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REVIEW ARTICLE

Type III secretion systems and pathogenicity islands

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Some bacterial pathogens have evolved by acquiring pathogenicity islands (PIs), which are clusters of genes encoding virulence traits. PIs encoding the secretion of effector molecules *via* type III secretion (TTS) systems have been discovered in several gram-negative pathogens. TTS systems are involved in contact-dependent secretion of virulence factors and can facilitate delivery of toxins directly into target cells. The expanding list of bacteria found to contain clusters of TTS genes includes members of the genera *Yersinia*, *Salmonella*, *Shigella*, *Escherichia*, *Pseudomonas*, *Bordetella*, *Burkholderia*, *Chlamydia* and a number of plant pathogens or symbionts. This review discusses the current knowledge of the role of TTS PIs in pathogenicity, the genetic organisation and evolution of such systems, and the potential for using TTS systems as targets for novel treatments.

Pathogenicity islands

There has been considerable interest for many years in addressing the question of how bacterial pathogens evolve. With the onset of modern molecular biological techniques it has become possible to shed some light on this question. In particular, the execution of numerous genome sequencing projects, with many more to come, will provide a mass of data concerning the current evolutionary position of bacterial pathogens. Much of our knowledge stems from the study of closely related bacteria associated with different modes of pathogenesis. By analysing organisms that are essentially similar, it is easier to identify and characterise differences that are associated with pathogenicity. For example, it appears that *Escherichia coli* and *Salmonella enterica* diverged from a common ancestor some 100 million years ago. The major differences now apparent between these organisms can be accounted for by the acquisition of a number of large DNA fragments termed loops [1]. Some loops encode metabolic functions or motility, but others are responsible for pathogenicity traits. The latter are now termed pathogenicity islands (PIs).

For some time it has been recognised that plasmids can carry clusters of genes responsible for encoding a virulence trait, and that such clusters play an important role in the evolution of pathogenicity. PIs have extended this notion to include other regions of bacterial genomes. PIs, found in both gram-positive and gram-negative bacteria, are defined on the basis of a number of characteristics: they are present in virulent strains of a species but absent in non-pathogenic variants; they can comprise large regions (up to 200 kb); they can carry multiple virulence genes; their G + C mol % content often differs from typical values for the rest of chromosome; they are flanked by direct repeats or insertion sequences (IS) and often have regions of DNA homologous to phage integrase genes, plasmid origins of replication or IS elements [2]. Well known examples of PIs include Pai I (encoding α-haemolysin) and Pai II (encoding α-haemolysin and P-related fimbriae) in uropathogenic *E. coli*. In enteropathogenic *E. coli* (EPEC) all genes required for the attaching and effacing (A/E) phenotype are encoded on a 35-kb type III secretion (TTS)-related PI called the locus of enterocyte effacement (LEE). It has been demonstrated that a cloned PI (the LEE) can confer upon strain K-12 the A/E phenotype [3]. An equivalent locus to the LEE has also been found in *Citrobacter rodentium*, the causative agent of transmissible murine colonic hyperplasia [4], and in *Cit. freundii* [5].

In *S. enterica* there are at least five PIs [6–10], including two that are associated with TTS systems. The best characterised of the *S. enterica* PIs is the 40-

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kb SPI-1 segment which encodes genes that enable invasion of non-phagocytic cells. These include 31 genes that encode the components of the Inv/Spa TTS system. Mutants in the Inv/Spa genes are defective in virulence when inoculated orally in mice, but remain fully virulent if inoculated intraperitoneally. This suggests that the primary role of SPI-1 genes is related to invasion of intestinal cells. A second virulence locus that encodes a TTS system in *S. enterica* [11], designated the Spi/Ssa system and encoded within the SPI-2 PI, is essential for causing systemic disease. Mutants in the SPI-2 TTS system are attenuated for mice when inoculated orally or intraperitoneally. SPI-2 also plays a role in enabling *S. enterica* to avoid NADPH oxidase-dependent killing by macrophages [12]. SPI-2 is similar in size to SPI-1 but differs in genetic organisation as well as function. The discovery of two functionally divergent TTS systems in the same bacterium is, at present, unique to *S. enterica*. Whilst SPI-1 is present in all of the subspecies comprising *S. enterica*, SPI-2 is not, which suggests that acquisition of SPI-2 by some salmonellae has been a more recent event, enabling them to cause systemic disease. The SPI-1 TTS system is similar to the plasmid-encoded Mxi/Spa TTS system of *Shigella flexneri*, which is responsible for invasion of eukaryotic cells and apoptosis of macrophages, and is also thought to play a role in cell-to-cell spread [13].

The structure of PIs generally, and the location of TTS PIs in particular (some plasmid-encoded some chromosomal), has led to the notion that bacterial pathogens may evolve by acquiring PIs that encode virulence traits by horizontal gene transfer. A gene encoding an SPI-1-dependent translocated protein was recently discovered to be present on a temperate phage in *S. enterica serotype* Typhimurium [14]. Thus, not only might genes encoding a secretion apparatus be acquired in a single evolutionary step, but additional genes for extra effector proteins could be added following lysogenic infection with bacteriophages. It is easy to imagine how the functions of TTS systems might diverge, depending on particular host–pathogen relationships.

