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Surface Display of the HPV L1 Capsid Protein by the Autotransporter *Shigella* IcsA

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Autotransporters have become attractive tools for surface expression of foreign proteins in Gram-negative bacteria. In this study, the *Shigella* autotransporter IcsA, has been exploited to express the human papillomavirus (HPV) type 16 L1 capsid protein in *Shigella sonnei* and *Escherichia coli*. The L1 gene was fused in-frame to replace the coding sequence of the IcsA passenger domain that is responsible for actin-based motility. The resultant hybrid protein could be detected by an anti-L1 antibody on the surface of *S. sonnei* and *E. coli*. In *E. coli*, the protein was expressed on the entire surface of the bacterium. In contrast, the protein was detected mainly at one pole of the *Shigella* bacterium. However, the protein became evenly distributed on the surface of the *Shigella* bacterium when the *icsP* gene was removed. Our study demonstrated the possibility of exploiting autotransporters for surface expression of large, heterologous viral proteins, which may be a useful strategy for vaccine development.

Keywords: autotransporter, gene fusion, surface expression, *Shigella sonnei*

Introduction

Autotransporters of Gram-negative bacteria contain the translocation domains that form a pore on the cell envelop, allowing the translocation of the passenger domain through the inner and outer membranes to the surface of the bacterium (Henderson *et al*., 1998; Jain *et al*., 2006). Autotransporters have recently emerged as an ideal vehicle for expression of non-native proteins on the bacterial surface. This strategy has been exploited for translocation analysis, vaccine development, enzyme activity studies and drug delivery using autotransporters from a number of bacterial species including AIDA-I, EspP, and Ag43 from *E. coli*, MisL from *Salmonella enterica*, the IgA protease of *Neisseria gonorrhoeae* and IcsA of *Shigella flexneri* (Suzuki *et al*., 1995; Kjærgaard *et al*., 2002; Rizos *et al*., 2003; Jose and Zangen, 2005; Skillman *et al*., 2005; Zhu *et al*., 2006). Notably, it has been shown that *Shigella* IcsA has the capacity to express larger foreign proteins such as PhoA and MalE originating from *E. coli* (Suzuki *et al*., 1995).

IcsA is the major virulence factor of *Shigella* and is localised to the surface of the bacterium where it is found concentrated at the old poles. Here, IcsA polymerises host cell actin and propels the bacteria forward, mediating intra- and intercellular dissemination (Kotloff *et al*., 2000; May and Morona, 2008). An advantage of IcsA as a vehicle for foreign protein translocation, is the polarization mechanism, which would be favourable for some antigens such as HPV L1 whose immunogenicity is dependent on the aggregation of monomers (Kirnbauer *et al*., 1992).

Despite previous successful applications (Suzuki *et al*., 1995), there remain uncertainties in using IcsA as a vehicle to express heterologous proteins. In previous studies, most of the foreign passenger proteins translocated by IcsA have been derived from Gram-negative bacteria (Suzuki *et al*., 1995). Thus far, no expression of viral antigens using autotransporters has been reported. Moreover, the largest protein tested in IcsA is the 47-kDa *E. coli* PhoA protein (Suzuki *et al*., 1995). Translocation of PhoA protein through the outer membrane was incomplete, presumably due to the tertiary structure of PhoA. Thus, the ability of IcsA to translocate larger proteins remains unknown. Further investigations are required to test larger foreign proteins with complex tertiary structures for translocation through this machinery.

In order to further exploit the IcsA autotransporter for foreign protein expression and to understand the translocation mechanisms, it was decided to investigate the ability of IcsA to translocate the HPV L1 capsid protein. As the major capsid protein, HPV L1 forms a complex tertiary and quaternary structure (Chen *et al*., 2000) and is larger than the previously reported foreign passenger (Suzuki *et al*., 1995). In this study, we have demonstrated that IcsA can efficiently drive the L1 capsid protein to the bacterial surface in both *E. coli* and *S. sonnei*. Additionally, polar expression of HPV16 L1 on the *Shigella* surface was observed, which appeared to be mainly dependent on the presence of the IcsP protease.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

The *S. sonnei* strain (20071599) was acquired from the Scottish *Salmonella* and *Shigella* Reference Laboratory (Glasgow, UK). Suicide vector pJCB12 (Turner *et al*., 2001) was kindly pro-

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vided by Dr. Arthur K. Turner from The Wellcome Trust Sanger Institute (Cambridge, UK). *E. coli* SM10 *λpir* (Simon *et al.*, 1983) and *S. sonnei* strains were routinely cultured on L-agar and L-agar containing Congo Red (0.1%), respectively, at 37°C. Antibiotics were used at the following concentrations: streptomycin, 100 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml. All chemicals were from Sigma unless otherwise indicated.

