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Plasmid 59 (2008) 86–101
PLASMID
www.elsevier.com/locate/yplas

Entry exclusion in the IncHI1 plasmid R27 is mediated by EexA and EexB

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Received 8 June 2007, revised 4 October 2007
Available online 14 January 2008
Communicated by Stuart Levy

Abstract

Conjugative plasmids have evolved entry exclusion mechanisms to inhibit redundant DNA transfer from donor cells into recipients harboring isogenic or closely related plasmids. This exclusion phenomenon has been documented in the incompatibility H group (IncH) plasmid R27. A cosmid library representing the majority of the large (180 kb) R27 plasmid was transformed into recipient cells and a conjugation assay identified that an operon located in the conjugative transfer region 2 (Tra2) of R27, the Z operon, mediated entry exclusion in the IncH plasmid. Reverse-transcriptase analysis revealed that the Z operon is comprised of four genes, *015*, *eexB*, *017*, and *eexA*. Sub-cloning of the individual genes located within the Z operon and subsequent screening for the entry exclusion phenotype determined that two genes, *eexA* and *eexB*, independently inhibit the entry of IncH-related plasmids. Bacterial fractionation studies predominantly localized the EexA protein to the cytoplasmic membrane, and the EexB protein to the outer membrane. Recipient cells expressing EexA and EexB were unable to exclude the entry of R27 plasmids harboring mutations within the IncH entry exclusion genes *eexA* and *eexB*. The IncH entry exclusion proteins EexA and EexB likely prevent redundant plasmid transfer by interaction with one another.

Keywords: Conjugation; Entry exclusion; Surface exclusion

1. Introduction

Horizontal DNA transfer enables the rapid dissemination of genetic information with evolutionary and medical implications. Bacterial conjugation is a mechanism of horizontal gene transfer requiring cell–cell contact (Willetts and Skurray, 1980). An early study of extrachromosomal inheritance in bacteria revealed that the conjugation frequency of F+ strains decreased significantly when the recipient was an F+ strain when compared to the conjugal mating frequencies when F− strains were the mat-

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doi:10.1016/j.plasmid.2007.11.004

ing recipients (Lederberg and Lino, 1956). This conjugative inefficiency is due to two processes, entry exclusion and plasmid incompatibility (Novick, 1969). Plasmid incompatibility is defined as the inability of two co-resident plasmids to be stably inherited in the absence of selective pressures (Novick, 1987). In contrast, entry exclusion functions by inhibiting the initial entry of DNA from donor cells, or by an outer membrane protein preventing cell-to-cell contact between cells harboring isogenic or closely related plasmids (Novick, 1969).

Entry exclusion (Eex) activity has been described in conjugative elements from a diverse array of incompatibility families including IncP (Haase et al., 1995, 1996), IncW (Llosa et al., 1994), IncF (Achtman et al., 1977, 1980), IncN (Pohlman et al., 1994), IncI (Hartskeerl and Hoekstra, 1984; Hartskeerl et al., 1986) and recently, IncJ (Marrero and Waldor, 2005, 2007). Notably, each conjugative element proficient in Eex activity can be grouped into one of two Eex classes: plasmids that encode one Eex polypeptide and plasmids that encode two independent polypeptides. RP4 is a well-studied member of the first Eex class of plasmids, as the IncPα plasmid encodes the lipoprotein TrbK, which is the only necessary determinant responsible for the Eex phenotype in this broad-host range plasmid (Haase et al., 1995). Cellular localization studies of cells harboring the RP4 plasmid revealed that TrbK is predominantly associated with the cytoplasmic membrane of the cell envelope. The mechanism by which TrbK elicits an Eex phenotype in recipient cells has been proposed to be due to an interaction between TrbK and the mating apparatus in donor cells containing a RP4-related plasmid (Haase et al., 1996).

The most intensively studied member of the second Eex class of plasmids in which two exclusion polypeptides elicit an Eex phenotype is the F plasmid. Similar to the TrbK protein of RP4, the F plasmid Eex protein TraS has been localized to the cytoplasmic membrane of F plasmid-containing cells (Achtman et al., 1979). Moreover, there is evidence to suggest that a similar interaction occurs between TraS and the stabilization protein TraG of the F plasmid mating apparatus (Frost et al., 1994; Audette et al., 2007). The F plasmid also encodes the lipoprotein TraT, a second independent exclusion protein, which localizes to the outer membrane of the cell and prevents stable mating pair formation (Perumal and Minkley, 1984; Sukupolvi and O'Connor, 1990).

IncHI plasmids move between different bacteria via a Type IV secretion system. This system consists of twelve to thirteen proteins which make up the mating-pair formation apparatus (Lawley et al., 2003). Identification of the proteins within R27 that mediate exclusion is the first step in elucidating the role they play in the overall procure of Type IV secretion.

Previous studies on the IncHI plasmid family have identified an Eex activity mediated by the IncHI1 plasmid pRG1251, however, the specific protein(s) responsible for this event were not identified (Taylor and Grant, 1977). The IncHI group is further subdivided into three subgroups: IncHI1, IncHI2, and IncHI3 based on their incompatibility with the *F*-factor, as well as resistance to tellurite and phage. Conjugative plasmids of the IncHI1 family encode multiple drug resistance in *Salmonella enterica* serovar Typhi, the causative agent of typhoid fever, thus increasing the difficulty in treating the bacteria-induced fever (Fica et al., 1997; Parkhill et al., 2001). Members of the IncHI2 plasmids are commonly responsible for antibiotic resistance in non-typhoidal *Salmonella* from humans and animals (Taylor et al., 1978). A unique feature of the IncHI plasmids is the temperature-sensitive nature of their mobilization with optimal conjugation of IncHI plasmids occurring at 30 °C, and reduced conjugation rates at 37 °C (Taylor and Levine, 1980). Recent transcriptional studies of genes essential for the transfer of the prototypical IncHI1 plasmid R27 indicate that the temperature-control is mediated at the transcriptional level (Alonso et al., 2005; Gunton et al., 2005).

The objective of this work was to identify the R27 determinants which mediate Eex activity in the IncHI plasmid family. A screen of a cosmid library containing the majority of the R27 plasmid DNA revealed that the Z operon, located in the Tra2 region of R27, was able to confer an R27 Eex phenotype to recipient cells. Subsequent subcloning of the Z operon identified two R27 entry exclusion proteins as EexA (previously named trhZ Lawley et al., 2003) and EexB (previously named orf016). RT-PCR analyses of the Z operon indicated that transcription of the R27 exclusion proteins is repressed at 37 °C, a temperature at which R27 conjugation is markedly decreased. The specificity of the exclusion action elicited by EexA and EexB on R27-related and non-related plasmids was determined using an exclusion assay. Bacterial fractionation studies revealed the cellular location of the R27 exclusion proteins EexA and EexB

within the inner and outer membrane, respectively. A possible mechanism for Eex in R27 is discussed.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and plasmids

Escherichia coli strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 30 °C (DY330R) or 37 °C (RG-11, JC3272) in Luria–Bertani (LB) broth (Difco Laboratories, Detroit, MI) with shaking or on LB agar plates. Antibiotics used in this study are listed with final concentrations as follows: ampicillin (100 μg ml^{-1}), kanamycin, (50 μg ml^{-1}), streptomycin (100 μg ml^{-1}), rifampicin (20 μg ml^{-1}), chloramphenicol (16 μg ml^{-1}), tetracycline (10 μg ml^{-1}), trimethoprim (12.5 μg ml^{-1}), and potassium tellurite (15 μg ml^{-1}).