TTS systems have been reported in a number of bacterial pathogens (Table 1). In many organisms, the G + C mol % content of the TTS system genes has been reported to be lower than the mean genomic value, suggesting that the ancestral TTS system genes may have evolved in a low G + C mol % host [6]. A TTS system has been identified in *Chlamydia psittaci*, a potential candidate for such a role [15, 16]. In addition to those TTS systems listed in Table 1, there have been reports suggesting the presence of one protein in *Campylobacter jejuni* that shares homology with type III secreted proteins associated with invasion [17], and in non-motile *Brucella* spp. open reading frames (ORFs) with similarities to TTS and basal body proteins have been found [18]. However, the presence of substantial or functional TTS PIs has not yet been established in these bacteria. In *Helicobacter pylori*, there have also been reports of genes sharing homology with TTS genes [19, 20]. In *Porphyromonas gingivalis* contact-dependent secretion of proteins has been observed [21], but there is no clear evidence of the mechanisms involved and attempts to identify TTS system structural genes were not successful. There have also been reports of a functional TTS gene cluster in the plant growth-promoting bacterium *Pseudomonas fluorescens* [22]. The TTS system was identified by in-vivo expression technology (IVET) that screened for rhizosphere and plant-induced genes and resembles the *Ps. syringae* system in gene organisation.

This laboratory has employed probes derived from

Table 1. Bacterial TTS systems

Bacteria	Location	Secreted proteins	Activity/function/effect on host cell
Animal pathogens			
EPEC	Chromosome	Esps	A/E lesion formation
		Tir	Binds to intimin
S. enterica SPI-1	Chromosome	Sops and Sips	Enterocyte invasion; induction of apoptosis
S. enterica SPI-2	Chromosome	SpiC	Invasion into tissues
Shigella spp.	Plasmid	Ipas	Induction of membrane ruffling and apoptosis; phagosomal lysis; cell invasion
Yersinia spp.	Plasmid	Yops	Cytotoxic; F-actin disruption; inhibition of phagocytosis; induction of apoptosis
Ps. aeruginosa	Chromosome	Exo-enzymes	ADP-ribosyltransferase; cytotoxic
Bor. bronchiseptica	Chromosome	Products of *bopD*, *bopN* and *bsc22*	Modulation of immune response by inhibition of NF-κB activation; induction of apoptosis
Bur. pseudomallei	Chromosome	Unknown	Unknown
Chlamydia psittaci	Chromosome	Unknown	Unknown
Plant pathogens			
Ps. syringae, *X. campestris*, *Erw. amylovora*	Chromosome	Harpins	HR
R. solanacearum	Plasmid	PopA1	HR
Plant symbiont			
Rhizobium sp. NGR234	Sym plasmid	Y4xL and NolX	Role in nodulation

characterised TTS gene clusters to probe for the presence of equivalent genes in gram-negative pathogens. This approach has led to the identification of a putative TTS gene cluster in *Burkholderia pseudomallei* [23, 24]. This approach has also been used to identify DNA homologous to TTS genes, in the cystic fibrosis pathogen *Bur. cepacia* (unpublished observation).

Secretion systems

Secreted proteins play important roles in the virulence of many pathogenic bacteria. The mechanisms by which proteins traverse bacterial membranes vary considerably. In gram-negative bacteria different types of secretion system have been implicated in the delivery of toxic effectors. They vary in a number of important properties, including complexity, dependence on a protein signal sequence (*sec*), and whether secretion across inner and outer membrane is achieved in a single step (Table 2). For example, the number of components involved in type I secretion is far fewer than in type II and type III secretion. However, type I secretion, like type III but unlike type II secretion, occurs in a single step. In the general secretory pathway (type II secretion), secretion across the inner membrane is dependent upon the presence of a *sec* signal sequence which is cleaved in the periplasm. The second step, secretion through the outer membrane, can then occur. There are some similarities between the respective secretion systems. Type I, II and III secretion are all driven by ATP hydrolysis, and the outer-membrane components of type II and type III secretion complexes are homologous, consisting of a multimeric protein. The designation 'type IV secretion' has been ascribed to more than one mechanism in the literature. Type IV secretion has been used to refer to a group of autotransporters with similarities to type II secretion, in that a *sec* sequence is required for export from the cytoplasm [25, 26]. However, this mechanism of secretion differs from type II secretion in that the information required for transport across the outer membrane resides within the protein sequence. It appears that such proteins can form a pore through which they pass [27]. The term type IV secretion is now more commonly employed to describe a system that appears to function primarily as a means for the transfer of DNA [28] (Table 2).

