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Mini review

Conserved virulence factor regulation and secretion systems in bacterial pathogens of plants and animals

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Abstract

Recent research has revealed the emergence of common themes in the molecular mechanisms of virulence in bacterial pathogens of plants and animals. In particular, the systems used for the global control of virulence factor synthesis and for the secretion of virulence determinants in diverse bacterial pathogens show strong conservation, implying evolutionary relatedness. Global control of virulence factor synthesis can be affected by a variety of environmental factors, bacterial 'hormones' and programmed genetic rearrangements.

Protein secretion in Gram-negative bacteria occurs via a number of targeting pathways. Type I and type III secretion systems mediate translocation across both the inner and outer membrane in a single step, while type II secretion proceeds via a periplasmic intermediate. Type II and type III secretion systems have been shown to target virulence determinants in both plant and animal bacterial pathogens.

Abbreviations: Cel = cellulase; EPS = extracellular polysaccharide; HL = homoserine lactone; OHHL = *N*-(3-oxohexanoyl)-L-homoserine lactone; PC = phenotypic conversion; Pel = pectate lyase; pv. = pathovar; spp. = species.

Introduction

Historically, studies of bacterial pathogens of plants and bacterial pathogens of animals and humans have been conducted in relative isolation. The hosts (plants and animals) are obviously quite different and so it seemed reasonable to presume that their respective pathogens would show little similarity. However, recent progress in the genetics and molecular biology of diverse bacterial plant and animal pathogens has revealed some remarkable similarities in the mechanisms of pathogenesis and virulence [e.g. see Kado and Crosa, 1994]. We will highlight aspects of the regulation and secretion of virulence factors in these bacteria to show that, irrespective of host, some bacterial pathogens of plants or animals use similar molecular strategies for the global control of virulence factor synthesis and for the secretion of these virulence factors to the extracellular environment or target host cell. The subject of conservation of strategies and mechanisms in bacterial pathogenicity is so large that this review cannot be comprehensive, but rather gives an introductory overview of the subject. Where appropriate, we cite references which detail further examples.

Global regulation of virulence factor synthesis in plant and animal pathogens

Bacterial pathogens must be able to sense and respond to changes in their immediate environment. This ability is critically important because, broadly speaking,

Table 1. Examples of global regulatory systems affecting virulence in bacterial pathogens of plants and animals

Signal	Organism	Effect	Mechanism
Plant pathogens			
Phenolics	*Agrobacterium tumefaciens*	*vir* gene expression	2-component system[1]
Sugars	*A. tumefaciens*	*vir* gene expression	2-component system[1]
Volatile molecule	*Pseudomonas solanacearum*	Exoproteins, EPS	'LysR' system[2]
N-acyl HL	*Erwinia carotovora*	Multiple exoenzymes	'LuxI/LuxR' system[3]
Iron	*Erwinia chrysanthemi*	Exoenzymes	'Fur-like' system[2]
Temperature	*E. carotovora*	Exoenzymes	Unknown
Osmolarity	*E. chrysanthemi*	Exoenzymes	Unknown
Plant extracts	*E. chrysanthemi*	Exoenzymes	Unknown
'Spontaneous'	*P. solanacearum*	Exoproteins, EPS	'PC' mutations
Animal Pathogens			
N-acyl HL	*Pseudomonas aeruginosa*	Exoenzymes	'LuxI/LuxR' system[3]
Cell density	*Salmonella* spp.	Virulence factors	'LysR' system[2]
Iron	*Corynebacterium diphtheriae*	Toxin	'Fur-Like' system[2]
Iron	*Vibrio cholerae*	Virulence factors	'LysR' system[2]
Calcium	*Yersinia* spp.	'Yops' (Exoproteins)	via LcrH 'repressor'[2]
Temperature	*Bordetella pertussis*	Virulence factors	2-component system[1]
Temperature	*Shigella flexneri*	Virulence factors	2-component system[1]
Osmolarity	*Vibrio cholerae*	Toxin, pili, other factors	ToxR system[2]
pH	*Vibrio cholerae*	Toxin, other factors	ToxR system[2]
Anaerobiosis	*Salmonella* spp.	Invasion	DNA supercoiling
'Spontaneous'	*Neisseria gonorrhoeae*	Pili	DNA rearrangement

[1] Usually involves a transmembrane sensor protein (which 'receives' the environmental signal) and an intracellular transcriptional activator (which when phosphorylated by the sensor can activate its target genes).
[2] These regulatory proteins define distinct families.
[3] Involves *N*-acyl homoserine lactones (synthesised by the LuxI homologues) which bind to the LuxR homologues, enabling them to act as transcriptional activators of specific genes.
For further information on these and other regulatory systems see Dorman and Ní Bhriain [1992, 1993]; Kado and Crosa [1994]; Mekalanos [1992]; Moxon et al. [1994]; Swift et al. [1994].

most pathogens have to survive in at least two niches; in their respective plant or animal hosts and in non-host environments. Also, during the course of pathogenesis within their hosts, pathogens often have to multiply within quite different cells, tissues and organs, each representing different microenvironments. This ability to adapt to different environments requires the expression (and repression) of different sets of both virulence and 'housekeeping' genes in a co-ordinated and tightly regulated fashion – 'global' regulation. Global regulation of multiple sets of genes can be controlled by many factors, some of which are environmental (see Table 1 for some examples and footnotes for more details). We have chosen well characterised systems as 'paradigms' of global regulation in bacterial pathogens. For convenience we have divided factors mediating the regulation of virulence into two classes; extrinsic factors, governed by environmental conditions, and intrinsic factors, under the control of the pathogens themselves.

