP-His$_6$ protein were used as controls.

The results in Figure 5.10 unexpectedly showed that the *virK* mutant strains ETRM306 and ETRM318 expressed equivalent levels of IcsA compared to the wild-type strain 2457T under the standardising conditions used in this study (Fig. 5.10 B, lanes 4 and 5 compared to lane 2 respectively). ETRM22 (2457T *icsP*::*kan*^{R}) also showed equivalent IcsA expression levels to wild-type (Fig. 5.10 B, lane 3). In contrast, all *rmlD* mutants showed increased IcsA expression levels (Fig. 5.10 B, lanes 6, 7, 8 and 9). These results contradict previous data that *rmlD* mutants produce similar amounts of IcsA as the wild-type control (Van Den Bosch *et al*., 1997), but agree with the observed increase in IcsA level observed on the cell surface of *rmlD* mutants (Van den Bosch & Morona, 2003). RMA2041 (2457T Δ*icsA*::*tet*^{R}) was used as

Fig. 5.10 Detection of IcsA expression in *S. flexneri virK* and *rmlD* mutants by Western immunoblotting

S. flexneri strains (as indicated below) were grown in LB at 37°C to an OD_{600} of ~0.2-0.4. Standardised whole cell samples were then electrophoresed on a SDS 15% polyacrylamide gel followed by Coomassie blue staining (**A**), and Western immunoblotting with anti-IcsA (**B**) and anti-IcsP (**C**). The sizes of the low molecular weight standards (Amersham) are indicated in kDa on the left. The size of the mature 120 kDa IcsA and the 36 kDa IcsP proteins are indicated on the right. The genotype of each strain is indicated in brackets after the strain name above each lane. RMA2041 (2457T Δ*icsA*::*tet*^{R}) was used as a negative control in (**A**), and purified IcsP-His_6 protein (IcsP-His_6) was used as a positive control in (**C**). Samples in each lane represent $5x10^7$ bacteria. The strains in each lane are as follows:

1. Marker
2. 2457T
3. ETRM22 (2457T *icsP*::*kan*^{R})
4. ETRM306 (2457T *virK*)
5. ETRM318 (2457T *virK*/*icsP*::*kan*^{R})
6. ETRM233 (2457T *rmlD*)
7. ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R})
8. ETRM320 (2457T *rmlD/virK*)
9. ETRM309 (2457T *rmlD*/*virK*/*icsP*::*kan*^{R})
10. RMA2041 (**B**)/Purified IcsP-His_6 control (**C**)


Marker
2457T
ETRM22 (icsP::kan^R)
ETRM306 (virK)
ETRM318 (virK/icsP::kan^R)
ETRM233 (rmlD)
ETRM240 (rmlD/icsP::kan^R)
ETRM320 (rmlD/virK)
ETRM309 (rmlD/virK/icsP::kan^R)
97.0
66.0
45.0
30.0
20.1
14.4
A
RMA2041 (icsA)
B
Western blot with anti-IcsA
120 kDa
IcsP-His6
C
Western blot with anti-IcsP
36 kDa
1 2 3 4 5 6 7 8 9 10

a negative control and showed no IcsA protein as expected (Fig. 5.10 B, lane 10). Coomassie blue staining showed an approximately even loading of bacterial cells in each lane (Fig. 5.10 A).

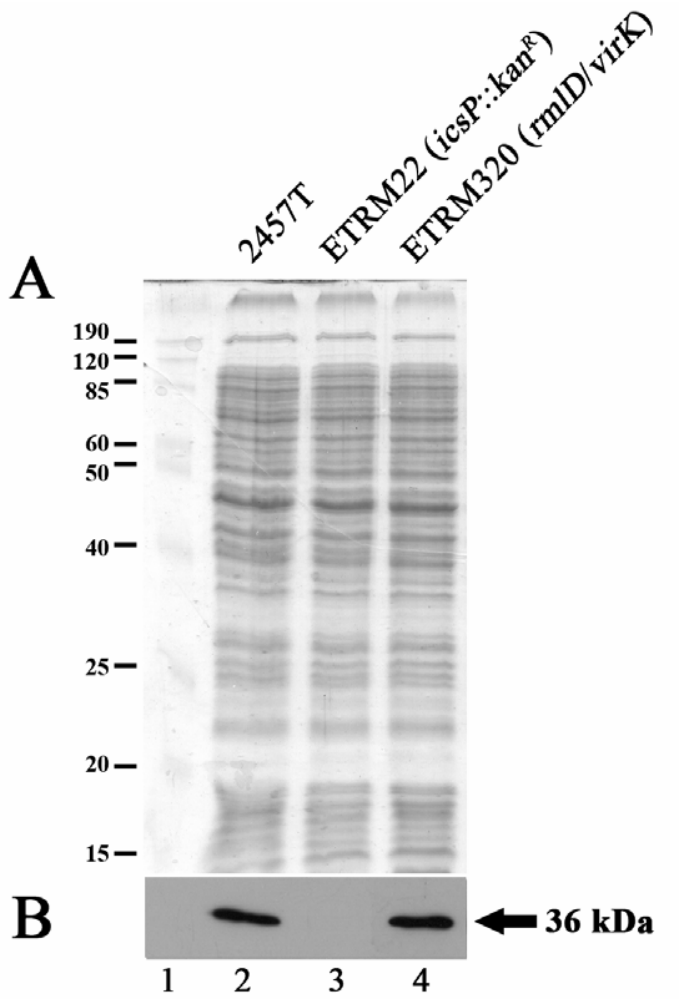
5.6 IcsP expression in *S. flexneri virK* and *rmlD* mutants

The expression of IcsP in the constructed *virK* and *rmlD* mutants was also investigated as studies from Wing *et al*. (2005) previously showed that the *virK*::Tn*10* mutant had increased IcsP expression by Western immunoblotting. The effect of the *rmlD* mutant on the expression level of IcsP has not been reported previously. The same whole cell samples from Section 5.5 above were subjected to SDS 15% PAGE and Western immunoblotting with anti-IcsP. The results in Figure 5.10C show that in contrast to data from Wing *et al*. (2005), equivalent levels of IcsP expression was observed for ETRM306 (2457T *virK*) compared to 2457T (Fig. 5.10 C, lanes 2 and 4). Similar levels of IcsP expression were also observed for ETRM233 (2457T *rmlD*) (Fig. 5.10 C, lane 6). Strains carrying a mutation in *icsP* (ETRM22 (2457T *icsP*::*kan*^{R}), ETRM318 (2457T *virK*/*icsP*::*kan*^{R}), ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}), and ETRM309 (2457T *rmlD*/*virK*/*icsP*::*kan*^{R})) showed no expression of IcsP protein by Western immunoblotting with anti-IcsP as expected (Fig. 5.10 C, lanes 3, 5, 7 and 9 respectively). ETRM320 (2457T *rmlD*/*virK*) appeared to show a slight increase in IcsP expression level compared to 2457T (Fig. 5.10 C, lanes 8), but in a repeated experiment this strain also produced equivalent levels of IcsP expression compared to 2457T (Fig. 5.11, lanes 2 and 4). Note that the IcsP protein band appears more intense in Fig. 5.11 than in Fig. 5.10 due to recent optimisation of the secondary antibody dilution used for Western immunoblotting and a suspected difference in the quality of the batch of Western blot substrate which was used to develop the western blot for Fig. 5.10 (hence, resulting in the observed weak IcsP signal).

Fig. 5.11 IcsP expression in *S. flexneri rmlD/virK* mutant by Western immunoblotting

S. flexneri strains 2457T, ETRM22 (2457T *icsP*::*kan*^{R}) and ETRM320 (2457T *rmlD/virK*) were grown in LB at 37°C to an OD_{600} of ~0.2-0.4 and whole cell samples were electrophoresed on a SDS 15% polyacrylamide gel followed by Coomassie blue staining (**A**), and Western immunoblotting with anti-IcsP (**B**). A different batch of chemiluminescence substrate was used in this experiment. The size of the 36 kDa IcsP protein is indicated on the right. The migration positions of the BenchMark Pre-Stained marker (M) (Invitrogen) are indicated on the left. Samples in each lane represent $5x10^7$ bacteria. The strains in each lane are as follows:

1. BenchMark Pre-stained marker
2. 2457T
3. ETRM22 (2457T *icsP*::*kan*^{R})
4. ETRM320 (2457T *rmlD*/*virK*)


2457T
ETRM22 (icsP::kanR)
ETRM320 (rmlD/virK)
A
190
120
85
60
50
40
25
20
15
B
36 kDa
1
2
3
4

5.7 Analysis of IcsP activity in *S. flexneri virK* and *rmlD* mutants

To investigate whether IcsP activity was affected in the *virK* and *rmlD* mutants, cell associated and soluble IcsA samples were prepared (Section 2.9.9) to determine whether the ability of IcsP to cleave IcsA into culture supernatants was affected. Samples were subjected to SDS 15% PAGE and Western immunoblotting with anti-IcsA. Figure 5.12 shows the presence of the full length 120 kDa IcsA protein in the whole cell samples of 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), ETRM233 (2457T *rmlD*), ETRM306 (2457T *virK*) and ETRM320 (2457T *rmlD*/*virK*) as expected (Fig. 5.12, lanes 1, 3, 5, 7 and 9). The presence of the ~95 kDa cleaved form of IcsA was observed in the supernatant sample of all samples (2457T, ETRM233, ETRM306 and ETRM320) carrying a functional *icsP* gene (regardless of the presence of a *virK* or *rmlD* mutation) (Fig. 5.12, lanes 2, 6, 8 and 10). IcsA fragments less than 95 kDa in size are degraded IcsA products. ETRM22 (2457T *icsP*) showed no cleaved form of IcsA in the supernantant sample as expected (Fig. 5.12, lane 4). The results suggest that *virK* and LPS structure have no effect on IcsP activity.

