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Examination of the cell sensitizing gene orf43 of ICE R391 suggests a role in ICE transfer enhancement to recipient cells

Patricia Armshaw and J. Tony Pembroke

Molecular and Structural Biochemistry Laboratory, Materials and Surface Science Institute, Department of Chemical and Environmental Sciences, University of Limerick, Limerick, Ireland

*Corresponding author: Molecular and Structural Biochemistry Laboratory, Materials and Surface Science Institute, Department of Chemical and Environmental Sciences, University of Limerick, Limerick, Ireland. Tel: +353-61-202491; Fax: +353-61-202568; E-mail: tony.pembroke@ul.ie

One sentence summary: Cell sensitizing conjugation gene enhances ICE transfer rates.

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ABSTRACT

SXT/R391 family of ICEs have been found to express an unusual function that enhances bacterial cell death post-UV irradiation. Previous analysis of ICE R391 found four core SXT/R391 ICE genes to be involved—orf96, orf90, orf91 and orf43. These genes functioned as part of a UV-inducible pathway, where upon exposure to UV, the levels of the Orf43 protein, a TraV homolog which we propose naming TraV\textsubscript{R391}, were upregulated, resulting in increased cell sensitization. Here, we examined the effect of orf43 overexpression and found it led to host cell permeabilization. The inducing agent for orf43, UV irradiation, is also known to increase the ICE R391 extrachromosomal form and apparent conjugative transfer rate. We demonstrated, via conjugative transfer deficient mutants, that orf43 overexpression alone restored a small level of ICE R391 transfer to recipient cells via an unknown mechanism other than conjugation. TraV homologs have been reported to function in conjugative transfer. However, TraV\textsubscript{R391} is the first homolog to cause UV-associated cell sensitization. TraV\textsubscript{R391} when overexpressed must contain a unique adaptation or function which results in cell lysis and decreased survival. A hypothesis for retaining such a detrimental effect may be in its role of enhancing ICE survival upon cell damage.

Key words: SXT/R391-like elements; type IV secretion systems; UV-sensitizing; cell escape mechanism

INTRODUCTION

ICE R391 [GenBank: AY090559] is a prototype member of the SXT/R391 family of enterobacterial integrative conjugative elements (ICEs), a group of some 89 elements that display both plasmid and transposon traits (Böltnet al., 2002; Bi et al., 2012). Such elements have been discovered extensively in epidemic Vibrio cholerae strains and encode antibiotic-resistance determinants, metal resistance and numerous adaptive traits (Taviani et al., 2009; Wozniak et al., 2009). ICEs transfer between host cells by utilizing a highly conserved plasmid-like type IV conjugation system (T4SS) that is self-encoded. They integrate site specifically into the prfC gene of recipient cells without disrupting the function of the gene and form a novel functioning of prfC hybrid gene which is part of ICE-encoded (Hochhut and Waldor 1999; McGrath and Pembroke 2004). As with other mobile elements, ICEs are thought to be maintained within the host as they provide adaptive or beneficial traits (Pembroke et al., 2002; Guglielmini et al., 2011). However, members of the SXT/R391 family of ICEs have also been found to encode an unusual...
Table 1. Bacterial strains, genetic elements and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/Phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157</td>
<td>F−, thr−1, araC14, leuB6, Δ(gpt-proA)62, lacY1, tpx-33, gsr−0, gntV44, galK2, lac−, lac−, lac−, lac−, hisG4, rfbC1, mgt-51, rpsS36, rpsL31(StrR), kdgK51, xylA5, mti-1, argE3, thi-1</td>
<td>E. coli genetic stock centre (CGSC), Yale University, New Haven, Connecticut, USA</td>
</tr>
<tr>
<td>TOP10</td>
<td>F−, mcrA0, Δ(mrr-bsdRMS-mcrBC), s080lacZ58(M15), ΔlacX74, recA1, araD139, Δ(araA-leu)7697, galU−, galK0, rpsL−(StrR), endA1, nupG−</td>
<td>Bio-Sciences, Dun Laoghaire, Dublin, Ireland.</td>
</tr>
<tr>
<td>NK5148</td>
<td>F−, thrA34::Tn10 (Teβ), lac−, Δ(rrnD-rrnE)1</td>
<td>CGSC</td>
</tr>
<tr>
<td>Plasmid/ICE</td>
<td>Genotype/Phenotype</td>
<td>Source</td>
</tr>
<tr>
<td>pBAD33-orf43</td>
<td>CmR, pT5A ori, pBAD, L-arabinose inducible, orf43</td>
<td>Armshaw and Pembroke (2013b)</td>
</tr>
<tr>
<td>ICE R391</td>
<td>KmR, HgR</td>
<td>Dr R.W. Hedges, Royal Postgraduate Medical School, London, UK.</td>
</tr>
<tr>
<td>ICE R391 Mutant</td>
<td>Genotype/Phenotype</td>
<td>Source</td>
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<tr>
<td>AB1157 R391 Aoorf40–orf41</td>
<td>ICE R391 orf40–orf41 deletion strain, AmR, UV−, tra−</td>
<td>Armshaw and Pembroke (2013b)</td>
</tr>
<tr>
<td>AB1157 R391 Aoorf43</td>
<td>ICE R391 orf43 deletion strain, AmR, UV−, tra−</td>
<td>Armshaw and Pembroke (2013b)</td>
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<tr>
<td>AB1157 R391 Aoorf50–orf91</td>
<td>ICE R391 orf50–orf91 deletion strain, AmR, UV−, tra−</td>
<td>Armshaw and Pembroke (2013b)</td>
</tr>
<tr>
<td>AB1157 R391 KOB</td>
<td>ICE R391 orf32 – orf42 (29575bp – 41527bp) deletion strain, AmR, UV−, tra−</td>
<td>Armshaw and Pembroke (2013a)</td>
</tr>
</tbody>
</table>

