The *Yersinia pseudotuberculosis* and *Yersinia pestis* toxin complex is active against cultured mammalian cells

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The toxin complex (Tc) genes were first identified in the insect pathogen *Photobacterium luminescens* and encode ~1 MDa protein complexes which are toxic to insect pests. Subsequent genome sequencing projects have revealed the presence of *tc* orthologues in a range of bacterial pathogens known to be associated with insects. Interestingly, members of the mammalian-pathogenic yersiniae have also been shown to encode Tc orthologues. Studies in *Yersinia enterocolitica* have shown that divergent *tc* loci either encode insect-active toxins or play a role in colonization of the gut in gastroenteritis models of rats. So far little is known about the activity of the Tc proteins in the other mammalian-pathogenic yersiniae. Here we present work to suggest that Tc proteins in *Yersinia pseudotuberculosis* and *Yersinia pestis* are not insecticidal toxins but have evolved for mammalian pathogenicity. We show that Tc is secreted by *Y. pseudotuberculosis* strain IP32953 during growth in media at 28 °C and 37 °C. We also demonstrate that oral toxicity of strain IP32953 to *Manduca sexta* larvae is not due to Tc expression and that lysates of *Escherichia coli* BL21 expressing the *Yersinia* Tc proteins are not toxic to *Sf9* insect cells but are toxic to cultured mammalian cell lines. Cell lysates of *E. coli* BL21 expressing the *Y. pseudotuberculosis* Tc proteins caused actin ruffles, vacuoles and multi-nucleation in cultured human gut cells (Caco-2); similar morphology was observed after application of a lysate of *E. coli* BL21 expressing the *Y. pestis* Tc proteins to mouse fibroblast NIH3T3 cells, but not Caco-2 cells. Finally, transient expression of the individual Tc proteins in Caco-2 and NIH3T3 cell lines reproduced the actin and nuclear rearrangement observed with the topical applications. Together these results add weight to the growing hypothesis that the Tc proteins in *Y. pseudotuberculosis* and *Y. pestis* have been adapted for mammalian pathogenicity. We further conclude that Tc proteins from *Y. pseudotuberculosis* and *Y. pestis* display differential mammalian cell specificity in their toxicity.

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INTRODUCTION

The high molecular mass (~1 MDa) toxin complexes (Tc's) are dominant secreted virulence factors of the insect pathogen and nematode symbiont *Photobacterium luminescens* (Bowen *et al.*, 1998; Bowen & Ensign, 1998). They

were originally described in *P. luminescens* subsp. *akhurstii* strain W14, where they were shown to comprise four native complexes encoded by the loci *tca*, *tcb*, *tcc* and *tcd*, with the individual genes assigned according to their order within each locus, e.g. *tcaA*, *tcaB*, *tcaC*, etc. (Bowen *et al.*, 1998).

Comparative sequence analysis has revealed the four loci to have a high degree of similarity that can be grouped into three genetic components: the *tcaAB/tcdA*-like or [A] components, the *tcaC/tcdB*-like or [B] components and

Abbreviations: Tc, toxin complex; TUNEL, TdT-mediated dUTP nick end-labelled.

A supplementary figure showing sequence alignments of Tc proteins is available with the online version of this paper.

the *tccC*-like or [C] components (French-Constant & Waterfield, 2006; Waterfield *et al.*, 2001b). The Tc's have previously been demonstrated to possess both oral and injectable toxicity to lepidopteran caterpillar pests (Blackburn *et al.*, 1998; Bowen *et al.*, 1998). However, the precise mechanism of how they elicit this toxic response remains to be determined. There is evidence to suggest that the Tc's cause massive cell death of the insect midgut; when the Tca complex was fed to first-instar caterpillar larvae of the model insect host *Manduca sexta*, cessation of feeding occurred within 24 h, followed by death after several days. Histopathological examination of the larvae showed that the columnar cells of the anterior midgut swell apically, extruding large cytoplasmic vesicles 3 h post-feeding. After 6 h, apical 'blebs' containing nuclei and vacuoles also occur. The disintegration of the epithelium is complete after 12 h (Blackburn *et al.*, 1998; Silva *et al.*, 2002).

Subsequently, heterologous expression of the individual Tc proteins in *Escherichia coli* revealed that the TcdA subunit (an [A]) alone was capable of demonstrating toxicity to *M. sexta* (Waterfield *et al.*, 2001a) but that it was significantly less toxic than the native complex. In addition, transgenic expression of *tcdA* in *Arabidopsis thaliana* also produced toxicity, although again this was not associated with the high levels of oral activity of the native protein complexes (Liu *et al.*, 2003). Subsequent work by Waterfield *et al.* (2005) and Sergeant *et al.* (2003) in *Xenorhabdus nematophilus* showed that when heterologously expressed, in order for high toxicity to be established not only must all three subunits be present but the [BC] subunit genes must be co-expressed in the same *E. coli* bacterial cytoplasm.

Tc orthologues have since been found in other Gram-negative bacteria including *Serratia entomophila* and *X. nematophilus* (Hurst *et al.*, 2000; Sergeant *et al.*, 2003) and have also been found in the Gram-positive bacterium *Paenibacillus* (Enright & Griffin, 2005). This implies that the Tc's may be important in insect pathogens. Interestingly, they have also been found in several *Yersinia* species which are not known to be insect pathogens (Parkhill *et al.*, 2001; Hinchliffe *et al.*, 2003; Chain *et al.*, 2004; Tennant *et al.*, 2005; Bresolin *et al.*, 2006a).

Three species of the genus *Yersinia* are pathogenic to humans. *Yersinia pestis*, transmitted by a flea, is the causative agent of bubonic plague, and *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*, which are intestinal pathogens, cause symptoms such as diarrhoea, fever and abdominal pain (reviewed by Wren, 2003). Interestingly, although *Y. pestis* and *Y. pseudotuberculosis* cause different diseases, they share 97% amino acid sequence identity. Indeed, *Y. pestis* is believed to have evolved from *Y. pseudotuberculosis* as little as 1500–20 000 years ago whereas *Y. enterocolitica* is far more distantly related (Achtman *et al.*, 1999).

For a comprehensive discussion on the evolution of the *Yersinia* Tc toxins see Waterfield *et al.* (2007); however, we will discuss these genes briefly below. To date, *tc* coding

sequences have only been identified in a very small percentage of *Y. enterocolitica* strains. Of these, *Y. enterocolitica* W22703 has orthologues to *tcaA*, *tcaB*, *tcaC* and *tccC*, which are located on a pathogenicity island, TcPAI, and are toxic to *M. sexta*. Insertion knockouts of *tcaA* resulted in a loss of activity, indicating that this gene is directly involved in the observed insecticidal toxicity. The *tc* genes of *Y. enterocolitica* W22703 were expressed 4.6-fold higher when the bacteria were grown at 10 °C compared to 37 °C (Bresolin *et al.*, 2006b), which suggests that *Y. enterocolitica* may use the Tc proteins in the soil environment or that the bacterium is hosted by an insect during its life cycle. Conversely, divergent *tc* gene orthologues identified in *Y. enterocolitica* strain T83 were not expressed at low temperature and *tc* gene knockouts resulted in an inability to colonize the rat gut or invade CHO cells (Tennant *et al.*, 2005). These findings suggest that different strains of *Y. enterocolitica* may use Tc proteins either in the soil environment against invertebrates or in the mammalian gut. The *tc* genes in these two strains are significantly divergent in gene sequence and operon structure (Waterfield *et al.*, 2007), suggesting that different Tc's are specific to different hosts.