2.2. Eex studies

All Eex matings (with the exception of those involving DY330R containing IncPα conjugative plasmid RP4 as a donor strain) were initiated from overnight cultures which were sub-cultured (1/20 dilution) and donor and recipient

Table 1
Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant genotype, phenotype, or characteristic[a]	Source or reference
E. coli		
DH5α	*supE44 lacU169 (ϕ80lacZΔM15) hsdRU recA1 endA 1 gyrA96 thi-1 relA 1*	Invitrogen
XL1-blue	*recA 1 endA 1 gyrA96 thi-1 hsdRU supE44 relA 1 lac [F′ proAB laclqZ AM 15 Tn 10]*	Stratagene
DY330R	W3110 *ΔlacU169 gal-490* λcl857 Δ(cro-bioA) temperature-susceptible revertant; Rif^r	Christie and Vogel (2000), Lawley et al. (2002)
RG11	Str^r *his rpsL (chlD-pgl) λ*^{s}	D.E. Taylor, unpublished
J53-1	pro met; Nal^r	Bachman (1972)
JC3272	Sm^r; F$^-$ *lac ΔX74 gal his trp lys rpsL tsx*	Achtman et al. (1971)
Plasmids		
R27	Inc HI1; Tc^r	Taylor and Levine (1980)
pAS251-2-3	lncHI2; Cm^r, Tc^r, Te^r	This study
pDT1942	Derepressed R27; R27::Tn/acZ	Waters et al. (1992)
pHH 1508a	Inc HI2; Cm^r Inc HI1; Te^r Tp^r Str^r	Bradley et al. (1982)
pRG1251 RP4	Inc HI1; Cm^r Inc P; Km^r	Taylor and Grant (1977), Haase et al. (1995)
R478	Inc HI2; Cm^r	Gilmour et al. (2004)
F	Inc F; Km^r	Frost et al. (1994)
pMS119EH/HE	Expression vector; Ptac-laclq; pMB1 origin of replication; Amp^r	Strack et al. (1992)
TrhO-EH	948 bp PCR product with EcoRI and BamHI cut sites in pMS119EH; Amp^r	This study
EexB-HE	840 bp PCR product with HindIII and BamHI cut sites in pMS119HE; Amp^r	This study
017-EH	522 bp PCR product with EcoRI and BamHI cut sites in pMS119EH; Amp^r	This study
EexA-EH	837 bp PCR product with EcoRI and BamHI cut sites in pMS119EH; Amp^r	This study
TrhO-EexA-EH	3147 bp PCR product with EcoRI and BamHI cut sites in pMS119EH; Amp^r	This study
EexBHis-HE	858 bp PCR product with HindIII and BamHI cut sites in pMS119HE; Amp^r	This study
EexAHis-EH	855 bp PCR product with EcoRI and BamHI cut sites in pMS119EH; Amp^r	This study
PJEG144	pDT1942 with cat in TrhO	Lawley et al. (2003)
PJEG142	pDT1942 with cat in EexB	Lawley et al. (2003)
PJEG139	pDT1942 with cat in orf017	Lawley et al. (2003)
pJEG141	pDT1942 with cat in EexA	Lawley et al. (2003)

[a] *Abbreviations:* Nal^r, nalidixic acid resistance; Rif^r, rifampicin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Amp^r, ampicillin resistance; Str^r, streptomycin resistance; Cm^r, chloramphenicol resistance; Tp^r, trimethoprim resistance; Te^r, tellurite resistance.

strains grown until the mid-log growth phase ($A_{550} = 0.4$–0.6). All recipient strains were induced with 0.4 mM isopropyl-β-D-thiogalactoside (IPTG-Rose Scientific) for 1 h. After IPTG induction, 0.1 ml of donor cells, 0.4 ml of recipient cells and 0.5 ml of LB media were mixed by inversion several times in a 1.5 ml Eppendorf tube and then left to incubate for 16 h at 30 °C. The only exception was for mating mixtures containing donor cells harboring the F or pHH1508a plasmids which were mated at 37 °C. Following incubation serial dilutions were set up for each mating (undiluted to 10^{-6} dilution) in phosphate buffered saline (PBS) and 100 μL was plated onto LB agar plates with the appropriate antibiotic selection. The plates were incubated at 37 °C for 16 h before enumeration. Eex indices (EI) were determined by dividing the transfer frequency of donor plasmids into plasmid-free recipients by the transfer frequency of the donor cells into recipients containing the plasmid of interest.

For the Eex studies involving DY330R containing IncPα conjugative plasmid RP4 as the donor, overnight cultures were sub-cultured (1/20 dilution) and grown at 37 °C until strains reached mid-log growth phase ($A_{550} = 0.5$–0.6). Subsequently, all recipient strains were induced with 0.4 mM IPTG. After IPTG induction, 0.5 ml of donor and recipient cells each were added and spread on an LB agar plate without antibiotic selection. Plates were incubated for 3 h at 37 °C to allow for bacterial conjugation. After 3 h of incubation, 1 ml of PBS solution was spread on individual agar plates. Plates were then tilted and the liquid was collected into a 1.5 ml Eppendorf tube. This was repeated twice and the collected cells and undiluted sample were adjusted to a volume of 3 ml. Serial dilutions were set up for each mating (undiluted to 10^{-6} dilution), plated on LB agar plates with the appropriate antibiotic selection and were incubated at 37 °C for 16 h before enumeration. EI levels were calculated as described above.

2.3. DNA manipulations, mutagenesis, and reverse transcriptase PCR (RT-PCR)

IncHI1 plasmid R27 DNA was isolated and purified with a Qiagen (Mississauga, Ont.) LargeConstruct Kit. Expression and cloning vectors were isolated and purified using either the Qiagen Midi-prep kit (Qiagen Inc.) or the Qiagen Spin Mini-prep kit (Qiagen Inc.). Standard recombinant DNA methods were carried out as described by Sambrook et al. (1989). Restriction endonucleases were used according to the manufacturer's instructions and digested DNA was analyzed by agarose gel electrophoresis. The drR27 mutants *eexA*, *trhO*, *orf017*, and *eexB* have been described previously (Lawley et al., 2003). RT-PCR of the Z operon was performed as described previously using the primers listed in Table 2 (Gunton et al., 2005). Briefly, total bacterial RNA was isolated using the RNeasy Midi Kit (Qiagen) and treated with Turbo DNase (Ambion) to remove any contaminating DNA. For RT-PCR experiments, 2 μg total RNA was used as a template in the Superscript II RT kit (Invitrogen) according to manufacturer's instructions. Negative controls without template and RNA that has not been subjected to retrotranscription were performed with each reaction. Positive controls utilizing R27 DNA were done with each pair of primers (Alonso et al., 2005).