StrR, streptomycin resistant; CmR, chloramphenicol resistant; KmR, kanamycin resistant; HgR, mercury resistant; AmR, ampicillin resistant; Teβ, tetracycline resistant. UV−, exhibits sensitizing phenotype; UV+, sensitizing phenotype abolished; tra−, transfer deficient mutant.

recA-dependent, UV-inducible function which decreases host cell survival after exposure to UV irradiation (Pembroke and Stevens 1984; Wang et al., 1996). The preservation of this function is unexpected as it is detrimental to the host cell.

For ICE R391, this phenotype and the molecular mechanism by which it occurs have been shown to be due to induction of a UV-inducible control loop (Armshaw and Pembroke 2013a,b). UV irradiation initiates the cleavage of ICE R391 encoded orf46 (a lambda cl-like repressor) in a recA-dependent manner (Beaber, Hochnut and Waldor 2004). This repressor cleavage results in the expression of orf50–orf91 which encodes a putative transcriptional enhancer, conserved in all SXT/R391 ICE elements and related to the flhC/D transcriptional regulator of flagellar synthesis (O’Halloran et al., 2007). The transcriptional enhancer has been found to upregulate orf43 expression and this appears to be the executioner of the UV-sensitizing effect. As orf43 is proposed to encode a TraV-like homolog (Bolnter et al., 2002; Alvarez-Martinez and Christie 2009; Bi et al., 2012) which functions in ICE conjugative transfer, its role in sensitization is unexpected.

The orf43 gene is one of the most highly conserved core genes (≥98% at the nucleotide level) within the SXT/R391 ICE family (Armshaw and Pembroke 2013b). It is proposed to be a TraV homolog as deletion of this gene abolishes ICE R391 transfer and bioinformatical analysis positions orf43 within an operon of other predicted transfer gene homologues—traL (orf39), traE (orf40), traK (orf41), traB (orf42), traV (orf43) and traA (orf44) (Bolnter et al., 2002; Armshaw and Pembroke 2013b).

To date, little is known about the function of TraV-like proteins (Alvarez-Martinez and Christie 2009). However, a TraV homolog has been structurally characterized as part of the membrane-spanning pore complex expressed from pKM101. This has given some insight into the localization and function of TraV-like proteins (Fronzes et al., 2009). Generally, they are thought to be outer membrane-associated lipoproteins that aid in conjugation that vary widely in terms of size, sequence conservation and that function as part of a complex stabilizing the mating pore required for DNA transfer between donor and recipient cells (Lawley et al., 2003; Alvarez-Martinez and Christie 2009; Fronzes et al., 2009). There is no data available on the mechanism by which the expression of the ICE R391 TraV homolog (TraV_{R391}) might cause UV sensitization and cell death (Armshaw and Pembroke 2013a).