Regulation of virulence in response to extrinsic factors

Temperature

There are many bacterial pathogens which regulate their virulence factors in response to temperature [e.g. see Mekalanos, 1992; Dorman and Ní Bhriain, 1992

and 1993 for examples]. In the animal pathogen *Bordetella pertussis* the *bvgA* and *bvgS* two component system controls expression of several virulence genes. This two component system (with a transmembrane 'sensor' protein and an intracellular 'activator' protein) itself responds to multiple environmental signals, including temperature, nicotinic acid and SO_4^{2-}. No examples of thermoregulation, mediated by two component systems, have been established in plant pathogenic bacteria. Two component systems have, however, been shown to regulate the expression of phytopathogenic virulence determinants, the best characterised example being *vir* gene regulation in *Agrobacterium tumefaciens*. VirA is an inner-membrane-associated histidine protein kinase which acts as a sensor of monosaccharides and phenolics in the environment. VirA activates VirG, in response to these signals, by phosphorylation of the VirG protein which then acts as a positive regulator of *vir* gene expression [Winans, 1992]. *Yersinia* species elaborate multiple virulence factors called 'Yops' (**Y**ersinia **o**uter **p**roteins), which are efficiently secreted (see below). The *yop* genes and various other virulence genes are carried by a large virulence plasmid and are co-ordinately thermoregulated via the *virF* (called *lcrF* in some species) product [Cornelis et al., 1989]. LcrF/VirF are in the 'AraC class' of transcriptional regulator proteins and appears to be the protein which senses and responds to temperature fluctuations [for review see Forsberg et al., 1994]. Other examples of thermoregulation in animal pathogens include the control of *ipa* or *inv* loci expression (affecting invasion of eukaryotic cells) in *Shigella flexneri* and the thermoregulation of Pap pilus expression in *Escherichia coli* [Mekalanos, 1992].

Several bacterial plant pathogens exhibit thermoregulation of virulence determinants. For example, in the soft rot pathogens *Erwinia carotovora* and *Erwinia chrysanthemi* the synthesis of multiple plant cell wall degrading enzymes (pectinases, cellulase and proteases) is subject to thermoregulation [Cooper and Salmond, 1993; Lanham et al., 1991; Hugouvieux-Cotte-Pattat et al., 1992]. Phaseolotoxin synthesis by *Pseudomonas syringae* pv. *phaseolicola* is also sensitive to temperature control [Rowley et al., 1993]. However, in none of the above examples of thermoregulation in plant pathogenic bacteria are the mechanisms understood at the molecular level.

Osmolarity

When bacterial pathogens invade their respective hosts they encounter significant variations in osmolarity. Thus it is not surprising that many bacterial pathogens have developed sensing systems to regulate the expression of virulence factors in response to osmotic stress. Expression of some *inv* (invasion) genes of *Salmonella typhimurium* is induced in response to changes in osmolarity, as are exopolysaccharide capsule in *Pseudomonas aeruginosa* and cholera toxin, pili and other virulence factors in *Vibrio cholerae* [Mekalanos, 1992]. Some pathogenicity genes (e.g. many of the *hrp* genes – **h**ypersensitive **r**esponse and **p**athogenicity) of phytopathogens such as *Erwinia amylovora*, *P. syringae* and *Xanthomonas* spp. respond to osmolarity [e.g. see Hutcheson et al., 1994; Willis et al., 1991]. Also, synthesis of some exoenzyme virulence factors of soft rot *Erwinia* spp. is osmosensitive [Hugouvieux-Cotte-Pattat et al., 1992].

Iron availability

Iron is frequently a limiting factor for colonization and virulence in the anaerobic conditions and neutral pH often encountered within a host. *Corynebacterium diphtheriae*, well characterised in this respect, regulates diphtheria toxin production as part of a global 'low iron regulon' [Schmitt and Holmes, 1991]. Also some Shiga-like toxins produced by certain enteropathogenic *E. coli* strains are regulated in response to low iron status [Calderwood and Mekalanos, 1987]. In the phytopathogen *E. chrysanthemi*, iron availability appears to affect virulence. These mutants are iron 'auxotrophs' *in planta* [Sauvage and Expert, 1994]

Regulation of virulence mediated by intrinsic factors

Small molecules

We are discussing small molecules under this heading because, although production may be affected by extrinsic factors and the molecules are found in the environment, their presence is determined by the pathogen itself. There is growing evidence that many bacteria, including plant and animal pathogens produce small, freely-diffusible signalling molecules. The main class of these molecules is the *N*-acyl homoserine lactones (N-Acyl HLs). These N-Acyl HLs are involved in the regulation of exoenzyme virulence factor synthesis in the opportunistic human pathogen *P. aerugi-*

nosa and the plant pathogen *E. carotovora* [Jones et al., 1993]. These signalling molecules are effective above a threshold concentration which is exceeded only at high cell density. Since the molecules are freely diffusible they act as bacterial hormones, regulating virulence gene expression as a 'community event'. Synthesis of the signalling molecules by various bacteria is mediated by homologues of the LuxI protein, the 'autoinducer synthetase' of *Vibrio fischeri*. LuxI makes *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) – the Lux autoinducer. OHHL is thought to bind to LuxR, allowing transcriptional activation of the *lux* operon, leading to bioluminescence. Homologues of both LuxI and LuxR are involved in OHHL-dependent signalling systems in *E. carotovora* and *P. aeruginosa* [Fuqua et al., 1994; Swift et al., 1994]. *A. tumefaciens* produces a conjugation factor similar in structure to OHHL [Zhang et al., 1993] and possesses a regulatory protein responsive to this chemical, TraR, which is a positive regulator of conjugal transfer of the Ti plasmid [Piper et al., 1993]. There is also evidence in *Pseudomonas solanacearum* for a volatile molecule acting as a global regulator of virulence factor synthesis although this molecule is not OHHL [Schell et al., 1994].