5.8 Surface distribution of IcsA in *S. flexneri virK* and *rmlD* mutants

To further characterise the *virK* and *rmlD* mutants constructed in this study, the distribution of IcsA on the surface of these mutants was investigated. As expected, based on the results obtained above, ETRM306 (2457T *virK*) displayed polar localisation of IcsA on the cell surface similar to 2457T (Fig. 5.13 A and C), while ETRM318 (2457T *virK*/*icsP*::*kan*^{R}) displayed IcsA on polar and lateral regions similar to that seen for ETRM22 (2457T *icsP*::*kan*^{R}) (Fig. 5.12 B and D). All strains carrying a mutation in *rmlD* i.e. ETRM233 (2457T *rmlD*), ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}), ETRM309 (2457T

Fig. 5.12 IcsA cleavage by *S. flexneri virK* and *rmlD* mutants

S. flexneri strains (as indicated below) were grown in LB at 37°C to an OD_{600} of ~0.2-0.4. For the detection of cleaved IcsA in culture supernatants, whole cell protein samples were obtained from the pellet of 1 ml culture and supernatant protein samples were obtained from 50 ml culture supernatants treated with 5% (v/v) TCA (Section 2.9.9). Samples were electrophoresed on SDS 15% polyacrylamide gels prior to Western immunoblotting with anti-IcsA. The size of the mature IcsA protein (120 kDa) and cleaved IcsA fragment (~95 kDa) is indicated on the right. Bands smaller than 95 kDa are degraded IcsA fragments and are not labelled. Lanes containing whole cell samples represent $5x10^7$ bacteria. Supernatant protein samples were prepared from 50 ml volumes of culture. Lanes are as follows:

1. 2457T whole cell
2. 2457T supernatant
3. ETRM22 (2457T *icsP*::*kan*^{R}) whole cell
4. ETRM22 (2457T *icsP*::*kan*^{R}) supernatant
5. ETRM233 (2457T *rmlD*) whole cell
6. ETRM233 (2457T *rmlD*) supernatant
7. ETRM306 (2457T *virK*) whole cell
8. ETRM306 (2457T *virK*) supernatant
9. ETRM320 (2457T *rmlD*/*virK*) whole cell
10. ETRM320 (2457T *rmlD*/*virK*) supernatant

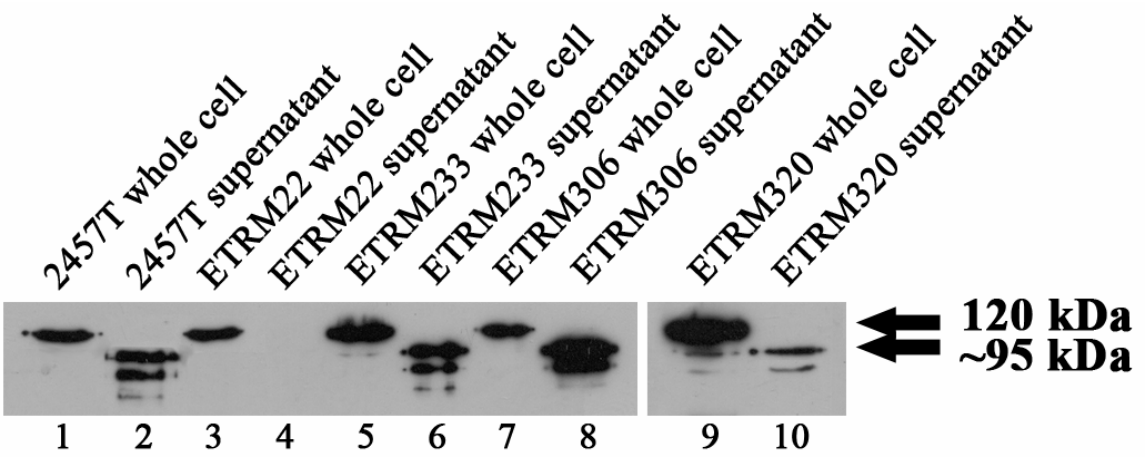

2457T whole cell
2457T supernatant
ETRM22 whole cell
ETRM22 supernatant
ETRM233 whole cell
ETRM233 supernatant
ETRM306 whole cell
ETRM306 supernatant
ETRM320 whole cell
ETRM320 supernatant
120 kDa
~95 kDa
1 2 3 4 5 6 7 8 9 10

Fig. 5.13 IF detection of IcsA localisation in *S. flexneri virK* and *rmlD* mutants

Strains were grown in LB at 37°C to an OD_{600} of ~0.2-0.4 and formalin fixed. Cell surface IcsA was detected by indirect IF staining with a rabbit anti-IcsA antibody and a donkey anti-mouse Alexa Fluor 488 IgG secondary antibody. Bacteria were observed by epi-fluorescence microscopy. Within each IF image an enlargement of a typical bacterium is shown. Scale bars represent 1 μm in size. The images are as follows:

A. *S. flexneri* 2a 2457T

B. ETRM22 (2457T *icsP*::*kan*^{R})

C. ETRM306 (2457T *virK*)

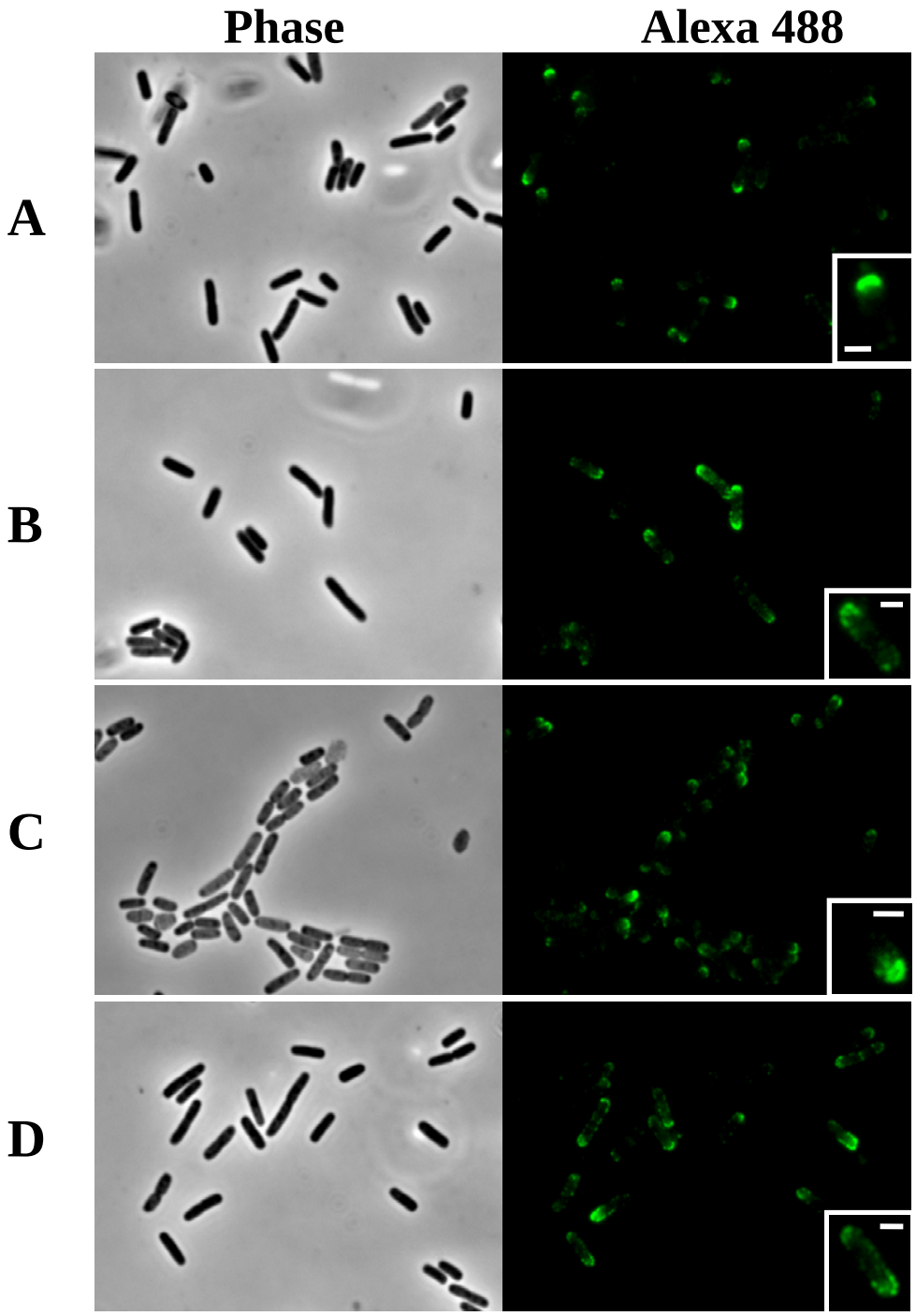
D. ETRM318 (2457T *virK*/*icsP*::*kan*^{R})

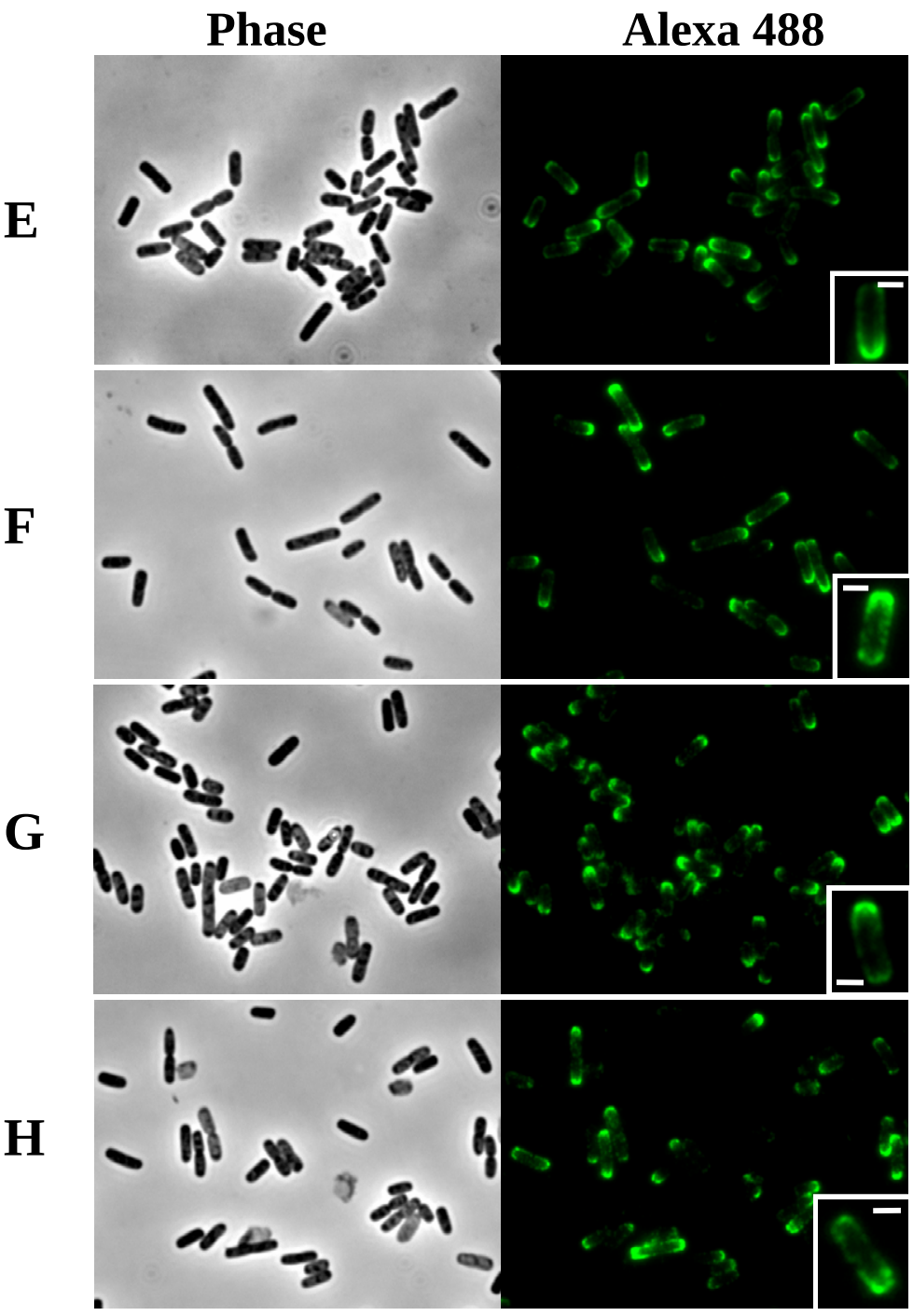
E. ETRM233 (2457T *rmlD*)

F. ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R})

G. ETRM320 (2457T *rmlD*/*virK* mutant)

H. ETRM309 (2457T *rmlD*/*virK*/*icsP*::*kan*^{R})


Phase
Alexa 488
A
B
C
D


Phase
Alexa 488
E
F
G
H

rmlD/*virK*/*icsP*::*kan*^{R}) displayed IcsA on polar and lateral regions of the cell surface as has been previously described for mutants carrying a mutation in *rmlD* (Van den Bosch & Morona, 2003) (Fig. 5.13 E, F, G and H).