Here, orf43 was initially cloned with its native UV-inducible promoter in a Δ orf43 (Table 1, AB1157 R391 Δorf4) deletion background to mimic natural expression. UV irradiation increased cell death rates at a rate comparable to ICE R391-containing cells. To examine how TraV_{R391} overexpression caused cell sensitization, the tightly regulated pBAD promoter was employed to allow controlled overexpression of the protein (Armshaw and Pembroke 2013b) and probe the cell damage resulting from orf43 overexpression. The effect of induced TraV_{R391} overexpression on cells was characterized via a number of methods including transmission electron microscopy (TEM), confocal scanning laser microscopy (CSLM) and dye uptake assays. A hypothesis for the conservation of such an apparently detrimental gene is discussed.
MATERIALS AND METHODS

Construction of pBAD33-orf43

The construction of pBAD33-orf43 was previously described (Armshaw and Pembroke 2013b). The orf43 gene sequence was amplified by PCR from AB1157 ICE R391 genomic DNA and cloned into pBAD33 via the XbaI and PstI restriction sites. The resulting clone was confirmed by DNA sequencing and confirmed to complement for the UV sensitizing phenotype in Δorf43 deletion strain. pBAD33 contains an arabinose inducible, glucose repressible pBAD promoter (Guzman et al., 1995).

Bacterial strains, elements and media

The bacterial strains, plasmids and ICE R391 deletion mutants utilized as part of this study are listed in Table 1. Strains were stored at −80°C in either Luria-Bertani (LB) broth or M9 minimal media containing 50% (v/v) glycerol. Media was supplemented with appropriate antimicrobial agents: ampicillin, 100 μg ml⁻¹; chloramphenicol, 25 μg ml⁻¹; kanamycin, 30 μg ml⁻¹; streptomycin, 100 μg ml⁻¹; mercuric chloride, 20 μg ml⁻¹; tetracycline, 20 μg ml⁻¹ as required. For all growth and analysis of strains containing pBAD33-orf43, M9 minimal media containing 0.4% (w/v) glycerol was used, with either 0.4% (w/v) glucose or 0.02–0.2% (w/v) L-arabinose to repress or induce gene expression respectively as previously described (Armshaw and Pembroke 2013b).

Generation of ICE R391 deletion strains

The generation of ICE R391 specific deletions was previously described (Armshaw and Pembroke 2013b). Deletions were generated by homologous recombination methods based on the λ Red recombination system (Murphy 1998; Chaveryo, Ghigo and d’Enfert 2000; Datsenko and Wanner 2000). All deletion mutations generated were ampicillin resistant and verified by PCR using primers that spanned the deleted regions and subsequent sequencing of these amplicons. The conjugative transfer ability of these deletion strains was analyzed [Table 1].

Trypan blue dye exclusion assay

Trypan blue dye exclusion assays were carried out according to the methods described (Kucsera, Yarita and Takeo 2000). Briefly, overnight cultures were washed twice with 0.85% NaCl and used as inoculate for M9 minimal media containing 0.4% glycerol and 0.4% trypan blue agar plate. Plates were incubated at 37°C until colony growth was evident. 0.2% L-arabinose solution was then used to coat colonies on plates to induce pBAD33-orf43 overexpression and plates were incubated at 30°C for a further 12 to 24 h until dye uptake was observable. Assays were repeated a minimum of three times for each strain tested. Representative results are displayed.

CSLM analysis

Bacterial cell damage was assessed using the Bacteria Live/Dead Staining Kit from PromoKine (Promokine, PK-CA707–30027). Live/Dead stained samples were visualized using a Meta 710 laser scanning confocal microscope excited with a 405 nm argon–krypton laser using appropriate standard filter (Carl Zeiss, Germany). Image processing was performed using the ZEN 2011 software package (Carl Zeiss, Germany). All confocal images were acquired with the same settings with respect to laser intensity and detector gain. Cultures of each test strain were grown for 12 h in M9 minimal media containing 0.4% glycerol with 0.4% glucose and appropriate antibiotics. Aliquots were washed twice with 0.85% NaCl and used as inoculate in inducing or non-inducing assay media as required. Strains were then incubated at 30°C, shaking at 200 rpm until an OD600nm of at least 0.2 was obtained. Aliquots were washed twice and resuspended in 0.85% NaCl and then stained with Calcein-AM green fluorescent nucleic acid stain which stains both intact and membrane damaged cells, and ethidium homodimer-III (EtD-III) red fluorescent nucleic acid stain which only stains membrane permeabilized cells using the standard manufacturer’s protocol for the Bacteria Live/Dead Staining Kit and visualized using the CSLM system described. All strains were analyzed a minimum of three times. Representative results are displayed.