2.4. Bacterial fractionation

The bacterial cell fractionation procedure was based on a previous study by Gauthier et al. (Gauthier et al., 2003). Briefly, DH5α cells harboring either $EexA_{His}$ cloned into pMS119EH or $EexB_{His}$ cloned into pMS119HE were grown overnight in LB media at 30 °C with shaking at 200 RPM. One millilitre of culture was diluted in 20 ml of LB media and grown for 3 h under the conditions described above. Upon reaching mid-log growth conditions ($A_{550} = 0.5$–0.6) the cultures were induced with 0.4 mM IPTG for 1 h. Cells were harvested, washed in phosphate-buffered saline and resuspended in 1 ml of sonication buffer (10 mM Tris, pH 7.0) with 1× complete protease inhibitor cocktail (Boehringer–Mannheim, Mannheim, Germany). Lysozyme was added to a final concentration of 10 μg ml^{-1}, and then cells were incubated at 4 °C for 30 min. The periplasmic fraction was collected after centrifugation of the lysozyme-treated cells (at 8000*g* for 10 min). The pellet was resuspended in 1 ml of sonication buffer and sonicated for 3 min on ice (30 s pulses, 10 s breaks, Fisher Sonicator 300). Unlysed cells were collected with centrifugation at 16,000*g* for 2 min, and the supernatant containing the cytoplasmic and membrane fractions was centrifuged at 100,000*g* for 1 h (Optima Max-E Ultracentrifuge, TLA 120.2 rotor, 350,000*g*). The supernatant containing the cytoplasmic fraction was collected and the membrane pellet was washed with sonication buffer. The pellet was resuspended in 0.5 ml of sonication buffer with 0.5% *N*-laurylsarcosine which selectively solubilizes the cytoplasmic membrane and the resuspension was rotated at room temperature for 30 min before centrifugation (100,000*g* for 1 h). The supernatant representing the cytoplasmic membrane fraction was collected and the pellet was washed with sonication buffer with 0.5% *N*-laurylsarcosine. The pellet, which was resuspended in 0.5 ml of sonication buffer with 0.5% *N*-laurylsarcosine and 0.1% sodium dodecyl sulfate (SDS) contained the outer membrane fraction. The antibodies that were used to ensure appropriate separation of fractions were anti-DnaK (Stressgen,Victoria, BC), anti-SecY (Minotech, Crete, Greece), and anti OmpA (Prasadarao et al., 1996).

2.5. Electron microscopy

Transmission electron microscopy (TEM) was performed as described previously (Maher et al., 1993). Briefly, transconjugant colonies were applied to freshly prepared Formvar-coated copper grids by briefly touch-

Table 2
Primers used in this study to clone the genes of the Eex operon and for transcriptional analysis of the Eex operon

Primer ID	Primer name	Sequence(5′–3′)[a]	Predicted product size (bp)
TrhO-EH	TrhO-GUN13	F: TGAATTCAATGGCATCCGAGCACGTCCAG	948
	TrhO-GUN14	R: TGGATCCTTATAATGTGGCCTCTATATTATTATC	
EexB-HE	EexB-GUN15	F: TAAGCTTATGCTTAAATTAGAACTG	840
	EexB-GUN16	R: TGGATCCTTAATCATTTTCAGTCCTCATAGTCG	
orf 017-EH	orf 017-GUN17	F: TGAATTCAATGATTAGAGTTTCAGC	522
	orf 017-GUN18	R: TGGATCCTTATGATTGCTCCTTTTGCAAAAATTC	
EexA-EH	EexA-GUN19	F: TGAATTCAATGAAATATATATTGACCG	392
	EexA-GUN20	R: TGGATCCTTATTGAACAGGGTGATGGGC	
EexBHis-HE	EexBHis-GUN188	R: TATAGGATCCTCAATGGTGATGGTGATGGTGATCATTTTCAGTCCTCATAGTCG	858
EexAHis-EH	EexAHis-JRU13	R: TATAGGATCCTCAATGGTGATGGTGATGGTGTTGAACAGGGTGATGGGC	
2–19 (RT-PCR)	orf 017	F: AGGCACTGATGTCTGGTTACTC	695
	EexA	R: CAACCGCTGCTGCATTACTGTC	
2–20 (RT-PCR)	orf 016	F: ACGGAATGTGGCTTGTTGATGG	930
	orf 017-orf 016	R: TGACGAATAGGTGCAACGACTCC	
2–21 (RT-PCR)	orf 016-TrhO	F: TTGCCCATCCGATTACACAGAC	662
	orf 016-TrhO	R: CGACCATCAACAAGCCACATTC	
Control (RT-PCR)	PfkA	F: GTGGCGGTACGTTCCTCGGTTCT	762
	PfkA	R: TTTTTCGCGCAGTCCAGCCAGTC	

[a] Restriction endonuclease sites are underlined.

ing the grids to isolated colonies. The samples were stained with the addition of 1% phosphotungstic acid staining solution (pH 7.0) with excess stain blotted away with filter paper. Grids were examined with a Philips model 410 transmission electron microscope.

2.6. Web-based computer programs

PSI-Blast (http://www.ncbi.nlm.nih.gov/BLAST/); ClustalW (Gonnet matrix, gap penalty = 10, extension penalty = 0.2; http://www.ebi.ac.uk/clustalw/); TMPred (http://www.ch.embnet.org/software/TMPRED_form.html); ScanProsite (http://ca.expasy.org/tools/scanprosite/); EMBOSS pairwise local alignment (Blosum40 matrix, gap penalty = 10, extension penalty = 0.5; http://ebi.ac.uk/emboss/align/); and SignalP (http://www.cbs.dtu.dk/services/SignalP/) were used for analysis.

3. Results

3.1. EexB and EexA mediate entry exclusion activity of the R27 plasmid

A plasmid-encoded Eex phenomenon has been observed within the incompatibility H plasmid family (Taylor and Grant, 1977). To identify the specific mediators of the exclusion event encoded by the IncHI1 plasmid R27, a cosmid library of the large conjugative plasmid (Newnham and Taylor, 1990) was screened for IncH exclusion activity (Table 3). The exclusion activity is reported as an EI, which is defined as the frequency of transfer of R27 to a plasmid-free recipient divided by the frequency of transfer of R27 to a recipient of interest; therefore a high EI indicates inhibition of R27 conjugative transfer. In the screen of the R27 cosmid bank for Eex activity, the highest EI level (EI = 210) was recorded when donor cells harboring R27 were mated with recipient cells containing the cosmid pJEG801 (Table 3). The pJEG801 cosmid contains the R27 coordinates 4.1–41.8 kb. This region of R27 encodes a portion of the transfer region 2 (Tra2) which is located from coordinates 2.1–38.0 kb (Lawley et al., 2003). Notably, lower EI levels of 28 and 16 were recorded when R27 was mated into recipients cells containing the cosmids pJEG800 and pJEG803, respectively; these two cosmids also encode regions of the R27 Tra2 sequence (Table 3).

The single cosmid containing regions of R27 Tra1 and pDT1693 demonstrated background levels of EI (EI = 0.8). It was not possible to screen the region of Tra1 extending from orf115 to *trhX* for R27 Eex activity. It seems likely that the presence of the origin of transfer (oriT) within Tra1 resulted in an inability to clone this region of the R27 Tra1 sequence (Newnham and Taylor, 1990; Lawley et al., 2003).