Construction of *icsA*-L1 translational gene fusion

To construct the gene fusion, 3 fragments were amplified. The first fragment included 312 bp of the *icsA* 5′-end coding sequence and the flanking upstream 550 bp non-coding sequence using primers 5′-CGGTACCCGGGGATCTAGTTATGTTTGATGTCTGCA-3′ and 5′-AGGCTCATAGTTCTAGATGCATGAGAGG-3′. The second fragment was the entire coding sequence of codon-optimized HPV16 L1 gene (Baud *et al.*, 2004) using primers 5′-CTAGAACTATGAGCCTGTGGCTGC-3′ and 5′-ATAGTACTCAGCTTCCTCTTCTTCC-3′. The third fragment including the coding sequence of *icsA*, using primers 5′-GGAAGCTGAGTACTATTCTGGCAGATA-3′ and 5′-CGACTCTAGAGGATCTCAGAAGGTATATTTCACAC-3′. All fragments were ligated sequentially into linearized pUC19 (Clontech, USA) using an In-Fusion™ Advantage PCR Cloning Kit (Clontech) to generate pUC19-*icsA*-in-L1 according to the manufacturer's instructions.

This construct (as illustrated in Fig. 1) was subcloned into the suicide vector pJCB12, which possesses functional *tra* genes for conjugation, to create pJCB12-*icsA*-in-L1. These clones were kept in *E. coli* SM10 λ*pir* which allowed the replication of R6K origin plasmids and servedas donor strains.

Construction of the recombinant *S. sonnei* strain carrying an IcsA-L1 fusion

For conjugation, a streptomycin-resistant *S. sonnei* derivative of strain 20071599 was selected as the recipient strain. Both donor and recipient strains were grown in LB until late-log phase, and then mixed at an equal ratio and spotted onto an LB plate (Turner *et al.*, 2001). After overnight incubation at 37°C, cells were resuspended using L-broth and plated out on to the L-agar containing 100 μg/ml streptomycin and 20 μg/ml chloramphenicol to select for transconjugates. Neither the donor SM10 λ*pir* nor the recipient strain could survive double antibiotic selection (results not shown). Survival indicated a single cross-over at the *icsA* locus and was confirmed by PCR using primers 5′-CGGTACCCGGGGATCTAGTTATGTTTGATGTCTGCA-3′ and 5′-CGACTCTAGAGGATCTCAGAAGGTATATTTCACAC-3′. This mutant was then grown in salt-free L-broth supplemented with 5% sucrose at 30°C overnight to select for the second cross-over, resulting in removal of the suicide vector sequence (Turner *et al.*, 2001, 2006). Sucrose-resistant colonies were analysed by PCR-sequencing to confirm the second cross-over and the presence of the *icsA*-L1 gene fusion.

Deletion of the *icsP* gene in *S. sonnei*

To delete the *icsP* gene, the λ Red recombination system was used (Datsenko and Wanner, 2000) using previously published primers. Briefly, pKD46 was transformed into *S. sonnei*, followed by selection using 100 μg/ml ampicillin at 30°C. Bacteria were grown in L-broth in the presence of L-arabinose to induce the expression of the lambda Red recombinase. A linear PCR product, amplified using the primers 5′-CCTTGTACTTGCACTTTGTGTACCAGCGATCTTTACTACACATGCTACCGTGTAGGCTGGAGCTGCTTC-3′ and 5′-GTGCACCAAGAGGCGTTTCCTTAATTTCTGAATTTACAGGCTCTTTTGCATGGGAATTAGCCATGGTCC-3′, containing a kanamycin-resistance cassette flanked by FLP and 50 bp of the 5′- and 3′-end coding sequences of the *icsP* gene at both ends. This product was electroporated into the *S. sonnei* strain and kanamycin used to select the transformants. The plasmid pKD46 was eliminated by incubation at 37°C. To cure the kanamycin maker, pCP20 was introduced into a kanamycin-resistant colony to elicit the recombination of flanking FLP sequences at both ends of the kanamycin cassettes. The resultant *iscP*-deletion mutant was confirmed by PCR-sequencing using primers 5′-CCTTGTACTTGCACTTTGTG-3′ and 5′-GTGCACCAAGAGGCGTTTCC-3′.

