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The Insect Toxin Complex of *Yersinia*

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Abstract. Many members of the *Yersinia* genus encode homologues of insect toxins first observed in bacteria that are insect pathogens such as *Photorhabdus*, *Xenorhabdus* and *Serratia entomophila*. These bacteria secrete high molecular weight insecticidal toxins comprised of multiple protein subunits, termed the Toxin Complexes or Tc's. In *Photorhabdus* three distinct Tc subunits are required for full oral toxicity in insects, that include the [A], [B] and [C] types, although the exact stochiometry remains unclear. The genomes of *Photorhabdus* strains encode multiple *tc* loci, although only two have been shown to exhibit oral and injectable activity against the Hawk Moth, *Manduca sexta*. The exact role of the remaining homologues is unclear. The availability of bacterial genome sequences has revealed the presence of *tc* gene homologues in many different species. In this chapter we review the *tc* gene homologues in *Yersinia* genus. We discuss what is known about the activity of the *Yersinia* Tc protein homologues and attempt to relate this to the evolution of the genus and of the *tca* gene family.

22.1 Introduction: What is the "Insect Toxin Complex"?

The "toxin complex" insecticidal genes were first identified in *Photorhabdus luminescens* W14, a Gram-negative insect-pathogenic member of the Enterobacteriacea, which is symbiotically associated with insect-pathogenic nematodes from the family *Heterorhabditidae*. The Tc's from *P. luminescens* W14 were shown to have both oral and injectable toxicity against caterpillar pests. These were originally separated into four native complexes each approximately 1MDa in size termed Tca, Tcb, Tcc and Tcd, which are respectively encoded by the four loci *tca, tcb, tcc* and *tcd*, each of which has multiple *tc* genes (Bowen et al. 1998). The *tcd* locus, which was shown to contain three genes, *tcdA1*, *tcdB1* and *tccC2* is now known to be part of a much larger pathogenicity island containing many tandem repeats of *tc* gene homologues (Waterfield et al. 2001). It should be noted that of the many different *tc* homologues in *P. luminescens*, only the *tca* and *tcd* loci encode proteins that have been demonstrated to be orally toxic to the moth *Manduca sexta* and it is assumed that the other homologues are specific either to different hosts or tissues. Tc operons usually have genes belonging to three homology types, '*tcdA/tcaAB/tccAB*', '*tcdB/tcaC*' and '*tccC*' types, or [A], [B] and [C] for simplicity. Tc gene operons can be classified into one of three types based on their gene complement, these being; (i) *tca* , containing [A]+[B], (ii) *tcd*, containing [A]+[B]+[C] and (iii) *tcc*, containing [A]+[C]. Figure 1 illustrates the four *tc* loci initially identified in *P. luminescens* strain W14 (ffrench-Constant and Waterfield 2006).

Initial heterologous expression experiments of genes from the *tca* and *tcd* operons in *Escherichia coli* suggested that the presence of all three distinct protein subunits is required for the formation of a complex with full oral toxicity to *M. sexta*.