Unlike *Y. enterocolitica* where a few strains have independently acquired pathogenicity islands containing *tc* orthologues, a single *tc* locus appears to be present in all *Y. pseudotuberculosis* and *Y. pestis* strains. The genome sequence of *Y. pestis* strain CO92 first revealed orthologues of *tcaA*, *tcaB*, *tcaC* and two copies of *tccC* with a further two orthologues of *tccC* located elsewhere in the genome. This locus has since been identified in all strains of *Y. pestis* sequenced so far or by comparative genomics using microarray analysis (Deng *et al.*, 2002; Hinchliffe *et al.*, 2003; Song *et al.*, 2004). Similarly, microarray analysis and sequencing of *Y. pseudotuberculosis* strains has shown that this locus is also present (Hinchliffe *et al.*, 2003; Chain *et al.*, 2004). However, in *Y. pseudotuberculosis* only one copy of *tccC* is associated with this locus (Chain *et al.*, 2004). The amino acid sequences of *Y. pestis* and *Y. pseudotuberculosis* Tc proteins are approximately 97% identical, with between 35 and 70% identity to the *P. luminescens* Tc's. It has been suggested that *Y. pseudotuberculosis* Tc's may be insecticidal toxins and that the divergence observed between *Y. pseudotuberculosis* and *Y. pestis* evolved to enable *Y. pestis* to persist within the rat flea *Xenopsylla cheopis* (Parkhill *et al.*, 2001; Wren, 2003; Waterfield *et al.*, 2004). However, while *Y. pseudotuberculosis* is able to colonize the midgut of the rat flea and cause chronic infection, displaying symptoms such as diarrhoea, the heterologously expressed *Y. pseudotuberculosis* TcaATcaB [A] and TcaCTccC [BC] were found to lack toxicity to the flea. Furthermore, knockouts of these *tc* genes in *Y. pseudotuberculosis* strains did not diminish flea toxicity (Erickson *et al.*, 2006, 2007). This adds weight to the hypothesis that the Tc's in *Y. pestis* and *Y. pseudotuberculosis* are not insecticidal but may instead be mammalian toxins. The Tc toxins of *Y. pestis* have been suggested to be

secreted via a type III secretion system indicative of immunomodulatory toxins although no functional data supporting this hypothesis have been published (Gendlina *et al.*, 2007).

Here we present what is believed to be the first investigation into the biological role of the *tc* genes of *Y. pseudotuberculosis* and *Y. pestis*. Our data provide evidence that the Tc's in *Y. pseudotuberculosis* and *Y. pestis* have no demonstrable insecticidal activity but show differential toxicity to cultured mammalian cells, indicating a role in mammalian pathogenicity.

METHODS

Bacterial strains and growth media. *Y. pseudotuberculosis* strains IP32953 and YPIII pIB1 were obtained from E. Carniel (Institute Pasteur, Paris, France) and H. Wolf-Watz (Umeå University, Umeå, Sweden) respectively. Other *Y. pseudotuberculosis* strains used for sequencing were obtained from H. Fukushima (Shimane Prefectural Institute of Public Health and Environmental Science, Matsue, Japan). All strains were maintained on CIN agar supplemented with *Yersinia* selective supplement (Oxoid) and cultures were grown in LB broth with appropriate antibiotics unless otherwise stated.

Cloning and sequencing of *Y. pseudotuberculosis* Tc coding sequences. Full-length *tcaA*, *tcaB* and *tcaC* coding sequences were PCR amplified from six different strains of *Y. pseudotuberculosis* and the *Y. pestis* KIM⁺ strain. Primers TcaA-Myc, TcaB-Myc and TcaC-Myc were used to amplify the coding sequences in conjunction with TcaA-STOP, TcaB-STOP and TcaC-STOP respectively (Table 1). PCRs were performed using Accuprime pfx DNA polymerase (Invitrogen) to ensure high fidelity and were cloned into the CMV-promoter-based eukaryotic expression vector RK5myc using *Bam*HI and *Xba*I, for later transfection into the Caucasian colon adenocarcinoma (Caco-2) cell line (Fogh *et al.*, 1977). Cloned products were then sequenced using the ABI Big Dye Terminator v3.1 cycle sequencing kit and an ABI 3730 DNA analyser.

Cloning of Tc coding sequences for functional heterologous expression. Construction of the functional *P. luminescens* W14 *tcaAB* and *tcdBC* expression strains is described elsewhere (Waterfield *et al.*, 2005). The *tcaAB* and *tcaCtccC* coding sequences from *Y. pseudotuberculosis* strain IP32953 or *Y. pestis* KIM⁺ were PCR amplified from the relevant genomic DNA using rTth DNA polymerase (Applied Biosystems) and cloned into the arabinose-inducible expression plasmid pBAD30. An artificial translation initiation sequence (AGGAGG) was substituted for the native sequence of the first gene in each of the operons in order to increase levels of expression in the *E. coli* host strain BL21. *Y. pseudotuberculosis* strain IP32953 *tcaAB* was amplified with primers YptbTA5 and YptbTB3 whilst *tcaCtccC* was amplified with primers YptbTC5 and YptbTD3. *Y. pestis* KIM⁺ *tcaAB* was amplified with primers YerArtSDtcaAF and YerArtSDtcaBR whilst KIM⁺ *tcaCtccC* was amplified with primers YerArtSDtcaC and YpKIMtccC1artSDR. These resulted in the generation of the expression plasmids pBAD30-*YptbA*, pBAD30-*YptbBC*, pBAD30-*YpeA* and pBAD30-*YpeBC*. All constructs were sequenced using the ABI Big Dye Terminator v3.1 cycle sequencing kit and an ABI 3700 DNA analyser.

Heterologous expression of Tc components. Expression and assays were performed essentially as described previously (Waterfield *et al.*, 2005). Briefly, glycerol stocks were streaked onto LB agar plates containing 100 µg ampicillin (Amp) ml⁻¹. Colonies were picked and

grown in 5 ml LB containing 0.4% glucose and Amp overnight at 30 °C, 300 r.p.m. Then 0.5 ml of cells was harvested and used to inoculate 50 ml LB + Amp and grown at 37 °C, 250 r.p.m. to OD₆₀₀ ~0.7. Cultures were induced with 0.4% arabinose and cooled to 15 °C, at which temperature they were grown for 24 h. Cells were harvested by centrifugation at 4000 r.p.m., 4 °C. The cell pellet was resuspended in 10 ml sterile 1 × PBS and physically lysed by sonication (45 mA, 10 s on/10 s off for a total 2 min on ice using a Branson 450 digital sonifier fitted with a tapered probe). Lysates were analysed by 6% SDS-PAGE to confirm the presence of the expressed Tc proteins. These same lysates were subsequently split in two: one half was used in insect oral bioassays, and the other for cytosolic preparation and in topical bioassays as described below.

Western blotting to determine expression and secretion of TcaCTccC [BC]. *Y. pseudotuberculosis* strain IP32953 was used to inoculate 5 ml LB broth at 28 °C overnight with shaking. Then 0.5 ml was harvested and used to inoculate 50 ml LB broth and grown at 37 °C, 28 °C or 10 °C for 72 h. One millilitre of cell suspension was harvested and the OD₆₀₀ adjusted to 1.5. The pellet was then harvested and resuspended in 0.05 ml SDS loading buffer (50 mM Tris/HCl, pH 6.8, 4%, w/v, SDS, 0.2% w/v bromophenol blue, 20% glycerol and 5% β-mercaptoethanol). The remaining cell suspension was harvested and supernatant was collected. The supernatant was subsequently concentrated using an Amicon Ultra 15 centrifugal concentrator (Millipore) and total protein determined using Bradford assay according to the manufacturer's instructions (Sigma) and adjusted to 4 mg ml⁻¹. Equal volumes of whole-cell extract and concentrated supernatant were subjected to 6% SDS-PAGE, then transferred to Immunoblot PVDF membrane (Bio-Rad). The membrane was incubated with an anti-*P. luminescens* TcdB rabbit polyclonal antiserum raised against the C-terminal peptide RRTSTGVPNYDETDC, followed by an anti-rabbit IgG alkaline-phosphatase-conjugated antibody (Sigma) and visualized with the BCIP/NBT liquid substrate system (Sigma).

