unfortunately passed away before the development of many technologies that have made *Stentor* a more tractable system, and much of their expertise was lost with them.

***What can we learn from studying* Stentor?** *Stentor coeruleus* is a uniquely tractable model for studying complex morphogenesis at the level of a single cell without needing to worry about external influences from neighboring cells that are present in metazoan development. Whether or not any of the mechanisms that regulate *Stentor* morphogenesis are conserved in metazoans remains an open question but further research might shed light on how complex single cells are organized. *Stentor* may also be useful for studying wound healing within cells, as it has the ability to maintain its integrity even after severe surgical manipulations. As a final example, *Stentor* could be useful as a model for memory at the level of a single cell. Work from David Wood has shown that *Stentor* possesses the ability to habituate to mechanical stimuli and can remain habituated over the course of hours, although no molecular mechanism for this phenomenon has been determined.

***What tools are available for studying* Stentor?** *Stentor coeruleus* is easily imaged on even the most basic microscopes at low magnification, where the majority of the cell structures can be resolved. As discussed previously, *Stentor* is amenable to microsurgical manipulation, including surgical removal of specific regions of the cell and even grafting of cell fragments onto other cells. Recently, RNA interference methodology has been adapted and shown to be an effective method for probing gene function in *Stentor*. This can be achieved by feeding the *Stentor* with bacteria expressing long double-stranded RNA corresponding to a gene of interest for 3–5 days.

What about classical genetics? Ciliate genetics has been developed to a high level in *Tetrahymena* and *Paramecium*, but has never been developed as a tool for *Stentor*, owing to the low frequency of mating under standard laboratory conditions. There are a handful of studies that describe mating in *Stentor coeruleus*, and show that isolates from different locations have the ability to form mating pairs that look similar to mating pairs from other ciliates. Previous studies on *Stentor*, and work from other ciliates, suggest that stressful conditions such as starvation or temperature shifts can induce conjugation but these protocols do not seem to be successful in our lab strain. We have seen cells with altered morphologies consistent with descriptions of pre-conjugation but mating pairs have never been obtained, possibly because standard laboratory cultures only contain cells of a single mating type. Interestingly, mating pairs have been seen in two different species of *Stentor* obtained from the wild after being isolated for 24–48 hours without additional food, so there is potential for conjugation in *Stentor coeruleus* by isolating cells from natural sources. Development of classical genetics in *Stentor* could be a very powerful tool for furthering *Stentor coeruleus* as a useful model organism.

Is there a* Stentor coeruleus *genome project? The macronuclear genome of *Stentor coeruleus* has been sequenced and assembled, and is currently being annotated in our lab. Once completed, the genome will be publicly accessible on the stentor.ciliate.org server.

Where can I find out more?

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Primer

Type III secretion system

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The type III secretion system (T3SS) is a membrane-embedded nanomachine found in several Gram-negative bacteria. Upon contact between bacteria and host cells, the syringe-like T3SS (Figure 1) transfers proteins termed effectors from the bacterial cytosol to the cytoplasm or the plasma membrane of a single target cell. This is a major difference from secretion systems that merely release molecules into the extracellular milieu, where they act on potentially distant target cells expressing the relevant surface receptors. The syringe architecture is conserved at the structural and functional level and supports injection into a great variety of hosts and tissues. However, the pool of effectors is species specific and determines the outcome of the interaction, via modulation of target-cell function.

The T3SS-mediated phenomenon of contact-dependent translocation of bacterial proteins into host cells was first reported for *Yersinia pestis* 20 years ago. Since then, studies have shown that bacteria use this system to communicate with organisms belonging to other kingdoms, including protists, fungi, plants and animals.

Both commensal and pathogenic bacteria express T3SSs. Nevertheless, the T3SS has received far more attention in microorganisms that cause disease in humans, livestock or crops because of the social and economic consequences. In fact, the T3SS is a crucial virulence factor in many bacteria, as highlighted by the reduced, if not abolished, virulence of bacterial mutants with impaired secretion. Table 1 gives an overview of model bacteria endowed with T3SSs.