5.9 Summary

This chapter has compared a 2457T *virK* mutant to wild-type 2457T, and examined the effect of *virK* and *rmlD* on IcsP and IcsA expression. The 2457T *virK* mutant (ETRM306) constructed in this study was found to form plaques equivalent in size to 2457T plaques on cell monolayers, express equivalent levels of IcsA and IcsP protein compared to 2457T in samples standardised by Western immunoblotting, and have no affect on IcsP's ability to cleave IcsA into culture supernatants. Additional F-actin comet tail staining of bacteria inside CV-1 cells showed that F-actin tail formation by the 2457T *virK* mutant was also similar to 2457T. These results show that *virK* does not affect *Shigella* intercellular spread as previously reported by Nakata *et al*. (1992).

A 2457T *rmlD* mutant (ETRM233) was also investigated and found to form no plaques on cell monolayers and displayed infrequent, short and distorted F-actin tails inside cells when compared to 2457T, as previously reported by other authors (Van den Bosch *et al.*, 1997; Van den Bosch & Morona, 2003). IcsP expression levels in the *rmlD* mutant were observed to be equivalent to 2457T, however IcsA expression was slightly increased compared to 2457T and this agrees with the increase levels of IcsA observed on polar and lateral regions of the cell surface of *rmlD* mutants (Van den Bosch *et al.*, 1997; Van den Bosch & Morona, 2003). IcsP activity also appeared to be unaffected in *rmlD* mutants (refer to Section 5.7).

In conclusion, the results in this chapter have shown that *virK* is not essential for *Shigella* intercellular spread. The difference in plaque sizes observed by Nakata *et al*. (1992) may be due to a polar effect exhibited by *virK*::Tn*10* upon adjacent genes in the *virK* operon, or a difference in the *S. flexneri* YSH6000T strain used compared to this study. There was also no effect on IcsP expression or activity observed for the *virK* mutant (ETRM306) constructed in this thesis, suggesting that *virK* may not be important in regulating IcsP as was suggested by Wing *et al*. (2005). The LPS profile of ETRM306 was also found to be no different to wild-type 2457T, suggesting that *virK* has no detectable effect on the structure of LPS. In contrast, *rmlD* was shown to affect *Shigella* intercellular spreading as previously reported by other authors (Charles *et al.*, 2001; Sandlin *et al.*, 1995; Sandlin *et al.*, 1996; Steinhauer *et al.*, 1999; Van den Bosch *et al.*, 1997)), and appeared to affect IcsA expression, but not IcsP expression or activity under the conditions used in this study.

Chapter 6 – Alternative substrates for IcsP

6.1 Introduction

Members of the Omptin family appear to be multifunctional (Section 1.6). OmpT for example, has been shown to cleave the antimicrobial protein protamine (Stumpe *et al.*, 1998), activate human plasminogen (Leytus *et al.*, 1981), degrade recombinant proteins (Goldberg *et al.*, 1997; Grodberg & Dunn, 1988; Laird *et al.*, 2004; Yam *et al.*, 2001), as well as cleave colicins as an important defence mechanism against other *E. coli* strains (Masi *et al.*, 2007). The Omptin proteases PgtE and Pla have been shown to cleave components of complement (Ramu *et al.*, 2007; Sodeinde *et al.*, 1988), activate plasminogen (Plg) and inactivate α_2-antiplasmin (α_2AP) (Kukkonen *et al.*, 2004). The activity of IcsP on these substrates has not been investigated.

In this chapter, alternative substrates for IcsP were investigated. Plg and α_2AP cleavage assays were performed on smooth and rough LPS *S. flexneri* strains expressing IcsP, and preliminary bactericidal assays using complement and protamine were also conducted. Finally, colicin sensitivity assays were carried out to investigate the ability of strains expressing IcsP to inactivate colicins.

6.2 IcsP activity against plasminogen and α_2AP

Plg is a proenzyme which circulates in plasma and is converted to the active serine protease plasmin by proteolytic activation. Plasmin in turn breaks down fibrin clots and helps to activate the classical complement pathway in humans. The serine protease α_2AP is a major

human plasmin inhibitor. Both Omptin proteases Pla and PgtE are known to activate Plg and inactivate α_2AP (Kukkonen *et al.*, 2001; Lahteenmaki *et al.*, 2005).

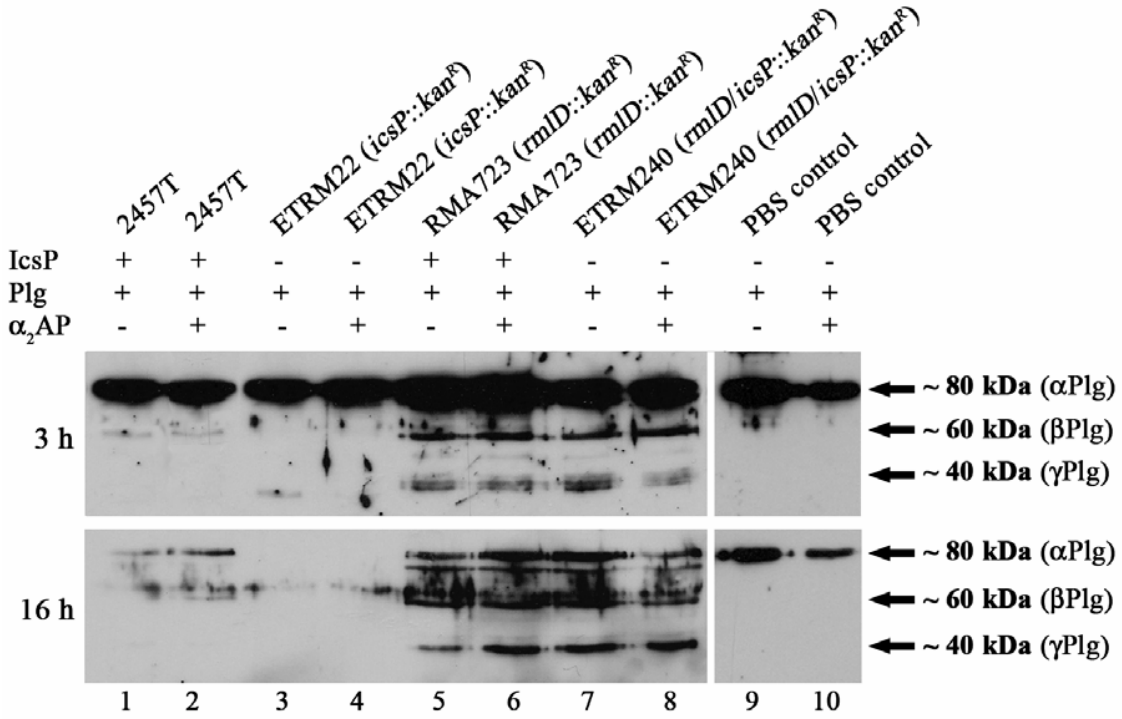
6.2.1 Analysis of IcsP activity against plasminogen

To investigate IcsP's activity against Plg, Plg cleavage assays were performed with and without α_2AP as described in Section 2.13. Incubation with α_2AP protein was included as a control to determine whether Plg could be cleaved by *S. flexneri* in the presence of α_2AP inhibition. The results in Figure 6.1 showed that Plg cleavage by *S. flexneri* strains 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), RMA723 (2457T *rmlD*::*kan*^{R}) and ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}) appeared to be unaffected by the presence or absence of α_2AP (Fig. 6.1, lanes 1-8). For smooth LPS strains 2457T and ETRM22, the mature ~80 kDa Plg protein appeared to be weakly cleaved by 2457T to give a ~60 kDa βPlg product (Fig. 6.1, lanes 1 and 2), but not by ETRM22 (Fig. 6.1, lanes 3 and 4). These results suggested that IcsP might have weak activity against Plg. However, analysis of the PBS control sample incubated with only Plg showed that a band of ~60 kDa in size was also found to be weakly present (Fig. 6.1, lane 9), and suggests that the observed activity against Plg by 2457T is not due to IcsP but due to an effect of incubation in PBS. When rough LPS strains RMA723 (2457T *rmlD*::*kan*^{R}) and ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}) were analysed, Plg was cleaved into βPlg and γPlg fragments by both strains (Fig. 6.1, lanes 5, 6, 7 and 8), showing that IcsP did not contribute to the observed activity against Plg. At 16 h incubation with Plg, similar results were obtained (as described above) for all *S. flexneri* strains (Fig. 6.1, 16 h western blot), however by this stage reduced amounts of Plg protein were observed in all strains (including the PBS control samples), suggesting that some protein degradation had occurred over the prolonged incubation period.