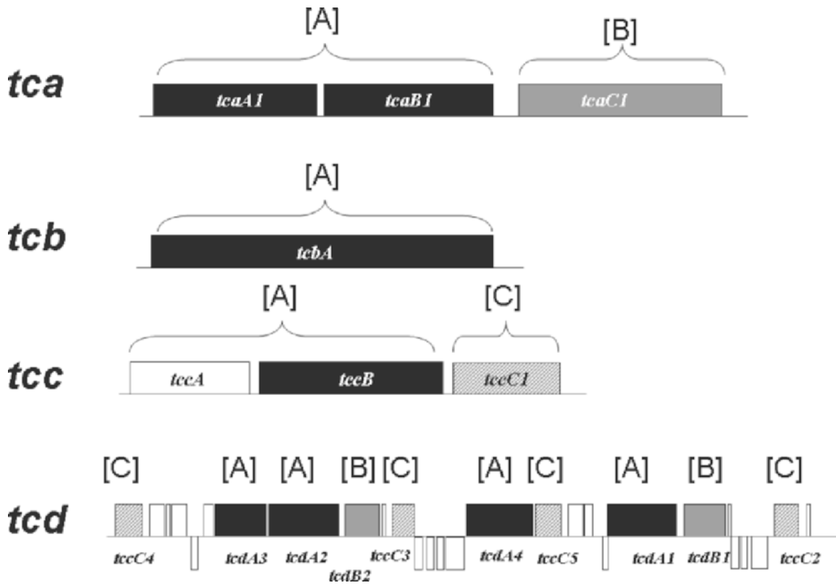

Fig. 1. The genetic organisation of four *tc* gene loci in *P. luminescens* W14. Boxes represent the open reading frames. For simplicity, the gene names are below the boxes and the homology-type of the *tc* gene is given above.

We now know that [A] and [B+C] subunits can both show toxicity independently, however *maximum* toxicity is only afforded by a complex of [A] and [B+C]. This makes a mature Tc structure a true "complex of toxins", although the exact stochiometry remains unclear (Waterfield et al. 2005).

22.2 Distribution of Toxin Complex Genes in Other Bacteria

Tc's have been demonstrated in other insect pathogens including the closely related *Xenorhabdus* (Morgan et al. 2001) (also a nematode symbiont) and the free living *Serratia entomophila*. (Dodd et al. 2006). However they have also been identified in a range of other bacteria. Not only in bacteria known to parasitise insects, such as *Pseudomonas entomophila* (Vodovar et al. 2006) and the Gram-positive *Paenibacillus* but also in cases where no such associations are known to exist. These include plant pathogenic members of the *Pseudomonas* (*syringae* and *fluorescens*), *Fibrobacter succinogens* and diverse *Burkholderia* species including the human pathogenic species. The presence of *tc* genes in diverse "soil associated" bacteria is highly suggestive that invertebrates may in fact play an as yet undefined role in the life histories of many of these bacteria. Interestingly a particular class of *tc*-homologue represented by a genetic fusion between a [B] and [C] like genes [B::C] are now

turning up in a much wider range of organisms including *Shewanella*, *Rodococcus*, *Desulfotomaculum*, *Ralstonia* and even the plant pathogenic fungus *Giberella*. Of particular interest here is the discovery of *tc* gene homologues in members of the *Yersinia* (Waterfield et al. 2001).

22.3 *Yersinia tc* Gene Homologues

Genome sequencing of different *Yersinia* isolates first identified homologues of the *tca* and *tcc* genes in both *Yersinia pestis* and *Yersinia pseudotuberculosis*. Subsequent studies have confirmed the presence of *tc*-like genes in other members of the genus including some *Yersinia enterocolitica* isolates, *Yersinia mollaretii* and *Yersinia frederiksenii*. Interestingly, as is also the case with *Photorhabdus* the types of *tc* genes and their genomic context are variable across the genus. Figure 2 illustrates a comparison of the common genomic "backbone" locus which contains *tc* gene homologues in several different *Yersinia* species. Note that all the chromosomal *tc* operons are inserted into an equivalent location on the common *Yersinia* genomic