The particular interest in TTS stems largely from evidence that host–pathogen interaction influences regulation of the secretion process, and that secreted proteins are often delivered directly into target cells. This triggering of secretion in response to the presence of host cells, termed contact-dependent secretion, helps to ensure that effectors are secreted only when required. This can make the systems difficult to study. Because of the nature of TTS, secreted proteins are often not produced in sufficient quantities to be readily detectable in culture supernates, and are secreted only when the right environmental conditions are in place.

One of the more interesting features of TTS systems is the fact that both animal and plant pathogens use them. The abilities of plant pathogens of the genera *Erwinia*, *Pseudomonas*, *Ralstonia* and *Xanthomonas* to cause various diseases in susceptible plant hosts depend on the presence of TTS system genes. In resistant plants, TTS systems induce a defence reaction called the hypersensitive response (HR), which is characterised by localised tissue necrosis and the production of antimicrobial substances at the site of infection. This response prevents spread of the infection. TTS genes in plant pathogens are often discussed in terms of their ability to elicit the HR, and many are designated *hrp* (*h*ypersensitive *r*esponse and *p*athogenicity). The ability of a pathogen to cause disease rather than elicit the HR depends on the presence of a matching pair of a dominant resistance gene (*R*) in the host and an avirulence gene (*avr*) in the pathogen. If the *avr* gene is mutated, the pathogen is often able to cause disease in a previously resistant plant. Introduction of the *R* gene into a previously susceptible plant will confer resistance, provided that the pathogen carries the matching *avr* locus [29, 30]. A TTS system has also been reported for the plant symbiont *Rhizobium* [31], where it apparently plays a role in the nodulation process. The similarities between plant and animal TTS systems have been confirmed by the fact that reciprocal

Table 2. Secretion systems for bacterial toxins

Type	Features	Examples
I	One-step; *sec*-independent	*E. coli* α-haemolysin; *Bor. pertussis* adenylate cyclase; *Ps. aeruginosa* protease
II	Two-step; *sec*-dependent, signal sequence cleaved in periplasm	*Ps. aeruginosa* exotoxin A, elastase, phospholipase C; *Klebsiella oxytoca* pullulanase; *Aeromonas hydrophila* amylase, protease
Autotransporters	Two-step; *sec*-dependent, signal sequence cleaved in periplasm	Gonococcal IgA protease; *Haemophilus influenzae* IgA proteases; *Serratia marcescens* serine protease; *Bor. pertussis* pertactin
III	One-step; *sec*-independent	*Yersinia* Yop proteins; *Salmonella* Sip proteins; *Shigella* Ipa proteins; EPEC Esp proteins
IV	Probably two steps; may be *sec*-like; related to DNA transfer system in *Agrobacterium*	*Bor. pertussis* toxin; *Legionella pneumophila*

secretion of effectors is possible [32, 33]. There has been a recent review addressing the subject of effectors secreted by plant pathogens [34].

TTS system apparatus

There have been several previous reviews on the subject of TTS [26, 35–38]. The best characterised TTS system is the highly conserved plasmid-encoded system of *Yersinia pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* [39, 40]. There are also specific reviews covering the TTS systems of *E. coli* [41], *S. enterica* [42–44] and *Shigella* spp. [45]. The present review will be confined to a brief summary of the TTS apparatus and some of the recent developments in this fast-moving and exciting area of research.

TTS PIs contain genes that encode proteins in a number of categories: structural, regulatory, chaperones and secreted. Whilst the majority of genes in individual TTS systems encode proteins with homologues in other systems, each respective system may contain genes not widely observed in other systems and often unique to itself. In particular, proteins involved in the formation of the secretion structure, or secreton, tend to be conserved throughout TTS systems. In contrast, the secreted proteins themselves differ greatly from one TTS system to another. Thus, within the structural constraints of the requirement for a functional secretion apparatus, evolution of TTS systems has generated considerable diversity. Table 3 summarises those proteins that are common throughout TTS systems and indicates their homology to the archetypal yersinia TTS proteins.

As well as sharing similarities with the outer-membrane component of type II secretion (a multimeric 'secretin'), some TTS proteins, specifically those associated with the inner membrane, share homology with components of the flagellar basal body. Indeed, there is one report of virulence factor secretion *via* the flagellar export apparatus of *Y. enterocolitica* [46]. TTS systems may have evolved from flagellar assembly systems. However, there have been no reports of TTS-like systems in gram-positive bacteria. Although chaperonins have been found in *Streptococcus pneumoniae*, no genes homologous to TTS structural genes have been reported [47]. Generally, secretion of proteins from gram-postive bacteria, usually involving a signal sequence, is less complex. The majority of secreted or surface proteins in streptococci, staphylococci and *Listeria monocytogenes* contain a signal sequence and a carboxy-terminal region comprising a number of particular features such as tandem repeats, a proline/glycine-rich segment, a conserved hexapeptide (LPXTGE), a hydrophobic stretch of *c*. 20 amino acids and a positively charged tail [48].