Two classes of T3SSs have been described: the flagellar and the non-flagellar T3SS, which is also termed the injectisome. This Primer will only discuss non-flagellar T3SSs.

Structure and assembly of the injectisome
The T3SS is an approximately 3.5 MDa complex that spans the

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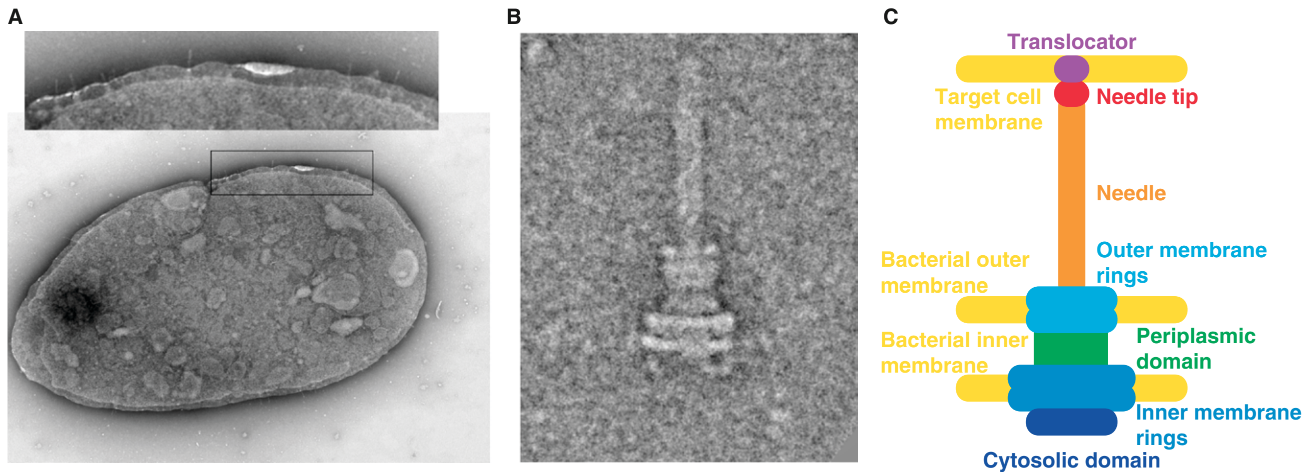

Figure 1. Appearance and organisation of the T3SS.
(A) In negatively stained, flagella-deficient *Salmonella enterica* serovar Typhimurium, membrane-embedded SPI-1 T3SSs can be observed all around the cell. The inset shows how injectisomes are embedded in the Gram-negative double membrane. (B) Purified, negatively stained SPI-1 T3SS. Electron micrographs in (A,B) are courtesy of Julia Mayr and Thomas Marlovits, IMP/IMBA Vienna, Austria. (C) Schematic representation of the T3SS and its major domains. The tip complex is not present in all species and has not been identified in T3SSs of plant-colonising bacteria, which are endowed with a pilus instead of a needle.

Gram-negative double membrane and protrudes into the extracellular space (Figure 1A,B): around 25 structural and ancillary proteins are required for its assembly. It encompasses an ATPase-containing cytoplasmic domain, membrane-integrated inner rings, a periplasm-traversing domain, membrane-integrated outer rings, a hollow filament establishing the connection between the bacterial cytosol and the target cell, and translocators forming a pore in the plasma membrane of target cells (Figure 1C). Additionally, the T3SS can possess a tip protein, which blocks secretion until host contact is established. The filament connecting the bacterial cytosol to the target cell is a pilus in microbes interacting with plants, but a needle in microbes that interact with other organisms. The outer membrane ring is related to secretins of type II secretion systems and type IV pili. After Sec- or Tat-dependent transport of secretion substrates from the bacterial cytosol to the periplasm, the type II secretion system transfers proteins across a multimeric pore complex (the secretin) to the extracellular milieu. Such a pore complex is also present in type IV pili, which can deliver proteins or DNA to target cells (similarly to T3SSs), but can also take up DNA from the extracellular milieu.