Expression levels of the heterologously expressed *Y. pseudotuberculosis* TcaCTccC (pBAD30-*YptbBC*), *Y. pestis* TcaCTccC (pBAD30-*YpeBC*) and *P. luminescens* TcdBTccC (pBAD30-*tcdBtccC*) were also determined by Western blotting.

Generation of *tcaAB::Km* insertion mutants. The mutation method was a variation on the λ-Red recombinase method (Datsenko & Wanner, 2000) and utilized plasmid pAJD434, which has been optimized for efficiency in *Yersinia* ssp. (Maxson & Darwin, 2004). Plasmids and primers used for the mutant generation are listed in Table 1. Gene-specific primers were designed to introduce a kanamycin resistance cassette into the intended coding sequence. These 70-mer oligonucleotides consisted of 50 bases of gene-specific sequence at the 5' end of the primer whilst the 3' end consisted of 20 bases designed to amplify the kanamycin cassette from the pUC4K plasmid. After optimization, the single PCR product was purified using a Qiagen PCR Purification kit and digested with *Dpn*I to remove the plasmid template.

Wild-type strains were electroporated with the λ-Red plasmid pAJD434 and grown at 28 °C in LB broth containing 0.8% (w/v) L-arabinose to maintain a high level of expression of the bacteriophage *red-gam* genes. Once cells had reached OD₆₀₀ ~0.7, they were incubated on ice for 20 min before being washed twice in ice-cold H₂O and resuspended with the 50 µl purified PCR product. Cells were then electroporated, transferred to a bijou bottle containing 500 µl LB + 0.8% (w/v) L-arabinose and incubated overnight at 28 °C before being plated onto selective plates. Mutations were verified by PCR and Southern blotting. Retention of the virulence plasmid pYV was confirmed by PCR and by colony appearance on CRMOX plates (Riley & Toma, 1989). pAJD434 is thermosensitive and thus retention of the plasmid was selected

Table 1. Bacterial strains, plasmids and primers used in this study

Regions of the mutagenesis primers YptbTcaAKanF and YptbTcaBKanR which anneal to the pUC4K vector are underlined.

Strain, plasmid or primer	Characteristics	Reference
Strains		
IP32953	<i>Y. pseudotuberculosis</i> serotype O:1b	Chain <i>et al.</i> (2004)
YPIII pIB1	<i>Y. pseudotuberculosis</i> serotype O:3	
IP32953 <i>tcaAB</i> ::Km	IP32953 Km ^r insertion mutant	This study
YPIII pIB1 <i>tcaAB</i> ::Km	YPIII pIB1 Km ^r insertion mutant	This study
Plasmids		
pAJD434	~0.7 kb <i>Pst</i> I Tp ^r fragment of p34s-Tp in pKD46 (Tp ^r Ap ^s)	Maxson & Darwin (2004)
pUC4K	Amp ^r Km ^r	Taylor & Rose (1988)
pBAD30	Amp ^r P _{BAD}	Guzman <i>et al.</i> (1995)
pBAD30- <i>tcdA</i>	Amp ^r P _{BAD} <i>P. luminescens tcdA</i> [A]	Waterfield <i>et al.</i> (2005)
pBAD30- <i>tcdBtccC</i>	Amp ^r P _{BAD} <i>P. luminescens tcdBtccC</i> [BC]	Waterfield <i>et al.</i> (2005)
pBAD30- <i>YptbA</i>	Amp ^r P _{BAD} <i>Y. pseudotuberculosis tcaAtcaB</i> [A]	This study
pBAD30- <i>YptbBC</i>	Amp ^r P _{BAD} <i>Y. pseudotuberculosis tcaCtccC</i> [BC]	This study
pBAD30- <i>YpeA</i>	Amp ^r P _{BAD} <i>Y. pestis tcaAtcaB</i> [A]	This study
pBAD30- <i>YpeBC</i>	Amp ^r P _{BAD} <i>Y. pestis tcaCtccC</i> [BC]	This study
Primers		
	Sequence	
YptbTcaAKanF	ACCGTATGGCAATGATCCCCGCACACTTGGTGTGAGTTTCTCGCAAGCTCAAGTCAGCGTAATGCTCTGC	
YptbTcaBKanR	TTATCTTTTTGGGTGCTATAACTGAGCAGTTCATCAACTGATGTTTGAAGCACAGGAAACAGCTATGACC	
TcaA-Myc	TTTGAGCTCACCATGGAGCAGAAGCTGATCTCCGAGGAGGACCTGGGATCCTTGCAAGATAATATCGACCATAC	
TcaA-STOP	TTTTCTAGAACTGATCGCTTCGGCAATAGG	
TcaB-Myc	TTTGAGCTCACCATGGAGCAGAAGCTGATCTCCGAGGAGGACCTGGGATCCATAATTCTGCCTACTAGCACTG	
TcaB-STOP	TTTTCTAGATATCAAGAACGGATGGTGTAG	
TcaC-Myc	TTTGAGCTCACCATGGAGCAGAAGCTGATCTCCGAGGAGGACCTGAGATCTCTGGAAAACAGTAAACAGCAAGTG	
TcaC-STOP	TTTTCTAGATACCCGTCATCTTTTCATGTTG	
YptbTA5	AAACTAGCTAGCTCGAGTAAGGAGGTAACATATGCAAGATAATATCGACCATACTG	
YptbTB3	TGACTCGAGCCCCGGGCGTTATCAGGAACGGATGGTGTAGCGAATA	
YptbTC5	CCCACCCGGGCTCGAGTAGGAGGACCCATATGGAAAACAGTAAACAGCAAGTGG	
YptbTD3	GGCTCGAGCGCATGCTTAATAAATTATAATGATGTAGAGTGTGTCTT	
YerArtSDtcaAF	CCCGAATTCAGGAGGTAACATATGCAAGATAATATCGACCATACTG	
YerArtSDtcaBR	GAGCCCCGGGCGTTATCAAGAACGGATGGTGTAGCGAATA	
YerArtSDtcaCF	GGCCCCGGGAGGAGGCCCATATGGAAAACAGTAAACAGCAAG TGG	
YpKIMtccC1artSDR	ATATTCTAGATTATCGATTCTGTTTGCTATATAAA

Fig. 1. Expression and secretion of *Y. pseudotuberculosis* TcaCTccC. (a) Western blot of *Y. pseudotuberculosis* wild-type strain IP32953 grown at 15 °C, 20 °C, 28 °C or 37 °C. Equal total protein concentrations as determined by Bradford assay of whole-cell pellet and concentrated supernatant were analysed. Samples were loaded as follows: M, standard size marker; 1, 15 °C whole-cell extract; 2, 15 °C supernatant; 3, 20 °C whole-cell extract; 4, 20 °C supernatant; 5, 28 °C whole-cell extract; 6, 28 °C supernatant; 7, 37 °C whole-cell extract; 8, 37 °C supernatant; C, BL21 pBAD30-YptbBC control. (b) Western blot showing levels of expression from *E. coli* BL21 transformed with (1) pBAD30-YpeBC, (2) pBAD30-YptbBC and (3) pBAD30-tcdBtccC.

against by growth of mutants at 37 °C in LB broth containing 2.5 mM CaCl_2 to prevent the loss of the virulence plasmid pYV. Complementation was performed by transformation of the pBAD30-YptbA plasmid in these mutant strains.