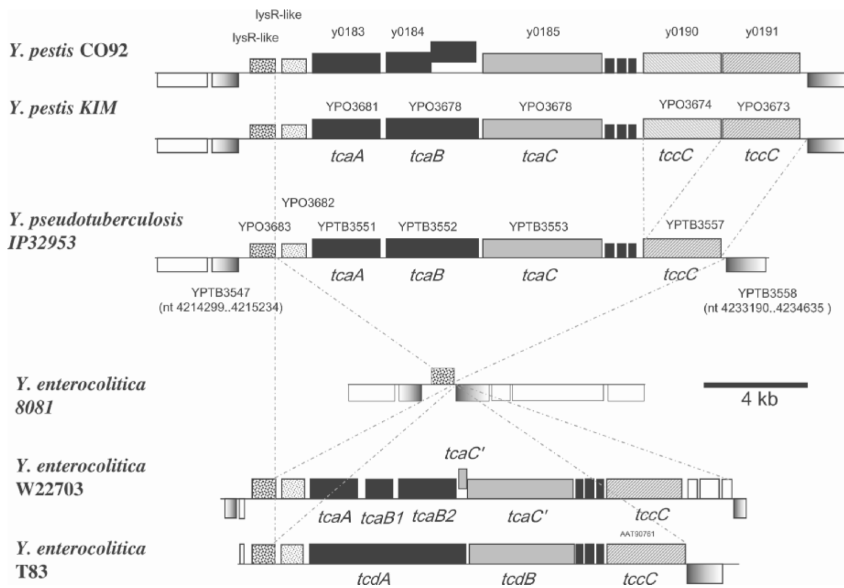

Fig. 2. The *tc* loci of the *Yersinia*. A single chromosomal locus contains the *tc* operons in diverse members of the *Yersinia*. *Y. pseudotuberculosis* and *Y. pestis* contain similar *tca* operons as expected. However *Y. enterocolitica* 8081 does not appear to have picked up this operon. Interestingly, this chromosomal locus must represent a 'hot spot' for *tc* gene horizontal acquisition as two other *Y. enterocolitica* strains have picked up different tc operon types.

backbone. This location is exemplified as between the genes YPTB3547 (nt 4214299..4215234) and YPTB3558 (nt 4233190..4234635) of the *Y. pseudotuberculosis* IP32953 genome.

22.3.1 The Chromosomal *tca* Loci of *Y. pseudotuberculosis*/ *Y. pestis* Clade

Y. pestis and *Y. pseudotuberculosis* isolates share a recent common ancestor (Achtman et al. 1999) which is reflected in conservation of the *tcaABC-tccC* gene arrangement ([A]+[B]+[C]). *Y. pestis* isolates on the other hand have a second *tccC* gene homologue and the suggestion of gene degradation in the *tcaAB* ([A]) genes, at least in the C092 isolate. Interestingly, *Y. mollaretii* also has a *tca* operon in this common genomic location, which is very similar to that of *Y. pseudotuberculosis*, suggesting that it is likely to have diverged from the common ancestor after the acquisition of the *tca* operon.

22.3.2 The *tc* Loci of *Y. enterocolitica*-like Strains

It is unlikely that the *tca*-locus is ancestral to the whole *Yersinia* genus as the equivalent *tc* genomic loci in *Yersinia bercovieri*, *Yersinia intermedia*, *Y. enterocolitica* 8081 and *Y. frederiksenii* are absent. We suggest that these strains maintain the ancestral genetic arrangement at this locus. Interestingly, *Y. enterocolitica* W22703 and *Y. enterocolitica* T83 do have *tc* operons at this location, but importantly they are dissimilar both to each other and to the *tca*-like operons of the *Y. pseudotuberculosis*-like isolates. What factors have "targeted" these different *tc* operons to this particular locus in the *Yersinia* backbone is unknown and difficult to explain. Furthermore, while *Y. frederiksenii* does not have this *tca*-locus, at least one isolate has acquired plasmid encoded close homologues of the *S. entomophila sepABC* genes (Dodd et al. 2006). This is more similar to a *tcd*-like locus, with the [A] subunit being encoded by a single continuous ORF, unlike the *tca* [A] elements which are split into two genes; *tcaA* and *tcaB*. The chromosomal *Y. enterocolitica* T83 *tc* operon also has a *tcd* organisation and in this case there is evidence to suggest they are involved in the colonisation of the mouse gut (Tennant, Skinner, Joe and Robins-Browne 2005). Conversely, the *tc* genes of *Y. enterocolitica* W22703 are only expressed at low temperatures (10°C) and have been shown to exhibit oral toxicity to insects. This operon, which has a *tca*-like organisation, also shows some evidence of undergoing degradation, with an apparent frameshift in the *tcaC* gene ([B] subunit) and the splitting of the *tcaB* gene into two separate ORFs. Experiments in this species suggest that the *tcaA* gene alone is responsible for mediating insect toxicity in this operon (Bresolin et al. 2006).

22.4 Biological Activities of the *tca* Genes From the *Y. pseudotuber*-culosis/Y. pestis Clade

The evidence from *Y. enterocolitica* suggests that *tc* genes can serve a role in either mammalian or invertebrate infections. We have therefore examined the role of the more conserved *tca*-like loci of the *Y. pseudotuberculosis/Y. pestis* clade.