Although TTS systems are notable for their ability to

Table 3. Comparisons of TTS system conserved proteins

			Identity/similarity (%)*										
Protein	Location	SwissProt ID	EPEC	SPI-1	SPI-2	Shig	Paer	Bord	Burk	Rals	Psy	Erw	Xanth	Rhi
YscC	OM	yscc_yeren	33/44	34/45	32/42	33/44	64/73	33	34/45	34/46	32/42	33/42	36/46	ND
YscD	IM	yscd_yeren	23/34	ND	ND	ND	45/56	21	29/42	ND	26/34	28/36	ND	ND
YscJ	Unknown	yscj_yeren	37/48	32/43	37/52	29/43	70/79	36	40/51	33/56	37/48	39/51	35/44	43/55
YscL	Unknown	yscl_yeren	ND	ND	26/40	ND	56/69	34	25/41	23/35	21/35	24/38	20/31	19/36
YscN	IM	yscn_yeren	48/59	45/54	54/62	43/54	82/85	64	53/62	56/65	51/60	51/60	58/67	54/65
YscQ†	Unknown	yscq_yerps	ND	38/46	35/45	25/33	ND	42/65	38/48	31/38	ND	27/36	ND	36/49
YscR	IM	yscr_yerps	46/59	47/60	54/65	45/59	ND	56/65	46/59	50/58	44/57	41/54	ND	46/56
YscS	IM	yscs_yerps	39/58	43/54	51/64	43/56	ND	51/65	43/58	35/48	45/54	47/58	ND	27/44
YscT	IM	ysct_yerps	32/46	31/43	33/45	28/41	ND	33	29/41	34/43	30/40	35/47	28/38	30/40
YscU	IM	yscu_yerps	37/50	35/51	36/49	33/48	ND	38	33/47	32/46	34/48	31/46	ND	30/40
YscV (LcrD)	IM	lcrd_yeren	46/60	43/58	43/57	40/57	ND	57	42/56	44/57	43/57	43/56	47/59	42/56

IM, inner membrane; OM, outer membrane.
*The table shows % identity/% similarity values for protein homologues of the Ysc proteins listed in the first column. Figures were obtained following alignment with the programme GAP from the GCG sequence analysis software package (Genetics Computer Group, University of Wisconsin, USA) with default settings. Identity indicates identical amino-acid residues whereas similarity indicates amino acids with similar properties. Only representatives of TTS systems for which substantial sequence information is accessible in SwissProt or PIR databases are shown. ND indicates that homologies are not known or have not been determined. Abbreviations are as follows: enteropathogenic *E. coli* (EPEC); *S. enterica* pathogenicity islands 1 and 2 (SPI-1 and SPI-2); *Sh. flexneri* (Shig); *Ps. aeruginosa* (Paer); *Bordetella* spp. (Bord); *Burk. pseudomallei* (Burk); *R. solanacearum* (Rals); *Ps. syringae* (P.sy); *Erw. amylovora* (Erw); *X. campestris* (Xanth); *Rhizobium* spp. (Rhi). For *Bordetella*, in some cases, only %identity values for *Bor. pertussis* are available (taken from reference [64]).
†YscQ data are based on alignments of the final C-terminal 80 amino acids only.

deliver secreted proteins directly into the target cell cytoplasm following cell contact, they are not necessarily deployed exclusively for this type of delivery. It has been reported that in *S*. Typhimurium the major proteins secreted into culture media are either flagellar proteins or the TTS system-associated secreted virulence factors SipA, SipB, SipC and InvJ [49]. In flagellar mutants, virulence factors were constantly secreted at higher levels. In *Yersinia* spp, TTS systems function not only to secrete proteins into target cells but also secrete virulence factors into the extracellular milieu [50].

An interesting consequence of TTS was reported in *Yersinia* spp., where after adherence of bacteria to receptors on the mammalian cell membrane, several Yop proteins are transported by TTS into the host cell cytoplasm. If epithelial cells are first infected with *Yersinia* spp. to permit delivery of Yops, subsequent infection with other invasive pathogens can be prevented [51]. Pre-infection with *Yersinia* spp. inhibits invasion of *Edwardsiella*, *Shigella* and *Listeria*, but not *Salmonella* spp. It was found that either YopE or YopH prevented invasion by *Listeria* spp., whereas only YopE prevented invasion by *Edwardsiella* and *Shigella* spp. YopH and YopE prevent macrophages from phagocytosing *Yersinia* cells by disrupting the host cell cytoskeleton and signal transduction pathways.