Fig. 6.1 Detection of Plg cleavage by *S. flexneri* strains

S. flexneri strains 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), RMA723 (2457T *rmlD*::*kan*^{R}) and ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}) were grown in LB at 37°C to an OD_{600} of 0.8 and incubated with 0.2 μg/ml Plg (with and without 0.4 μg/ml α_2AP) at 37°C (Section 2.13). Samples taken at 3 h and 16 h were centrifuged (to remove bacteria), and supernatant samples were solubilised and electrophoresed on a SDS 15% polyacrylamide gel followed by Western immunoblotting with anti-Plg. The genotype of each strain incubated with (+) Plg and with (+)/without (-) α_2AP is indicated in brackets after the strain name above each lane. PBS buffer incubated with Plg protein (with and without α_2AP) were used as positive controls. The presence of the mature αPlg (~80 kDa), and cleaved βPlg (~60 kDa) and γPlg (~40 kDa), forms of Plg are indicated on the left. The strains in each lane are as follows:

1. 2457T (Plg)
2. 2457T (Plg + α_2AP)
3. ETRM22 (*icsP*::*kan*^{R}) incubated with Plg
4. ETRM22 (*icsP*::*kan*^{R}) incubated with Plg + α_2AP
5. RMA723 (*mlD*::*kan*^{R}) incubated with Plg
6. RMA723 (*rmlD*::*kan*^{R}) incubated with Plg + α_2AP
7. ETRM240 (*rmlD*/*icsP*::*kan*^{R}) incubated with Plg
8. ETRM240 (*rmlD*/*icsP*::*kan*^{R}) incubated with Plg + α_2AP
9. PBS incubated with Plg
10. PBS incubated with Plg + α_2AP


2457T
2457T
ETRM22 (icsP::kanR)
ETRM22 (icsP::kanR)
RMA723 (rmlD::kanR)
RMA723 (rmlD::kanR)
ETRM240 (rmlD/icsP::kanR)
ETRM240 (rmlD/icsP::kanR)
PBS control
PBS control
IcsP + + - - + + - - - -
Plg + + + + + + + + + +
α2AP - + - + - + - + - +
3 h
16 h
~ 80 kDa (αPlg)
~ 60 kDa (βPlg)
~ 40 kDa (γPlg)
~ 80 kDa (αPlg)
~ 60 kDa (βPlg)
~ 40 kDa (γPlg)
1 2 3 4 5 6 7 8 9 10

6.2.2 Analysis of IcsP activity against α_2AP

To investigate IcsP activity against α_2AP, strains 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), RMA723 (2457T *rmlD*::*kan*^{R}) and ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}) were incubated with α_2AP for 3 h as described in Section 2.14. The results in Figure 6.2 show that IcsP is unlikely to have activity against α_2AP, as the mature ~70 kDa α_2AP protein appeared to be cleaved into the ~50 kDa fragment by all strains with and without IcsP (Fig. 6.2, lanes 1, 3, 5 and 7). The PBS buffer control sample (without bacteria) (Fig. 6.2, lane 9), and strains incubated without α_2AP protein (Fig. 6.2, lane 2, 4, 6 and 8) showed no cleaved product as expected. Samples of strains incubated with α_2AP were also taken after 16 h, but prolonged incubation appeared to completely degrade the α_2AP protein in all samples (data not shown). The results suggest IcsP does not have activity against α_2AP.

6.3 Antimicrobial assays

6.3.1 Analysis of IcsP activity against complement

Complement is a component of the innate immune system consisting of a series of proteins which circulate inactively in the serum. Activation of these proteins enables them to damage the membranes of pathogenic organisms, and destroy pathogens or facilitate their clearance from the host. The Omptin proteases Pla of *Y. pestis* and PgtE of *S. enterica* are known to cleave complement proteins (Ramu *et al.*, 2007; Sodeinde *et al.*, 1992). The activity of IcsP against complement was hence investigated by incubating strains in guinea pig and human serum (10% (v/v) final concentration) as described in Section 2.14.1.1. Viable counts were determined at 30 min intervals. The results obtained from incubation with guinea pig serum are summarised in Figure 6.3 A. Rough LPS strains RMA723 (2457T *rmlD*::*kan*^{R}) and ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}) were sensitive to the activity of complement with 0%

Fig. 6.2 Detection of α_2AP cleavage by *S. flexneri* strains

S. flexneri strains 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), RMA723 (2457T *rmlD*::*kan*^{R}) and ETRM240 (2457T *rmlD*/*icsP*) were grown in LB at 37°C to an OD_{600} of 0.8 and incubated with and without 0.4 μg/ml α_2AP for 3 h at 37°C (Section 2.14). Mixtures were then centrifuged (to remove bacteria), and supernatant samples were solubilised and electrophoresed on a SDS 15% polyacrylamide gel followed by Western immunoblotting with anti-α_2AP. The genotype of each strain incubated with (+) and without (-) α_2AP is indicated in brackets after the strain name above each lane. PBS buffer incubated with α_2AP protein only was used as a positive control. The presence of the mature (70 kDa) and cleaved (50 kDa) forms of α_2AP are indicated on the right. The strains in each lane are as follows:

1. 2457T incubated with α_2AP
2. 2457T incubated with PBS
3. ETRM22 (*icsP*::*kan*^{R}) incubated with α_2AP
4. ETRM22 (*icsP*::*kan*^{R}) incubated with PBS
5. RMA723 (*rmlD*::*kan*^{R}) incubated with α_2AP
6. RMA723 (*rmlD*::*kan*^{R}) incubated with PBS
7. ETRM240 (*rmlD*/*icsP*::*kan*^{R}) incubated with α_2AP
8. ETRM240 (*rmlD*/*icsP*::*kan*^{R}) incubated with PBS
9. PBS incubated with α_2AP

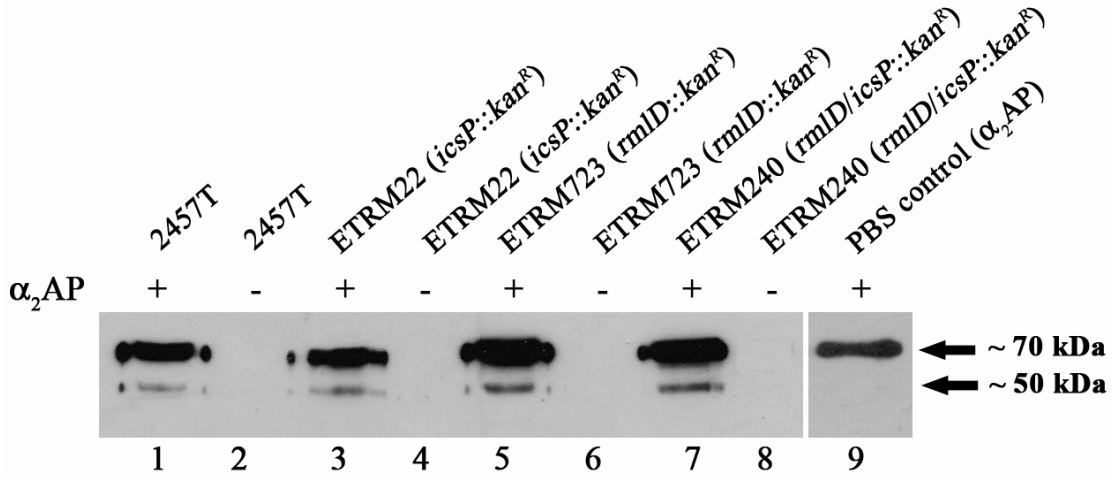
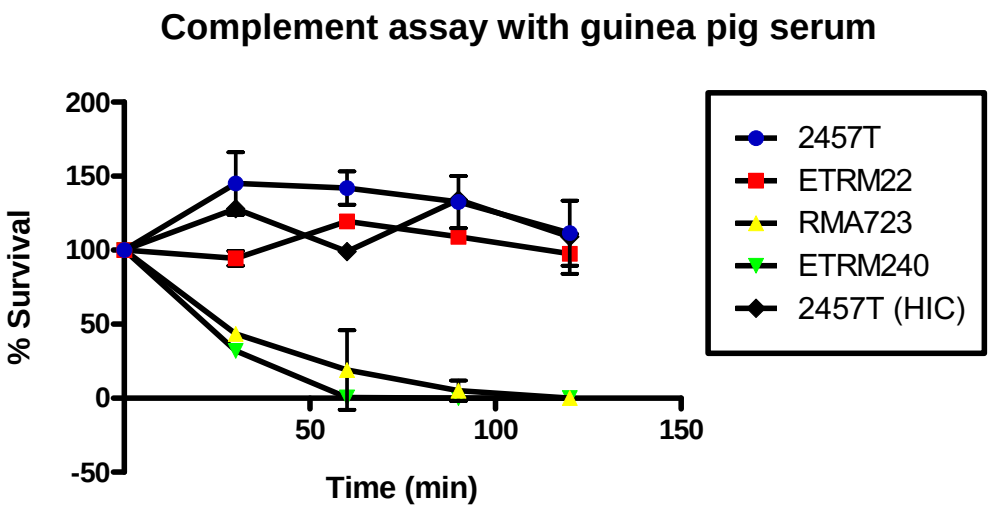

2457T
2457T
ETRM22 (icsP::kan^R)
ETRM22 (icsP::kan^R)
ETRM723 (rmlD::kan^R)
ETRM723 (rmlD::kan^R)
ETRM240 (rmlD/icsP::kan^R)
ETRM240 (rmlD/icsP::kan^R)
PBS control (α2AP)
α2AP
+ - + - + - + - +
~ 70 kDa
~ 50 kDa
1 2 3 4 5 6 7 8 9

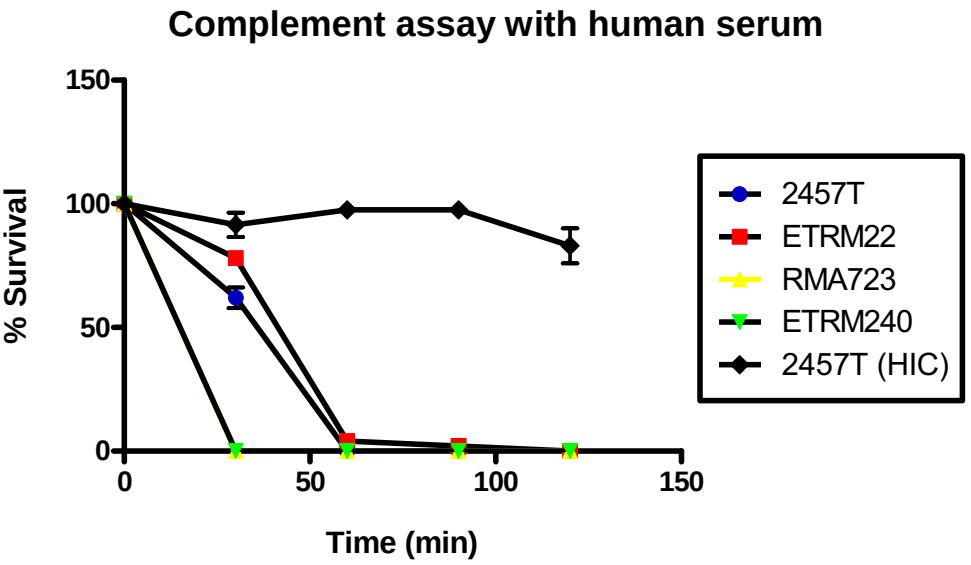
Fig. 6.3 Survival of *S. flexneri* strains in 10% serum

S. flexneri strains 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), RMA723 (2457T *rmlD*::*kan*^{R}) and ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}) were grown in LB at 37°C with aeration to an OD_{600} of 0.8. Strains were then serially diluted to $1x10^6$ cells/ml in PBS (containing Cml) and supplemented with 2 mM $MgCl_2$. Guinea pig serum (**A**) and human serum (**B**) were added to 10% and mixtures incubated at 37°C for 2 h. Samples were taken at 30 min intervals for viable counts (refer to Section 2.14.1.1). Strain 2457T was incubated with heat inactivated complement (HIC) as a control. Data points represent percentage survival (mean $\pm$ standard deviation, n=2 assays).