To identify the individual genes encoding the R27 Eex phenotype the cosmid clone containing the smallest portion of the Tra2 region which demonstrated exclusion activity was further characterized. The clone pJEG800 contains the R27 sequence corresponding to the coordinates 4.1–

Table 3
R27 Cosmid library screen for entry exclusion activity

Cosmid clone[a]	Coordinates[b]	Restriction enzyme[c]	Tra region encoded[d]	Transfer frequency[e]	EEX index[f]
No insert	—	—	—	1.9×10^{-4}	1
pJEG800	4.1–28.0	XbaI	Tra2	6.8×10^{-6}	28
pJEG801	4.1–41.8	XbaI	Tra2	0.9×10^{-6}	210
pJEG802	41.8–51.3	XbaI	—	3.2×10^{-4}	0.6
pDT1693	111.4–155.6	SalI	Tra1	2.5×10^{-4}	0.8
pDT1602	144.1–173.6	SalI	—	3.9×10^{-4}	0.5
pJEG803	164.4–28.0	XbaI	Tra2	1.2×10^{-5}	16

The EEX index is an average index derived from at least two independent experiments.

[a] Cosmids were constructed using pHC79 and pUCD5 and introduced by electroporation into RG192-2 cells.

[b] The R27 map coordinates of the sequence contained within the cosmid library. The coordinates are from the R27 GenBank sequence (NC_002305).

[c] Restriction endonuclease used to generate the R27 fragments.

[d] The presence of sequence encoding partial Tra1 regions (98.1–117.3) or Tra2 regions (2.1–38.0) within the cosmid clones are indicated.

[e] Transfer frequency is listed as transconjugants per donor. The transfer frequency represents the average frequency from at least two independent experiments.

[f] EEX index is calculated by dividing the transfer frequency of cells harboring an empty cosmid with the transfer frequency of cosmid clones containing R27 fragments.

28.0 kb including the R27 genes *orf004* to *orf023*. Within this region, four contiguous genes comprise the Z operon; *trh*O, orf016, orf017, and *trhZ* are flanked by the double partitioning determinants of R27 (Lawley and Taylor, 2003). Notably, the Z operon is present in all three R27 cosmid clones (pJEG800, pJEG801, and pJEG803) which showed Eex activity. To ascertain the potential role this operon may play in IncHI Eex, the region encoding *trh*O-*trh*Z was cloned into the IPTG-inducible expression vector pMS119HE (Fig. 1). When *trh*O-trhZ was expressed in recipient cells and mated with donors harboring the R27 plasmid, EI levels of 3001 were observed. Sub-cloning of the individual genes and screening for R27 Eex activity resulted in the identification of *orf16* and *trhZ* as R27 exclusion proteins (Fig. 1). Due to the discovery of an Eex function mediated by *trh*Z and *orf*16, we changed the nomenclature of these R27 genes to *eex*B (*e*ntry *ex*clusion) and *eexA*, respectively.

Interestingly, there was a significant difference in the R27 EI levels mediated by *eexB* and *eexA*. Whereas the entire regions of *trh*O-*eex*A and *eexA* were able to exclude the R27 plasmid at an EI of ~3000, the *eexB* gene and the *trhO-orf017* region conferred a higher R27 EI of >10,000 (Fig. 1). These data indicate the IncHI1 plasmid R27 encodes two functional Eex proteins which inhibit the redundant conjugative transfer of this genetic element.

3.2. Temperature-dependent transcription of Z operon

A hallmark feature of the IncHI plasmids is optimal conjugative transfer at 25–30 °C with reduced transfer ability at 37 °C (Taylor and Levine, 1980). To determine the effect of temperature on the transcription of the R27 exclusion genes *eexA* and *eexB*, RT-PCR analysis of the Z operon was performed. Total RNA was extracted from cells at 30 and 37 °C harboring wild-type (WT) R27 or R27 derepressed for transfer (drR27) (Maher et al., 1991). Primers were designed to amplify adjacent genes in the Z operon transcript (Table 2 and Fig. 2a). For

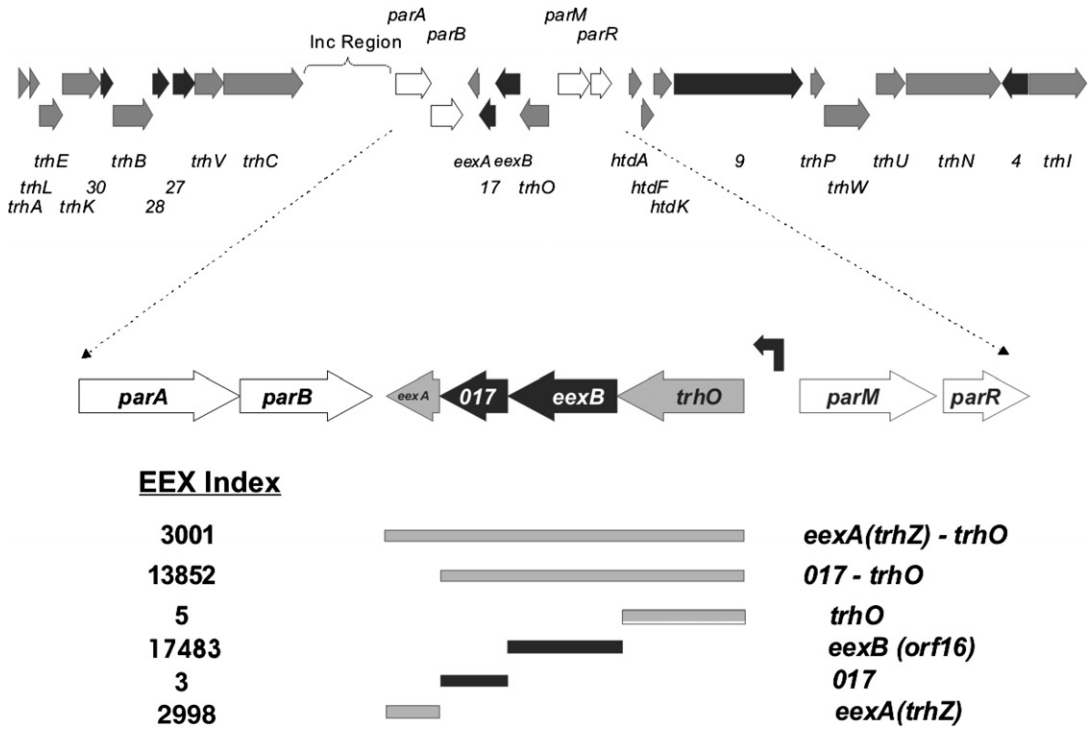

Fig. 1. Open reading frame map of the Tra2 region of R27, with an enlarged map of the operon encoding the entry exclusion proteins EexA and EexB of R27. Gray orfs indicate genes involved in the conjugative transfer of R27 and the black orfs indicate genes that are not essential for transfer. White orfs indicate the partitioning genes of the R27 that encode stability determinants. Stipples orfs indicate genes that are involved in the regulation of R27 transfer. The black bent arrow represents a proposed promoter region upstream of the TrhO-EexA operon. The gene nomenclature has the former name of *eexA* (*orf16*) and *eexB* (*trhZ*). EEX index is calculated by dividing the transfer frequency of DY330R cells harboring an empty plasmid with the transfer frequency of plasmid containing the indicated gene(s). The EEX index is an average index derived from three independent experiments.