Oral toxicity of *Y. pseudotuberculosis* strains to *M. sexta*. *Y. pseudotuberculosis* wild-type and insertion mutants were grown in 5 ml LB at 28 °C overnight with shaking. An aliquot (0.5 ml) was harvested and used to inoculate 50 ml of the same medium and grown at 28 °C for 72 h ($\text{OD}_{600}=1.5$). Aliquots (200 μl ; 2×10^8 cells) of these whole cultures were then topically applied to 1 cm^3 blocks of artificial wheatgerm *M. sexta* diet. Treated food blocks were allowed to dry at room temperature for 20 min before two neonate larvae were applied to each food block and incubated at 25 °C for 7 days to allow the larvae to feed. After this time the larvae were weighed and scored for mortality.

Similarly lysates from 50 ml cultures of BL21 cells expressing pBAD30-YptbA, pBAD30-YptbBC, pBAD30-tcdA or pBAD30-tcdBtccC were prepared as described above. Equal volumes of pBAD30-YptbA and pBAD30-YptbBC lysates were mixed as were equal volumes of pBAD30-tcdA and pBAD30-tcdBtccC lysates. These were then incubated at room temperature for 20 min before 200 μl aliquots were applied to food blocks as above.

Topical application to cultured cell lines. Lysates prepared as described above were centrifuged at 20 000 r.p.m., 4 °C for 20 min (Beckman Coulter) in sterile 50 ml Oakridge centrifuge tubes. Supernatants were collected. Concentration was determined by SDS-

PAGE and Bradford assay (Sigma), and samples were adjusted to 4 mg ml^{-1} . Caco-2 cells were seeded into six-well plates containing borosilicate glass coverslips (BDH) and grown for 24 h in 2 ml Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10 % fetal calf serum, 1 % non-essential amino acids (Sigma) and 1 % penicillin and streptomycin at 37 °C until cells were confluent. A 250 μl aliquot of BL21 TcaATcaB lysate was combined with an equal volume of TcaCTccC lysate (TcdBtccC for *P. luminescens*) and incubated at room temperature for 20 min. This mixture was then diluted with 500 μl serum-free DMEM and added to cells refreshed in 1 ml DMEM before incubation for 24 h at 37 °C with 95 % air/5 % carbon dioxide (v/v). After incubation, cells were washed with PBS, fixed with 4 % paraformaldehyde and permeabilized with 0.02 % Tween 20. Cells were washed three times with PBS before being incubated with either 50 μg FITC-phalloidin ml^{-1} or 50 μg TRITC-phalloidin ml^{-1} (Sigma) for 1 h at room temperature in the dark, followed by 10 min incubation with 0.12 mg Hoechst 33258 DAPI stain (Sigma) ml^{-1} . After washing twice with PBS and twice with dH_2O , coverslips were mounted in Dako Cytomation fluorescent mounting medium, examined and photographed with an Olympus BX61 microscope. These conditions were similar for the NIH3T3 mouse fibroblast cell line except fetal calf serum was replaced with calf serum. Sf9 (Invitrogen) cells were treated as above except cells were incubated in Grace's medium with amino acids, yeastolate and hydrosylate (Invitrogen) and supplemented with 25 μg gentamicin (Sigma) ml^{-1} and incubated at 28 °C in air.

In situ apoptosis detection. Heterologously expressed BL21 Tc lysates were applied to Caco-2 cells as described above. After the 24 h incubation at 37 °C, cells were stained via TdT-mediated dUTP nick end label (TUNEL) following the manufacturer's instructions (Roche Applied Science), followed by Hoechst 33258 DAPI stain, before being mounted and visualized as described.

Mammalian cell transfection. Caco-2 cells were seeded into six-well plates containing borosilicate glass coverslips (BDH) and grown for 24 h in 2 ml DMEM (Invitrogen) supplemented with 10 % fetal calf serum, 1 % non-essential amino acids (Sigma) and 1 % penicillin and streptomycin and grown at 37 °C, 95 % air/5 % carbon dioxide (v/v). These conditions were similar for the NIH3T3 mouse fibroblast cell line except that fetal calf serum was replaced with calf serum. The RK5myc mammalian expression vector containing the Tc fusion and an N-terminal Myc epitope tag (see 'Cloning and sequencing of *Y. pseudotuberculosis* Tc coding sequences') were co-transfected with a GFP-fusion construct, using GeneJuice Transfection reagent (Novagen), according to the manufacturer's protocol. The GFP-actin construct chosen was the pEGFP-actin, a mammalian expression vector expressing the EGFP-actin fusion protein of the human codon optimized variant green fluorescent protein and the gene encoding human cytoplasmic β -actin (Clontech). After 24 h the samples were fixed with 4 % paraformaldehyde (w/v) in PBS, permeabilized with 0.2 % Triton X-100 and blocked with four drops of Image-iT FX signal enhancer (Molecular Probes). Cells were labelled with monoclonal anti-Myc primary antibody (Invitrogen), followed with Alexa Fluor 594 goat anti-mouse dye-labelled secondary antibody (Molecular Probes) to detect the Myc protein. The cell nucleus was visualized by incubating in Hoechst 33258 DAPI stain (Sigma), at a final concentration of 0.12 mg ml^{-1} in PBS for 10 min at room temperature in the dark. Slides were washed twice in PBS followed by two washes in water. Coverslips were mounted in Dako Cytomation fluorescent mounting medium and examined and photographed with an Olympus BX61 microscope.

RESULTS

Sequence alignment of *Yersinia* Tc proteins

In order to investigate the genetic diversity of *tc* genes in different serotypes of *Y. pseudotuberculosis*, we sequenced

paralogues from several strains, as described. Sequence alignments of TcaA, TcaB and TcaC revealed a reasonable degree of conservation (see Supplementary Fig. S1a, b and c, available with the online version of this paper). However, we do see differences in the level of conservation for the different proteins of the complex. TcaC is the most conserved of the three protein sequences, showing 96 % identity between even the most diverse orthologues. The TcaA proteins show a higher degree of variation, which appears to be mainly in the C-terminal regions and may suggest some selective diversification. TcaB shows the highest degree of diversification, especially in the region between amino acids 201 and 426.

Expression and secretion of the Tc

Previous RT-PCR results have demonstrated that the Tc components are preferentially transcribed at 30–37 °C. In order to show that the proteins are actually produced at these temperatures and are secreted by wild-type *Y. pseudotuberculosis*, we performed SDS-PAGE and Western blot analysis using a cross-reactive rabbit antiserum raised against the TcdB component of *P. luminescens* (Fig. 1a). Expression and secretion of the Tc was observed at 30 °C and 37 °C, with the complex being present in whole-cell extracts and supernatants. These data demonstrate that the proteins are secreted during growth in normal media and the size of the observed protein suggests that the secreted complex contains all the Tc components as expected.

Western blotting was also used to determine the relative levels of protein from *E. coli* BL21 expressing *Y. pseudotuberculosis* TcaCTccC, *Y. pestis* TcaCTccC and *P. luminescens* TcdBTccC from pBAD30 expression vectors (Fig. 1b). The TcaC and TccC proteins form a complex which runs at approximately 150 kDa, and is observable at comparable levels for all lysates.

Oral toxicity of *Y. pseudotuberculosis* to *M. sexta*

Oral toxicity to *M. sexta* has previously been observed for *Y. pseudotuberculosis* strain IP32953 but not for other *Y. pseudotuberculosis* strains tested (Waterfield *et al.*, 2007). In order to determine if this toxicity could be attributed to Tc proteins, a *tcaAB::Km* insertion mutant was generated in strain IP32953 and in the non-orally toxic strain YPIII pIB1. Wild-type and mutant strains were then fed to *M. sexta* neonates as described. As can be seen in Fig. 2(a), there was no observable difference in oral toxicity between the wild-type strains and their knockouts. Contrary to previous findings, no increase in mortality of caterpillars was observed for *Y. pseudotuberculosis* IP32953 wild-type (5 %) or YPIII pIB1 wild-type (10 %). However, there is a small increase in mortality for both the *Y. pseudotuberculosis* IP32953 *tcaAB::Km* mutant (15 %) and YPIII pIB1 *tcaAB::Km* mutant (20 %) when compared to the negative control (10 %).