A


Complement assay with guinea pig serum
% Survival
200
150
100
50
0
-50
50
100
150
Time (min)
2457T
ETRM22
RMA723
ETRM240
2457T (HIC)

B


Complement assay with human serum
% Survival
150
100
50
0
0
50
100
150
Time (min)
2457T
ETRM22
RMA723
ETRM240
2457T (HIC)

survival at 120 min, while smooth LPS strains 2457T and ETRM22 (2457T *icsP*::*kan*^{R}) showed resistance to the activity of complement with ≥90% survival throughout the course of the experiment. 2457T incubated with heat inactivated complement (HIC) only was used as a control and showed >90% survival throughout the course of the experiment as expected. The experiment was performed twice with consistent results. Overall, the presence or absence of IcsP in strains did not appear to have an effect on the strain's sensitivity to guinea pig serum complement under the experimental conditions used.

Incubation with human serum showed a greater bactericidal effect on the strains than incubation with guinea pig serum. The results summarised in Figure 6.3 B showed that all *S. flexneri* strains were susceptible to human complement, with 0% survival of smooth LPS *S. flexneri* strains observed by 120 min, and complete killing (0% survival) of rough LPS strains by 30 min. 2457T incubated with HIC was used as a control and showed >90% survival throughout the course of the experiment as expected. The experiment was performed twice with consistent results. The results suggest that *S. flexneri* strains are sensitive to human complement under the conditions used in this study and that IcsP appears to have no effect on the *S. flexneri*'s susceptibility to complement.

6.3.2 Analysis of IcsP activity against protamine

IcsP shares most sequence similarity to the Omptin proteases OmpT and OmpP. OmpT in particular, has been shown to be associated with complicated urinary tract diseases by cleaving protamine, a highly basic antimicrobial peptide that is secreted by epithelial cells of the urinary tract (Stumpe *et al.*, 1998). To investigate whether IcsP had activity against protamine, strains 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), RMA723 (2457T *rmlD*::*kan*^{R}) and ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}) were incubated with protamine (10 µg/ml final

concentration) as described in Section 2.14.1.2. Viable counts were determined at 30 min intervals and the results obtained are shown in Figure 6.4. The experiment was performed twice with consistent results. All strains appeared to show sensitivity to protamine with >75% killing by 2 h. Note that the concentration previously used by Stumpe *et al*. (1998) to test OmpT activity against protamine was 100 mg/l (which is equivalent to 100 μg/ml). The low concentration of protamine used in this study was intended to first determine whether *S. flexneri* strains were sensitive to protamine in general. Overall, the results suggest that *S. flexneri* are sensitive and that IcsP does not have activity against protamine in this assay.

6.4 Analysis of IcsP activity against colicins

Colicins are plasmid-encoded antibacterial toxins produced by *E. coli* that kill other *E. coli* cells (Masi *et al.*, 2007). OmpT has been shown to cleave a number of colicins which include colicin E1 and E2 (Cavard & Lazdunski, 1990). To determine if IcsP was active against colicins, the double layer test method of colicin sensitivity assay was performed as described in Section 2.15 on a number of *S. flexneri* strains expressing IcsP and *E. coli* K-12 strains expressing arabinose induced $IcsP^{HA}$ (refer to Table 6.1 and 6.2 for list of strains tested). Rough LPS *S. flexneri* strain ETRM240 (2457T *rmlD*/*icsP*::kan^R) was electroporated with pBAD30::$icsP^{HA}$ and pBAD30 to give ETRM243 (2457T *rmlD*/*icsP*::kan^R [pBAD30::$icsP^{HA}$]) and ETRM245 (2457T *rmlD*/*icsP*::kan^R [pBAD30]) respectively, and both were included in the assay as rough LPS control strains for smooth LPS strains ETRM117 (2457T *icsP*::kan^R [pBAD30::$icsP^{HA}$]) and ETRM118 (2457T *icsP*::kan^R [pBAD30]). Strains were cross-streaked against the colicin E1-producing *E. coli* strain K53 (Table 6.1) and the colicin E2-producing *S. sonnei* strain P9 (Table 6.2).

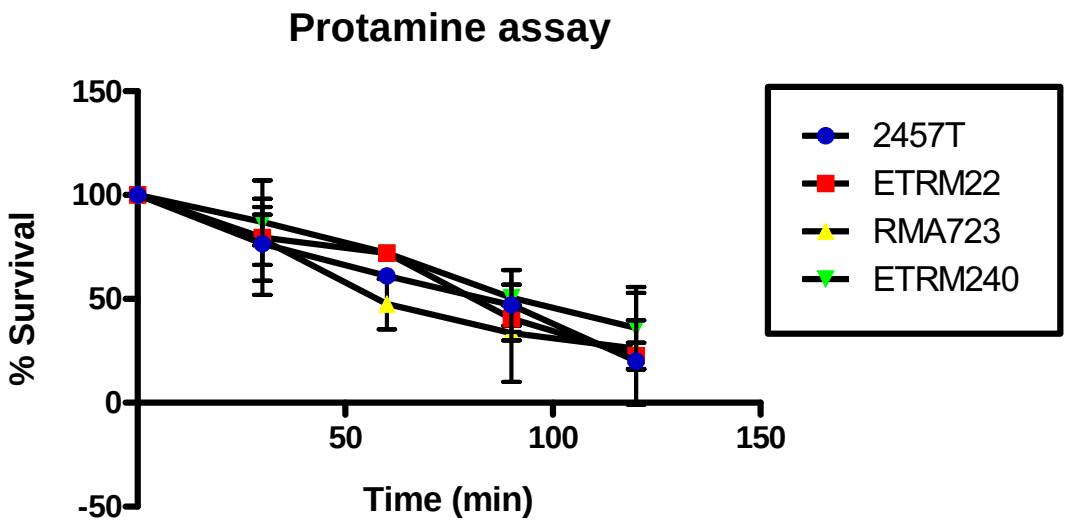


Fig. 6.4 Survival of *S. flexneri* strains against protamine

S. flexneri strains 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), RMA723 (2457T *rmlD*::*kan*^{R}) and ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}) were grown in LB at 37°C with aeration to an OD_{600} of 0.8. Strains were then serially diluted to $1x10^6$ cells/ml in MOPS buffer and protamine was added to 10 µg/ml before mixtures were incubated at 37°C for 2 h (refer to Section 2.14.1.2). Samples were taken at 30 min intervals for viable counts. Data points represent percentage survival (mean ± standard deviation, n=2 assays).

Table 6.1 Colicin E1 sensitivity assay

Cross-streak strains	Protein expressed	LPS	Growth Inhibition (mm)	Relative sensitivity
S. flexneri				
WT	IcsP	smooth	0	resistant
M90T	IcsP	smooth	0	resistant
RMA723	IcsP	rough	1	weak inhibition
ETRM22	-	smooth	0	resistant
ETRM29	-	smooth	0	resistant
ETRM31	IcsP	smooth	0	resistant
ETRM108	IcsP	smooth	0	resistant
ETRM112	IcsP	smooth	0	resistant
ETRM114	-	smooth	0	resistant
ETRM117	$IcsP^{HA}$	smooth	0	resistant
ETRM118	-	smooth	0	resistant
ETRM240	-	rough	1	weak inhibition
ETRM243	$IcsP^{HA}$	rough	1	weak inhibition
ETRM245	-	rough	1	weak inhibition
E. coli K-12				
UT5600	-	rough	3	sensitive
ETRM156	$IcsP^{HA}$	rough	3	sensitive
ETRM158	-	rough	3	sensitive
ETRM168	$IcsP^{HA}$	smooth	0	resistant
ETRM170	-	smooth	0	resistant

Table 6.2 Colicin E2 sensitivity assay

Cross-streak strains	Protein expressed	LPS	Growth Inhibition (mm)	Relative sensitivity
S. flexneri				
WT	IcsP	smooth	1	weak inhibition
M90T	IcsP	smooth	1	weak inhibition
RMA723	IcsP	rough	6	sensitive
ETRM22	-	smooth	1	weak inhibition
ETRM29	-	smooth	1	weak inhibition
ETRM31	IcsP	smooth	1	weak inhibition
ETRM108	IcsP	smooth	1	weak inhibition
ETRM112	IcsP	smooth	1	weak inhibition
ETRM114	-	smooth	1	weak inhibition
ETRM117	$IcsP^{HA}$	smooth	1	weak inhibition
ETRM118	-	smooth	1	weak inhibition
ETRM240	-	rough	6	sensitive
ETRM243	$IcsP^{HA}$	rough	6	sensitive
ETRM245	-	rough	6	sensitive
E. coli K-12				
UT5600	-	rough	5	sensitive
ETRM156	$IcsP^{HA}$	rough	< 21	hypersensitive
ETRM158	-	rough	< 21	hypersensitive
ETRM168	$IcsP^{HA}$	smooth	0	resistant
ETRM170	-	smooth	0	resistant

6.4.1 Analysis of IcsP activity against colicin E1

The results obtained when strains were cross-streaked against the colicin E1-producing *E. coli* strain K53 are summarised in Table 6.1. All smooth LPS strains (regardless of the presence or absence of IcsP) were resistant to colicin E1 (0 mm zone of inhibition), while rough LPS *S. flexneri* strains RMA723 (2457T *rmlD*::*kan*^{R}), ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}), ETRM243 (2457T *rmlD*/*icsP*::*kan*^{R} [pBAD30::*icsP*^{HA}]) and ETRM245 (2457T *rmlD*/*icsP*::*kan*^{R} [pBAD30::*icsP*]) showed a slight inhibitory effect (~1 mm zone of inhibition) at the intersection. Rough LPS *E. coli* K-12 strains UT5600, ETRM156 (UT5600 [pJRD215][pBAD30::*icsP*^{HA}]) and ETRM158 (UT5600 [pJRD215][pBAD30]) showed a greater sensitivity to colicin E1 (3 mm zone of inhibition).