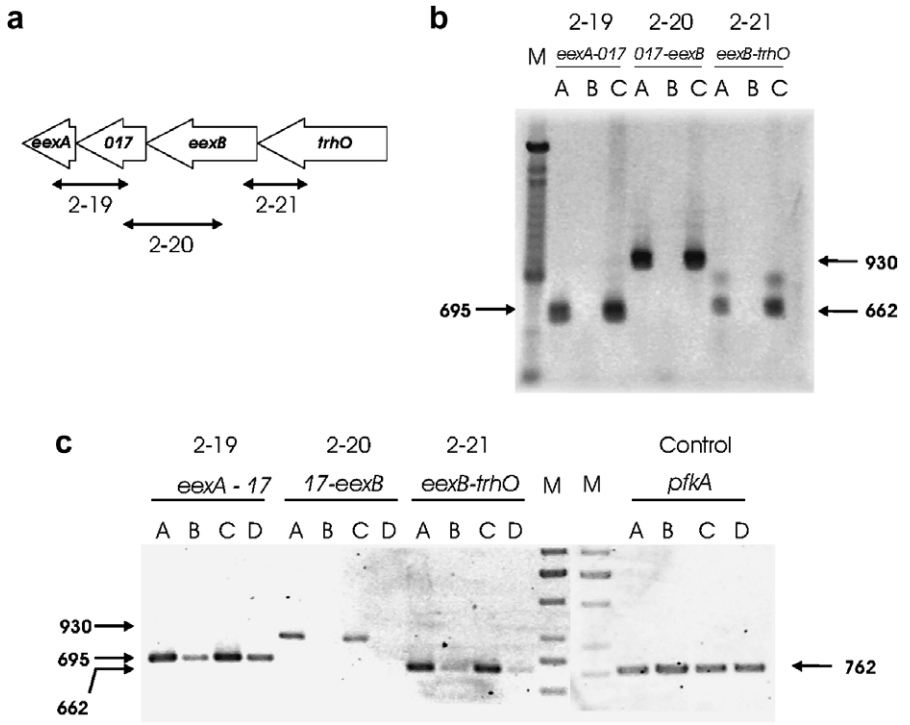

Fig. 2. Operon map and reverse transcriptase analysis of the Z operon. (a) Operon map of the *trhO-eexB-017-eexA* R27 genes where arrows indicate the region amplified by the RT-PCR primers 2–19, 2–20, and 2–21. (b) Confirmation of the *trhO-eexA* operon structure by RT-PCR analysis of the co-transcribed R27 genes *trhO-eexB-017-eexA*. For each primer set, three lanes are shown (A) RT-PCR using an RNA template isolated from cells harboring R27 grown at 30 °C, (B) negative control of the same RNA template with no reverse transcriptase, and (C) positive control of a DNA template from cells containing R27. (c) Characterization of the transcriptional profile of R27 *trhO-eexA* operon using RT-PCR with RNA from cells harboring (A) WT R27 grown at 30 °C, (B) WT R27 grown at 37 °C, (C) derepressed R27 grown at 30 °C, and (D) derepressed R27 grown at 37 °C. *pfK* corresponds to phosphofructose kinase which is an internal control.

each reaction, control DNA was used to ensure accurate amplification by the primers 2–19, 2–20, and 2–21 (Fig. 2b). In the absence of reverse transcriptase there was no evidence of contamination by the primers, however, when the polymerase was present there was amplification of the Z operon transcript (Fig. 2b).

When the primers 2–19, 2–20, and 2–21 were added to RNA extracted at 30 and 37 °C from cells harboring drR27 or WT R27, lower amplification of the Z operon transcript was observed (Fig. 2c). Although RT-PCR is a semi-quantitative technique, there was a difference in the Z operon transcript levels at 30 and 37 °C, with higher levels of transcript being observed at 30 °C. As a control to ensure that the levels of RNA template used for the RT-PCR reactions were equivalent, the *E. coli* housekeeping gene *pfkA*, encoding phosphofructokinase, was amplified (Fig. 2c). There was no difference in the level of *pfkA* amplification of RNA extracted from cells grown at 30 and 37 °C harboring WT R27 or drR27.

3.3. Effect of R27 entry exclusion proteins on the transfer of related and non-related conjugative plasmids

Previous studies have shown the natural ability of IncHI plasmids to exclusively mate with less related plasmids (Taylor and Grant, 1977). The specificity of the Eex activity mediated by EexA and EexB was determined by mating recipient cells expressing the R27 Eex proteins with donor cells harboring R27-related and non-related plasmids. The IncHI1 R27 had been found to elicit an EI of 3001 when mixed with recipient cells expressing the Eex protein EexA (Fig. 1). Similarly, when donor cells contained the closely related IncHI1 plasmid pRG1251, the EexA encoding recipients were able to exclude the donors to an EI of 1781 (Table 4). When the donor cells harbored the IncHI2 plasmids pAS251-2-3 and R478, the EI levels decreased to 640 and 526, respectively. The EI levels decreased further to 207 when donor cells

containing the IncHII plasmid pHH1508a were mixed with recipient cells expressing EexA. Non-related IncP RP4 plasmid and IncF F plasmid were only slightly inhibited in conjugation with recipient cells encoding EexA, as the EI levels were 10 and 19, respectively (Table 4).

When EexB was tested with the Eex assay, the trend noted with EexA of decreasing EI levels corresponding to increasing donor plasmid diversity was not observed. Whereas donor cells containing the R27 IncHI1 plasmid elicited EI levels of 17483 when mixed with recipient cells expressing EexB, the EI levels were markedly lower at 461 when the donor cells harbored closely related IncHI1 plasmid pRG1251. The EexB mediated EI levels associated with the IncHI2 plasmids pAS251-2-3 and R478 were decreased further at 209 and 95. Surprisingly, the most distantly related IncH mobile element in this study, the IncHII plasmid pHH1508a, obtained the second highest EI of 1353, when mixed with EexB containing cells. The two plasmids not related to the IncH family, the RP4 and the F plasmid, were both conjugally inhibited by EexB to varying levels; the F plasmid registered an EI of 353 and RP4 elicited an EI of only 39 (Table 4).

3.4. Localization of EexA and EexB in the bacterial cell

In order to determine the cellular location of the R27 Eex proteins EexA and EexB, a bacterial fractionation experiment was performed. The bacterial cells were fractionated into cytoplasmic, cytoplasmic membrane, periplasmic, and outer membrane fractions by treatment with lysozyme, sonication, ultracentrifugation and selective solubility with the detergent sarkosyl. The fractionation method used in this study exploits the differential lipid composition of the inner and outer membranes to separate the bacterial cell envelope; solubilization of the cytoplasmic membrane with sarkosyl has been shown to be an effective means of separating the membranes of *E. coli* (Filip et al., 1973; Nikaido, 1994; Gauthier et al., 2003).

Characterization of plasmid-encoded Eex proteins has revealed that this family of proteins commonly contains signal sequences and hydrophobic domains implying membrane-association. The bacterial fractionation data of R27-encoded Eex proteins determined that $EexB_{His}$ predominantly localized to the outer membrane of the cell (Fig. 3a). Small amounts of $EexB_{His}$ were detected in the cytoplasmic membrane fraction with no detectable protein in the cytoplasmic or periplasmic fractions. Conversely, $EexA_{His}$ was found primarily in the cytoplasmic membrane fraction with trace amounts of protein in the outer membrane fraction (Fig. 3b). The cytoplasmic and periplasmic fractions contained no detectable $EexA_{His}$. These data suggest that the R27 Eex proteins function in different regions of the bacterial cell envelope.