Genetic variation

Genetic variation may be either spontaneous, or a response to a specific environmental signal, i.e. programmed. Both can affect fitness or immunoevasion of subpopulations of the pathogen in particular environments. It has been known for some time that some animal pathogens such as gonococci, meningococci, *Haemophilus* and *Borrelia* can vary antigenically at high frequency [e.g. see Moxon et al., 1994; Swanson et al., 1992]. In *Neisseria gonorrhoeae*, pilus antigenic variation is controlled by spontaneous genetic re-arrangements in the *pil* loci, and outer membrane opacity proteins (Opa) vary by translational frameshifting in the *opa* genes due to pentanucleotide repeated sequences [Moxon et al., 1994; Swanson et al., 1992]. In the plant pathogen *P. solanacearum*, gross phenotypic conversions (PC) occur by virtue of spontaneous mutation in the *phcA* regulatory gene [Brumbley et al., 1993] which globally regulates the synthesis of multiple virulence factors [Schell et al., 1994]. PC therefore explains the old observation of 'spontaneous' variation in the virulence of morphologically different colonies of *P. solanacearum* [Brumbley et al., 1993; Schell et al., 1994].

Final comments on global regulatory systems

It is clear that there are multiple mechanisms involved in global regulation of virulence factor production in bacterial pathogens of plants and animals. In addition to those highlighted above, anaerobiosis, catabolite repression and stress (pH, heat shock, starvation etc.) also affect the global regulation of virulence [for examples see Mekalanos, 1992; Dorman and Ní Bhriain, 1992 and 1993; Dorman, 1994]. Some regulatory mechanisms in animal pathogens are modulated by changes in DNA topology [Dorman and Ní Bhriain, 1993], but the importance of this in plant pathogens is currently unknown. The sheer multiplicity of factors influencing the similar global control mechanisms of both plant and animal pathogens highlights the strong convergence of regulatory themes in these bacteria.

Secretion systems in bacterial plant and animal pathogens

As mentioned above, many bacterial plant and animal pathogens secrete enzymes and/or toxins. Since non-secreting mutants have reduced virulence, a knowledge of secretion is important if pathogenicity is to be understood. By analyzing various secretion-deficient mutants, genes encoding 'secretory apparatus' proteins were identified. These proteins, sometimes referred to as 'traffic wardens' [Salmond and Reeves, 1993], are thought to control the 'flow and location' of macromolecules, most commonly proteins, during translocation across bacterial membranes. There are a number of different secretion pathways [for reviews see Wandersman, 1992; Pugsley, 1993a; Salmond and Reeves, 1993; Salmond, 1994], only some of which will be discussed here. Type I secretion is accomplished via a one-step mechanism. This secretion system is widespread in bacterial pathogens of animals and humans, with the best characterised example being for the secretion of α-haemolysin by uropathogenic *E. coli*. The only well characterised type I system in phytopathogenic bacteria is that for the secretion of proteases by *E. chrysanthemi*. However, since protease secretion is not required for pathogenicity [Dahler et al., 1990], the type I system will not be discussed in this review of virulence determinants common to plant and animal pathogens.