22.4.1 Sequence of *tc* Genes From Different *Y. pseudotuberculosis* Strains

Microarray analysis of 10 diverse strains of *Y. pseudotuberculosis* revealed a high level of variability in *tcaB* and *tcaC* (Hinchliffe et al. 2003). We used Hi-fidelity PCR to clone *tc* ORFs from a range of different *Y. pseudotuberculosis* strains. Amino acid alignment of these sequences revealed that TcaC was reasonably well conserved between strains, but TcaB showed significant variation, especially in the region between amino acids 201-426. TcaA also showed some variability with several different C-terminal regions. These observations suggest that the *tc* genes may be under diversification selection (Hares et al. 2006).

22.4.2 Insect Oral Toxicity of *Y. pseudotuberculosis* Strains

In order to determine if any of the *Y. pseudotuberculosis* strains were capable of oral toxicity against model insects we tested them with our *M. sexta* (hawk moth) neonate oral bioassay. In order to replicate the experiments done with *Y. enterocolitica* W22703 (Bresolin et al. 2006), we tested aliquots of whole cultures grown at 10, 30 and 37°C in liquid culture (Fig. 3). We used whole live cultures as it is unknown how (or when) the *tca* genes are expressed in these strains. For example; the Tca could be secreted free into the growth medium, as in the case for both Tca and Tcd in *P. luminescens* W14, or it may remain cell associated, as in the case for Tcd in *P. luminescens* TT01 (unpublished data). Furthermore it is also possible that more specific factors, such as host cell contact, is required to trigger *tca* expression.

Interestingly we saw weak oral toxicity of several strains but no obvious correlation with growth temperature between strains (Hares et al. 2006). Obviously many different strain specific factors could contribute to these minor deleterious effects on the insects, but in no case did we observe strong oral toxicity, such as is mediated by *P. luminescens* W14 Tca. It remains possible that the *tca* genes were not being expressed in these experiments so we decided to sub-clone them for controlled heterologous expression in *E. coli* as has been done for *Photorhabdus tc* toxin genes.

22.4.3 Heterologous Expression and Insect Toxicity of *Y. pseudo*-tuberculosis and Y. pestis tca Genes

The *tca* genes from the sequenced *Y. pseudotuberculosis* IP32953 strain were PCR amplified and cloned into the arabinose inducible expression plasmid pBAD30. The

tcaAB genes were cloned as a single fragment (the [A] subunit), but with the native SD sequence of *tcaA* replaced by an artificial optimal consensus SD sequence. In addition, the *tcaC-tccC* fragment was PCR cloned, again replacing the native *tcaC* SD with an artificial sequence (the [BC] subunit). The *Y. pestis* KIM *tc* genes were also cloned for heterologous expression in *E. coli* using the same strategy.

Manduca neonate oral bioassays suggested only very weak oral toxicity of the heterologously produced IP32953 Tc proteins when all components were present, *i.e.*; [A]+[B+C] (Fig. 4). This level was similar to that seen when whole cultures were tested. As a control, heterologously expressed Tc toxin from *P. luminescens* can be seen to be highly orally toxic to *M. sexta*. Note the *tc* genes from *Y. pestis* also show little or no effect on the *Manduca*, although their expression level was lower (Hares et al. 2006). Interestingly, while heterologously expressed *Photorhabdus* Tc toxins are also highly toxic to the rat flea vector of *Y. pestis*, *Xenopsylla cheopis*, those of either *Y. pseudotuberculosis* or *Y. pestis* KIM strains have no deleterious effect when included in blood meals. Conversely, live *Y. pseudotuberculosis* cells can cause a lethal infection when fed to the flea, although this is not affected by deletion of the *tca* loci. Similarly, a *tca* mutant in *Y. pestis* does not appear to affect