Similarly, no oral toxicity or mortality was observed when neonates were fed food blocks impregnated with cell lysates

from *E. coli* BL21 containing *Y. pseudotuberculosis* [A]+[BC]. Cell lysates from *E. coli* BL21 containing *P. luminescens* [A]+[BC] were used as a positive control and produced a dramatic reduction in weight gain and 60 % caterpillar mortality (Fig. 2a).

Toxicity of *Y. pseudotuberculosis* Tc's to *Spodoptera fragiperala* insect cell line Sf9

Lysates from *E. coli* BL21 expressing the [A] and [BC] components from *Y. pseudotuberculosis* and *Y. pestis* were compared to lysates from *E. coli* BL21 expressing the [A] and [BC] components from *P. luminescens* when topically applied to the insect cell line Sf9 over a 24 h period. Cells were stained for F-actin and cell nuclei and were examined using fluorescence microscopy. Marked actin condensation (86 %) and apoptotic nuclei (94 %) were observed when BL21 lysates containing *P. luminescens* Tc proteins were topically applied to cells, whereas limited effects were observed for BL21 lysates containing *Y. pseudotuberculosis* (34 % and 36 %) and *Y. pestis* (22 % and 27 %) Tc proteins (Fig. 2b and c). These were comparable to the vector control (28 % and 27 % respectively).

Effect of *Y. pseudotuberculosis* wild-type and *tcaAB::Km* mutants topically applied to the Caco-2 cell line

Incubation of wild-type *Y. pseudotuberculosis* strains IP32953 and YPIII pIB1 with Caco-2 cells produced similar phenotypic results to those observed for the lysates of BL21 cells expressing *Y. pseudotuberculosis* Tc proteins (Fig. 3). Vacuolation was observed in 26.5 % and 29 % of cells for wild-type *Y. pseudotuberculosis* strains IP32953 and YPIII pIB1 respectively, whereas the cytosolic preparations produced vacuolation in 29 % of Caco-2 cells whilst only 4.4 % of control cells demonstrated this phenotype. Interestingly, the *Y. pseudotuberculosis* strains IP32953 and YPIII pIB1 *tcaAB::Km* mutants produced significantly less vacuolation than wild-type (6.6 % and 11.8 %), which indicates that the Tc's are involved in the cellular toxicity exhibited by *Y. pseudotuberculosis*.

Effect of the Tc toxins on Caco-2 and NIH3T3 cell lines

Lysates from *E. coli* BL21 expressing the [A] and [BC] components from *Y. pseudotuberculosis*, *Y. pestis* and *P. luminescens* were topically applied to the mammalian gut cell line Caco-2 and the mouse fibroblast cell line NIH3T3 over a 24 h period. Cells were stained for F-actin and cell nuclei and were examined using fluorescence microscopy. As shown in Fig. 3, lysates containing *Y. pseudotuberculosis* [A]+[BC] induced marked rearrangement of the actin cytoskeleton in Caco-2 cells, causing membrane ruffling (Fig. 3a) and vacuolation (Fig. 3b) as well as nuclear fragmentation. Membrane vacuoles were observed in 41 % of cells whereas no significant effects were observed for

Fig. 2. Effect of Tc's on insects. (a) Oral bioassay of wild-type *Y. pseudotuberculosis* strains IP32953 and YPIII pIB1 and their respective *tcaAB::Km* knockouts. The histogram bars show the relative weight gain of *M. sexta* neonate larvae fed with cell lysates added to artificial diet. Weight gain of different treatments is expressed relative to a pBAD30 BL21 (negative control; always the value of 1) and standard errors of each mean are shown. Columns: 1, BL21 pBAD30 control; 2, BL21 lysates containing *P. luminescens* [A]+[BC]; 3, BL21 lysates containing *Y. pseudotuberculosis* [A]+[BC]; 4, YPIII pIB1; 5, YPIII pIB1 *tcaAB::Km*; 6, IP32953; 7, IP32953 *tcaAB::Km*. The black line shows the percentage of dead caterpillars. (b) Fluorescence micrographs of *Sf9* insect cells topically treated with cytosolic preparations of A, BL21 pBAD30 control; B, BL21 lysates containing *P. luminescens* [A]+[BC]; C, BL21 lysates containing *Y. pestis* [A]+[BC]; or D, BL21 lysates containing *Y. pseudotuberculosis* [A]+[BC]. Cells were stained for F-actin with FITC-phalloidin, and for nuclei with Hoechst 33258. Arrows indicate typical cells observed (A, C) or apoptotic nuclei with heavily condensed actin (B). (c) Graph representing percentage of cells with actin condensation (white bars) and apoptotic nuclei (grey bars), where n =minimum of 300 cells. Columns: 1, BL21 pBAD30 control; 2, BL21 lysates containing *Y. pseudotuberculosis* [A]+[BC]; 3, BL21 lysates containing *Y. pestis* [A]+[BC]; 4, BL21 lysates containing *P. luminescens* [A]+[BC].

lysates containing *P. luminescens* [A]+[BC] (13.5 %) or *Y. pestis* [A]+[BC] (6 %) compared to the vector control (4 %) (Figs 3d, e and f). Again we see maximal activity only when the [A] and [BC] components are combined prior to topical application (data not shown). Conversely, lysates containing *Y. pseudotuberculosis* [A]+[BC] did not appear to have any marked effect on NIH3T3 cells (Fig. 4a), whereas lysates containing *Y. pestis* [A]+[BC] caused marked levels of actin condensation (56.8 %) and nuclear fragmentation (36.5 %), (Fig. 4c), compared to the control

(4.8 % actin condensation and 11.25 % nuclear fragmentation). Interestingly, lysates containing *P. luminescens* [A]+[BC] also caused punctuate actin and actin condensation (65.4 %) and nuclear fragmentation (37 %) (Fig. 4b).

To investigate if the *Y. pseudotuberculosis* and *Y. pestis* Tc cell intoxication effects observed led to either necrosis or apoptosis (programmed cell death), lysates containing *Y. pseudotuberculosis* and *Y. pestis* [A]+[BC] were topically applied to the respective cell lines followed by TUNEL

Fig. 3. Fluorescence micrographs of Caco-2 cells topically treated with (a, b) BL21 lysates containing *Y. pseudotuberculosis* [A] + [BC], (c) BL21 lysates containing *P. luminescens* [A] + [BC], (d) BL21 lysates containing *Y. pestis* [A] + [BC], (e) IP32953, (f) IP32953 *tcaAB*::Km, (g) YPIII pIB1, (h) YPIII pIB1 *tcaAB*::Km or (j) BL21 pBAD30 control. Cells were stained for F-actin with FITC-phalloidin or TRITC-phalloidin, and for nuclei with Hoechst 33258. Arrows indicate membrane ruffles (a), actin vacuoles (b), multinucleation (e) and actin condensation (g) typically seen in Caco-2 cells treated with *Y. pseudotuberculosis* containing TC's or heterologously expressed *Y. pseudotuberculosis* [A] + [BC]. (h) Punctuate actin seen with cells treated with any *Y. pseudotuberculosis* strain. (i) Graph representing percentage of cells with vacuoles/membrane ruffles (white bars) and nuclei fragmentation (grey bars), where n =minimum of 300 cells. Columns: 1, BL21 pBAD30 control; 2, BL21 lysates containing *Y. pseudotuberculosis* [A] + [BC]; 3, BL21 lysates containing *Y. pestis* [A] + [BC]; 4, BL21 lysates containing *P. luminescens* [A] + [BC].