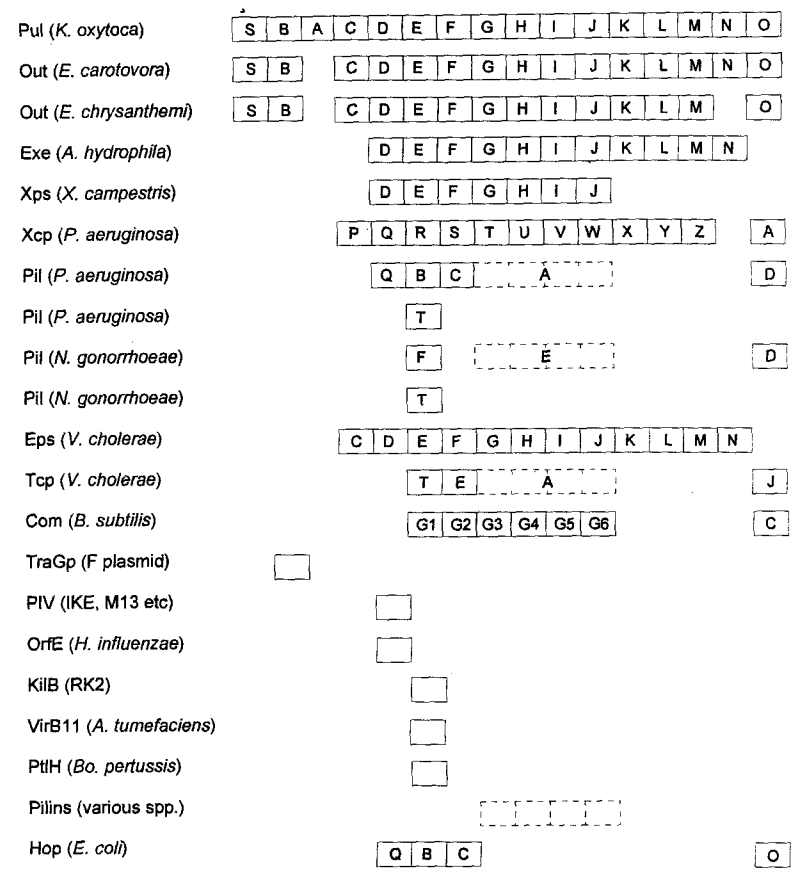

Fig. 1. Type II trafficking components. Type II trafficking proteins are aligned on the basis of their similarity with the proteins encoded by the *pul* gene cluster of *K. oxytoca*. It should be noted that this does not represent the gene cluster organization for some of the systems. Pilins, indicated by dotted boxes, show limited similarity to the PulG-J proteins, confined to the region around the consensus MePhe cleavage site. Since these sites are present in each of the PulG-J proteins, it is not possible to align pilins with particular Pul proteins. PilA (*P. aeruginosa*), PilE (*N. gonorrhoeae*) and TcpA (*V. cholerae*) have therefore simply been shown as similar to the PulG-J group of proteins. The various species of pathogenic bacteria possessing MePhe pili (but not known to possess other components of type II secretory systems) are not listed here, but examples are given in the text. Sequence accession numbers/source: *K. oxytoca*: PulS & PulB (M29097), PulC-O (M32613); *E. carotovora*: OutS & OutB (Wharam, unpubl. obs.), OutC-O (X70049); *E. chrysanthemi*: OutS & OutB (X65265), OutC-M, OutO (L02214); *A. hydrophila*: ExeD-N (X66504); *X. campestris*: XpsD (S92688), XpsE-J (X59079); *P. aeruginosa*: XcpP & XcpQ (X68594) XcpR-X (X62666), XcpY & XcpZ (X56183), PilQ (L13865), PilB-D (M32066), PilT (M55524). N.B. XcpA is the same protein as PilD; *N. gonorrhoeae*: PilD, PilF & PilT (L10291); *V. cholerae*: TcpT, TcpE & TcpJ (X64098), EpsE (M96172), EpsM (L13660), EpsC & EpsD, EpsF-L, EpsN (Overbye LJ, Sandkvist M and Bagdasarian M, 94th ASM meeting 1994, abstracts book p. 133); *B. subtilis*: ComG1-G6 (M22854), ComC (M30805); *E. coli*: TraGp (M59763), KilB (X59971), HopO (M27176), HopQ, HopB & HopC [see Hobbs and Mattick, 1993]; Filamentous phage: pIV e.g. from IKE: (X02139); *H. influenzae*: ORFE (M62809); *A. tumefaciens*: VirB11 (X53264); *Bo. pertussis*: PtlOrfH (L10720).

Type II secretion

Type II secretion proceeds in two steps, via a periplasmic intermediate, as demonstrated for *Klebsiella oxytoca* [Pugsley, 1993a] and *E. chrysanthemi* [He et al., 1991b]. The first step, involving cleavage of a leader peptide, is dependent on a 'translocase' complex of SecA, D, E, F and Y and soluble chaperone proteins [Wickner et al., 1991] which direct proteins to the periplasm. Although this process is commonly referred to as 'secretion', 'export' is preferred, since this avoids confusion with the process of true secretion into the culture supernatant. The involvement of the Sec apparatus in type II secretion has been demonstrated by functionally reconstituting the type II secretion system of *K. oxytoca* in *E. coli*. Sec^- strains of *E. coli* are incapable of directing extracellular enzymes to the supernatant [Pugsley, 1993a].

The genes required for the second step of secretion across the outer membrane of various Gram-negative bacteria encode families of homologous proteins and are usually organised into clusters, not normally linked to the structural gene(s) for the secreted protein(s). Exceptions are the *pul* cluster of *K. oxytoca* which includes the gene for pullulanase [Pugsley, 1993a] and the *out* gene cluster of *E. carotovora* which includes two structural enzyme genes [Wharam, unpubl. obs.]. Some type II trafficking components are shown in Fig. 1, where proteins are arranged according to their similarity with the proteins encoded by the *pul* gene cluster of *K. oxytoca.* These Pul proteins, required for the secretion of pullulanase, constitute the best characterised example of a type II secretion system [for review see Pugsley, 1993a]. Secretion of pullulanase (PulA) requires the *pulC-O* and *pulS* gene products. When *pulA* is expressed in *E. coli* the protein is targeted to the periplasm by the Sec apparatus, but does not reach the culture supernatant, suggesting that *E. coli* lacks the type II secretion system [Pugsley et al., 1986]. The only type II systems yet successfully reconstituted in *E. coli* are the Pul [d'Enfert et al., 1987] and the *E. chrysanthemi* Out [He et al., 1991a] systems.

Some idea of the diversity of type II secretion systems can be gained from Fig. 1. The Pul apparatus of *K. oxytoca* (Pugsley, 1993a] is unusual in that it seems to secrete only pullulanase, while most systems secrete multiple enzymes and/or toxins. Examples include the secretion of pectinases (Pel), cellulases (Cel) and polygalacturonase by the Out system [He et al., 1991a; Reeves et al., 1993] of phytopathogenic *Erwinia* spp.; aerolysin, amylase and acyltransferase by the opportunistic animal pathogen *Aeromonas hydrophila* [Jiang and Howard, 1992]; endogluconase, pectinases and proteases by *Xanthomonas campestris* pathovar *campestris*, causal agent of black rot in crucifers [Dums et al., 1991]; and elastase, lipase, phospholipase C, alkaline phosphatase and exotoxin A by *P. aeruginosa* [Bally et al., 1992].

In *P. aeruginosa*, *Pseudomonas putida*, *N. gonorrhoeae* and *V. cholerae* (Tcp proteins; [Iredell and Manning, 1994]], Pul homologues are involved in pilus assembly. The significance of the observation that some components of type II secretion systems are similar to MePhe pilins is discussed below. MePhe pilins are found in many bacterial animal pathogens including *Pseudomonas* spp., *N. gonorrhoeae*, *Bacteroides nodusus*, *Moraxella bovis*, *Moraxella lacunata* and *Eikenella corrodens*.