TEM analysis

TEM was performed using a JEOL JEM-2011 transmission electron microscope (JEOL UK Ltd, Herts AL7 1LT, UK) operating at an accelerating voltage of 200 kV. Images were recorded with a Gatan DualVision 600™ CCD camera (Gatan GmbH, München, Germany) and analyzed using the Gatan Digital Micrograph version 3.6.5 software. Strains were cultured as described for CSLM analysis. Samples were prepared as per the standard manufacturer’s protocol (Electron Microscopy Sciences, Hatfield, PA, USA). Samples were washed once in 10 mM HEPES and resuspended in fixative solution (2.5% formaldehyde, 100 mM sodium cacodylate buffer, pH 7.4). Fixative solution was prepared by addition of 0.66 ml of 38% formaldehyde solution to 9.34 ml 100 mM sodium cacodylate buffer, pH 7.4. Samples were left to fix at room temperature for a minimum of 4 h, fixed cells were washed and resuspended in 100 mM sodium cacodylate buffer, pH 7.4. An equal volume of 2.5% phosphotungstic acid staining solution was then added to samples. Cell samples were incubated in staining solution for 1 min and then resuspended in 100 mM sodium cacodylate buffer, pH 7.4. 10 μl droplets of stained cell samples were placed on formvar coated TEM grids from Electron Microscopy Sciences (Hatfield, PA, USA), air dried and analyzed using the TEM system described. All strains were analyzed a minimum of three times. Representative results are displayed.

Conjugation rate determination

Overnight cultures were washed twice with 0.85% NaCl and used to inoculate inducing or non-inducing media as required and grown at 30°C without antibiotics, shaking at 200 rpm until an optical density at 600 nm of 0.4 was reached. Bacterial conjugation was then carried out according to the methods described (McGrath et al., 2005). Briefly, a donor to recipient ratio of 5:1 was used. The mating mixes were incubated at 30°C without agitation for 16 to 24 h. Samples were washed with 0.85% (w/v) NaCl and dilutions plated on appropriate transconjugant selective media (tetracycline for recipient strain, kanamycin for ICE R391 and ampicillin for deletion mutants) and incubated at 30°C for up to 24 h.Recipient strain used in all cases was Escherichia coli N35148. As a control, the donor and recipient cultures were also spread on the transconjugant selective media. Frequency of transfer was determined as the number of transconjugants per final number of recipient cells. Transconjugant colonies were cultured again in selective antibiotics (tetracycline, kanamycin, ampicillin) and verified by PCR with appropriate primers to contain ICE R391 and any ICE R391 deletion mutations that were
present in the donor strain used. Transfer rates were calculated from a minimum of three separate conjugation experiments. The limit of detection of the assay was a transfer rate of less than $10^{-10}$.

**RESULTS**

**TraV$_{R391}$ overexpression causes cell membrane damage**

The trypan blue dye exclusion assay (Fig. 1) determined that the blue dye was excluded from cells unless TraV$_{R391}$ overexpression was induced via pBAD33-orf43. This suggested that TraV$_{R391}$ overexpression was affecting membrane integrity in some manner. Further, CSLM analysis of fluorescently stained cells (Fig. 2, Table 2) demonstrated that overexpression of TraV$_{R391}$, again via pBAD33-orf43, allowed the membrane impermeable red dye (EtD-III) to penetrate into over 90% of the cell population. This indicated that TraV$_{R391}$ overexpression resulted in permeabilization of the cell membrane.