As a control for the appropriate fractionation of the bacterial cell components, the isolated fractions were probed with anti-DnaK, anti-SecY and anti-OmpA antibodies (Fig. 3c). DnaK is a cytoplasmic heat shock protein (Hsp) (Liberek et al., 1988). The DnaK protein was detected in the cytoplasmic fraction with trace amounts in the soluble periplasmic fraction (Fig. 3c). The SecY protein is a member of the cytoplasmic membrane-associated Sec system (Akiyama and Ito, 1985). SecY was found predominantly in the cytoplasmic membrane fraction.

Table 4
Specificity of the entry exclusion process mediated by EexA and EexB

Donor strain containing[a]:		Exclusion indexes when[b] recipients harbor[c]:		
Plasmid	Incompatibility group	pMS119EH	EexA-EH	EexB-HE
drR27	IncHI1	5	3001	17483
pRG1241	IncHI1	8	1781	461
pAS251-2-3	IncHI2	4	640	209
R478	IncHI2	2	526	95
pHH 1508a	IncHII	1	207	1353
F pro lac::Tn5	IncF	1	19	353
RP4	IncP	1	10	39

[a] *E. coli* DY330R served as the donor strain.
[b] EI is calculated by dividing the transfer frequency of cells harboring an empty plasmid with the transfer frequency of plasmid expressing either EexA or EexB. The EEX index is an average derived from three independent experiments.
[c] *E. coli* RG11 served as the recipient strain.

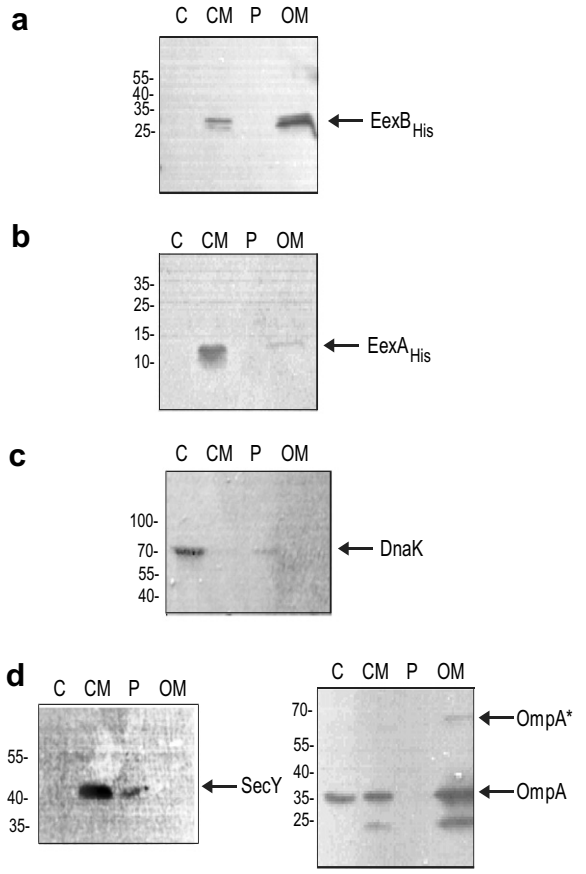

Fig. 3. EexA and EexB localize to the inner and outer membranes, respectively. Bacteria were fractionated into cytoplasm (C), cytoplasmic membrane (CM), periplasm (P), and outer membrane (OM) fractions with an equal amount of each fraction volume loaded on a SDS–PAGE and transferred to a nitrocellulose membrane. (a and b) Western blots were probed with anti-His monoclonal antibody. (c) Western blots were probed with anti-DnaK monoclonal antibody, anti-SecY and anti-OmpA antisera to ensure appropriate separation of fractions. The OmpA* corresponds to a ~60 kDa protein detected by the anti-OmpA antisera in the outer membrane (De Mot et al., 1992).

(Fig. 3c). The OmpA protein is a porin-like integral membrane protein found in the outer membrane of many Gram-negative organisms (De Mot et al., 1992). The 35 kDa OmpA was primarily detected in the inner and outer membrane fractions.

3.5. A potential mechanism for entry exclusion with R27

R27-encoded exclusion may occur via interaction of the entry exclusion proteins in donor and recipient cells; consequently, for exclusion to occur the donor and the recipient cells would each have to possess the Eex proteins. Functional and mutational analyses of the Tra2 region of R27 determined that the R27 Eex genes *eexA* and *eexB* were not essential for the conjugative transfer of the derepressed IncHI1 plasmid, however, mutations in *eexA* greatly reduced the conjugation frequency (Lawley et al., 2003). Cells harboring drR27 plasmids with insertional mutations in the Eex genes were mixed with recipient cells containing pMS119EH with no insert, or pMS119EH/HE expressing EexA or EexB. As an experimental control, donor drR27 with mutations in another gene located in the Z operon *orf017* was included in this study. Derepressed R27 (dR27) contains a Tn10 insertion in the *htdA* gene (Lawley et al., 2003). Conjugative transfer with R27 is very low at 5×10^{-5} transconjugants/donor (Lawley et al., 2003). When R27 knockout strains are used, this transfer frequency is even lower, making determination of exclusion indexes more difficult. dR27 has been used previously in conjugation experiments with knockout mutations (Lawley et al., 2003).

When donor cells harboring drR27 plasmid with mutations in *orf017* were mated with recipient cells expressing EexA, the resulting EI levels of 2013 were similar to the EI levels of 3001 obtained with WT drR27 (Table 5). Conversely, when the donor cells contained the drR27 mutant *eexB*, the EI level decreased to 126. Notably, only background levels of EI were observed when the donor plasmid did not encode for the EexA protein (Table 5). These data suggest that when the donor plasmid drR27 lacks the EexA protein, the IncH plasmid is not inhibited in conjugation with recipient cells expressing the R27 exclusion protein EexA. Next the experiment was repeated with recipient cells expressing EexB proteins. A significant decrease in EI to background levels was observed (from 17483 to 8) when the R27 donor plasmid had an insertional mutation in the *eexA* gene (Table 5). When donor cells containing drR27 mutants *eexB*, and *orf017* were mated with recipient cells expressing EexB, the dilution plating required for the conjugation assay resulted in massive clumping of the transconjugant cells on the LB plates. The presence of EexB in the recipient cells consistently resulted in this cell morphology when mated with the two aforementioned drR27 mutants, even after repeated mating attempts. Therefore it was impossible to assess the EI levels elicited by EexB with these drR27 mutants. Electron microscopic analysis of transconjugant cells harboring R27 mutants *orf017* and *eexB* revealed heavily

Table 5
Entry exclusion by EexA and EexB requires that donor cells contain R27 exclusion protein(s) for exclusion activity

Plasmid in recipient cells[a]	dR27	Eex indicies[b] when donor strain DY330R harbors dR27 with mutations in		
		EexB	orf017	EexA
Vector alone	5	5	3	2
EexA-EH	3001	126	2013	5
EexB-HE	17483	N/A[c]	N/A[c]	8

[a] *E. coli* RG11 served as the recipient cell.
[b] EI is calculated by dividing the transfer frequency of cells harboring an empty plasmid with the transfer frequency of plasmid expressing either EexA or EexB. The EEX index is an average index derived from three independent experiments.
[c] N/A corresponds to "not available" as the transconjugant colonies had a clumped morphology which made enumeration impossible.

negatively stained regions on cell surfaces (Fig. 4b). The transconjugant cells harboring the R27 mutant *orf017* and a vector expressing EexB showed an elongated phenotype which was not observed when EexB was absent from the RG11 cells (Fig. 4c). To ensure that the clumping effect and elongated phenotype were not an artifact of the *E. coli* RG11 strain, the matings were also done with *E. coli* strain JC3272 as the recipient strain. The clumping effect was seen with these matings as well, ruling out that it was a strain-specific phenomenon. In order to determine whether this phenotype was due to over-expression of the EexB protein, the matings were also done without IPTG induction of EexB. The "leaky" expression of EexB in the recipient was sufficient to produce the clumping effect. This result suggests that over-expression is not the sole cause of the clumping phenotype. As EexB was determined to be an outer membrane protein, the presence of this entry exclusion protein on the cell surface appears to cause phenotypic changes in transconjugants with certain drR27 mutants.