There are some other Pul homologues which are involved in the trafficking of DNA rather than proteins. VirB11 is an autophosphorylating ATPase, required for the transfer of the *A. tumefaciens* Ti plasmid [Christie et al., 1989], while the *E. coli* plasmid-encoded proteins KilB (RK2) and TraGp (F plasmid) are involved in conjugal transfer. VirB11 and KilB appear to be highly conserved, suggesting that the mechanisms of Ti plasmid transfer and F plasmid conjugation may be similar. There are also Pul homologues involved in competence for DNA uptake in *Bacillus subtilis* (Com) and *Haemophilus influenzae* (OrfE). These homologues, involved in the transfer of DNA, may form a distinct subset of type II trafficking proteins.

Locations and functions of components of type II secretory systems

Cell fractionation studies have shown that almost all of the Pul proteins are thought to be located in the inner membrane, with only one or two in the outer membrane and one soluble in the cytoplasm [Pugsley, 1993a]. These findings, surprising for proteins involved in the second step of secretion across the outer membrane, were supported by topology mapping of the *E. carotovora* Out proteins [Reeves et al., 1994]. One theory is that the proteins interact to form a (perhaps transient) transmembrane structure.

It is possible to predict the functions of some proteins of the type II secretory systems. PulE and its homologues (OutE, etc.) are cytoplasmic and have consensus nucleotide binding sites, typical of ATPases, which are essential for their functioning [Turner et al., 1993; Possot and Pugsley, 1994]. These proteins

might either couple energy from ATP hydrolysis, or phosphorylate and hence activate another component of the secretory apparatus. Interestingly, *P. aeruginosa* has several PulE homologues, each having a different function in either secretion or pilus assembly [see review Hobbs and Mattick, 1993].

Another type II trafficking component identified in *P. aeruginosa* is the XcpA/PilD protein, a peptidase with homologues in other type II systems. This processes the N-terminal signal sequence of the PilA pilin precursor, leaving an N-terminal Phe residue which is then methylated, hence the term MePhe pili [Hobbs and Mattick, 1993]. The consensus MePhe peptidase cleavage site is conserved in the proteins of the PulG-J family (themselves similar to each other), some of which have been shown to be processed by the respective XcpA/PilD (e.g. PulO) homologue [Bally et al., 1992; Pugsley, 1993b]. The PulO homologues are polytopic inner membrane proteins. The catalytic site is located in the cytoplasm [Reeves et al., 1994] where the MePhe cleavage event occurs. The processed G-J 'pseudopilins' are thought to remain anchored in the inner membrane by a stretch of hydrophobic residues, with the majority of the protein in the periplasm.

A role of the PulD homologues has been proposed following studies of filamentous phage assembly [for review see Russel, 1991]. The phage protein pIV (similar to PulD) forms a multimer of 10–12 monomers, thought to form an outer membrane pore (expected to be gated), large enough to allow extrusion of phage proteins [Kazmierczak et al., 1994].

Specificity of type II secretion systems

Of all the type II trafficking components, those capable of functioning in the widest range of backgrounds are the peptidases, but even amongst these some divergence/specialisation seems to have occurred [Dupuy et al., 1992]. Secretion does not normally occur in heterogeneous (or even heterospecific, in the case of *Erwinia* spp.) systems [He et al., 1991a; Py et al., 1991]. It is a paradox that some proteins secreted by the same system, for example Pel, and Cel secreted by the Out apparatus of *E. carotovora*, show no sequence similarity to each other, yet homologous proteins such as the Pels of *E. carotovora* and *E. chrysanthemi* are not secreted when expressed heterospecifically. This suggests that the recognition process is highly complex.

Since proteins fold in the periplasm before being secreted [Pugsley, 1993a], and disulphide bond for-