Detection of L1 protein by indirect immunofluorescence imaging

For *E. coli* and *S. sonnei* strains, bacteria were harvested at mid-log phase on glass-slides and fixed by passing through a flame. Cells were permeabilized with 0.1% Triton X-100 (in PBS) for 3 min and blocked with 5% BSA (in PBS) for 30 min at room temperature. Bacteria were then incubated in 5% BSA with mouse anti-HPV16 L1 monoclonal antibody (Abcam, UK, Cat No 289-16981) overnight at 4°C, washed and incubatedwith goat anti-mouse antibody conjugated to TRITC (Invitrogen, USA) for 1 h. Coverslips were mounted with Anti-Fade solution (Invitrogen) and images analysed with a Leica Confocal microscope (Leica, Germany).

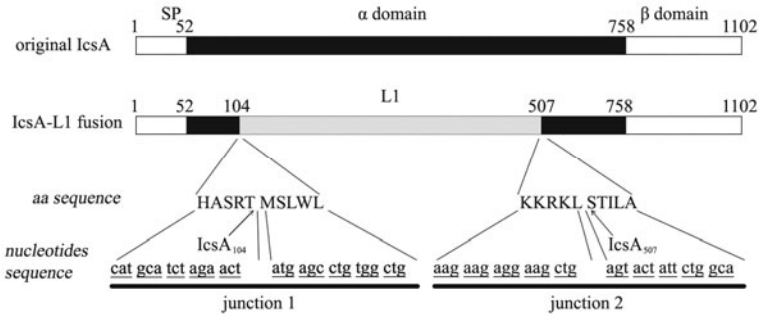

Fig. 1. Schematic presentation of IcsA and IcsA-L1 fusion. $IcsA_{105\text{-}506}$ was replaced with HPV16 L1 in a translational fusion; i.e. the HPV16 L1 gene was placed in-frame with the coding sequences at the upstream and downstream junctions.

Gentamycin-killing assay

HEp-2 (ATCC CCL-23) cells were cultured in Dulbecco's minimal essential medium (DMEM) (Sigma-Aldrich, USA) containing 10% fetal bovine serum with 5% CO_2 at 37°C, and assays carried out as described previously (Lucchini *et al*., 2005). Briefly, *S. sonnei* strains from mid-exponential phase were added to the cells at an MOI (multiplicity of infection) of 10 and bacteria centrifuged at 2,000 rpm for 10 min at room temperature to enhance invasion. The infection was allowed to proceed for 40 min, then the cells were washed with PBS. Media containing gentamicin (50 µg/ml) was added and incubated for 2 h. Cells were then washed with PBS and lysed with 0.1% Triton X-100 in water. The cell lysates were plated onto LB agar to quantify CFU.

Protease accessibility and immunoblotting

Cells were harvested from 20 ml of mid-log bacterial culture (approximately OD_{600}=0.8), washed twice with PBS and resuspended in 1 ml of PBS. Samples were treated with 100 µl/ml proteinase K (Tiangen, China) or mock treated with distilled water for one hour at 37°C, then 4 mM phenylmethanesulfonylfluoride (PMSF) was added to terminate the proteinase treatment. Cells were spun down and resuspended in 100 µl SDS sample buffer and boiled for 5 min. A 10-µl sample was subsequently separated using SDS-PAGE and transferred onto nitrocellulose membranes (Invitrogen). Membranes were blocked with 5% skimmed milk in PBS and incubated with mouse anti-HPV16 L1 monoclonal antibody (Abcam, Cat No 289-16981), then washed and incubated with a secondary goat anti-mouse antibody conjugated to HRP (Abcam). Signals were generated with Enhanced Chemiluminescence Detection reagents (Sigma, USA), and image was captured with a GENEGNOME system (Gene Company Limited, Hong Kong).

Results

Construction of the HPV16 L1-*icsA* gene fusion

Using the in-fusion procedure described in 'Materials and Methods', the coding sequence for residues 105-506 (Charles *et al*., 2001) of the *S. sonnei* IcsA was replaced with the HPV16 L1 gene to produce a translational fusion. As depicted in Fig. 1, the entire HPV16 L1 gene was in the middle of *icsA* and replaced the coding sequence 313–1518 bp of the IcsA passenger domain.