4. Discussion

Within large bacterial populations prokaryotic genomes are in a continuous state of flux due to a massive reservoir of DNA upon which to draw. Bacterial conjugation is a significant method of DNA transfer. For a recent review of the mechanics of conjugation, see Chen et al. (2005). Mobile genetic elements, however, encode barriers that inhibit the initial entry of DNA into recipient cells (Thomas and Nielsen, 2005). The Eex process inhibits the transfer of conjugative elements into recipient cells harboring isogenic or closely related mobile elements (Novick, 1969). In addition to preventing the unnecessary energy expenditure of redundant conjugative transfer, Eex determinants have been found to confer serum resistance on host cells (Moll et al., 1980) and have been proposed to break apart mating pairs releasing transconjugant cells to act as new donor cells (Thomas and Nielsen, 2005).

Here we describe two proteins which mediate Eex within the prototypical IncHI plasmid R27. Eex was initially documented within the IncHI family in 1977 (Taylor and Grant, 1977), and a cosmid screen of R27 has revealed that the Eex phenotype was conferred by EexA and EexB encoded within the Z operon located in the Tra2 region of this conjugative plasmid. The R27 cosmid library bank contained the majority of the R27 plasmid, however, the presence of an oriT sequence within the Tra1 region resulted in difficulties with cloning adjacent regions (Newnham and Taylor, 1990; Lawley et al., 2002). The cosmid library did not include 10 orfs located within this section of the Tra1 region, of which six orfs have been assigned a functional role in conjugation (Lawley et al., 2002). In the existing literature on Eex, no gene mediating Eex has been observed to be encoded outside the defined transfer region (Achtman et al., 1980; Hartskeerl et al., 1986; Llosa et al., 1994; Pohlman et al., 1994; Haase et al., 1995). Nevertheless, we cannot exclude the possibility that one or more of the uncharacterized Tra1 orfs may be involved in the Eex function of R27.

The functional and mutational characterization of the Tra2 region of R27 revealed that *eexA* and *eexB* are not essential for the conjugative transfer of the IncHI1 plasmid (Lawley et al., 2003). However, conjugative transfer in *eexA* mutants is significantly reduced when compared to WT transfer levels (Lawley et al., 2003). The non-essential nature of the R27 Eex proteins in conjugation is consistent with the Eex determinants that have been characterized (Frost et al., 1994; Haase et al., 1996; Marrero and Waldor, 2005). A lipoprotein motif that has

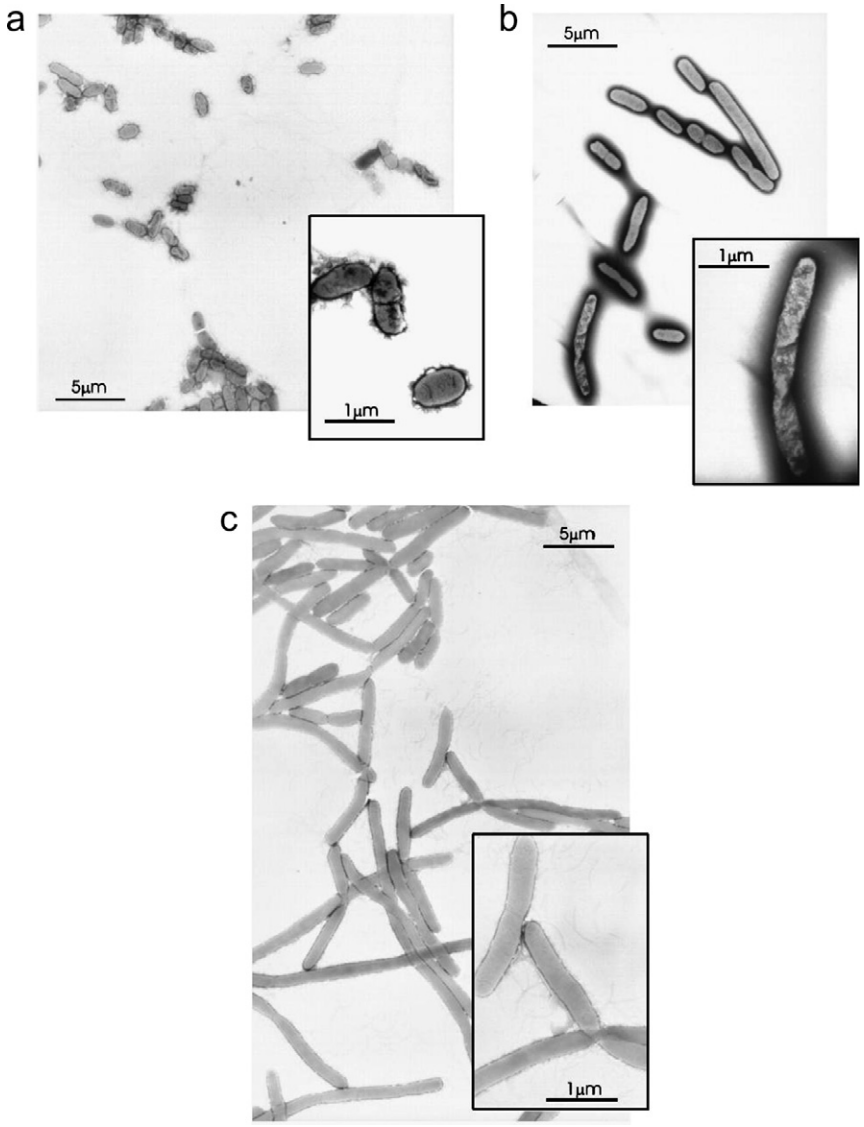

Fig. 4. Electron microscopic images of RG11 transconjugant cells expressing the entry exclusion protein EexB from pMS119HE. The RG11 EexB-HE cells were mated with DY330R cells containing (a) drR27 and dR27 containing mutation in; (b) *eexB*, and (c) o*rf017* (magnification 2000×). The inset images were collected at a magnification of 10,000×.

been associated with Eex determinants encoded by other plasmids (Frost et al., 1994; Haase et al., 1996; Pohlman et al., 1994; Llosa et al., 1994), however, neither EexB or EexA contains this motif. Notably, mutational analysis of the RP4 Eex TrbK lipoprotein motif has identified that a functional lipoprotein motif is not required for Eex activity (Haase et al., 1995). The Eex proteins of the R27 plasmid both contain predicted transmembrane domains preceding a putative signal sequence. The majority of Eex determinants contain predicted transmembrane domains and localization studies of the IncP and IncF Eex components have confirmed an association with the cellular envelope (Achtman et al., 1977; Haase et al., 1996; Marrero and Waldor, 2005). Bacterial fractionation studies on the EexB and EexA proteins of R27 indicated that the EexA protein primarily associates with the cytoplasmic membrane, whereas EexB was predominantly located in the outer membrane. The well-characterized TraT protein from the F plasmid was also identified as an outer membrane protein (Achtman et al., 1977) and has been proposed to prevent the formation of mating aggregates. The

TraT protein has been designated a surface exclusion protein. Likewise, EexB may also be designated as such. Nonetheless, we have followed the proposal by Novick et al. in which all exclusion proteins are classified as Eex proteins (Novick, 1969).