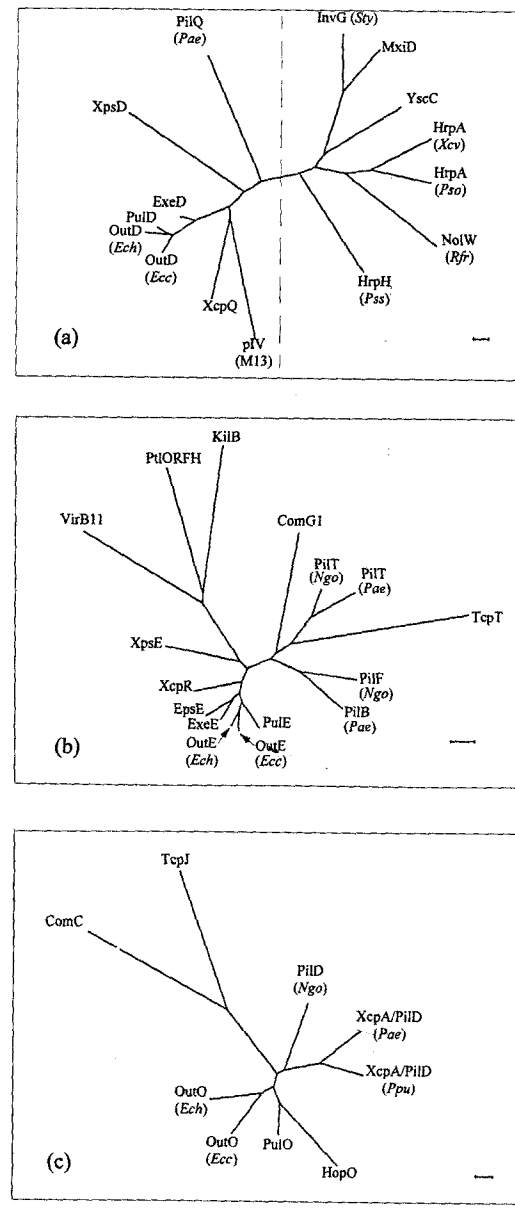

Fig. 2. Dendrogram showing the relationships between members of the; (a) PulD family of 'gate' proteins, (b) PulE-like proteins (ATPases), (c) PulO, peptidase homologues. The dashed line in (a) highlights the division of the dendrogram into type II (left) and type III (right) trafficking components. The scale bar in the lower right represents 10 PAM units. The dendrograms are adapted from the output of the PhyloTree analysis program at the E-mail server of the Computational Biology Research Group in Zurich, Switzerland (E-mail address, cbrg@inf.eth.ch). Organism abbreviations: *Ecc* = *Erwinia carotovora* subsp. *carotovora*, *Ech* = *Erwinia chrysanthemi*, *Ngo* = *Neisseria gonorrhoeae*, *Pae* = *Pseudomonas aeruginosa*, *Ppu* = *Pseudomonas putida*, *Pso* = *Pseudomonas solanacearum*, *Pss* = *Pseudomonas syringae* pv. *syringae*, *Rfr* = *Rhizobium fredii*, *Sty* = *Salmonella typhimurium*, *Xcv* = *Xanthomonas campestris* pv. *vesicatoria*. Sequence accession number: *Pss* HrpH (A45243). For other accession numbers see the legend of Fig. 1 and Table 2.

mation is required [Bortoli-German et al., 1994], a 'patch' signal might be recognised. In theory a species-specific 'discriminator' could exist and recognise self-proteins, targeting them for secretion across the outer membrane. Species-specific recognition of diverse proteins might be explained if this discriminator was intolerant of minor changes in the sequence of secreted proteins.

Similarity of various components of type II secretory systems

Groups of Pul homologues (see Fig. 1) were compared by computer analysis to create dendrograms. Both the PulE and PulO homologues (Fig. 2b and 2c) fall into three subgroups containing: general secretory proteins (Pul, Out etc), pilus assembly proteins and other, more distantly related proteins (e.g. those for the translocation of DNA). Most interesting is the dendrogram of PulD homologues (Fig. 2a). Approximately half the proteins shown (those on the left) are involved in type II secretion, pilus assembly or filamentous phage morphogenesis (i.e. discussed above). Some of the remainder, although involved in secretion, are encoded by genes mapping to clusters which do not encode other Pul homologues. These proteins function in a different type of secretory apparatus (discussed below). The fact that PulD homologues appear to be required in both systems probably reflects the importance of this protein, predicted to form a gated channel in the outer membrane.

Type III secretion

A further class of secretion system, which targets a group of proteins lacking amino-terminal signal sequences, has begun to be recognised as wide-spread in both Gram-positive and Gram-negative bacteria. This pathway is of interest as virulence factors are targeted, via type III systems, in a variety of plant and animal pathogens.

Hrp and secretion of elicitors

The Gram-negative phytopathogens *Erwinia*, *Pseudomonas* and *Xanthomonas* have gene clusters of 25–40 kb which are involved in the elicitation of the **h**ypersensitive **r**esponse and **p**athogenicity (*hrp*) *in planta* [Willis et al., 1991]. The hypersensitive response (HR) is the reaction of non-host (or resistant) plant tissue to infiltration of high numbers of hrp^+ bacteria. The HR is characterised by rapid and localised plant cell death which restricts movement of the pathogen and reduces further bacterial growth.

The HR requires that the bacterial phytopathogen has functional *hrp* genes and is able to secrete elicitors which mediate the HR [Bonas, 1994]. The three proteinaceous elicitors characterised to date, dependent on the function of *hrp* genes for their targeting, are $harpin_{Ea}$ from *Erwinia amylovora* [Wei et al., 1992], $harpin_{Pss}$ from *P. syringae* pv. *syringae* [He et al., 1993] and PopA1 from *P. solanacearum* [Arlat et al., 1994]. Targeting of harpins is thought to be *sec*-independent as none of the proteins possess a classical N-terminal signal sequence.

Mutations of the *hrp* cluster, initially defined by a series to Tn*5*-induced mutants of *P. syringae* pv. *phaseolicola*, gave rise to a pleiotropic phenotype which included the loss of HR on non-host plants, reduced pathogenicity and restricted bacterial growth in host plants. The transposon mutants could be complemented by a single cosmid clone which suggested that the genes involved were clustered. Further transposon mutagenesis and subcloning allowed detailed analysis of this cluster [Rahme et al., 1991]. The *hrp* genes were found in a 22 kb region which consisted of seven complementation groups (*hrpL*, *hrpAB*, *hrpC*, *hrpD*, hrp*E*, hrp*F* and *hrpSR*) and transcriptional organisation was established [Rahme et al., 1991]. Subsequently, *hrp* mutants have been isolated from many *P. syringae* pathovars [for review see Willis et al., 1991]. Sequence analysis of *hrp* genes from plant pathogenic bacteria has revealed similarities (Table 2) to various gene products involved in bacterial virulence [Fenselau et al., 1992; Genin et al., 1992; Gough et al., 1992, 1993; Van Gijsegem et al., 1993]. Many of the predicted protein products share similarity with the *ysc/lcr* (**Y**op **s**ecretion, **l**ow Ca^{2+} **r**esponse) gene products of yersiniae [Forsberg et al., 1994] and the *mxi/spa* gene products of *S. flexneri* [Venkatesan et al., 1992; Sasakawa et al., 1993]. These proteins (Table 2) are type III 'traffic wardens' [Salmond and Reeves, 1993].