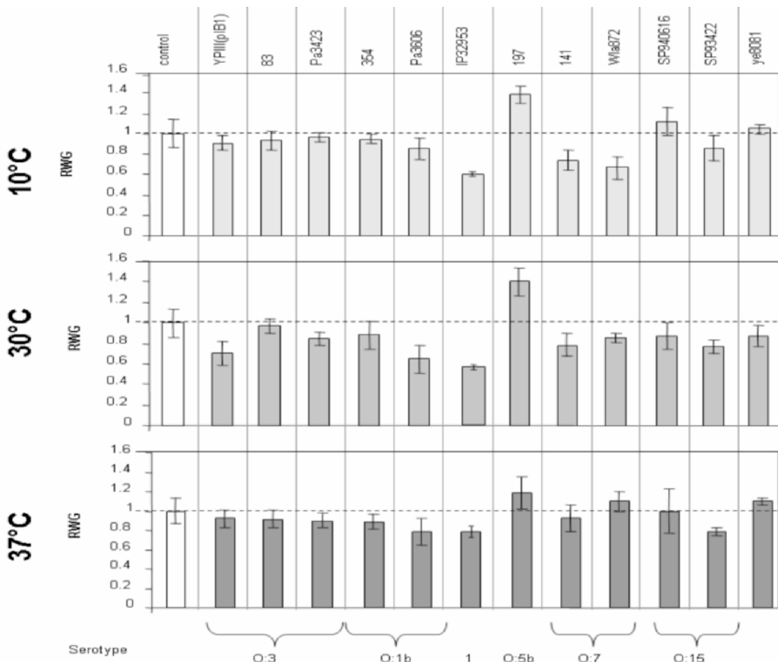

Fig. 3. Oral toxicity of overnight cultures of *Y. pseudotuberculosis* (and *Y. enterocolitica* 8081) to *M. sexta*. Toxicity relative to a control *E. coli* strain is shown (RWG).

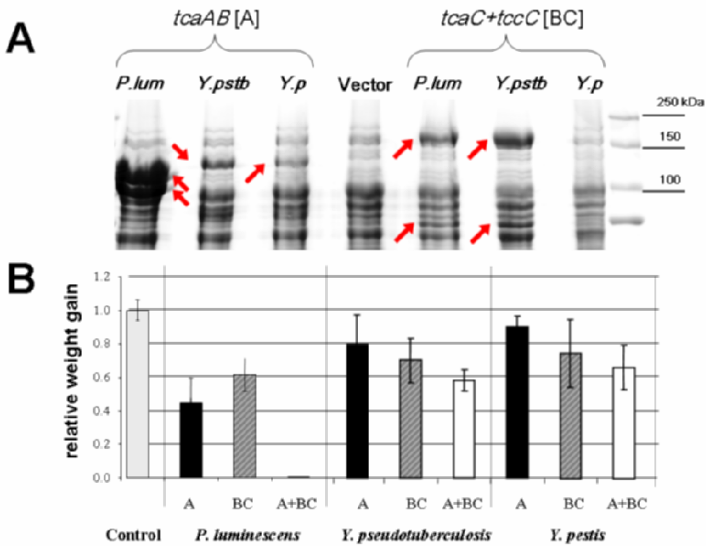

Fig. 4. (A) SDS-PAGE of cell lysates of heterologously expressed Tc proteins in *E. coli* BL21 at 15°C from *P. luminescens* (*P.lum*), *Y. pseudotuberculosis* strain IP32953 (*Y. pstb.*) and *Y. pestis* strain KIM10+ (*Y. p.*). Tc proteins are indicated by arrows. (B) *M. sexta* oral bioassay data of heterologously expressed Tc protein. The Y-axis represents mean larval weight gain relative to a vector control treatment (RWG).

the formation of biofilm on the insect proventriculus, although they are expressed after the formation of the biofilm. It has been suggested that they may play a role upon the initial entry into the mammalian host (DL Erickson and BJ Hinnebusch, personal communication). Together these observations suggest no obvious role for the *Y. pseudotuberculosis/Y. pestis* Tca in bacterial/insect interactions.