A comparative analysis of these cells with those treated by isopropyl alcohol (IPA) was carried out (Fig. 2e). IPA causes cell dehydration resulting in membrane disruption and cell lysis (McDonnell and Russell 1999). TraV$_{R391}$ overexpressing cells appeared as larger higher intensity (trapping EtD-III within cells) fluorescing units compared to those treated by IPA. In contrast, the IPA-treated cell samples appeared to contain mainly lysed cells.

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**Figure 1.** Trypan blue dye exclusion assay showing the effect of TraV$_{R391}$ overexpression on cell colonies. Top row: un-induced colonies; bottom row: colonies after induction and 12–24 h incubation at 30°C. From left to right: (a) AB1157, (b) AB1157 pBAD33-orf43, (c) AB1157 ICE R391, (d) AB1157 ICE R391 Δorf43 and (e) AB1157 R391 Δorf43 pBAD33-orf43. Induction of pBAD33-orf43 caused cytotoxic TraV$_{R391}$ overexpression which compromised cell membrane integrity allowing absorption of the trypan blue dye into the host cell.

**Figure 2.** CSLM fluorescence images showing the effect of TraV$_{R391}$ overexpression on membrane permeability. Calcein-AM (green) and EthD-III (red) were used to stain cells. Intact viable cells fluoresce green and cells with permeabilized membrane fluoresce red. For A–D, top row shows Calcein-AM excited cells, middle row EthD-III excited cells and bottom row combined view. From left to right: (a) TOP10, (b) TOP10 pBAD33-orf43, (c) TOP10 pBAD33-orf43 induced and (d) TOP10 after IPA incubation. For (e) Top row shows the combined view of Calcein-AM excited and EthD-III excited induced TOP10 pBAD33-orf43 cells and bottom row IPA-treated TOP10.
overexpression was induced via pBAD33-orf43 overexpression. Resulting in no observable ICE R391 transfer upregulation by UV irradiation such as AB1157 R391 KOB which showed had no detectable ICE transfer above this limit. However, when TraV$_{R391}$ overexpression was induced via pBAD33-orf43, ICE R391 transfer to recipient cells was observed in these tra$^-$ strains (Table 3; AB1157 R391 $\Delta$orf43). This indicated that there was some detectable ICE transfer occurring by a mechanism other than conjugation in these strains. Addition of DNase to AB1157 R391 $\Delta$10 pBAD33-orf43 conjugation mixtures prevented the formation of transconjugants [unpublished]. This indicated that the orf43-promoted ICE transfer process may include the release of circularized ICEs through cell membrane disruption. How this released ICE R391 DNA may be taken up by recipient cells is unclear.

Deletion of orf43 resulted in no observable ICE R391 transfer even after pre-exposure to UV irradiation (Table 3; AB1157 R391 $\Delta$14) to increase the level of circularized ICE R391 for transfer (O’Halloran et al., 2007). However when orf43 was present and expressed in the conjugative transfer deficient donor strains, either natively on the ICE or overexpressed on pBAD33-orf43 (Table 3; AB1157 R391 $\Delta$14 pBAD33-orf43, AB1157 R391 $\Delta$10 pBAD33-orf43, AB1157 R391 $\Delta$11), there was observable ICE R391 transfer to recipient cells. Since the AB1157 R391 $\Delta$10 ($\Delta$orf40-orf44) deletion abolished genes necessary for conjugation, this small level of ICE transfer is likely occurring by another unknown mechanism.

In any deletion strain that prevented native orf43 upregulation by UV irradiation such as AB1157 R391 KOB which

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Calcein-AM (‘Live’)</th>
<th>% EthD-III (‘Dead’)</th>
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</thead>
<tbody>
<tr>
<td>TOP10 (‘Live’ control)</td>
<td>98.21</td>
<td>1.79</td>
</tr>
<tr>
<td>TOP10 pBAD33</td>
<td>98.57</td>
<td>1.43</td>
</tr>
<tr>
<td>TOP10 pBAD33-orf43</td>
<td>95.66</td>
<td>4.34</td>
</tr>
<tr>
<td>TOP10 pBAD33-orf43 (0.2% L-arabinose)</td>
<td>6.59</td>
<td>93.41</td>
</tr>
<tr>
<td>TOP10 (‘Dead’ control)</td>
<td>10.3</td>
<td>89.7</td>
</tr>
</tbody>
</table>

Mean fluorescent intensity for both Calcein-AM and EthD-III for five fields of view was averaged to estimate the percentage of the cell population permeabilized by the induction of pBAD33-orf43 overexpression in TOP10 cells. A ‘Live’ control was analyzed to ensure that sample preparation had minimal effect on cellular membrane integrity. The ‘Dead’ control was prepared by incubating TOP10 cells in 70% isopropyl alcohol (IPA) for 60 min before sample preparation.