For its assembly, the T3SS exports its own periplasmic and extracellular components: secretion is hierarchical, starting with subunits that are closer to the base. Peptidoglycan-degrading enzymes assist the T3SS with its insertion into the periplasmic mesh. Needle length is determined by molecular rulers and lies within the nanometre range, whilst pili are several micrometres long to allow for penetration of the thick wall of plant cells. Furthermore, to facilitate pilus insertion the T3SS translocates enzymes termed harpins, which modify the plant cell wall.

Regulation of secretion

Secretion signal in effectors

After injectisome assembly, a substrate switch allows for the secretion of effectors, which can be delayed with respect to assembly. At the molecular level, the substrate switch is poorly understood. In most species, the injectisome is assembled long before encounter with a target cell, but in some species assembly takes place just before injection of effectors. To what extent a hierarchy of injection exists for co-expressed effectors and how it is regulated is poorly understood.

T3SS effectors do not have a specific secretion signal. Rather, the amino-acid composition of a large amino-terminal region of the effector seems crucial to the recognition of secretion substrates. Secreted hybrid proteins can be constructed by fusing the portion of the effector gene encoding the amino terminus to a gene of interest. The amino-terminal secretion domain can be swapped between effectors from distinct species, e.g. in closely related *Chlamydophila caviae* (which causes conjunctivitis in guinea pigs) and *Chlamydia trachomatis*. Strikingly, the injectisome of *Shigella* and *Yersinia* can transport heterologously expressed *Chlamydia* effectors. Similarly, *Pseudomonas syringae* and *Yersinia* effectors are translocated by the T3SS of *Dickeya dadantii* upon heterologous expression in *Escherichia coli*. These findings indicate that the mechanism of substrate recognition is conserved between distantly related species.

Translocation of effectors through the injectisome

In the bacterial cytosol, effectors are bound to cognate chaperones. The cytosolic ATPase detaches and unfolds secretion substrates, as the secretion channel is too narrow to accommodate folded proteins. It is still debated whether secretion is energised by the proton-motive force or by ATPase-dependent ATP hydrolysis (or both). Effectors are translocated through the injectisome with their amino terminus first. In *Salmonella* and *Shigella* the addition of a carboxy-terminal domain that cannot be unfolded traps secretion substrates inside the channel, plugging the injectisome.

Table 1. Overview of model bacteria endowed with T3SS(s).

Organism	Host	Outcome
Bordetella bronchiseptica	Domestic mammals	Respiratory infections
Bordetella pertussis	Humans	Whooping cough
Burkholderia pseudomallei	Mammals	Melioidosis
Chlamydia trachomatis	Humans	Urogenital infections, trachoma
Chlamydophila pneumoniae	Animals	Respiratory infections
Citrobacter rodentium	Mice	Gastrointestinal infections
Dickeya dadantii	Plants	Soft rot
Enterohaemorrhagic *Escherichia coli* (EHEC)	Humans, calves, piglets	Gastrointestinal infections
Enteropathogenic *Escherichia coli* (EPEC)	Humans, cattle, horses	Gastrointestinal infections
Pseudomonas aeruginosa	Plants, animals	Opportunistic infections, pulmonary infections in cystic fibrosis patients
Pseudomonas syringae	Plants	Bacterial speck, canker
Ralstonia solanacearum	Plants	Bacterial wilt
Rhizobium spp.	Legumes	Root nodule formation
Salmonella enterica	Animals	Gastrointestinal infections, typhus
Shigella spp.	Humans	Gastrointestinal infections
Vibrio parahaemolyticus	Humans, fish	Gastrointestinal infections
Xanthomonas campestris	Plants	Canker, black rot, bacterial spot disease
Xanthomonas oryzae	Rice	Blight
Yersinia enterocolitica	Humans, swine	Gastrointestinal infections
Yersinia pestis	Rodents, humans	Plague
Yersinia pseudotuberculosis	Humans	Gastrointestinal infections

The principal hosts and the outcome of these interactions are indicated (hosts acting as carriers only are omitted). In species that infect plants the host range is often extremely broad. The host range is restricted by pathovars in plant pathogens and serovars in animal pathogens, such as *Salmonella*, but pathovars and serovars were not detailed for the sake of simplicity.