6.4.2 Analysis of IcsP activity against colicin E2

When strains were cross-streaked against the colicin E2-producing *S. sonnei* strain P9, a greater level of killing against the strains tested was observed (Table 6.2). This time, only the *E. coli* K-12 smooth LPS strains ETRM168 (UT5600 [pRMA154][pBAD30::*icsP*^{HA}]) and ETRM170 (UT5600 [pRMA154][pBAD30]) showed complete resistance to the effect of colicin E2 (0 mm zone of inhibition), while all other smooth LPS strains showed an inhibitory effect against colicin E2 (1 mm zone of inhibition) (Table 6.2). Greater sensitivity to the effect of colicin E2 was observed in the rough LPS strains: *S. flexneri* strains ETRM723 (2457T *rmlD*::*kan*^{R}), ETRM240 (2457T *rmlD*/*icsP*::*kan*^{R}), ETRM243 (2457T *rmlD*/*icsP*::*kan*^{R} [pBAD30::*icsP*^{HA}]) and ETRM245 (2457T *rmlD*/*icsP*::*kan*^{R} [pBAD30]) showed a zone of inhibition of 6 mm; *E. coli* K-12 strain UT5600 showed a zone of ~5 mm. Unexpectedly, rough LPS *E. coli* K-12 strains ETRM156 (UT5600 [pJRD215][pBAD30::*icsP*^{HA}]) and ETRM158 (UT5600 [pJRD215][pBAD30]) showed hypersensitivity to colicin E2 compared to the parent strain UT5600, whereby complete inhibition was observed except for a number of resistant colonies (Table 6.2). These results

suggest that the pBAD30 vector in these strains has an effect on the strain's sensitivity to colicin E2. Overall however, IcsP did not appear to be a contributing factor to the resistance or sensitivity of these strains against colicin E2, as all strains expressing IcsP/IcsP^{HA} showed the same result as control strains without IcsP/IcsP^{HA}. The presence of Oag had an effect on sensitivity as previously reported (van der Ley *et al.*, 1986a).

6.5 Summary

This chapter has examined the activity of IcsP against various substrates known to be cleaved by other members of the Omptin family. This included investigating IcsP activity against Plg, α_2AP, complement, protamine, and colicins E1 and E2. Analysis of IcsP activity against Plg showed that cleavage of Plg was observed in rough LPS *S. flexneri* strains with and without IcsP, suggesting that IcsP did not play a contributing role to the observed cleavage. Smooth LPS strains did not show significant cleavage of Plg. The results suggest that another protease with increased expression in rough LPS *S. flexneri* might be contributing to the observed activity against Plg. The addition of α_2AP to inhibit Plg cleavage also appeared to have no effect, as rough LPS strains still cleaved Plg in the presence of α_2AP. When smooth and rough LPS strains were incubated with α_2AP alone, a cleaved product was observed by both strains, suggesting that *S. flexneri* inactivated α_2AP under the conditions used in this study. IcsP did not play a contributing role to the observed cleavage of α_2AP as all *S. flexneri* strains showed cleaved product regardless of the absence of IcsP.

Bactericidal assays with complement from guinea pig serum showed that *S. flexneri* strains with rough LPS were sensitive to complement and smooth LPS strains were resistant. However IcsP appeared to have no effect on the strain's sensitivity to complement overall. When human serum was used, all *S. flexneri* (regardless of whether they possessed rough or

smooth LPS) showed sensitivity to complement, and again, IcsP did not appear to affect the strains' sensitivity to complement. IcsP activity against protamine was subsequently investigated, and all *S. flexneri* strains (with and without IcsP) showed sensitivity to protamine. Finally, the activity of IcsP against colicins was investigated but the results showed little difference between colicin sensitivity between strains with and without IcsP. The presence of Oag did however, show a major protective role against colicins as previously reported (van der Ley *et al.*, 1986a). The results suggest that IcsP has no significant activity against these substrates.

Overall this chapter has looked at IcsP activity against a range of known Omptin cleavable substrates, and the results obtained suggest that IcsP is inactive against all of them. These results may be explained by the fact that IcsP bears less than 60% identity to all Omptins, and does not appear to be closely related to the other members of the Omptin family, as shown in the phylogram in Fig. 1.5.

Chapter 7 – Discussion

7.1 Introduction

Omptin proteases are a family of multifunctional enterobacterial surface proteases which share at least 56% sequence identity and similar structure. Most have been implicated in bacterial pathogenesis and in particular IcsP, is an Omptin protease which cleaves the OM IcsA protein known to be essential for *S. flexneri* virulence. However the pathogenic relevance of IcsP per se remains unclear. Despite the general consensus that IcsP is required for the exclusive polar localisation of IcsA observed in *S. flexneri,* the distribution of IcsP itself on the cell surface has not been characterised before. Furthermore, previous studies have suggested that VirK may also affect IcsP expression and activity. Since IcsP shares most similarity to OmpT, the best biochemically characterised protease of the Omptin family, the activities of OmpT (as well as other Omptin members) may provide insights into the biology of IcsP.

7.2 Characterisation of *icsP* mutants and the role of IcsP in *S. flexneri* cell-to-cell spread

Several studies have analysed *Shigella icsP* mutants (Egile *et al.*, 1997; Shere *et al.*, 1997; Steinhauer *et al.*, 1999). Egile *et al*. (1997) found that a *S. flexneri* 5a M90T *sopA* (*icsP*) mutant did not cleave IcsA into culture supernatants and displayed IcsA on all regions of the cell surface. Similarly, Shere *et al*. (1997) found that a *S. flexneri* 2a 2457T *icsP* mutant also displayed a marked reduction of IcsA cleavage into culture supernatants and detectable IcsA over the entire bacterial surface. In Section 3.2, mutagenesis of *icsP* in both 2457T and M90T

was undertaken and found to prevent cleavage of IcsA and the release of IcsA into culture supernatants, resulting in the display of IcsA on polar and lateral regions of the cell surface, confirming these earlier reports (Egile *et al.*, 1997; Shere *et al.*, 1997).

7.2.1 Role of IcsP in *Shigella* cell-to-cell spread

Previous data have shown that the absence of IcsP in *S. flexneri* appears to either have no effect on the size of plaques formed by 2457T serotype 2a strains (Shere *et al.*, 1997) or to decrease the size of plaques formed by M90T serotype 5a strains (Egile *et al.*, 1997). Shere *et al*. (1997) proposed that this inconsistency was potentially related to the differences between the two *S. flexneri* serotypes used to construct the *icsP* mutants, and that the molecular mechanisms of cell-to-cell spread between the two serotypes might differ slightly. However the results presented in this thesis showed that *icsP* mutants constructed in both backgrounds did not differ in plaque formation. On HeLa cells, both ETRM22 (2457T 2a *icsP*::*kan*^{R}) and ETRM108 (M90T 5a *icsP*::*kan*^{R}) showed no change in the size of plaques formed in comparison to the wild-type of both strains (Section 3.4.1). The result obtained here for the 2457T *icsP* mutant constructed in this study supports the results observed by Shere *et al*. (1997) for their 2457T *icsP* mutant and HeLa cells. Additional staining of F-actin inside HeLa cells infected with wild-type and *icsP* mutants showed no abnormality in F-comet actin tail formation (contradicting the data of Egile *et al*. (1997) where abnormal F-actin tails were observed inside HeLa cells for a M90T *sopA* (*icsP*) mutant). The decrease in plaque size observed by Egile *et al*. (1997) when *icsP* was mutated may more likely be related to the Caco-2 cell line used to perform the plaque assays.

In contrast, IcsP does appear to affect intra- and intercellular spreading of *S. flexneri* inside CV-1 cells. Both ETRM22 (2457T 2a *icsP*::*kan*^{R}) and ETRM108 (M90T 5a *icsP*::*kan*^{R}) formed plaques with an increase in size on CV-1 cells. CV-1 cells are African Green monkey

kidney fibroblast cells often used to look at aspects of cellular invasion by *S. flexneri* strains. Interestingly Shere *et al*. (1997) reported a 27% increase in the speed of their 2457T *icsP* mutant's ABM inside PtK2 cells compared to wild-type. The presence of IcsP in *S. flexneri* may therefore limit *S. flexneri* bacterial dissemination within the host and reduce the extent of Shigellosis, a disease which is known to be self-limiting (Section 1.3). Since IcsA is the only known substrate for IcsP, IcsP may specifically function to limit IcsA and its effect on cell-to-cell spread.

7.3 Surface distribution of IcsP

It has been suggested that IcsP may be distributed all over the cell surface of *S. flexneri* (d'Hauteville *et al.*, 1996; Shere *et al.*, 1997; Steinhauer *et al.*, 1999), but the cell surface distribution of IcsP (or any other member of the Omptin family) has not been experimentally determined. It is interesting to note that few OM proteins have had their distributions discerned. Of those that have, IcsA, *E. coli* LamB and Iss have been shown to be either polarly localised (Goldberg *et al.*, 1993a), distributed in a helical pattern (Gibbs *et al.*, 2004), or detectable all over the cell surface with no distinct pattern (Lynne *et al.*, 2007), respectively.

In this thesis, a HA-tagged IcsP ($IcsP^{HA}$) was constructed and expressed under pBAD control in smooth and rough LPS backgrounds of *S. flexneri* and *E. coli* K-12, followed by IF detection to determine the protein's surface distribution. Characterisation of $IcsP^{HA}$ (Section 4.3.3) showed that it was functional for IcsA cleavage, and localised to the OM when cell fractionation was performed by sucrose density gradient centrifugation (Fig. 4.7 and 4.9). Unexpectedly, cell fractionation by Triton/$MgCl_2$ treatment did not show localisation of $IcsP^{HA}$ to the OM, and this may attributed to a slight difference in the structural conformation

of the IcsP^{HA} protein, allowing it to solubilise in Triton/$MgCl_2$ buffer. Detection of IcsP and IcsP-His_6 by IF with anti-IcsP and anti-His_6 was also attempted but not achieved, as previously reported by Steinhauer *et al*. (1999) who used anti-IcsP antibodies.

7.3.1 IcsP^{HA} distribution in smooth and rough LPS strains

The distribution of the OM protein IcsA has previously been shown to be masked by the presence of LPS Oag (Morona & Van Den Bosch, 2003). Taking this into account, the distribution of IcsP^{HA} was investigated in both smooth and rough LPS backgrounds of *E. coli* K-12 *ompT* and *S. flexneri* 2a VP^{-ve} strains. Anti-HA revealed IcsP^{HA} to be punctate and randomly distributed across the cell surface. Further analysis by 3D deconvolution of both smooth and rough LPS backgrounds suggested a banded distribution which might be similar to that of OM protein LamB (Gibbs *et al.*, 2004) and possibly other OM proteins described by Ghosh and Young (2007).