Dispersed cell debris that fluoresced with lower intensity. This indicated that the stain was not retained within IPA-treated cells. From this analysis, it was determined that it was likely that TraV$_{R391}$ overexpressing cells were aggregated.

To visualize the nature of this aggregation following overexpression of TraV$_{R391}$ from pBAD33-orf43, we utilized TEM analysis (Fig. 3). Induced cells were observed to predominately aggregate compared to controls. Cells within aggregates displayed a lower level of stain contrast. Some aggregated cells were observed to lose clearly definable rod-shaped cell structure and have a ghost-like transparent appearance. Such an appearance has been correlated with loss of cytoplasmic content (Regev-Yochay et al., 2007). An extracellular material was observed around cells, which trapped unknown electron dense particles and may indeed be composed of lysed cells debris which may aid in the aggregation of cells that had not yet lysed. The observations described are suggestive that under typical UV-induced conditions, TraV$_{R391}$ overexpression may cause increased cell death rates by causing damage to cell membrane integrity.

**Overexpressed TraV$_{R391}$ is beneficial for ICE R391 post-UV irradiation survival**

TraV$_{R391}$ overexpression was found to damage cell membrane integrity. Conjugative transfer assays of a range of transfer deficient ICE R391 deletion mutants (tra$^-$) were carried out to determine if overexpression of TraV$_{R391}$, either natively on the ICE R391 chromosome or overexpressed through pBAD33-orf43, would allow some ICE R391 transfer to recipient cells. This analysis was carried out to determine if cell membrane damage by TraV$_{R391}$ could be advantageous for ICE survival post-UV exposure.

Table 3 presents data on the apparent conjugative transfer rates of ICE R391 transfer deficient deletion mutants (tra$^-$). It was observed that upon exposure to UV irradiation, the transfer rate of ICE R391 (Table 3; AB1157 R391) increased as previously documented (O’Halloran et al., 2007). This has previously been shown to be due to the increased levels of the circular form of ICE R391 available for transfer through the orf59/91-mediated increased expression of orf4 encoded excisionase (O’Halloran et al., 2007). This was confirmed as we observed no UV-induced increase in transfer in orf4 deletion strains (Table 3; AB1157 R391 $\Delta$orf4).

The limit of detection of the assay was found to be $10^{-10}$ per donor cell. All tra$^-$ mutants without overexpressed TraV$_{R391}$ screened had no detectable ICE transfer above this limit. However, when TraV$_{R391}$ overexpression was induced via pBAD33-orf43, ICE R391 transfer to recipient cells was observed in these tra$^-$ strains (Table 3; AB1157 R391 $\Delta$10 pBAD33-orf43). This indicated that there was some detectable ICE transfer occurring by a mechanism other than conjugation in these strains. Addition of DNase to AB1157 R391 $\Delta$10 pBAD33-orf43 conjugation mixtures prevented the formation of transconjugants [unpublished]. This indicated that the orf43-promoted ICE transfer process may include the release of circularized ICEs through cell membrane disruption. How this released ICE R391 DNA may be taken up by recipient cells is unclear.

Deletion of orf43 resulted in no observable ICE R391 transfer even after pre-exposure to UV irradiation (Table 3; AB1157 R391 $\Delta$14) to increase the level of circularized ICE R391 for transfer (O’Halloran et al., 2007). However when orf43 was present and expressed in the conjugative transfer deficient donor strains, either natively on the ICE or overexpressed on pBAD33-orf43 (Table 3; AB1157 R391 $\Delta$14 pBAD33-orf43, AB1157 R391 $\Delta$10 pBAD33-orf43, AB1157 R391 $\Delta$11), there was observable ICE R391 transfer to recipient cells. Since the AB1157 R391 $\Delta$10 ($\Delta$orf40-orf44) deletion abolished genes necessary for conjugation, this small level of ICE transfer is likely occurring by another unknown mechanism.