22.4.4 Temperature Dependence of *tca* Gene Transcription in *Y. pseudotuberculosis* IP32953

Reverse transcriptase (RT) PCR was used to determine the temperature dependence of *tc* gene transcription in *Y. pseudotuberculosis* IP32953. Interestingly, unlike the *tc* genes of *Y. enterocolitica* W22703, we saw no expression of the *tcaABC* genes at 15°C, but we did see transcription of the *tccC* gene. When the temperature was increased to either 30 or 37°C the *tcaABC* genes were also transcribed (Hares et al. 2006). This pattern of expression argues that the Tca complex is not expressed at "soil", temperatures but may be expressed in a mammalian host. The presence of the *tccC* transcript at low temperature suggests that either it has a regulatory role or it is also used for an independent function. In order to test the hypothesis that the

Y. pseudotuberculosis IP32953 Tc toxins are active in the mammalian gut we tested their effect on cultured caucasian colon adenocarcinoma (CACO-2) cells.

22.4.5 Activity of the *Y. pseudotuberculosis* Toxin Complex on Human Gut Cells

Heterologously expressed Tc toxins from *P. luminescens* W14, *Y. pseudotuberculosis* IP32953 and *Y. pestis* KIM+ were topically applied to cultured human gut cells to determine if they had any effect. Interestingly, the *Y. pseudotuberculosis* Tca caused initial membrane ruffling leading to the formation of many small vacuoles, (reminiscent of hyper pinocytosis) in over 41% of the cells examined (Fig. 5). This effect was seen only when the full [A]+[BC] complex was used. The Tca from *P. luminescens* W14 (13.5%) and *Y. pestis* (6%) had no strong effect, giving results comparable to an *E. coli* cytosolic negative control preparation (5%) (Hares et al. 2006).

Taking these observations together, we hypothesize that the *tca* operon in the *Y. pseudotuberculosis* lineage has been adapted to function in the mammalian host. In the case of *Y. pseudotuberculosis* (and the Tc's of *Y. enterocolitica* T83) they likely serve to "modify" the gut epithelium and in the case of *Y. pestis*, they may be involved in the initial invasion of the mammalian host after transmission by fleas.

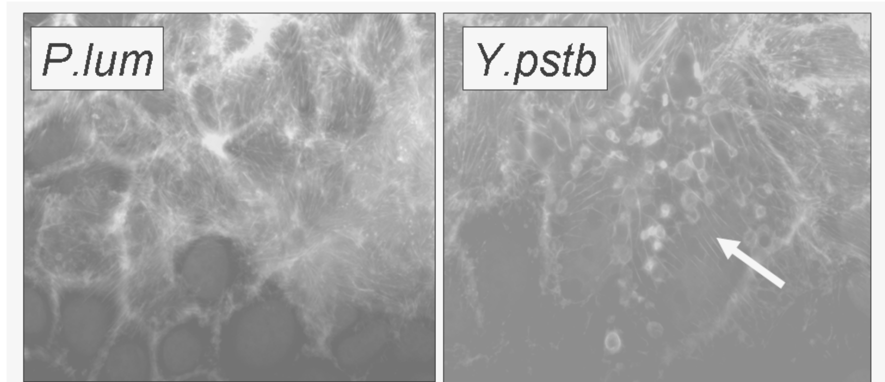

Fig. 5. Cytosolic preparations were made from heterologously expressed *P. luminescens* W14 and *Y. pseudotuberculosis* IP32953 *tcaAB* ([A]) and *tcaC+tccC* ([BC]) and topically applied to confluent mammalian CACO-2 gut cells. Cells were fixed and nuclei stained with Hoechst and the actin stained with phalloidin-rhodamine (B) Vacuoles and actin depolarisation were observed in ~40% of the cells for *Y. pseudotuberculosis* (arrow).