Real-time imaging of injection using fluorescently labelled *Shigella* effectors or fluorescent-protein-tagged *Salmonella* chaperones, which were expressed in target cells to track their recruitment to the site of injection, has shown that secretion is completed within a few minutes of contact with the target cell. The speed of secretion approaches 7–60 molecules per second.

Activation of T3SS during infection

Bacteria sense environmental and host-derived cues to determine and coordinate the stage of infection with T3SS activity. Changes in temperature, pH, oxygen tension, extracellular Ca^{2+} concentration, bile salts, and contact with the host cell can positively or negatively regulate transcription of T3SS genes and initiation of secretion. For example, in *P. syringae*, expression of the T3SS is induced by certain plant metabolites (which, for instance, are absent in resistant hosts). Similarly, the presence of bile, indicating ingestion by the host, stimulates T3SS-dependent virulence in *Vibrio parahaemolyticus*. In *Shigella*, an increase in oxygen tension, reflecting the passage from the anaerobic gut lumen to the oxygenated mucosal surface, induces the expression of tip and translocator proteins, allowing secretion. The use of fluorescent reporters to monitor T3SS activity in real time at different stages of *Shigella* infection showed that secretion is active during *Shigella* entry into intestinal epithelial cells, quickly shut off in the cytoplasm, and then switched on again to invade neighbouring cells.

Some species express multiple T3SSs, e.g. *Salmonella enterica* serovar Typhimurium and *V. parahaemolyticus* have two, whereas *Burkholderia pseudomallei* has three. These different T3SSs might each be active at particular stages of infection or might each support injection into distinct hosts. Observation of the temporal and spatial *in vivo* expression pattern of *Salmonella* Typhimurium T3SSs — *Salmonella* pathogenicity island 1 and 2 (SPI-1 and SPI-2) — with a fluorescent single-cell reporter revealed that invasion-associated SPI-1 is active in the intestinal lumen in epithelium-contacting and vacuole-dwelling microbes, whilst survival-associated SPI-2 is turned on in deeper tissue layers.

Evolution and distribution of the T3SS

Genomic organisation of T3SS genes

Genes encoding the structural components of the injectisome, the regulators of T3SS expression/assembly, chaperones and secreted protein effectors generally colocalise within the genome. In pathogens, T3SS genes can be located on chromosomal pathogenicity islands — e.g. the locus of enterocyte effacement (LEE) in enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) and *Citrobacter rodentium* — or on large virulence plasmids, as is the case for *Shigella*, *Yersinia* and *Ralstonia solanacearum*.

Acquisition of T3SS genes

Many species have acquired entire operons of T3SS genes by horizontal transfer, as indicated by flanking repeat regions and the distinct G+C content. In contrast, in Chlamydiales and *R. solanaecearum* T3SS genes are very ancient and vertically transmitted, with horizontal gene transfer accounting for the acquisition of only a few effectors.

As a result of horizontal gene transfer, homologous effectors are most frequently found in related species sharing similar niches. Nevertheless, homologous effectors have also been described in distantly related species colonising distinct hosts. For example, effectors belonging to the YopJ family, which are characterised by a catalytic His–Glu–Cys triad that confers acetylation activity, were identified in *Yersinia* spp. and later reported in *V. parahaemolyticus* VopA, *Salmonella* Typhimurium AvrA, *R. solanacearum* PopP2, and *P. syringae* HopZ1 and AvrBsT.

Under evolutionary pressure, effectors can arise *de novo* by combining a desired activity with the capacity of being secreted. Two distinct mechanisms for the emergence of effectors have been proposed. ‘Terminal reassortment’ involves the addition of an existing secretion domain to a gene of interest as a result of genome rearrangements, yielding chimeric gene products. Alternatively,