Some progress has been made in elucidating the actual structures produced by TTS systems to facilitate direct secretion into target cells. In 1998, it was reported that in *S. enterica* the TTS apparatus forms a supramolecular structure (termed a needle complex) visible by electron microscopy [52]. The needle complex spans inner and outer membranes and comprises a cylindrical base with a thin, needle-like structure projecting outwards from the cell, in many ways resembling the basal body of the flagellar apparatus. Such complexes have also been observed in *Sh. flexneri* [53], but not as yet in *Yersinia* spp. In EPEC, a filamentous organelle implicated in translocation of TTS secreted proteins has also been reported. The structure is on the bacterial surface during the early stage of A/E lesion formation and forms a physical bridge between the bacterium and the infected eukaryotic cell surface [54, 55]. It has been demonstrated that salmonella needle structures are formed in the presence of cultured epithelial cell lines or murine intestinal cells [56]. Interestingly, invasion-deficient *S*. Typhimurium strains carrying mutations in components of SPI-1 exhibited filamentous appendages similar to those on wild-type *S*. Typhimurium when adhering to epithelial cells, demonstrating that SPI-1 is not an absolute requirement for the formation of such appendages.

Phenotypes associated with TTS effectors

Although the secretion apparatus exhibits great similarity amongst the TTS systems of different bacteria, the proteins secreted are responsible for producing very different responses in an infected host. Often it is not a simple matter to analyse the effect of an individual secreted protein, as the stimulation or interference with host cell functions can be due to a combination of effectors. Furthermore, some secreted proteins do not act as effectors *per se* but are involved in the secretion process and may be required for the effective delivery of genuine effectors. Moreover, because of the nature of TTS, whereby there is delivery of secreted proteins directly into the cytoplasm of target cells, secreted proteins are not often readily identified in culture media. However, detection in culture medium has proved possible in some cases. A number of TTS secreted proteins in *Ps. aeruginosa* were identified by analysing extracellular proteins after growth under conditions already known to stimulate the secretion of another TTS system-secreted protein, exo-enzyme S [57]. This requirement for some prior knowledge of regulation considerably limits the effectiveness of a direct approach to studying secreted proteins.

Despite the difficulties inherent in studying TTS systems, the functions of a number of effector proteins have been resolved. In animal pathogens these phenotypes include modulation of cell invasion, A/E lesion formation and various cytotoxic effects leading to inhibition or apoptosis of phagocytic cells. Often multiple proteins that provoke a variety of responses are secreted from the same TTS apparatus. In *Ps. aeruginosa*, exo-enzyme S functions as an ADP-ribosylating toxin that interferes with GTP-binding proteins involved in actin organisation [58–60]. Other secreted proteins include ExoT (another ADP-ribosyltransferase) and ExoY (an adenylate cyclase [61]). A genetic construct of *Ps. aeruginosa* was used to analyse the biological effects of individual type III-secreted proteins on Chinese hamster ovary (CHO) cells. Whereas ExoS, ExoY and ExoT caused alterations in cell morphology, ExoU activity was associated with acute cytotoxicity [62]. Indeed, it has been reported that ExoU, and a second unidentified effector, induce apoptosis of macrophages and epithelial cells [63]. TTS proteins also play a role in inhibition of internalisation of *Ps. aeruginosa* by epithelial cells, a mechanism that may play a role in host defences against *Ps. aeruginosa* cytotoxicity [64].

The *bvgAS* virulence control system regulates the TTS system in *Bordetella bronchiseptica* [65]. A deletion in one of the genes, *bscN*, led to decreased cytotoxicity towards cultured macrophage cell lines and was used to demonstrate that TTS is required for persistent colonisation of the trachea in a rat infection model. Recent evidence suggests that the *Bor. bronchiseptica* TTS system is involved in modulation of immune responses and induction of apoptosis, and some secreted proteins have been identified [66]. Importantly, none of the previously described virulence factors synthesised by *Bordetella* spp. are thought to be

secreted by TTS [65]. We have demonstrated the presence of TTS system genes in 27 animal isolates of *Bor. bronchiseptica*, associated with various clinical signs [67]. Although this suggests that TTS is not an important factor in influencing variations in virulence between strains, it is possible that differences in the expression of *bsc* genes, rather than the presence or absence of the genes, may occur. Some evidence for this has been reported [65]. Analysis of genomic sequence data has confirmed the presence of an equivalent TTS gene cluster in *Bor. pertussis* [68].

Studies of the secreted proteins of *Yersinia* spp. have been assisted by the fortuitous fact that its TTS system can be induced artificially by growth of the organism at 37°C in the absence of calcium. This low-calcium response (LCR) has been exploited to identify the substrates of TTS in this organism. In *Yersinia* spp., 14 different proteins are known to be secreted by TTS, of which only a subset have been identified and characterised as effectors. Amongst the better characterised protein effectors are YopE and YopH, which disrupt macrophage function by different mechanisms, YopP (YopJ), which induces macrophage apoptosis, and YopT, which has a cytotoxic effect in HeLa cells and macrophages [40]. Interestingly, there is some evidence of functional conservation between the type III-secreted proteins of different bacteria. The N-terminal domain of YopE exhibits sequence similarity with *Ps. aeruginosa* ExoS [69]. Indeed, to some extent, the TTS systems of *Yersinia* spp. and *Ps. aeruginosa* are interchangeable [58], suggesting a close relationship between the two systems. In addition, it has been demonstrated that *Y. pseudotuberculosis* can secrete the *Sh. flexneri* IpaB protein and that *S.* Typhimurium can secrete YopE [70]. It has also been noted that a type III-secreted protein from *S. enterica* shares homology with a secreted protein from the plant pathogen *Xanthomonas campestris* [71].