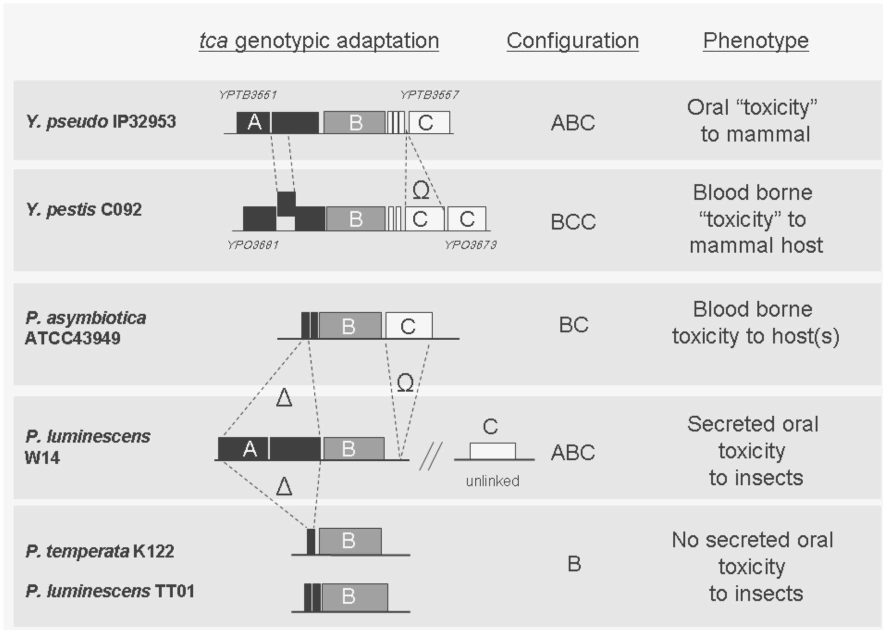

Fig. 6. A model showing the re-modeling of the tca operon in *Photorhabdus* and the *Y. pseudotuberculosis* clade of the *Yersinia*. We suggest that the loss of [A] components and the acquisition of [C] genes is related to changing selection pressures from different phenotypic requirements. Δ=deletion, Ω=insertion.

22.5 Conclusion: The Evolution of *tca* Loci

The presence of phage-associated genes in most *tc* operons and the location of the *sepABC* genes of *S. entomophila* on the conjugative plasmid pADAP (Hurst et al. 2000), suggests possible mechanisms of horizontal transfer. The demonstration that the *tc* genes of *Y. pseudotuberculosis* and *Y. enterocolitica* T83 are active against mammalian gut cells also indicates that these toxins can be adapted to more than just insect toxicity.

A comparison of the *tca* operons of the *Yersinia* with those of the *Photorhabdus* together with an understanding of the life histories of these bacteria may provide clues to the selection pressures that are driving the evolution of these complex toxins. Limited micro-array data (Marokhazi et al. 2003), genome sequencing and the analysis of *tc* containing cosmids from examples of the three different species of *Photorhabdus*; *P. luminescens* (W14 and TT01), *Photorhabdus temperata* (which are both insect pathogens) and *Photorhabdus asymbiotica* (which infects insects and man - Gerrard et al. 2006) has allowed us to build up a picture of the distribution and evolution of *tca* genes in this genus. Data suggests that the ancestral *Photorhabdus* possessed an intact *tca*-like operon. As the three species diversified there has been

degradation of the [A] component of the *tca* operon in all but a sub-clade of *P. luminescens* (containing strain W14).

Interestingly, the ancestor of *Yersinia* does not appear to have contained the *tca* operon; as exemplified in the genome of *Y. enterocolitica* 8081. However, as the lineage has diversified, different strains have acquired different *tc* genes, apparently adapted to different functions. The *Y. pseudotuberculosis*-like strains picked up a *tca* -like operon that appears to be adapted to mammalian hosts. The high level of variability in the Tca proteins of *Y. pseudotuberculosis* strains may therefore be explained by the selective pressure of the mammalian immune system that can lead to variability in surface expressed or secreted antigens. As in *Photorhabdus*, there is a suggestion that the [A] component is degenerating in the *Y. pestis* sub-clade (CO92). In addition both these species have also acquired an additional [C] component at this locus (Fig. 6). Interestingly, the life histories of both *Y. pestis* and *P. asymbiotica* involve systemic infections in mammals (vectored by a flea or nematode), and it is tempting to speculate that the loss of [A] and the creation of functional [BC] components reflect this adaptation.

22.6 Acknowledgements

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