Conservation of type III secretory apparatus proteins

Pathogenic yersiniae contain a common 70–75 kb plasmid encoding Yops which are secreted and act in the evasion of the primary immune response. Secretion of the Yops is *sec*-independent as they do not possess N-

Table 2. Proteins showing similarity to type III secretion systems

Yersiniae	*S. flexneri*	*P. solanacearum*	*X. campestris*	*E. carotovora*	*R. fredii*	Flagella proteins	*S. typhimurium*
YscC	MxiD (29)[1]	HrpA (34)	HrpA1 (24)		NolW (30)		InvG (28)
YscJ	MxiJ (27)	HrpI (34)	HrpB3 (38)		NolT (43)		
YscL		HrpF (23)					
YscN	Spa47 (43)	HrpE (57)	HrpB6 (60)			FliI (49)	SpaL (52)
YscO	Spa13 (20)						SpaM (21)
YscP	Spa32 (16)						SpaN (21)
YscQ	Spa33 (26)	HrpQ		MopA (26)		FliN (30)	SpaO (24)
YscR	Spa24 (46)	HrpT	Orf2 (50)	MopC (40)		FliP (40)	SpaP (47)
YscS	Spa9 (42)			MopD (33)		FliQ (36)	SpaQ (45)
YscT	Spa29 (26)			MopE (22)		FliR (23)	SpaR (29)
YscU	Spa40 (32)	HrpN (31)				FlhB (34)	SpaS (33)
LcrD	MxiA (39)	HrpO (44)	HrpC2 (41)			FlhA (39)	InvA (41)
LcrF	MxiE	HrpB (19)					InvF

[1] Figure denotes percentage sequence identity to the appropriate yersiniae proteins.
Sequence accession numbers/source: Yersiniae: YscC (M83986), YscJ (M74011), YscL (M74011), YscN (L23522), YscQ-S (L22495), YscO-U (L25667), LcrD (M77014), LcrF (M86690) *S. flexneri*: MxiD (X67206), MxiJ (M98390), Spa47–40 (D13663), MxiA (S40939) *P. solanarcearum*: HrpA (M99632), HrpI (M99632), HrpN (S35250), HrpO (M99633), HrpB (Z14153), HrpE (see Woestyn et al., 1994), HrpF (see Van Gijsegem et al., 1994) *X. campestris*: HrpA1 (M99173), HrpB3 (M99175), HrpB6 (M99174), Orf2 (S27742), HrpC2 (M99176) *E. carotovora*: MopA-E (X72969) *R. fredii*: NolT, NolW (L12251) Flagella: FliI (M62408), FliN (M24463), FliP-R (L22184), FlhA (X63698), FlhB (S34714) *S. typhimurium*: InvG (X75302), SpaL-S (X73525), InvA (M90846). Additional information from Ginocchio et al. [1994].

terminal signal sequences. The genes encoding Yops are scattered on the plasmid but are controlled as a regulon. Genes required for the regulation and secretion of Yops are clustered in a 20 kb region of the plasmid. These genes (variously named *lcr*, *vir* and *ysc*) have been characterised in detail [Forsberg et al., 1994].

S. flexneri is a major cause of bacterial dysentery. Invasion of epithelial cells of the colonic mucosa is a prerequisite for the elaboration of the disease. Among the plasmid-encoded invasion gene products required for pathogenicity are the **i**nvasion **p**lasmid **a**ntigen gene products (Ipa proteins) which, like the Yop proteins of *Yersinia* spp., lack a signal sequence. The genes involved in Ipa targeting have been localised to a gene cluster involved in the surface **p**resentation of invasion plasmid **a**ntigens (*spa*) and **m**embrane **e**xpression of **i**nvasion plasmid antigens (*mxi*) [Hale, 1991; Sasakawa et al., 1993; Venkatesan et al., 1992].

Flagella structural proteins are also targeted in a *sec*-independent manner and genes thought to be required for this process are scattered in several regions of both the *E. coli* and *S. typhimurium* chromosomes [Macnab, 1992]. Candidate targeting proteins include FlhA, FliI, FliN and possibly FliP, FliQ and FliR.

In addition to those mentioned above, there are several other systems which share similarity to this family of proteins (Table 2) including the Spa and Inv proteins of *S. typhimurium* required for bacterial internalization [Groisman and Ochman, 1993; Ginocchio et al., 1994] and the Mop proteins required for motility and pathogenicity in *E. carotovora* [Mulholland et al., 1993].

Characteristics of type III trafficking components

A number of gene products are required for the functioning of this class of targeting systems. The main systems considered are Hrp in *P. solanacearum* and Ysc in yersiniae. **HrpA/YscC** – YscC is predicted to be outer-membrane-associated (similar to the PulD putative 'gate' protein, see Fig. 2a) with multiple transmembrane regions [Michiels et al., 1991]. **HrpI/YscJ** – YscJ is an outer membrane-associated lipoprotein predicted to be covalently bound, at its C-terminal lysyl residue, to the peptidoglycan layer [Michiels et al., 1991]. **HrpF/YscL** – These two proteins are reported to be similar [Van Gijsegem et al., 1993]. **HrpE/YscN** – YscN is a member of the ATP-driven proton translocase family typified by FliI [Woestyn et al., 1994]. **HrpQ/YscQ** – YscQ is similar to the flagel-