However, it is clear that in TTS systems the signal for secretion does not reside within the protein sequence. It was demonstrated by Anderson and Schneewind [72] that the signals of the yersinia secreted proteins YopE and YopN resided in the first 15 codons, yet no common amino acid or peptide sequence could be identified among the secretion signals. Neither point mutants nor frame-shift mutations that completely altered the peptide sequences of these signals prevented secretion. The authors concluded that the signal that leads to the TTS of Yop proteins was encoded in their mRNA rather than the peptide sequence [72]. Similar work has been carried out on YopQ [73] and YopH [74]. This suggests some coupling of translation of a secreted protein with its export [75]. Anderson *et al.* [32] cloned the TTS machinery of *Erwinia chrysanthemi* in *E. coli* and demonstrated that the recombinant system recognised the secretion signals of YopE and YopQ. In addition, *Ps. syringae* AvrB and AvrPto, could be secreted by the recombinant *Erwinia* machine, or the *Yersinia* spp. TTS system. The authors mapped AvrPto sequences sufficient for the secretion of reporter fusions in *Yersinia* spp. and identified an mRNA secretion signal. They went on to propose that 11 conserved components of TTS system machines may recognise signals that couple mRNA translation to polypeptide secretion. Comparisons between the *Yersinia* spp. conserved proteins (designated Ysc) and equivalent proteins from different TTS systems are presented in Table 3.

Organisation of TTS genes

For several years, different laboratories working on separate and diverse pathogens were identifying important TTS-related pathogenicity genes without necessarily realising that equivalent systems operated elsewhere. For this reason, the nomenclature of TTS system genes has evolved to a level of diversity that can be both daunting and confusing. Those working with plant pathogens have settled on the designation *hrc* for genes represented in all phytopathogen TTS systems (for *hrp* conserved) [76]. There have been attempts to unify the naming of all genes and proteins with equivalent functions, but such attempts are thwarted to some extent by the fact that each individual TTS system contains elements not necessarily represented in other systems. It has been suggested that all TTS system nomenclature should be unified under the designation *sct* (for secretion and cellular translocation) [26], but it is not clear that this suggestion has been heeded to any significant degree.

Although the genes encoding TTS systems are clustered together either on a plasmid or in a chromosomal PI, the order of genes varies between systems (Fig. 1). In addition, whilst some genes are represented in all TTS systems, others are apparent only in related groups of bacteria, or are unique to a particular TTS system. There are two major blocks of genes in which the gene order is conserved in the majority of TTS systems (equivalent to *yscIJKL* and *yscNOPQRSTU* in *Yersinia* spp.). In *Bor. bronchiseptica* these blocks are linked together [65, 67] but their relative positions vary from one TTS system to another (Fig. 1). Although it is believed that TTS systems are acquired in intact genetic blocks, considerable degrees of variation in conservation of proteins have been observed. Indeed there are a number of examples where genes are conserved in relative location but exhibit low sequence homologies [26]. For example, the predicted proteins encoded by *yscO* and *yscP* and their equivalents in other TTS gene clusters share only low homology even when the equivalent genes are located in the same position with respect to other better conserved genes, such as *yscN* and *yscQRS*.

The TTS systems of various bacteria can be subgrouped to a certain extent on the basis of gene