staining. This reveals the presence of DNA strand breakage indicative of apoptosis. Approximately 26 % of the *Y. pseudotuberculosis* Tc-intoxicated Caco-2 cells were positive by TUNEL stain, which was significantly above background (0.8 % of cells) (Fig. 5a and b). The *Y. pestis* Tc-intoxicated

NIH3T3 cells also showed signs of apoptosis with 19.4 % of cells positive by TUNEL stain compared to 2.9 % for the negative control (Fig. 5d). Despite many of the Tc-intoxicated cells being TUNEL positive, not all nuclear aberrations seen were, which suggests either that the Tc's

Fig. 4. Fluorescence micrographs of NIH3T3 cells topically treated with (a) BL21 lysates containing *Y. pseudotuberculosis* [A]+[BC], (b) BL21 lysates containing *P. luminescens* [A]+[BC], (c) BL21 lysates containing *Y. pestis* [A]+[BC], or (d) BL21 pBAD30 control. Cells were stained for F-actin with FITC-phalloidin, and for nuclei with Hoechst 33258. Arrows indicate punctuate actin (red arrows; b), or actin condensation (white arrows; b and c). (e) Graph representing percentage of actin condensation (white bars) and nuclear fragmentation (grey bars), where n =minimum of 300 cells. Columns: 1, BL21 pBAD30 control; 2, BL21 lysates containing *P. luminescens* [A]+[BC]; 3, BL21 lysates containing *Y. pseudotuberculosis* [A]+[BC]; 4, BL21 lysates containing *Y. pestis* [A]+[BC].

can induce a combination apoptosis and necrosis, or that we are visualizing different time points in the same process, resulting from 'asynchronous' intoxication.

Intracellular expression of IP32953 Tc proteins in Caco-2 cells

To further verify that the Tc proteins were responsible for the observed cellular toxicity we transiently expressed each protein in cells using a method previously described for *P. luminescens* (Waterfield *et al.*, 2005). Briefly, each *tc* coding sequence (*tcaA*, *tcaB* and *tcaC*) from *Y. pseudotuberculosis* IP32953 was cloned into the pRK5myc mammalian expression construct. The proteins were then expressed in the cytoplasm of Caco-2 cells as cMyc-N-terminal fusions after co-transfection with a fused gfp::actin reporter construct (pEGFP-actin). Cells were then stained with an anti-cMyc tag antibody and Hoechst 33258 DAPI stain as described in Methods before visualization by fluorescence microscopy.

After 24 h the Myc-tagged *Y. pseudotuberculosis* TcaA protein accumulated in the cytoplasm and caused 'membrane ruffling' in the actin cytoskeleton of 18.7% cells (control 9.3%). However, most cells (64.3%) showed a heavily condensed actin cytoskeleton (control 7.3%) with 12% showing nuclear fragmentation (Fig. 6b, f, j and n), which was not observed in cells transfected with pEGFP-

actin alone (0%) (Fig. 6a, e, i and m). These two phenotypes may represent early- and late-stage intoxication effects, as transfection and 24 h incubation does not give synchronous cell effects. Transient expression of TcaB produced a very distinctive phenotype (Fig. 6c, g, k and o). Heavy actin condensation was observed in 78.8% of cells, with membrane ruffling/vacuolation observed at the edges of the actin cytoskeleton in the remainder of cells (21%). The Myc-tag accumulated in the cytoplasm. More interestingly, multinucleation was observed in 66.7% of cells. Transient expression of TcaC also produced a highly distinctive phenotype, with large actin vacuoles and nuclear fragmentation (28% of cells had vacuoles compared to 2% observed in the controls) (Fig. 6d, h, l and p), whilst the cMyc-tagged protein accumulated in the nucleus. Non-synchronous effects were again observed, with 53.5% of cells showing heavy condensation (7.3% in controls). Reassuringly, the cytotoxic effects seen with *tcaA*, *tcaB* and *tcaC* resembled the effects seen by topical application of the BL21 lysates containing the [A]+[BC] complex.

Intracellular expression of *Y. pestis* KIM⁺ Tc proteins in NIH3T3 cells

Intracellular expression of *Y. pestis* KIM⁺ Tc proteins in NIH3T3 cells also produced similar effects to those

Fig. 5. TUNEL staining of intoxicated cells to measure apoptosis levels. Cells were TUNEL stained to determine nuclear strand breaks, which indicate apoptosis (a, b and d). The nuclei were co-stained with Hoechst 33258 DAPI stain. (a) Negative control, (b) BL21 lysates containing *Y. pseudotuberculosis* [A] + [BC] on Caco-2 cells; (d) BL21 lysates containing *Y. pestis* [A] + [BC] on NIH3T3 cells. Arrows indicate co-stained cells indicative of apoptotic and TUNEL-positive cells. However, co-staining is not seen in all cases, suggesting a combination of necrosis and apoptosis. (c) Graph showing that ~26 % of cells were TUNEL positive for *Y. pseudotuberculosis*, compared to 0.8 % in the negative control and ~46 % cells in the positive control. For *Y. pestis*, 19.4 % of cells were TUNEL positive compared to 2.9 % in the negative control and 75 % in the positive control. White bars, Caco-2 cells; grey bars, NH3T3 cells.

observed for topical applications. TcaA accumulated in the cytoplasm and caused either membrane ruffling (23 %, control 0.47 %) or actin condensation (21 %, control 1.4 %), with 19 % showing nuclear fragmentation (Fig. 7b, f, j and n). Transient expression of TcaB resulted in actin breakdown (64.3 %) and apoptosis (21 %), with the Myc-tagged protein co-localizing to the cytoplasm (Fig. 7c, g, k and o). Expression of TcaC caused actin condensation in the majority of cells (74 %). Membrane ruffling and membrane blebbing were observed in some cases (6.7 % and 3.6 %). Interestingly, the Myc-tag protein appeared to co-localize in small aggregated blobs around the nuclear membrane (Fig. 7d, h, l and p).

DISCUSSION

Until recently the Tc proteins found in several bacterial species were always thought to be insecticidal toxins and often with a strict host specificity. The Tc's from *P.*

luminescens W14 are orally toxic to a wide range of insects, whilst orthologues found in *X. nematophilus* and *Serratia entomophila* are orally toxic to *Pieris brassicae* or the grass grub *Costelytra zealandica*, respectively (Hurst *et al.*, 2000, 2007). Tc orthologues have also been found in the Gram-positive bacterium *Paenibacillus*, the causative agent of American foulbrood in honeybees. In this genus, the *tc* genes are in many cases tightly linked to crystal-toxin genes, more typical of Gram-positive insect pathogens such as *Bacillus thuringiensis*. Finally, a small number of *Y. enterocolitica* strains from biotypes 2, 3 and 4 have been shown to carry *tcaA*, *tcaB*, *tcaC* and *tccC* genes, which are orally toxic to *M. sexta* (Bresolin *et al.*, 2006a, b). The discovery that a divergent *tc* locus present in some *Y. enterocolitica* biotype 1A strains was required for colonization of the rat gut was the first example of a non-insecticidal role for these proteins (Tennant *et al.*, 2005).