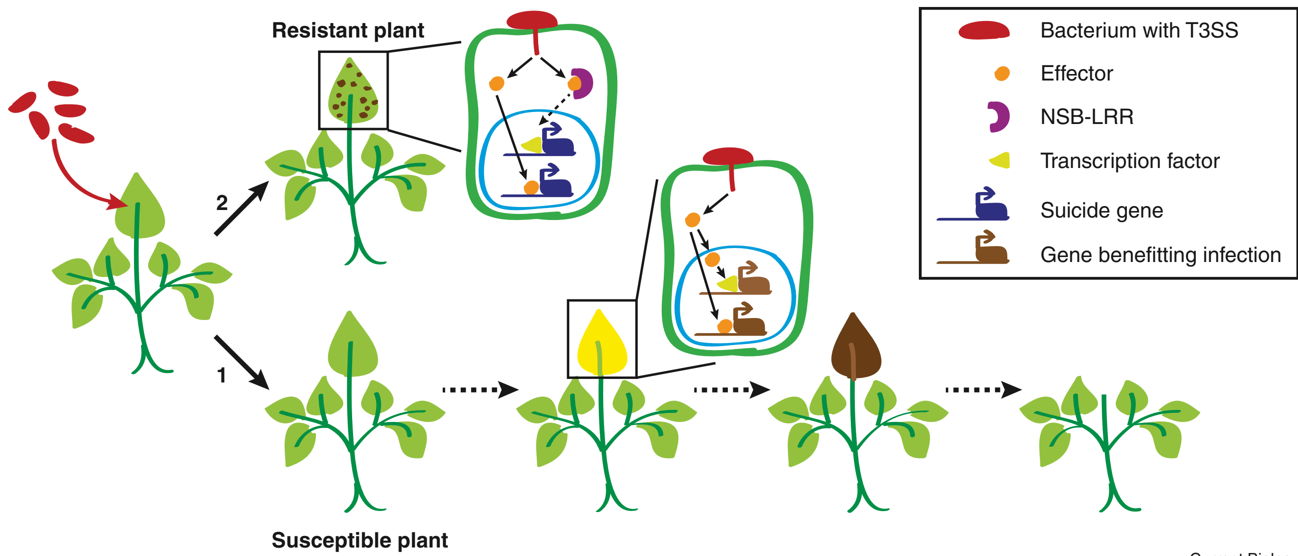

Figure 2. Immune recognition and immune response to T3SS-expressing bacteria in susceptible and resistant plants.
(1) When bacteria expressing a T3SS inject cells of susceptible plants, detection of bacterial components activates a protective immune response, promoting target cell survival. Concomitantly, effectors alter target cell physiology to the benefit of bacteria, e.g. by directly acting as transcriptional activators or by modifying host transcription factors to change gene expression patterns (lower inset). Depending on whether plant defence or bacterial virulence mechanisms prevail, infection with a pathogen can progress to disease. (2) In contrast, detection of injected effectors in resistant plants leads to rapid target cell death. Effectors can be sequestered by promoters of suicide genes or by nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins, which trigger a signalling cascade. This activates transcription of suicide genes, resulting in circumscribed necrotic lesions and prevention of bacterial spread (upper inset). Similar molecular defence and immune evasion mechanisms allow for the establishment of bacteria–plant symbiosis.

the accumulation of point mutations in the intergenic space preceding a gene of interest can lead to the development of a secretion sequence and the emergence of effectors.

Of note, mutations disrupt T3SS operons in several organisms, abolishing the expression of functional injectisomes. For example, in uropathogenic *E. coli* (UPEC) and the model laboratory *E. coli* strain K12 the T3SS is not operational, and in EHEC only one of the encoded T3SSs is active.

Identification of effector genes

Bacterial genomes encode dozens of effectors, many of which have not yet been characterised. The lack of a specific signal sequence in secretion substrates complicates the identification of effector genes. Several methods to identify effector genes have been employed *in silico*, including searches for homology to described effectors, colocalisation of genes within operons encoding known T3SS components, mapping of binding sites for T3SS transcriptional regulators in gene regulatory sequences, proximity to genes encoding T3SS chaperones, and the enrichment of certain amino acids in the amino-terminal region.