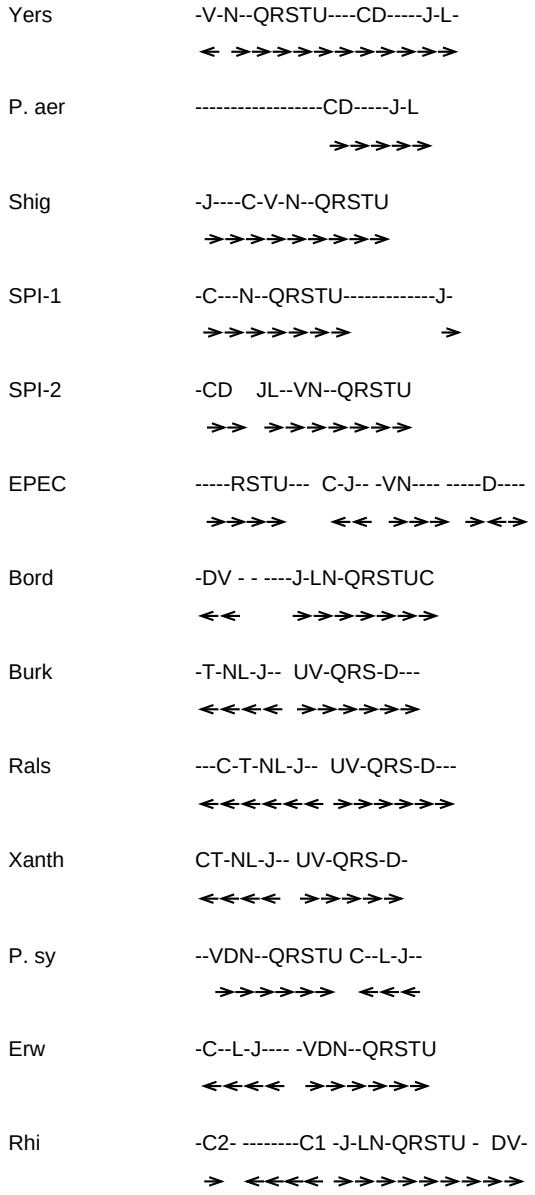

Fig. 1. Organisation of TTS conserved genes. The figure shows the relative locations of TTS genes in different bacteria. The letters used relate to genes encoding proteins equivalent to proteins carrying the recognised Ysc designations (as outlined in Table 3). In *Rhizobium* spp., two proteins are responsible for the functional equivalent of YscC. In *Bur. pseudomallei*, the location of the gene encoding a homologue of YscC is not yet known. In *Ps. aeruginosa*, information is available for only part of the TTS system gene cluster. Dashes (-) indicate the presence of other genes. Arrows indicate the direction of transcription. Abbreviations are as follows: *Yersinia* spp. (Yers); *Ps. aeruginosa* (P.aer); *Shigella* spp. (Shig); *S. enterica* pathogenicity islands 1 and 2 (SPI-1 and SPI-2); enteropathogenic *E. coli* (EPEC); *Bordetella* spp. (Bord); *Burk. pseudomallei* (Burk); *R. solanacearum* (Rals); *X. campestris* (Xanth); *Ps. syringae* (P.sy); *Erw. amylovora* (Erw); *Rhizobium* spp. (Rhi).

organisation. Such groups would include: (i) *S. enterica* SP-I and *Shigella* spp.; (ii) *Yersinia* spp. and *Ps. aeruginosa*; (iii) *R. solanacearum*, *X. campestris* and *Bur. pseudomallei*; (iv) *E. amylovora* and *Ps. syringae*.

A comparison of the TTS genes of all available representatives of the β-subdivision of proteobacteria (*Bor. bronchiseptica*, *R. solanacearum* and *Bur. pseudomallei*) suggests that the gene order in *Bor. bronchiseptica* shares greater similarity with bacteria of the γ-subdivision, such as *Yersinia* spp. Individual predicted protein homologies are also best between *Bor. bronchiseptica* and *Yersinia* spp. [67]. The TTS genes in the plant pathogen *R. solanacearum* and the human pathogen *Bur. pseudomallei* share a striking level of organisation with respect to gene order ([23] and unpublished observations). In these organisms, the region either side of the equivalent to *yscQRS* differs significantly from that of *Bor. bronchiseptica*. This diversity amongst ostensibly related bacteria suggests that the β-subdivision proteobacteria did not all acquire TTS system genes from a common ancestor.

In most cases reported to date, the G + C mol % contents of TTS genes are lower than the surrounding genome, an indication of acquisition by horizontal transmission from a host with a lower G + C mol % content. Even when the difference in G + C mol % content does not appear to be significant, closer analysis provides support for the notion of gene transfer. In *Bur. pseudomallei* and *Bor. bronchiseptica*, G + C mol % levels in TTS genes are only 2–3% lower than overall G + C mol % contents for these organisms. However, localised dips in G + C mol % content are apparent in the most conserved genes, such as those equivalent to *yscRS* (7–8% lower) [23, 67]. The lower G + C mol % content apparent in genes encoding the more conserved proteins may reflect the fact that structural constraints have resulted in less evolutionary change in such genes when compared with other areas of the TTS gene cluster

Regulation of TTS genes

The *Yersinia* spp. TTS system is the best known example whereby growth conditions can be used to induce TTS, but there are other examples of environmental factors inducing TTS. The *Sh. flexneri* Mxi-Spa TTS system, responsible for secretion of Ipa proteins, which are involved in the entry of bacteria into epithelial cells, is activated upon contact of bacteria with eukaryotic cells. Bahrani *et al*. [77] identified a group of chemical compounds, including Congo red, Evans blue and direct orange, which were able to induce secretion of Ipa proteins by bacteria suspended in phosphate-buffered saline. The authors suggested that the effect may be due to specific hydrophobic interactions similar to those involved in binding of Congo red to amyloid proteins. It was further reported that whereas the presence of Congo red led to increased transcription of some TTS system genes (*virA* and four *ipaH* genes), others were not induced (the *ipaBCDA* and *mxi* operons) [78]. Transcription of the *virA* and *ipaH* genes was also transiently activated

upon entry into epithelial cells. Thus, differential control of transcription of genes encoding secreted proteins was demonstrated.