Recent transcriptional studies on the R27 plasmid (Alonso et al., 2005; Forns et al., 2005) have determined that at 33–37 °C, temperatures that are non-permissive for conjugative transfer of IncHI plasmids (Taylor and Levine, 1980), there is global repression of essential transfer genes. This global repression can be mediated by plasmid or host-encoded Hha or H-NS (Forns et al., 2005). Although the reason for this temperature sensitivity is unknown, it is possible that it is an important mechanism for the host bacteria. This allows for conjugative transfer in the soil, water or other resevoirs, while not allowing mating in the physiological temperatures where pathogenesis is more important to the bacterial host. Transcriptional analysis of the Z operon, encoding the R27 Eex proteins, revealed that the global repression induced by elevated temperatures reduced the expression of the Z operon. The Z operon is flanked by the double partitioning modules of R27 and is transcribed in the opposite direction from the remaining two Tra2 operons. It is noteworthy that the Z operon, encoding proteins non-essential for the conjugative transfer of R27, appears to be regulated in the same temperature sensitive manner as the operons encoding essential R27 transfer proteins.

An Eex assay was established to determine the levels of exclusion mediated by recipient cells expressing R27-encoded EexA or EexB, when mated with donor cells harboring R27-related and non-related plasmids. It would be expected that a donor cell containing a closely related IncH plasmid would elicit a high EI when mixed with recipient cells expressing the R27 exclusion proteins; conversely, donor cells harboring non-related plasmids such as IncP or IncF plasmids could mate with the aforementioned recipient cells with minimal inhibition, thereby registering a low EI level. When recipient cells expressing EexA were mated with donor cells harboring R27-related and non-related plasmids, there was a direct correlation between the increase in evolutionary distance from the R27 plasmid and the decrease in EI levels. Conversely, this trend was not apparent with the EexB protein. Surprisingly, when the donor cells harboring the IncHI2 plasmid R478 were mated with recipient cells expressing EexB the EI levels of 95 were significantly lower than the EI level of 353 elicited by the non-related IncFI F plasmid. A search of the NCBI database for homologs of the EexA and EexB identified orf016 and orf018 from the IncHI2 plasmid R478. Whereas EexA is closely related to the orf016 from R478 (80.5% identity over an alignment length of 128 residues), EexB has only limited homology to the R478 orf018 (40.6% identity over an alignment length of 281). Moreover, the homology between EexB and orf018 is lower in the C terminal region of the proteins (32.1% identity over an alignment length of 100 residues). The C terminal region of Eex proteins has been demonstrated to be the domain which determines the specificity of the exclusion activity (Haase et al., 1996; Marrero and Waldor, 2005). The limited homology in the C terminal region between EexB and orf018 from R27 and R478, respectively, may be a factor in the decreased ability of the EexB plasmid to exclude the R478 during Eex activity assays.

Although a number of Eex determinants have been identified in a wide array of incompatibility groups, little is known about the mechanism by which the Eex proteins confer the exclusion phenotype to host cells. Studies on the TraS protein of the F plasmid and Eex determinants from the conjugative elements SXT and R391 have revealed that these cytoplasmic membrane proteins interact with the well-conserved mating pair formation protein TraG (Anthony et al., 1999; Marrero and Waldor, 2005). The TrbK protein from RP4 was also proposed to interact with a component of the mating pair formation complex (Haase et al., 1996). TrbK and the R391/SXT-encoded Eex proteins were not required in the donor cell to elicit an Eex event. Conversely, when donor cells containing an R27 *eexA* mutant were mated with recipients expressing EexA, there was minimal exclusion activity (EI = 5) as the mating frequency was equivalent to that obtained with the R27 EexA mutant mated with recipients containing empty vector (EI = 2). Similar results were obtained when the *eexA* mutant was utilized as a donor plasmid with recipient cells expressing EexB (EI = 8). Further evidence to support the association between the R27 Eex proteins is that the R27 EexB mutant was only minimally excluded from recipients expressing EexA (EI = 126). These putative Eex interactions between R27 proteins may explain the major discrepancy in the EI levels elicited by EexA and EexB. When the entire Z operon was expressed from pMS119EH, EI levels of 2998 were obtained, similar to the EI level observed with

donors expressing EexA alone (EI = 3001). However, when the *eexA* gene was excluded from the operon, the EI levels drastically increased to 13852, which is closer to the EI levels elicited by EexB alone (EI = 17483). In future studies it will be necessary to determine if the cytoplasmic membrane-associated EexA regulates the expression, or perhaps localization, of EexB in the outer membrane when the two R27 Eex proteins are co-expressed.

The abnormal cellular morphology that is observed in some of the transconjugants may also provide clues as to the mechanism of surface exclusion in the IncHI1 plasmids. The presence of EexB on the outer membrane of recipient cells caused changes in the cells of the transconjugants where the donors had mutations in specific genes. It is possible that these proteins play an alternate role in conjugation. It has been theorized that the surface/entry exclusion proteins may be involved in the breakdown of the mating pairs (Thomas and Nielsen, 2005). The presence of the clumped and elongated cells may show which protein interactions the EexB outer membrane protein is involved with and how these proteins function within the type four secretion system. Future studies could determine how widespread this effect is within the Tra2 region and to elucidate how the surface exclusion proteins are interacting.

Together, these data permit us to speculate on the mechanism involved in R27 Eex. A successful interaction between the outer and cytoplasmic membrane Eex components results in the exclusion of the incoming plasmid. A potential problem is that this model requires the cytoplasmic membrane proteins of donor and recipient cells to interact. A similar problem exists with the proposed interaction between the cytoplasmic membrane Eex and TraG proteins of the F and R391/SXT conjugative elements (Audette et al., 2007). The composition and architecture of the type four secretion system remain elusive; there is increasing evidence that the conjugative pore enables proteins within the cytoplasmic membrane to interact through translocation of certain conjugative proteins between mating cells (Anthony et al., 1999). A recent study on the F plasmid delimitated a region of TraG that is involved in Eex, however, attempts to isolate a TraG–TraS complex have not been successful (Audette et al., 2007). An alternative possibility is that the appressed donor and recipient membranes allow their respective cytoplasmic membrane proteins to come into close proximity as there is evidence that an intermediate fraction representing both cytoplasmic and outer membrane components is formed by the type four secretion system (Grahn et al., 2000; Marrero and Waldor, 2005).

Acknowledgments

We thank Joanne Simala-Grant and Marc Couturier for critical reading of the manuscript. We are grateful to Matthew Gilmour and Laura Frost for informative discussions on Eex, and to Richard Sherburne for assistance with electron microscopy.

This work was supported by Grant MOP6200 to D.E.T. from the Canadian Institutes of Health Research (CIHR). J.E.G was a recipient of a training award from the Alberta Heritage Foundation for Medical Research (AHFMR). J.E.R.U. was a recipient of a summer studentship from the National Science and Engineering Council (NSERC). D.E.T. is a Scientist with AHFMR.

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