T3SS and bacteria–host relationship

The T3SS was probably devoted to interactions with protists and fungi before the emergence of plants and animals. Chlamydiales provide an excellent model to study the evolution of the T3SS in the light of the bacteria–host relationship. This order includes commensal and pathogenic obligate intracellular T3SS-bearing organisms. Genome analysis highlighted T3SSs in ancient chlamydial symbionts of protozoa, indicating that initially the T3SS was a fitness factor that has adapted to virulence only under selective pressure following encounter with new hosts.

T3SSs are found in ‘accidental’ pathogens, acting as a fitness factor in harmless relationships with their principal hosts and as a virulence factor in ‘accidental’ hosts. Accidental pathogens can exist in the environment as free-living microbes or in association with organisms that they do not harm, but occasionally they encounter accidental hosts, on whom they inflict damage: infection of accidental hosts does not usually promote the dissemination of the pathogen. For example, the leech symbiont *Aeromonas veronii* uses its T3SS to evade phagocytosis by hemocytes during colonisation of the digestive tract, where it contributes to the breakdown of blood. However, *A. veronii* is an accidental pathogen of fish and mammals, e.g. in tsunami victims or following leech therapy.

Importantly, species that are resistant to potentially pathogenic T3SS-bearing microbes can become carriers. For example, adult cattle and sheep are reservoirs of the human pathogen EHEC. Similarly, *V. parahaemolyticus* belongs to the normal flora of bivalve shellfish, but causes food-borne gastroenteritis in humans.

Host specificity of T3SS

Some bacteria use their T3SS(s) to infect very diverse hosts. Plant pathogens can have extremely broad host spectra, e.g. *Xanthomonas campestris* pathovar (pv.) *campestris* infects Brassicaceae and *R. solanacearum* targets over 200 plant species (although there is considerable variation in the effector repertoire of the various pathovars). As a result of T3SS, *P. aeruginosa* strain P14 is pathogenic to such diverse organisms as *Arabidopsis thaliana*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Mus musculus*. The closely