Porter and Dorman [79] conducted a series of experiments to elucidate the regulatory cascade controlling expression of the *Sh. flexneri* TTS virulence gene regulon. A primary regulator, VirF, is a positive regulator of both a secondary regulatory gene, *virB*, and the structural gene *icsA*. The product of the *virB* gene in turn activates transcription of the genes coding for the invasion proteins and the TTS system. The regulatory system is controlled by multiple environmental signals. The pattern of regulation ensures that energetically wasteful expression of the structural genes does not occur under inappropriate conditions. However, regulatory genes are expressed at sufficient levels to ensure a rapid response when inducing conditions arise. The *virF*, *virB* and structural genes display different characteristics in response to stimuli such as growth medium osmolarity, pH and variations in DNA superhelicity. Such variations would allow fine-tuning of the response under inducing conditions.

A similar regulatory hierarchy is apparent in EPEC, where the expression of LEE genes depends upon the presence of a functional integration host factor (IHF). IHF appears to activate expression of a number of other genes, including *ler*. The Ler protein, in turn, is involved in activating the expression of the LEE genes *escJ*, *escV*, *tir*, *eae*, *espB* and *espF* [80].

It has also been demonstrated that invasion genes in *S.* Typhi are regulated by factors such as osmolarity, pH and oxygen levels [81, 82]. *S.* Typhimurium SPI-2 genes are induced by Mg^{2+} deprivation and phosphate starvation, conditions likely to represent the environmental cues encountered by *S.* Typhimurium inside the phagosome of infected host cells. Deiwick *et al*. [83] reported that the induction of SPI-2 gene expression is modulated by the global regulatory system PhoPQ and is dependent on SsrAB, a two-component regulatory system encoded by SPI-2. Early transcription of *ssrA*, after entry into macrophages, is activated by the OmpR protein, the regulatory component of a two-component system involved in response to changes in osmolarity. It has been demonstrated that growth in minimal medium at pH 4.5 induces SPI-2 gene expression in wild-type but not *ompR* mutant strains [84]. TTS by *S.* Typhimurium SPI-1 can also be induced by a change of pH [85]. Interestingly, there is overlap between the regulatory systems for SPI-1 and flagellar assembly. When the flagellar regulatory genes *flhDC* or *fliA* were mutated, strains of *S.* Typhi were found to be severely deficient in macrophage cytotoxicity and entry into epithelial cells [86].

Vallis *et al*. [87] reported that the secretion of ExoS by *Ps. aeruginosa* was induced under tissue-culture growth conditions when *Ps. aeruginosa* was in contact with CHO cells or after growth in tissue-culture medium with serum. Secretion of ExoS and other proteins can also be induced artificially by growth in the presence of the chelator nitrilotriacetic acid [57]. Similarly, effector proteins from *Ps. syringae* can be secreted in culture, with secretion strongly affected by pH and temperature [88].

In *Bor. bronchiseptica* the TTS genes are co-regulated with most other known virulence genes, under the control of the two-component BvgAS signal transduction system [65]. Secretion can be induced in response to growth at 37°C (Bvg^+). In contrast, growth at or below 26°C, or in the presence of high concentrations of nicotinic acid or $MgSO_4$ (Bvg^-), excludes the production of almost all of the known virulence factors of *Bordetella* spp. Instead, *Bor. bronchiseptica* produces flagella and becomes motile [89].

Wider perspectives

It seems unlikely that all TTS systems that play an important role in pathogenicity have been discovered. Indeed, the numerous bacterial genome sequence projects may lead to further discoveries. The conserved nature of TTS structural genes should make homologous regions easy to identify. Whilst TTS systems have revealed fascinating insights into bacterial pathogenesis and the evolution of virulent strains, the most important long-term consequence of the study of TTS systems may be the development of novel approaches to counteract pathogens. Several groups have investigated the use of TTS protein targets for vaccine development. The best known example is LcrV (the *Yersinia* V antigen), itself a secreted protein implicated in the regulation of effector secretion. LcrV is protective for mice in both active and passive immunisation against *Y. pestis* [90, 91]. This has led to the development of recombinant vaccines in which the V antigen is used singly or in combination with other antigens such as the *Y. pestis*-specific protein F1 [92, 93]. YopE, YopK and YopN proteins are antigenic but, unlike LcrV, proved non-protective in a murine model [94].

Because of these experiences in *Yersinia* spp., other workers have sought to target LcrV homologues in other bacteria. Unfortunately, these are not always present. However, a homologue (PcrV) has been discovered in *Ps. aeruginosa*. Protection against lung injury due to challenge with *Ps. aeruginosa* has been reported following active and passive immunisation with PcrV [95]. The antibodies were shown to inhibit TTS.

TTS systems are also being evaluated as a means to deliver desirable antigens directly into host cells. Russmann *et al*. [96] used the TTS system of avirulent *S.* Typhimurium strains to deliver viral epitopes to the

host cell cytosol. Because of the stimulation of class I-restricted immune responses, vaccinated animals were protected against lethal infection. These findings allow a glimpse of the possible developments that may follow the further study and characterisation of TTS systems in bacterial pathogens.

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