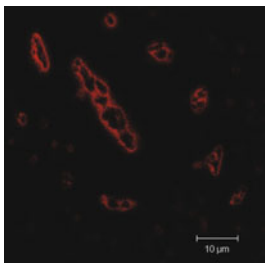

Fig. 2. Detection of IcsA-L1 hybrid protein in *E. coli*. The *icsA*-L1 gene fusion was cloned in pUC19 and transformed into *E. coli* DH5α. Transformed bacteria were collected from mid-log phase and fixed on the slides. The hybrid protein was detected by anti-L1 antibody followed by goat anti-mouse antibody conjugated with TRITC (red). Scale bar indicates 10 µm.

Detection of L1 in DH5α *E. coli*

By immunofluorescence microscopy, L1 was detected on the surface of the DH5α *E. coli* using an anti-L1 antibody (Fig. 2). This confirmed that the viral capsid protein can be translocated through the inner and outer membranes by IcsA. It has been noted that although the two polar targeting regions of IcsA were intact in our construct, L1 showed an even distribution on the surface of *E. coli* rather than a polar concentration (Fig. 2).

Detection of L1 in *S. sonnei*

For expression of the *icsA*-L1 gene fusion in *S. sonnei*, the construct was integrated into the virulence plasmid through homogenous recombination using the suicide vector pJCB12. The resultant *S. sonnei* strain was then subjected to immunofluorescence imaging (Fig. 3). In contrast to the even distribution of the fluorescence on *E. coli*, the fluorescent signals were most intense at one pole of recombinant *S. sonnei* (arrows, Fig. 3).

Assessment of IcsP in polar expression of IcsA-L1 fusion protein in *S. sonnei*

IcsP has been reported to be involved in surface expression of IcsA previously (d'Hauteville *et al*., 1996; Egile *et al*., 1997). To investigate the role of IcsP in polar expression, the λ Red recombination system was employed to remove the *icsP* gene in the recombinant *S. sonnei* strain that carried the *icsA*-L1 gene fusion. The *icsP* gene was first replaced by a kanamycin cassette flanked by FLP sequences. Insertion of the cassette was confirmed by PCR (Fig. 4A). The kanamycin cassette was then removed by recombination between the FLP sequences. After removal of the *icsP* gene, the IcsA-L1 hybrid protein appeared to be more evenly distributed

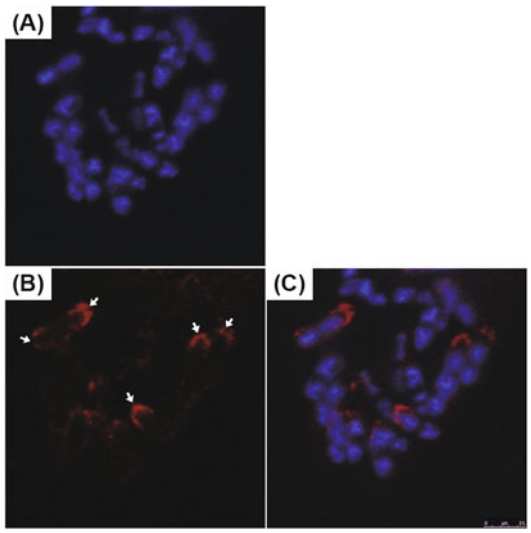

Fig. 3. Detection of IcsA-L1 hybrid protein in *S. sonnei*. The *S. sonnei* strain harboring the *icsA*-L1 fusion was fixed and the presence of the hybrid protein was detected as described in 'Materials and Methods'. The host cell nuclei were stained blue by DAPI (A). IcsA-L1 hybrid protein was stained red by TRITC and typical polar aggregation of the protein isindicated by arrows (B). A merged image of (A) and (B) is shown in (C). Scale bar in (C) indicates 2.5 µm.