In any deletion strain that prevented native orf43 upregulation by UV irradiation such as AB1157 R391 KOB which

![Figure 3. Representative TEM images showing effect of TraV$_{R391}$ overexpression on cell integrity. TOP10 pBAD33-orf43 induced cells appeared to aggregate, lose defined cell structure and secrete an unknown material that trapped surrounding electron dense particles or intracellular precipitate. Some cells on extremities of aggregate material appeared to have maintained defined rod-shaped cell structure (most likely uninduced cells). On closer inspection of aggregates, it appeared that cells within have lost their defined structure and stain contrast (transparent ghost-like appearance of cells).](image-url)
removes the predicted orfs90/91 control site in front of orf43 (Armshaw and Pembroke 2013a) or AB1157 R391 ∆26 which removed orfs90/91 (Armshaw and Pembroke 2013a), there is no observable ICE R391 transfer to recipient cells. This is the case even though UV irradiation also increases the levels of the excised circular form of the element available for transfer (O’Halloran et al., 2007).

**DISCUSSION**

The orf43 gene is one of the most highly conserved core genes of the SXT/R391 family of ICEs. Previously, it has been shown that its overexpression is cytotoxic (Armshaw and Pembroke 2013a,b). Here, we demonstrated that this most likely occurs by a mechanism that causes disintegration of the cell membrane. Given that UV-induced TraVorf43 overexpression is cytotoxic, the question arises: Why is the orf43 gene so highly conserved and maintained if it is conditionally lethal to host cells?

Mobile elements are known to be selfish DNA elements that prioritize their own spread and survival between host genomes. Therefore, we propose that orf43 is highly conserved both to ensure its role in the conjugative transfer system and upon overexpression, to enhance ICE R391 transfer or survival from damaged cells by causing cell lysis. We hypothesize that upon UV damage or indeed other forms of induced cellular stress that would activate RecA, orfs90/91 and subsequently orf43 and orf4 are induced, enhancing ICE R391 escape from the damaged host cell in what we term a ‘trap door’ mechanism.

According to O’Halloran et al., after UV irradiation, orfs90/91 increases the expression of orf4 (jej), the ICE excisionase that increases the level of the circular intermediate form of ICE R391 for transfer. This results in the provision of increased copies of the element for release (O’Halloran et al., 2007). Cell lysis at this stage, mediated by orf43, would allow rapid escape of these circular forms, allowing them to potentially act as targets for transformation into suitable recipients. Evidence to further support this hypothesis is presented in Table 3 where the ability of overexpressed TraVorf43 to promote transfer of ICE R391 transfer-deficient mutants is presented.

It should be noted that the transfer rates observed in Table 3 appear to be quite low compared to normal ICE R391 transfer rates. Expression of pBAD33-orf43 in an orf43 deletion mutant (Table 3; AB1157 R391 ∆14) does complement for ICE transfer to some degree but it does not return rates back to the expected native level (1.54 × 10⁻⁷ versus 5 × 10⁻⁵). This lack of full complementation may be due to the fact that overexpression of TraVorf43 in pBAD33-orf43 would presumably be higher than is natively produced even after UV irradiation upregulation. As TraVorf43 overexpression is cytotoxic, this level may inhibit maximal potential conjugative transfer rates from being achieved. Induction of TraVorf43 to levels above that which is normally present in the cell could cause significant donor cell lysis, preventing the normal conjugation mechanism from occurring even though all conjugative proteins required are present.

In AB1157 R391 ∆11, where two of the conjugation genes, orf40 and orf41, are deleted but the orf43 gene and its upstream control site are maintained, the average transfer rate after UV irradiation exposure is 2.48 × 10⁻⁷. This may be the most accurate indication of the contribution of orf43-mediated cell lysis to the overall post-UV irradiation ICE R391 transfer rate. However once again, many factors may have contributed to lowering this rate below the actual native levels. For instance, optimal dosage of UV irradiation, length of donor-recipient incubation time and the environment tested may all have a significant effect on this transfer rate.

In conclusion, from the data presented, we hypothesize that overexpression of TraVorf43, while detrimental to host cell survival, is beneficial for ICE survival following UV irradiation. We propose that this system is maintained to act as a stress inducible ‘trap door’ mechanism for enhancing element escape from damaged cells.
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