The recent increase in sequencing of bacterial genomes has led to the discovery of *tc* orthologues in a multitude of

Fig. 6. Intracellular transient expression of *tc* toxin genes from *Y. pseudotuberculosis* IP32953 in Caco-2 cells. (a–d) Actin visualization with pEGFP. (e–h) Detection of cMyc-proteins, visualized with Alexa Fluor 594 goat anti-mouse dye-labelled secondary antibody (Molecular Probes). (i–l) Nuclei were visualized with Hoechst 33258 DAPI stain. (m–p) Merge. (a, e, i and m) Control cells transfected with pEGFP alone. Control shows normal actin and nuclear morphology with no cMyc co-localization. (b, f, j and n) Cells transfected with pRK5myc-*tcaA* and pEGFP-actin. *tcaA* shows membrane ruffling which results in actin condensation (arrows; b). (f) RK5myc-*tcaA* tag shows localization of myc-tagged *tcaA* and pEGFP-actin (n). Nuclei are normal (j). Note the intense yellow staining corresponding to co-localization of myc-tagged *tcaA* and pEGFP-actin (n). (c, g, k and o) Cells transfected with pRK5myc-*tcaB* and pEGFP-actin. *tcaB* expression results in membrane ruffling/vacuoles and actin condensation (c and o). Interestingly, as observed in topical applications, multinucleation is observed in ~70 % of cells (k and o). The RK5myc-*tcaB* appears to co-localize with the cytoplasm, although this is difficult to determine in heavily condensed cells. (d, h, l and p) Cells transfected with pRK5myc-*tcaC* and pEGFP-actin. RK5myc-*tcaC* shows large vacuoles (white arrow; d and h). Here the RK5myc-*tcaC* tag co-localizes to the nucleus (h), and causes nuclear aberrations (l and p).

species with no known association with insects, including *Pseudomonas* and *Burkholderia* spp. Genome sequencing and microarray analysis has also revealed the presence of *tc* orthologues in all strains of the closely related mammalian pathogens *Y. pestis* and *Y. pseudotuberculosis* (Hinchliffe *et al.*, 2003; Parkhill *et al.*, 2001). Neither of these species is known to be toxic to insects and *Y. pestis* actually requires an insect vector for its transmission. Infected fleas do eventually die from colonization by *Y. pestis*, though this is thought to be due to starvation caused by the blockage of

the proventriculus preventing blood meal from reaching the stomach rather than any direct toxic effect. Interestingly, microarray analysis revealed a level of divergence in the all of the *tc* genes between *Y. pestis* and *Y. pseudotuberculosis* (Hinchliffe *et al.*, 2003; Parkhill *et al.*, 2001). In *X. nematophilus*, differing insect host specificities have been traced to two paralogous copies of the [A] component (XptA1 and XptA2), suggesting that relatively minor differences in [A] contribute to toxin specificity. This led to the hypothesis that the Tc's of *Y. pseudotuberculosis*

Fig. 7. Intracellular transient expression of *tc* toxin genes from *Y. pestis* KIM⁺ in NIH3T3. (a–d) Actin visualization with pEGFP. (e–h) Detection of cMyc-proteins, visualized with Alexa Fluor 594 goat anti-mouse dye-labelled secondary antibody (Molecular Probes). (i–l) Nuclei were visualized with Hoechst 33258 DAPI stain. (m–p) Merge. (a, e, i and m) Control cells transfected with pEGFP alone. Control shows normal actin and nuclear morphology with no cMyc co-localization. (b, f, j and n) Cells transfected with pRK5myc-*tcaA* and pEGFP-actin. *tcaA* shows actin condensation (b and n) and nuclear fragmentation (j and n). RK5myc-*tcaA* tag shows localization to the cytoplasm (f). Note the intense yellow staining corresponding to co-localization of myc-tagged *tcaA* and pEGFP-actin (n). (o, g, k and o) Cells transfected with pRK5myc-*tcaB* and pEGFP-actin: condensation is observed (c and o) as well as apoptotic nuclei (o and k; arrows). The RK5myc-*tcaB* appears to co-localize with the cytoplasm, although this is difficult to determine in heavily condensed cells. (d, h, l and p) Cells transfected with pRK5myc-*tcaC* and pEGFP-actin. RK5myc-*tcaC* shows membrane ruffling (white arrows) and actin condensation (yellow arrow; d and p). Here the RK5myc-*tcaC* tag shows localization to the nucleus (h).

might be insecticidal toxins that had then diversified during the evolution of *Y. pestis* in order to prevent toxicity in the flea. *Y. pseudotuberculosis* is often found in soil, where it could have the opportunity to interact with insect larvae. In order to determine the extent of the divergence we PCR amplified and cloned the individual *tc* genes from several *Y. pseudotuberculosis* strains of diverse origin and serotype (Table 1) and compared them to published *Y. pestis* gene sequences. Protein sequence alignment (Supplementary Fig. S1a) confirmed the conservation of TcaA across a range of *Y. pseudotuberculosis* serogroups. Differences observed were

mainly localized to the C-termini of these proteins, suggesting that they are under a diversification selection. TcaC was also highly conserved (Supplementary Fig. S1c), whilst TcaB was least conserved (Supplementary Fig. S1b), particularly within the region between amino acids 201 and 426. The diversity within these sequences suggests either diversity between strains or selective pressure for diversification such as the evasion of the mammalian immune system. As yet little is known about the structural and functional regions of Tc proteins in any species; thus we can only speculate as to the basis of this diversity.

We have previously shown that wild-type strains representing five different serogroups of *Y. pseudotuberculosis* were not orally toxic to *M. sexta* larvae (Waterfield *et al.*, 2007), with only strain IP32953 causing any reduction in larval weight gain. These results suggested that either: (i) *Y. pseudotuberculosis* does not encode orally active insecticidal Tc toxins and some other factor is responsible for any toxicity observed, (ii) insect-active Tc toxins are encoded but not expressed, or (iii) the Tc proteins are insect active but not specifically toxic to *M. sexta*. In order to determine if the slight oral toxicity observed for strain IP32953 could be attributed to Tc expression, a *tcaAB::Km* insertion knockout was constructed for this strain and also for strain YPIII pIB1. As shown in Fig. 2, no differences in oral toxicity were observed between the wild-type and the respective insertion knockout strains. In order to further rule out any toxicity of the Tc proteins to *M. sexta* we cloned the appropriate coding sequences and expressed them in the BL21 strain of *E. coli*. Cytosol preparations of expressing cells were fed to neonate larvae as described. These heterologously expressed *Y. pseudotuberculosis* Tc proteins did show limited oral toxicity against *M. sexta* neonates (Fig. 2); however, analysis by two-tailed *t*-test showed that any toxicity seen was not significant (*P* values are as follows: [A]=0.9064, [BC]=0.9064, [A]+[BC]=0.1346), unlike *P. luminescens* [A] (0.0017) [BC] (0.0087) and [A]+[BC] (<0.0001). In addition, we also demonstrated that heterologously expressed *Y. pestis* [A]+[BC] (0.3127) is not toxic to *M. sexta* (data not shown). These results were despite equal concentrations of proteins being fed to *M. sexta* as determined by Western blot.

Further evidence that the *Yersinia* Tc toxins are not insecticidal comes from their lack of toxicity towards cultured insect cells. When cytosolic preparations were topically applied to the *S. fragiperala* insect cell line Sf9 only heterologously expressed *P. luminescens* [A]+[BC] showed marked rearrangement of the actin cytoskeleton and nuclear apoptosis (Fig. 2). Both heterologously expressed *Y. pseudotuberculosis* and *Y. pestis* [A]+[BC] were comparable to the control. This apparent lack of insecticidal toxicity is further backed up by the fact that *Y. pseudotuberculosis* Tc gene knockouts show similar toxicity towards the flea as wild-type bacteria (Erickson *et al.*, 2006, 2007; Hinnebusch *et al.*, 2002).