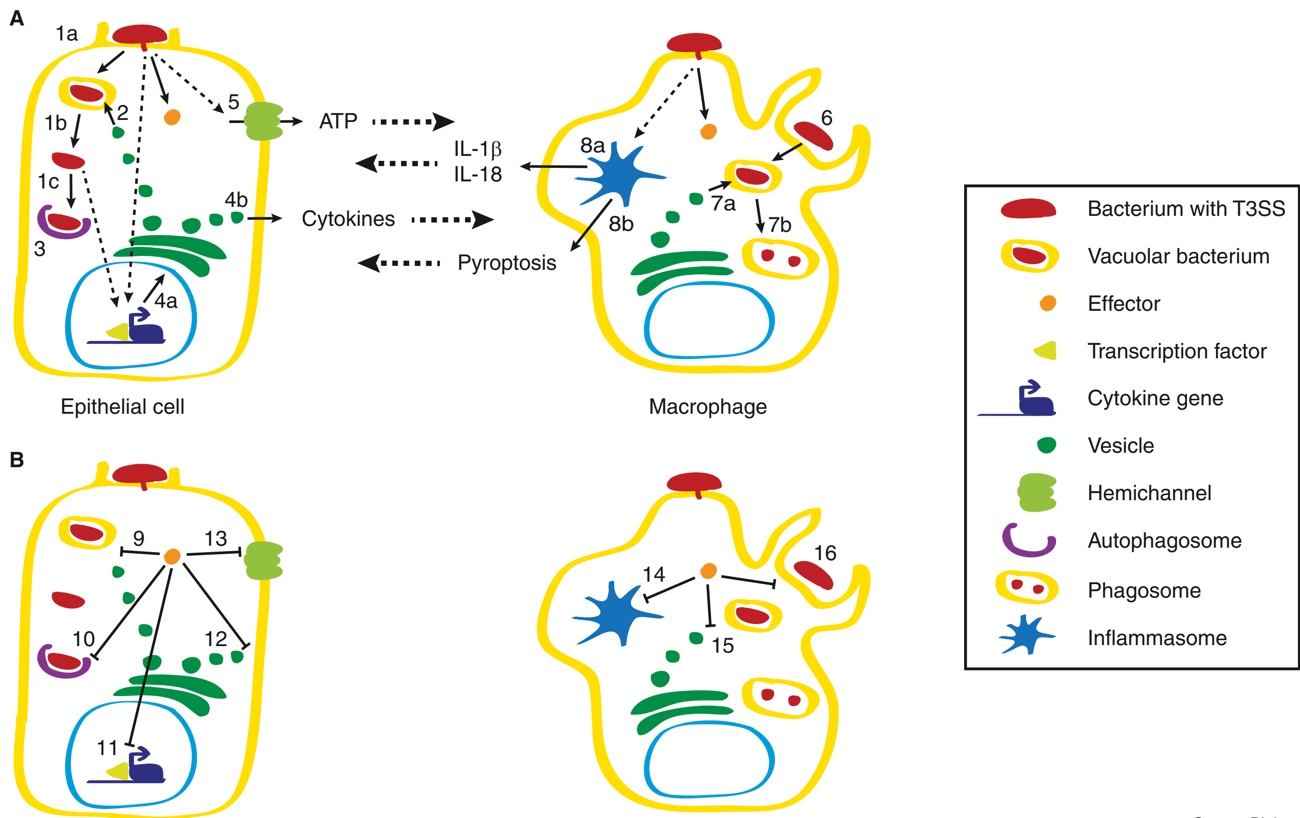

Figure 3. Immune recognition, elimination and bacterial evasion mechanisms in mammalians as exemplified in epithelial cells and macrophages infected with T3SS-expressing bacteria.

(A) Epithelial cells are frequently infected by pathogens (1a): in some cases, cellular uptake of these bacteria occurs via the T3SS. Certain invasive pathogens reside within a vacuole after entry (1b), whilst others escape into the epithelial cytosol (1c). Epithelial cells eliminate vacuolar and cytosolic bacteria by lysosomal (2) and autophagic degradation (3), respectively. In epithelial cells detection of T3SS-dependent injection or of intracellular pathogens (thin dashed arrows) activates the production of pro-inflammatory cytokines (4a,b) and secretion of the endogenous danger signal ATP (5), which alerts other host cells, e.g. macrophages (thick dashed arrows). Macrophages are immune cells specialised in phagocytosing (6) and digesting microorganisms (7a,b). Some pathogens infect macrophages as a result of the T3SS. In macrophages detection of T3SS-dependent injection (thin dashed arrows) activates the inflammasome, leading to secretion of IL-1β and IL-18 (8a) and pyroptotic cell death (8b), which alerts other host cells, e.g. epithelial cells (thick dashed arrows). (B) Bacteria interfere with the immune response in order to establish an infection and survive. For example, in infected epithelial cells T3SS effectors can block delivery of hydrolytic enzymes to bacteria-containing vacuoles (9), as well as inhibit autophagosomal degradation (10), transcription (11) and secretion of pro-inflammatory cytokines (12), and ATP secretion (13). In macrophages T3SS effectors can block inflammasome activation (14), phagolysosomal degradation (15), and phagocytosis (16).

related pathogens EHEC, EPEC and *C. rodentium* have 22 core effectors in common, but express several species-specific effectors that likely fine-tune infection mechanisms and host specificity. How bacteria use their pool of effectors and possibly multiple T3SSs to adapt to distinct hosts and lifestyles is poorly understood at the molecular level.

Immune response to injection

Injection of effectors results in transfer of bacterial products into the cytosol of target cells. Moreover, several pathogens use their T3SS to invade host cells or tissues, thereby introducing bacterial components. In animals and plants alike, detection of such bacterial components — pathogen-associated molecular patterns (PAMPs) — in normally sterile compartments triggers an immune response through cognate pattern recognition receptors (PRRs).

Immune response in plants

In plants, activation of PRRs initiates PAMP-triggered immunity (PTI). The hallmarks of PTI are callose deposition, reduced vascularisation of infected tissue, and protection against disease and cell death. Strikingly, resistant plants specifically sense T3SS activity as a result of the expression of resistance genes, resulting in protective effector-triggered immunity (ETI), also known as the hypersensitive response. ETI causes local, rapid programmed cell death to prevent the spread of microorganisms (Figure 2). ETI-eliciting effectors are often termed avirulence factors because of the resulting phenotype, i.e. absence of disease.