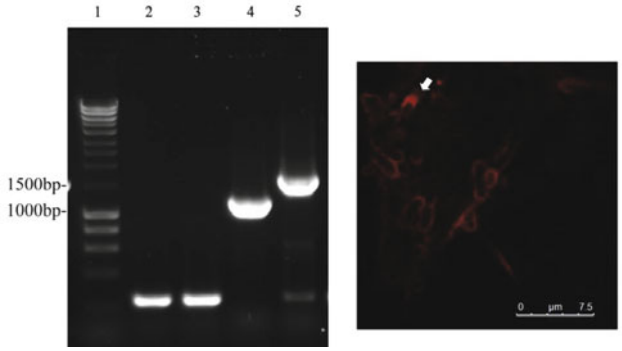

Fig. 4. Removal of *icsP* and detection of IcsA-L1 in *S. sonnei*. (A) PCR analysis of the *icsP* constructs. PCR was performed using *icsP*-specific primers. Lanes: 1, Hyperladder I (Bioline); 2, and 3, PCR products after removal of *icsP*; 4, PCR product of the intact *icsP* gene; 5, PCR product of *icsP*-kanamycin cassette. (B) Distribution of IcsA-L1 hybrid protein after removal of IcsP. Bacteria that expressed the IcsA-L1 fusion protein were revealed by immunofluorescent imaging using anti-L1 antibody followed by goat anti-mouse monoclonal antibody conjugated with TRITC (red). Arrow indicates the occasional polar display of L1 on the *S. sonnei* surface. Scale bar in (B) indicates 7.5 μm.

on the surface of the bacteria as indicated by the fluorescenceimages (Fig. 4B). However, stronger fluorescence signals were occasionally (~10%) detected at one pole of the bacterium (arrows, Fig. 4B).

The translocation efficiency of the IcsA-L1 fusion protein in *S. sonnei*

Proteinase K treatment was used to assess the translocation efficiency of the IcsA-L1 fusion in the recombinant *S. sonnei*. Whole bacterial cells were either treated with proteinase K or mock treated with water, and then the crude lysates were analysed by Western blot with the mouse monoclonal α-HPV16 L1 antibody. A specific 130-kDa band, corresponding to the IcsA-L1 fusion protein, was detected in all crude lysates (Fig. 5). Semi-quantitative analysis was carried out by measuring the optical density of the protein bands using Image-Pro Plus 6.0 software. In the presence of the functional IcsP, 90% of the fusion protein was removed by proteinase K, suggesting that only 10% of the fusion protein remained inside the cells. In the absence of IcsP, the proportion of the

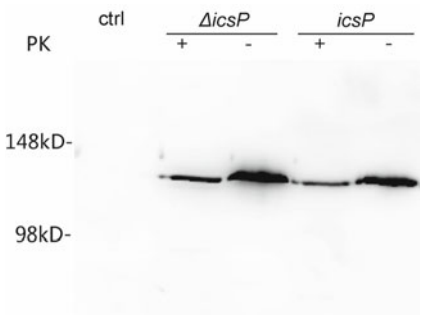

Fig. 5. Immunoblotting analysis of the proteinase K accessibility of the IcsA-L1 protein. Strains with intact (*icsP*) or *icsP*-deletion (*ΔicsP*) were treated with proteinase K (PK+) or mock-treated (PK-) with distilled water and analyzed by Western blot using anti-HPV16 L1 monoclonal antibody. Wild-type *S. sonnei* was set as negative control (ctrl).

protease-removable fusion protein was reduced to 65%, suggesting that 35% of fusion protein remained inside the cell. These results strongly suggest that IcsP is required for efficient surface expression of the IcsA-L1 fusion protein in *S. sonnei*.