IF labelling of IcsP^{HA} was also observed in the majority of rough LPS bacteria but not in all smooth LPS bacteria; only approximately half the number of smooth LPS bacterial cells appeared to fluorescent. Attempts to increase cell surface IF labelling of IcsP^{HA} in smooth LPS strains by extending the time of arabinose induction had no effect (Section 4.4.4). Since the levels of IcsP^{HA} expression in smooth and rough LPS strains (in both *E. coli* K-12 *ompT* and *S. flexneri* 2a VP^{-ve} backgrounds) after arabinose induction were identical as determined by Western blotting with anti-HA (Fig. 4.12), the difference observed in IF between smooth and rough LPS strains does not appear to be a result of a difference in the expression levels of IcsP^{HA} following arabinose induction.

7.3.2 Sf6 TSP treatment enhances detection of IcsP^{HA}

The one clear difference between smooth and rough LPS strains is the presence of Oag chains in the smooth LPS strains. One way to determine the effect of LPS Oag chains on the detection of IcsP^{HA} in smooth and rough LPS strains would be to shorten Oag chains by hydrolysis with Sf6 phage TSP. However, Sf6 phage TSP is only effective on strains with X or Y serotype LPS (Lindberg *et al.*, 1978). It is reported here for the first time the construction of a *yfdI* mutant in *E. coli* K-12 which, upon introduction of a plasmid carrying *S. flexneri* Oag biosynthesis genes, expressed smooth LPS of Y serotype specificity (Table 4.2), thereby allowing treatment with Sf6 phage TSP. IF labelling revealed an increase in the proportion of smooth bacteria which showed IcsP^{HA} labelling following treatment with Sf6 phage TSP (Fig. 4.19). These results suggest that IcsP is present across the entire surface of the bacterial cell, but is masked in *S. flexneri* and *E. coli* K-12 strains expressing smooth LPS because the presence of Oag chains prevents antibody binding to IcsP^{HA}. This type of protein masking by LPS Oag has also been shown for the *S. flexneri* protein IcsA (Morona & Van Den Bosch, 2003), and several other OM proteins (van der Ley *et al.*, 1986a; van der Ley *et al.*, 1986b).

7.3.3 Punctate IcsP^{HA} distribution at low arabinose induction

Following induction with 0.2% (w/v) arabinose, rough and smooth LPS strains displayed punctate and randomly distributed IcsP^{HA} labelling across the cell surface (Section 4.4.5). It was of interest to determine whether reducing the expression of IcsP^{HA} on the cell surface displayed the same distribution. In Section 4.4.5, the rough LPS *E. coli* K-12 *ompT* strain ETRM156 (UT5600 [pBAD30::*icsP*^{HA}]) strain also showed a punctate distribution of IcsP^{HA} when induced with 0.003% (w/v) arabinose (Section 4.4.3). Further deconvolution analysis of this (0.003% [w/v] arabinose-induced) strain showed that this labelling was punctate and slightly banded in distribution; similar to what was seen for the deconvolved images of both rough and smooth LPS bacteria induced at 0.2% (w/v) arabinose (Fig. 4.12). Importantly, induction of IcsP^{HA} at 0.003% (w/v) arabinose was found to be approximately equivalent to

native wild-type expression levels of IcsP in 2457T by Western immunoblotting (Fig. 4.16). This suggests that IcsP also has a punctate and slightly banded distribution on the cell surface in wild-type *S. flexneri*.

It is therefore proposed in this thesis that IcsP is distributed in a punctate manner all over the cell surface, with the protein concentrated in certain areas, resulting in a banded pattern across the bacterial cell as shown in Fig. 7.1. Previous authours have also proposed that IcsP contributes to the polar localisation of IcsA (d'Hauteville *et al.*, 1996; Egile *et al.*, 1997; Shere *et al.*, 1997; Steinhauer *et al.*, 1999). This distribution of IcsP is adequate to cleave laterally localised IcsA on the cell surface and reinforce IcsA polarity at the old pole, which is essential for *Shigella* virulence.

7.3.4 Helical distribution of LPS in *S. flexneri* 5a strains

In addition to investigating the distribution of IcsP in this thesis, the distribution of LPS was investigated in *S. flexneri* serotype 5a strain M90T (PE856). Interestingly, M90T had a helical distribution of LPS across the cell surface (Section 4.6) when the group 3,4 LPS antisera was used. This was observed for both *in vitro* (LB medium) and *in vivo* (CV-1)-grown bacteria. Further labelling of two other *S. flexneri* 5a strains (PE647 and PE780 (a M90T strain from a second source)) was performed and showed a similar helical distribution of LPS as seen in M90T (Fig. 4.20 and 4.21). Deconvolution analysis further supported these observations (Fig. 4.20).

Previous studies by Hartman *et al*. (1996) using monoclonal antibodies specific for *S. flexneri* Oag described a patchy distribution of LPS on the surface of *S. flexneri* 2a, a finding consistent with electron microscopy by Lehane *et al*. (2005), when antibodies directed against *S. flexneri* 2a Oag were used. However attempts to stain the LPS of *S. flexneri* 2a with group

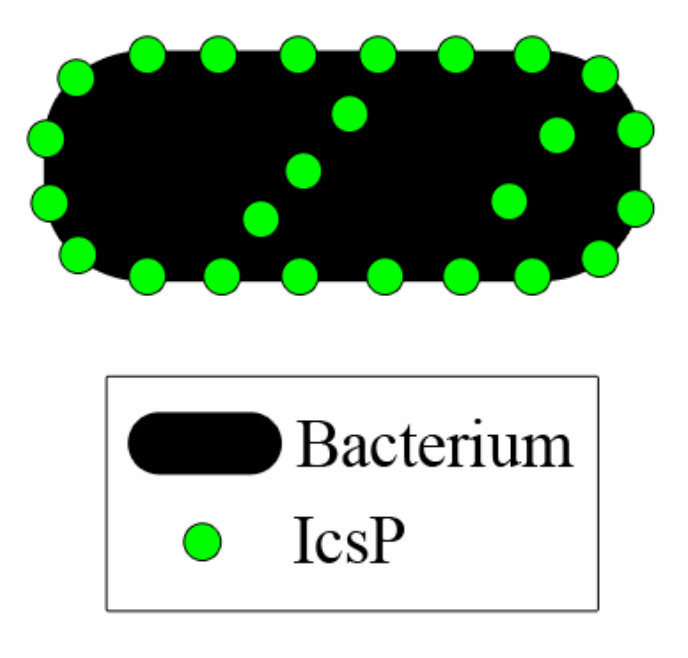


Fig. 7.1 Model for the distribution of IcsP on the cell surface of *S. flexneri*

IcsP (shown as green dots) is distributed in a punctate manner all over the cell surface, with IcsP concentrated in certain areas following a banded pattern across the bacterium (shown in black).

3,4 anti-LPS Oag resulted in an even distribution of LPS labelling across the cell surface, with no distinguishable pattern (Fig. 3.8). This suggests a possible difference in the configuration of LPS in serotype 5a strains compared to *S. flexneri* 2a strains. NMR data from Clement *et al*. (2003) have shown that the LPS structure of serotype 5a strains adopt a right-handed, three-fold helix with the branched glycosyl residues pointing outwards. The glucosylation of Y serotype Oag to give serotype 5a Oag is predicted to induce a transition from a linear to helical conformation with the glucose residue exposed on the exterior of the helix, forming a more compact structure than non-glycosylated LPS (West *et al.*, 2005). The labelling of serotype 5a strains observed here could be due to this predicted, compacted conformation of LPS. Glycosyl residues arranged in a certain conformation in the LPS structure of *S. flexneri* serotype 5a may result in the detection of an organised, helical pattern of LPS distribution across the cell surface of *S. flexneri* by the group 3,4 LPS Oag antisera used in this thesis. Presumably serotype 2a Oag does not have this conformation.

7.3.5 LPS and IcsP^{HA} distribution in *S. flexneri* 5a

To further investigate the distribution of LPS and punctate distribution of IcsP^{HA}, double labelling with anti-HA and group 3,4 anti-LPS was also performed on a M90T 5a VP^{-ve} strain expressing IcsP^{HA}. The results showed a degree of co-localisation of LPS with IcsP^{HA} in some bacterial cells, but this was not evident in the majority of cells (Section 4.6.3). Interestingly, co-localisation appeared to occur where the LPS helices encountered the punctate distribution of IcsP^{HA} (Fig. 4.21). These results are reminiscent of a model recently proposed by Thiem *et al*. (2007) whereby the positioning of chemosensory clusters in *E. coli* was shown to be marked by periodically positioned ring-like or short helical structures along the length of the bacterial cell. IcsP and LPS may also be distributed in this way, and areas where co-localisation is observed may be due to the occasional overlap of the banded/helical structures.

This section has examined the distribution of IcsP on the cell surface of *S. flexneri* and *E. coli* K-12 and found it to be punctate and potentially banded in pattern across the cell surface. We propose that the difference in the proportion of cells which displayed $IcsP^{HA}$ labelling between rough and smooth LPS bacteria is that Oag chains mask IcsP, hence preventing its detection by antibodies in the majority of smooth cells. Interestingly, the helical distribution of IcsP and of smooth LPS on the surface of *S. flexneri* serotype 5a bacteria, is consistent with a growing body of evidence that is showing that cell wall biosynthesis has an underlying organisation (Aaron *et al.*, 2007).

7.4 Characterisation of *S. flexneri virK* and *rmlD* mutants

It has been reported that *virK*, a gene reported to affect the intracellular spread of *S. flexneri*, affects the expression and activity of IcsP (Nakata *et al.*, 1992; Wing *et al.*, 2005). Furthermore LPS has been found to be essential for the enzymatic activity of OmpT (Kramer *et al.*, 2000; Kramer *et al.*, 2002) as well as affecting other members of the Omptin family such as Pla and PgtE (Kukkonen & Korhonen, 2004; Kukkonen *et al.*, 2004), and perhaps therefore may also affect IcsP. To investigate this, a *virK* and an *rmlD* mutant (as well as a *virK*/*rmlD* double mutant) were constructed in this study (Section 5.2).

7.4.1 Reassessment of *virK* mutant phenotype

A *virK* mutant has been previously shown to display smaller sized plaques and have decreased IcsA expression compared to wild-type (Nakata *et al.*, 1992). The intracellular spreading defect observed in this *virK* mutant was later shown to potentially result from increased IcsP expression and associated enhanced cleavage of IcsA (Wing *et al.*, 2005). In Chapter 5, analysis of a *S. flexneri* 2a 2457T *virK* deletion mutant contradicted these results. When the *virK* mutant was used to infect CV-1 and HeLa cells, no defect in *Shigella* cell-to-

cell spread was observed; the plaques formed by *virK* mutants were not significantly different to wild-type (Section 5.4.1 and Section 5.4.2) and the *virK* mutant inside CV-1 and HeLa cells showed normal F-actin tail comet formation (Section 5.4.3). When the expression of IcsA and IcsP in the *virK* mutants was investigated in standardised samples by Western immunoblotting, the results obtained showed no difference in the expression levels of IcsA and IcsP compared to the wild-type strain (Section 5.5 and 5.6).