la gene product FliN which is found in the hook-basal body and is thought to be involved in the switch in direction of flagellum rotation. **HrpT/YscR** – YscR is predicted to be an inner-membrane-associated polytopic protein with four membrane-spanning domains [Fields et al., 1994] and may be required for secretion and for full induction of secreted proteins. A mutant of *S. typhimurium spaP* (similar to *yscR* – see Table 2) has been functionally complemented with the *spa24* gene from *S. flexneri* [Groisman and Ochman, 1993]. **YscS** – This gene product is similar to FliQ [Fields et al., 1994] and is thought to be an inner-membrane-associated protein. FliQ is required in the early stages of flagellum biogenesis [Macnab, 1992]. **YscT** – The sequence of this protein is similar to Spa29, FliR and MopE [Bergman et al., 1994]. FliR, required for flagellum biogenesis, is an extremely hydrophobic protein predicted to have at least five membrane-spanning domains. **HrpN/YscU** – These proteins are predicted to be inner-membrane-associated with five membrane-spanning domains [Gough et al., 1993; Bergman et al., 1994].

Type III trafficking components involved in both secretion and regulation have also been characterised. **HrpO/LcrD** – LcrD is a 77 kDa inner-membrane-associated protein which is predicted to have 8 membrane-spanning domains and a large C-terminal cytoplasmic domain [Plano et al., 1991]. In an *lcrD* mutant, expression of LcrY, YopB and YopD was greatly reduced while expression of YopH and YopM was increased. **HrpB/LcrF** – The *lcrF* (or *virF*) gene product is predicted to be a transcriptional activator related to the AraC regulator [Cornelis et al., 1989]. HrpB of *P. solanacearum* appears to be a positive regulator of expression of five of the six transcriptional units of the *hrp* cluster [Genin et al., 1992].

Based on published analyses (see above) and computer predictions [Mulholland, unpubl. obs.] the locations of the Ysc proteins mentioned above can be postulated. Figure 3 shows a composite representation of these data. Two of the proteins are probably cytoplasmic, six inner-membrane-, and two outer-membrane-associated.

The complexity and wide-spread distribution of the type III system suggests that there may have been extensive and quite recent horizontal transfer of these genes between bacteria. However, Van Gijsegem et al. [1994] noted that when the DNA sequences encoding type III systems are compared, there are large discrepancies in GC content and codon usage. GC content and codon usage of a type III system is consistent with

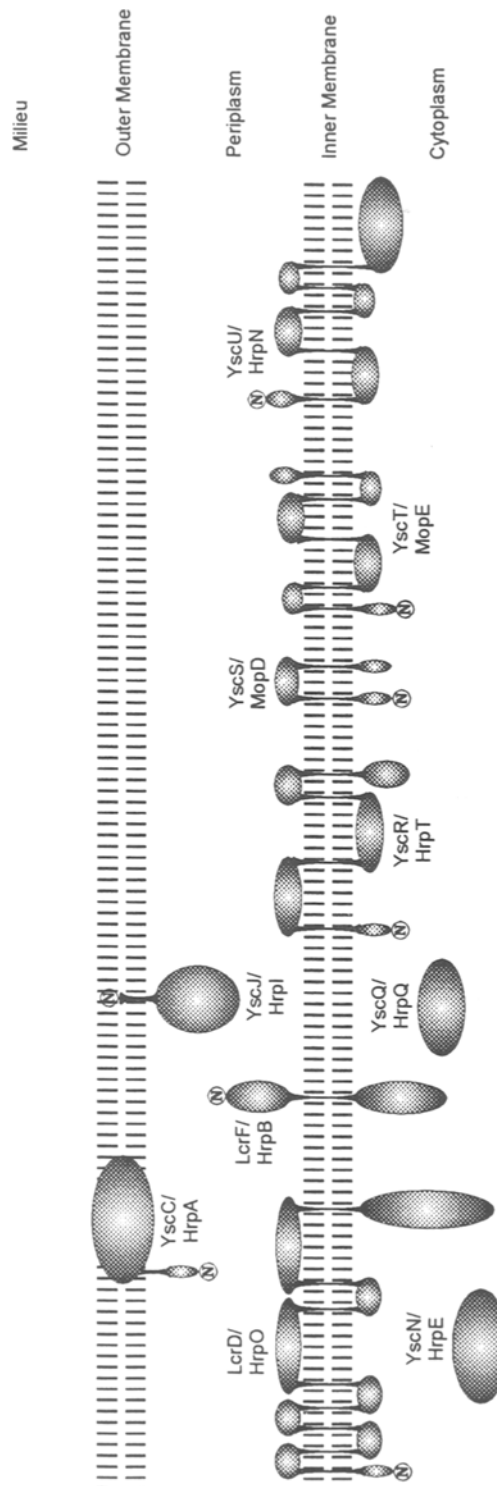

Fig. 3. Predicted locations and transmembranous regions of Ysc & Lcr proteins of yersiniae. The location of the N-terminus of a protein is indicated by the letter N. These data comprise published analyses of the Ysc and Lcr proteins (see text for details) as well as numerous computer predictions (Mulholland, unpubl. obs.). The names of *P. solanacearum* Hrp (or *E. carotovora* Mop) homologues of these proteins are also shown.

other genes of that particular bacterium. This would mean that they are of ancient origin and that the gene products have been constrained in their evolution, possibly because of protein-protein interactions required for their function.

Final comments

It is now apparent that some bacterial pathogens of plants and animals employ similar molecular mechanisms for the regulation and secretion of their virulence factors. As the molecular genetic information from various bacterial pathogens increases, the universal nature of such regulatory and secretory systems in prokaryotes should become progressively more evident. Thus, it seems highly likely that current advances in the molecular dissection of plant and animal pathogenicity determinants will pave the way towards an understanding of common principles concerning the fundamental, molecular bases of bacterial virulence.

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