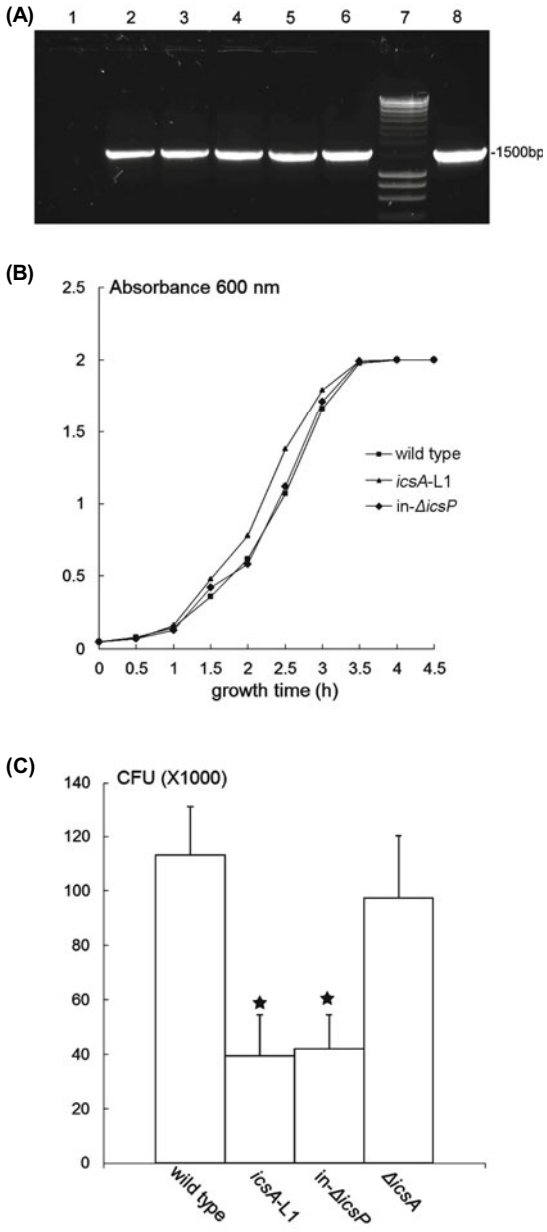

Fig. 6. (A) Detection of L1 gene by PCR using HPV16 L1-specific primers. Lanes: 1, no PCR amplification from wild-type *S. sonnei* genome; 2–6, PCR products from the 20th, 40th, 60th, 80th, and 100th generations of the *icsA*-L1 recombinant strain, respectively; 7, Hyperladder I (Bioline); and the positive control from the primary recombinant strain is set in lane 8. (B) Growth curve of the wild-type *S. sonnei* strain and the recombinant strains expressing IcsA-L1 fusion protein in the presence (*icsA*-L1) or absence (*in-ΔicsP*) of IcsP. Y axis indicates the optical density (OD600 nm) of the cultures. X axis indicates time (h). (C) Intracellular CFU recovered from cell lysates 2 h after cell invasion. Three independent experiments were carried out and one representative set of data is presented. All samples were in triplicate and error bars are shown. Stars (★) indicate statistical significance between the indicated groups with the wild type ($P < 0.001$).

The stability of *icsA*-L1 gene fusion in recombinant *S. sonnei* and the fitness of the strains

To test the stability of the L1 gene in the recombinant *S. sonnei* strain, we passaged the strain on L-ager every 12 h for 5 days, which roughly corresponds to 100 generations. The presence of the L1 gene in the genome of the recombinant *S. sonnei* was confirmed by PCR at 20, 40, 80, and 100 generations (Fig. 6A). Compared to the wild-type parental strain, integration of the L1 gene did not have any adverse effect on growth, either in the presence or absence of the *icsP* gene (Fig. 6B). The gentamycinkilling assay was used to evaluate invasiveness of the recombinant *S. sonnei* strains, with a multiplicity of infection of 10. Two hours post invasion, cells were lysed to recover intracellular CFU. Compared to the wild-type parental strain, the recombinantstrains (both in the presence or absence of *icsP*) had significantly reduced intracellular CFU. However, deletion of the *icsA* gene did not affect the intracellular CFU at this time point (Fig. 6C).

Discussion

Surface expression of foreign proteins by autotransporters has been of great interest in vaccine development. In this study, the autotransporter of *Shigella* IcsA, was employed to express the HPV16 capsid L1 protein. Expression of HPV L1 protein in Gram-negative bacteria has previously been problematic; bacterial hosts are either defective in growth (Aires *et al*., 2006) or frequently lost plasmids that express L1-protein (our unpublished observation). Here, the main passenger domain of IcsA ($IcsA_{105-506}$) was replaced with the entire HPV16 L1 gene as a translational fusion. The construct appeared to be stably integrated into the virulence plasmid in *S. sonnei* (Fig. 6A). Expression of the fusion protein was driven by the native *icsA* promoter and did not cause a growth defect as shown in Fig. 6B. Importantly, the fusion protein was found on the surface of both *S. sonnei* and *E. coli* demonstrating the transporter is still functional (Figs. 2 and 3).