It is hypothesised that the difference observed between the *virK* mutant phenotype in this study and that of previous *virK* mutant's phenotype, is due to the nature of the mutation inactivating *virK*. The *virK* mutant constructed by Nakata *et al*. (1992) was made by random insertion of a Tn*10* transposon into the *virK* gene, and this may have resulted in a polarity effect on other genes present in the *virK* locus (Fig. 1.7). Although complementation analysis was undertaken, the complementary DNA also contained DNA from genes adjacent to *virK*. In contrast, the *virK* mutant in this study was made by directed mutagenesis to precisely delete *virK*, avoiding the potential for a polarity effect on adjacent genes. Additionally, the *virK* mutant by Nakata *et al*. (1992) was made in the *S. flexneri* YSH6000T 2a strain, which may differ slightly to the *S. flexneri* 2457T 2a strain used in this study. Our findings overall suggest that *virK* is not a *Shigella* virulence determinant as proposed in the current literature and that *virK* may not affect IcsP as proposed by Wing *et al*. (2005). There are no homologs of VirK in 2457T or any other sequenced *S. flexneri* strains, but a close homologue in *S. typhimurium* has been shown to confer resistance against macrophage microbiocidal mechanisms (Brodsky *et al.*, 2005; Detweiler *et al.*, 2003).

7.4.2 Assessment of *rmlD* mutant phenotype

An *rmlD* mutant is unable to synthesize Oag due to a block in dTDP-rhamnose synthesis (Van den Bosch *et al.*, 1997)) and consequently produces rough LPS. Rough LPS mutants display

high levels of IcsA on polar and lateral regions of the cell surface and are capable of invading mammalian cells but form no plaques and display a defect in F-actin comet tail formation (Van den Bosch *et al.*, 1997). It is unknown whether LPS Oag affects IcsP, however there is evidence to suggest that Omptins have a dual interaction with LPS: Kramer *et al*. (2000, 2002) found that denatured OmpT could only be refolded into an active form by the addition of LPS, while Pla and PgtE were only functionally active in wild-type, or recombinant bacteria lacking the Oag chains (Kukkonen *et al.*, 2004), respectively.

The *S. flexneri* 2a 2457T *rmlD* mutant constructed in this thesis (Section 5.2.2) was found to display IcsA on polar and lateral regions of the cell surface, was unable to form plaques on cell monolayers, and displayed infrequent defected F-actin comet tails, as previously described by Van Den Bosch *et al*. (1997). Further characterisation of this mutant showed that there was no difference in IcsP expression compared to wild-type (Section 5.6), and it was still able to cleave and release IcsA into culture supernatants (Section 5.7). Based on these results, it appears that LPS Oag does not affect the expression and activity of IcsP. Since IcsP has only 40-60% identity to the other members of the Omptin family known to have some interaction with LPS, it may be that IcsP is different and may alternatively be affected by other components of LPS, such as lipid A.

7.5 IcsP activity against other known Omptin protease substrates

To further investigate the biology of IcsP, smooth and rough LPS strains expressing IcsP were tested against a range of known Omptin substrates (Section 6). Although Omptin proteases vary in their reported functions (Kukkonen & Korhonen, 2004), some Omptin members have been shown to be capable of proteolytic activity against similar substrates, such as Plg, complement, and antimicrobial proteins (Beesley *et al.*, 1967; Kukkonen *et al.*, 2001;

Kukkonen & Korhonen, 2004; Kukkonen *et al.*, 2004; Lahteenmaki *et al.*, 2001; Lahteenmaki *et al.*, 2005; Leytus *et al.*, 1981; Ramu *et al.*, 2007; Sodeinde *et al.*, 1992).

7.5.1 IcsP activity against plasminogen

Omptin proteases Pla and PgtE expressed in recombinant rough LPS *E. coli* have been shown to activate Plg into plasmin (Kukkonen *et al.*, 2001; Kukkonen *et al.*, 2004). Plg activation by Pla in particular, has been reported to be directly involved in the pathogenesis of *Y. pestis* infection, as Plg-deficient mice are a hundred-fold more resistant to *Y. pestis* infection than normal mice (Goguen *et al.*, 2000). OmpT has also been shown to activate Plg but very poorly in recombinant *E. coli* (Kukkonen *et al.*, 2001). IcsP in *S. flexneri* 2457T was not found to display proteolytic activity against Plg under the conditions used in this thesis (Section 6.2.1) in either rough or smooth LPS strains.

Rough LPS *S. flexneri* strains (carrying an *rmlD* mutation) did show increased proteolytic activity against Plg (Fig. 6.1), and this was observed regardless of the presence or absence of IcsP (Section 6.2.1). Since expression of IcsP is equivalent in both smooth and rough LPS *S. flexneri* strains (see Section 5.6), the increased activity observed against Plg is likely related to some other *S. flexneri* protein which may have increased proteolytic activity in rough LPS strains. Pic for example, is a protease encoded by the chromosomal *pic* gene in both *S. flexneri* and enteroaggregative *E. coli* which has been shown to display *in vitro* mucinolytic activity, serum resistance and haemagglutination, and may be an ideal protease to investigate with respect to Plg activity (Al-Hasani *et al.*, 2001; Henderson *et al.*, 1999). To determine whether the *Shigella* determinant that cleaves Plg in rough LPS strains is associated with the virulence plasmid, it would be ideal to compare virulence plasmid positive and VP^{-ve} strains.

7.5.2 IcsP activity against α_2-antiplasmin and complement

Two other substrates known to be cleaved by *Y. pestis* Pla and *S. enterica* PgtE include α_2-antiplasmin (α_2AP) and complement (Kukkonen *et al.*, 2001; Lahteenmaki *et al.*, 2005; Ramu *et al.*, 2007; Sodeinde *et al.*, 1992). α_2AP is the main circulating inhibitor of plasmin (the activated form of Plg), and plasmin activity is important in fibrinolysis and in cellular migration. Cleavage of α_2AP is suggested to promote uncontrolled proteolysis by plasmin in the human host. Uncontrolled proteolysis contributes to the invasive character of plague (Kukkonen *et al.*, 2001), and is suggested to enhance cellular migration associated with *Salmonella* infection (Lahteenmaki *et al.*, 2005). However, IcsP did not appear to have activity against α_2AP in this study (Section 6.2.2), as α_2AP was cleaved by both smooth and rough LPS *S. flexneri* regardless of the presence or absence of IcsP (Fig. 6.2).

Other proteolytic targets for Pla and PgtE are complement components. Human complement is a complex system of plasma proteins and activation of the complement cascade leads to opsonization of foreign microbes, release of chemotactic peptides, and disruption of bacterial cell membranes (Walport, 2001a; Walport, 2001b). Pla of *Y. pestis* has been reported to cleave the C3 component of serum complement (Sodeinde *et al.*, 1992), while PgtE of *S. enterica* has recently been reported to cleave the C3b, C4b, and C5 components of complement (Ramu *et al.*, 2007). However, IcsP appeared to have no activity against complement (present in guinea pig and human serum) in this study as there was no observed difference in the serum complement resistance of *S. flexneri* strains with and without IcsP (Section 6.3.1). There was however, a difference observed between smooth and rough LPS *S. flexneri* strains against guinea pig serum and this is likely due to the presence of very long type Oag chains in the LPS structure of smooth *S. flexneri* bacteria. Such chains which have previously been shown to confer resistance to the bactericidal activity of serum (Hong & Payne, 1997). Unlike Hong and Payne (1997) however, the *S. flexneri* smooth and rough LPS

strains tested in this thesis were all sensitive to the bactericidal activity of human serum, and the difference in results obtained may be due to differences in complement concentrations used.

The absence of significant IcsP activity against α_2-antiplasmin and complement found in this study suggests that IcsP is unlikely to play a role in *S. flexneri* interaction with the host Plg or complement system. However *S. flexneri* had Plg activity (in rough LPS strains) and α_2AP activity (in both smooth and rough LPS strains) which is currently cryptic.

7.5.3 IcsP activity against OmpT substrates protamine and colicins

The Omptin protease OmpT, which is one of the proteases which bears most identity to IcsP, has been shown to have activity against protamine and colicins. Stumpe *et al*. (1998) showed that *E. coli* strains carrying an *ompT* deletion were hypersensitive to protamine compared to strains carrying *ompT* on a plasmid, suggesting that OmpT may protect cells against antimicrobial cationic peptides excreted by epithelial cells in the urinary tract. Antimicrobial assay on *S. flexneri* strains in this study showed they were sensitive to protamine regardless of the presence of absence of IcsP (Section 6.3.2). The results suggest an unlikely role for IcsP against cationic antimicrobial agents.

OmpT activity against colicins has been described by Cavard *et al*. (1990). Colicins are plasmid-encoded antibacterial toxins that are produced by *E. coli* to target other *E. coli* strains, and OmpT has been shown to cleave colicins A, E1, E2 and E3 (Cavard & Lazdunski, 1990). IcsP activity against colicins E1 and E2 was investigated in this study using the double layer test method, but no difference was observed between *S. flexneri* and *E. coli* strains with and without IcsP (Section 6.4), suggesting that IcsP does not cleave colicins. Rough LPS strains in general however, appeared to be more susceptible to the effects of colicin than

smooth LPS strains, and this supports data observed by van der Ley *et al*. (1986), whereby the presence of Oag in the LPS structure of smooth LPS *E. coli* strains was shown to prevent access of colicins to OM protein receptors.

The results of this section suggest that IcsP may be functionally different to other Omptins. This may be reflected in the fact that IcsP only shares at most, 58% identity with the other Omptin members, and does not appear to be closely related to them as shown by the phylogram in Fig. 1.6. IcsP did show an affect on *Shigella* cell-to-cell spread (see Section 3.4.1). The guinea pig model recently described by Shim *et al*. (2007) has been shown to display symptoms of bacillary dysentery similar to those seen in humans without any requirement for antibiotic treatment or starvation of the animals. This may therefore be an ideal animal model to further investigate this aspect as the extent of rupture and destruction to the intestinal epithelium which may be caused by an *icsP* mutant (compared to a wild-type strain) has not been investigated before.

7.6 Conclusion

This thesis has found that IcsP appears to affect *Shigella* intra- and intercellular spreading. Investigation into the distribution of IcsP on the cell surface found that IcsP localisation was punctate and dispersed in a banded pattern across the cell surface of smooth and rough LPS strains. Inactivation of the *Shigella virK* gene had no effect on virulence, as determined using the plaque assay, and also no effect on IcsP expression, contradicting previous reports. The *rmlD* mutation which prevents Oag synthesis was found to have no effect on IcsP expression or activity. Finally, IcsP was found to be inactive against known Omptin protease substrates (Plg, α_2AP, complement, protamine and colicins), suggesting that IcsP is distinctly different to other Omptins and may only have a unique role in *Shigella* IcsA cleavage.

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