Western blots carried out with *Y. pseudotuberculosis* wild-type strain IP32953 grown at temperatures varying from 15 °C to 37 °C demonstrate the expression and secretion of these toxins at 28 °C and 37 °C, whereas there is only a low level of expression at 20 °C or 15 °C (Fig. 1). Gels were loaded with similar amounts of total protein and therefore the cell supernatant from cultures grown at 15 °C had to be concentrated far more than other samples in order to achieve this, which may account for the presence of a band in the 15 °C supernatant. Indeed, previous RT-PCR results indicated that there was no expression at 15 °C and thus the Tc complex present in this culture may be an artefact

from the initial starter culture, which was grown at 28 °C. Most importantly these Western blots demonstrate that the toxin complex of strain IP32953 is secreted during growth in normal media at all temperatures. This indicates a mechanism independent of the plasmid-borne type III secretion system, which has been previously reported to be responsible for secretion of this complex and is only expressed at 37 °C (Gendlina *et al.*, 2007). In certain tropical climates there is the potential for bacterial interactions with insects to occur at environmental temperatures of 30 °C or more. Therefore there is the possibility that the *Y. pseudotuberculosis* and *Y. pestis* Tc proteins are specifically toxic to certain insect species which are as yet unidentified. This seems unlikely in the case of *Y. pestis*, which is not known to survive in the environment and only colonizes mammalian hosts and its flea vector. Thus a more likely scenario is that the Tc toxins have evolved to become toxic to the mammalian host.

In order to investigate this hypothesis, lysates from BL21 cells containing *Y. pseudotuberculosis* [A]+[BC] and *Y. pestis* [A]+[BC] were topically applied to the mammalian cell lines Caco-2 and NIH3T3. As shown in Fig. 4, *Y. pseudotuberculosis* [A]+[BC] caused dramatic rearrangement of the actin cytoskeleton of Caco-2 cells, causing membrane ruffles and vacuoles similar to the effects described for CNF1 (Boquet, 2001; Fiorentini *et al.*, 1988). Interestingly, approximately 7% of cells were also multinucleate, compared to 2% of controls. Surprisingly, no toxic effects were seen with the *Y. pseudotuberculosis* Tc's when applied to NIH3T3 cells. Conversely lysates of BL21 cells containing *Y. pestis* [A]+[BC] showed limited cytopathic effects on Caco-2 cells but produced dramatic rearrangement of the actin cytoskeleton and nuclear fragmentation in NIH3T3 cells (Fig. 5). These effects on NIH3T3 cells were also seen with lysates of BL21 cells containing *P. luminescens* [A]+[BC] which is perhaps surprising because *P. luminescens* is not a mammalian pathogen and the Tc's are thought to act on insect midgut. This suggests that the Tc's of *P. luminescens* have a general toxic activity and therefore the evolution of mammalian-specific Tc proteins in *Yersinia* ssp. is not a huge evolutionary step. As previously discussed, host specificity has been attributed to the [A] component of the complex. Unlike the TcdA1, XptA1 and SepA [A] components, which are single long polypeptides (designated the *tcd* configuration), the [A] components of *Y. pseudotuberculosis* and *Y. pestis* are split into two polypeptides, encoded by the *tcaA* and *tcaB* genes (designated the *tca* configuration). In *P. luminescens* the TcaB protein contains an RDG motif at amino acid position 557–560 which is a QGD motif in *Y. pseudotuberculosis*, and in *Y. pestis* the equivalent homology position is 570–572. The presence of RDG motifs has led to speculation that a host integrin may be the toxin receptor on the cell surface. The differences between *P. luminescens* TcaB and *Y. pseudotuberculosis* TcaB could be attributed to this; however, *Y. pestis* TcaB also has QDG and exerts its effects on different cells to the *Y. pseudotuberculosis* Tc

proteins. Thus it can be assumed that although this motif may contribute to binding, it does not determine cell specificity. Rather, the high level of divergence seen in the N-terminal region of TcaB (Supplementary Fig. S1b) could contribute to the specificity observed. Further analysis of the effects of the Tc's on different cell types and structure-function characterization of TcaB will be required to fully understand the cell specificity seen here.

Delineation of the phenotypic effects was determined by transient expression of individual *Y. pseudotuberculosis* and *Y. pestis* TcaA, TcaB and TcaC proteins in Caco-2 or NIH3T3 cells (Figs 6 and 7). This confirmed that the Tc's are capable of causing significant rearrangement of the actin cytoskeleton. TcaA localized to the cytoplasm and induced actin condensation with membrane ruffling (*Y. pseudotuberculosis*) and nuclear fragmentation (*Y. pestis*). This observation is in contrast to the *P. luminescens* *tcaA* gene, which, when expressed in NIH-3T3 cells, had no obvious adverse effect on the cells' F-actin distribution (Waterfield *et al.*, 2005). These results suggest TcaA alone can be toxic, which has been previously reported for *Y. enterocolitica* strain W22703, where TcaA alone is responsible for oral toxicity to *M. sexta* (the *tcaB* gene contains a frameshift). Transient expression of TcaB also caused heavy actin condensation with the protein again accumulating in the cytoplasm (Figs 6 and 7). More interestingly, multinucleation was observed in Caco-2 cells expressing the *Y. pseudotuberculosis* TcaB protein (Fig. 6). Multinucleation has only previously been described with CNF1, which also causes actin rearrangement (Caprioli *et al.*, 1983; Fiorentini *et al.*, 1988). These phenotypic effects of CNF1 can be attributed to the permanent activation of Rho-GTPases (Fabbri *et al.*, 2002; Fiorentini *et al.*, 1997). Further work is now being carried out to determine if the Tc's have a similar mode of action. Transient expression of *Y. pseudotuberculosis* TcaC also produced a very unusual phenotype involving nuclear fragmentation and extensive vacuolation of the cytoplasm (Fig. 6d, h, l and p). In addition, the cMyc-tagged TcaC appeared to accumulate in the cell nucleus. Interestingly, the transient expression of the N terminus of the *Photobacterium* *tcdB* (an orthologue of *tcaC*) in NIH3T3 fibroblasts also localized in the cell nucleus, suggesting that this family of proteins represent nuclear-active toxins. The N-terminal domains of TcaC [B] and TcdB [B] share a high percentage identity to the *Salmonella* plasmid-borne virulence factor, SpvB. In SpvB, this domain is responsible for export across the outer membrane of the Gram-negative cell, suggesting this domain may have general membrane-crossing abilities. The large cytoplasmic vacuoles observed (Fig. 6d and p) are similar to those produced by the *Helicobacter pylori* VacA toxin and the CNF1 toxin of *E. coli*. The vacuoles produced by CNF1 intoxication originate from excessive pinocytosis caused by the inappropriate activation of Rho, Rac and Cdc42 (Boquet, 2001; Fabbri *et al.*, 2002; Falzano *et al.*, 1993), whereas VacA vacuoles originate from late endosomal compartments (Papini *et al.*, 1994). Further work will

be required to determine the origin of the *Y. pseudotuberculosis* TcaC-derived cytoplasmic vacuoles. Expression of TcaC from *Y. pestis* did not cause vacuoles but did cause massive actin condensation (Fig. 7d, h, l and p). Interestingly, it too accumulated at the nuclear periplasm in what look like vesicles, supporting the theory that this domain is involved in export.

In conclusion, we have shown that *Y. pseudotuberculosis* (and *Y. pestis*) Tc proteins appear to have no apparent oral toxicity against the model insect *M. sexta* and are only transcribed at temperatures relevant to expression in the mammalian host, and that these proteins are secreted. In addition, we have demonstrated that the Tc proteins from *Y. pseudotuberculosis*, *Y. pestis* and *P. luminescens* have the ability to cause significant effects on cultured mammalian cells and our results suggest cell specificity. Finally, intracellular transient expression of different *tc* subunit genes in the cytoplasm of mammalian cells reproduces the phenotypes seen by topical application of the protein and suggests an unusual mode of action. Taken together, these observations suggest that the *Y. pseudotuberculosis* *tc* genes have been adapted for activity against the mammalian gut whereas *Y. pestis* has been adapted to other mammalian cell types, while those of *P. luminescens* are adapted for the insect gut, but do show toxicity to mammalian cells, indicating a common mechanism.

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