In previous studies, IcsA has been shown to transport large foreign proteins including MalE and PhoA from *E. coli* to the surface of *Shigella* (Suzuki *et al*., 1995). Nevertheless, HPV16 L1 poses a greater challenge to the IcsA autotransportation system due to its viral origin, large size and complex structure (Olcese *et al*., 2004). Expression of the IcsA-L1 fusion protein in *S. sonnei* was achieved by integration of the gene fusion into the virulence plasmid to replace the original *icsA* gene. IcsA-L1 fusion protein could be translocated to the surface of the bacterium at relatively high efficiency (~90%) in recombinant *S. sonnei* (Fig. 5), confirming the capacity of the IcsA autotransporter to translocate large and complex heterologous proteins. Like the native passenger domain of the IcsA, L1 was predominately located atone pole and diffused laterally along the sides of the rod-shaped cell toward the other pole (Fig. 3). The polar distribution pattern of the foreign antigen on the surface of bacteria may have significant influence on its immunogenicity. It is plausible that the polar aggregation may be favorable for HPV16L1 to form pentamers, the basic unit of VLPs, which has been shown to be highly immunogenic (Kirnbauer *et al*., 1992).

The surface localisation of L1 was also observed when the *icsA*-L1 gene fusion was expressed in *E. coli* DH5α. Interestingly, the fusion protein was found to be evenly distributed on the *E. coli* surface (Fig. 2), which was somewhat surprising as the fusion protein had the two polar targeting regions of IcsA that have been shown to be important for the polar distribution of *Shigella* IcsA (Charles *et al*., 2001). Previous analysis has shown that the polar display components of IcsA in *S. sonnei* are also present in *E. coli* with the exception of the long-chain LPS, which is essential to maintain the polar distribution of IcsA on the surface of Gram-negative bacteria (Sandlin *et al*., 1996; Jain *et al*., 2006). Since *E. coli* DH5α has only the "core" LPS component (Chart *et al*., 2000), we speculate that the absence of long-chain LPS may be the critical determinant that prevented the polar targeting domains of IcsA to be functional in *E. coli* DH5α.

The different distribution patterns of the IcsA-L1 in *E. coli* and *S. sonnei* led us to explore other possible patterns that may provide more options for presenting heterologous antigens. We attempted to manipulate the display of HPV16 L1 through the disruption of IcsP as the IcsP cleavage site was kept intact in the IcsA-L1 construct. The role of IcsP in the regulation of IcsA distribution is controversial. Egile *et al*. (Egile *et al*., 1997) reportedthat IcsP plays an essential role in the polar localization of IcsA. However, a later study demonstrated that virulence plasmid-borne determinants (including IcsP) are not responsible for unipolar localization of IcsA (Sandlin and Maurelli, 1999). In our study, removal of IcsP generally resulted in the loss of the polar localisation and the distribution of the protein over the entire bacterial surface, resembling the localisation seen in *E. coli* (Fig. 4A). Our results are largely consistent with those reported by Egile *et al*. (1997) and d'Hauteville *et al*. (1996). However, it is also clear that IcsP is not the only determinant in the polarlocalization of IcsA as the IcsA-L1 fusion protein was occasionally located on one pole in some *icsP*-deleted bacteria (arrow, Fig. 4B), indicating that other factors are also involved in the polar distribution of IcsA, as shown by Sandlin and Maurelli (1999). Theoretically, even distribution of foreign antigens over the bacterial surface might facilitate the interaction between the immunogen and host cells, which may be immunologically advantageous. We observed that IcsP did play a role in surface expression of the fusion protein (Fig. 6), which implied that the uncleaved fusion IcsA-L1 on the surface hindered the further translocation of newly produced proteins.

The stability of the heterologous gene and its influence on the host strain is of great importance for this autotransporter system. The L1 gene could be detected even after 100 generations without any selective pressure, a major advantage over other plasmid-based expression systems. Deletion of *icsA* resulted in only a slight drop in intracellular CFU 2 h after invasion of HEp-2 cells. However, surprisingly, strains carrying the fusion construct had a significant drop in intracellular CFU (Fig. 6C). The slight drop of the intracellular CFU by the *ΔicsA* strain was presumably due to its defect in intercellular spread. It is assumed that this defect would be more pronounced at later time points. However, the reason for the significant drop in intracellular CFU in the *icsA*-L1 fusion is currently unknown. As with the *ΔicsA* mutant,

strains expressing the fusion protein should be defective in both intra- and inter-cellular dissemination. Additionally, the surface-expressed fusion protein may form structures that inhibit initial invasion or intracellular dissemination.

In summary, our study demonstrated the feasibility of using IcsA to present the large, complex HPV16 L1 protein on the surface of Gram-negative bacteria. We demonstrate that IcsP is a critical factor required for polar distribution of the hybrid protein in *S. sonnei*. Evaluation of the advantages of surface expression of the viral protein in eliciting immune responses is currently underway.

Acknowledgements

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