A major mechanism of effector detection by the plant cells depends on nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins, which bind to a range of effectors or host structures that have been modified by effectors. An ensuing conformational change in NBS-LRRs initiates ETI. Another mechanism depends on transcription activation-like (TAL) effectors, which are virulence factors

that activate transcription of host genes that are beneficial to infection. As a protective response, resistant plants have acquired binding sites for TAL effectors in the promoters of suicide genes, which results in the sequestration of TAL effectors from genes that are beneficial to infection and the upregulation of suicide genes instead (Figure 2, upper inset). For example, the *X. campestris* pv. *vesicatoria* effector AvrBs3 is recognised by alleles of the pepper resistance gene *Bs3*. Host–microbe co-evolution has created highly polymorphic repertoires of resistance genes and effector variants, which contribute to host specificity, e.g. different *R. solanacearum* effectors induce ETI in different plants — AvrA induces ETI in tobacco, PopP1 in petunia and PopP2 in *A. thaliana* ecotypes.

Immune response in animals

In animals, activation of PRRs triggers an innate immune response, culminating in inflammation, which protects the host by removing the source of danger and initiating healing, although it can inflict damage if excessive. An inflammatory reaction involves coordinated communication between several immune and non-immune cell types (Figure 3) and promotes adaptive immunity. Adaptive immune mechanisms protect against re-infection by recognising and eliminating previously encountered microorganisms.

Macrophages are essential immune cells that phagocytose intruding microbes. Macrophages sense T3SS activity upon injection, which activates the NLRC4 inflammasome, leading to the release of the cytokines interleukin 1β (IL-1β) and IL-18 and eventually resulting in pyroptosis, a form of programmed cell death that augments inflammation (Figure 3). Unlike plant cells, which detect a specific type of matching effector, the inflammasome adaptor protein NAIP interacts with a conserved component of the injectisome. Indeed, the cytosolic presence of the inner rod proteins of various bacteria, e.g. *Salmonella* Typhimurium PrgJ, *B. pseudomallei* BsaK, EHEC EprJ, EPEC EscI, *S. flexneri* MxiI and *P. aeruginosa* PscI, have all been reported to activate the inflammasome. Similarly, *Shigella* IpaB and *Salmonella* SipB, which are translocator proteins within the injectisome, induce macrophage pyroptosis.

Because the immune response can be detrimental to microbes, a vital function of the T3SS in bacteria interacting with plants and animals is to counteract immune recognition and clearance, as discussed below.

Molecular activities of effectors

At the molecular level, effectors are endowed with an impressive variety of activities (occasionally more than one activity per effector) and act upon very diverse substrates. Molecular activities frequently mimic host molecules and can be found in other prokaryotic factors (e.g. bacterial protein toxins). Generally, the T3SS reprograms target cells through modification of host components, signalling or transcriptional profiles.

Most effectors are enzymes, such as: GTPase-activating proteins (GAPs), guanine nucleotide exchange factors (GEFs), serine/threonine kinases, tyrosine phosphatases, phosphoinositide phosphatases, acetyl-transferases, N-acetyl-D-glucosamine-transferases, methyl-transferases, mono-ADP-ribosyltransferases, E3 ubiquitin ligases, deubiquitinases, SUMO proteases, cysteine proteases, zinc metalloproteases, deamidases, and transglutaminases.

Some effectors act through domains that mediate interactions with host molecules. For example, phytopathogen TAL effectors translocate into nuclei, bind to promoters via a central amino-acid repeat domain and reprogram gene expression in favour of infection (Figure 2, lower inset). *X. campestris* pv. *vesicatoria* AvrBs3 activates transcription of the *UPA20* gene in pepper, which causes host cell hypertrophy. Incidentally, TAL effectors are exploited for genome editing: TALEN restriction enzymes are obtained by fusing an engineered TAL effector chromatin-binding domain with a DNA-cleaving domain.

Function of effectors in infection

At the (patho)physiological level, the main functions of the T3SS are to establish and maintain a bacterial niche (e.g. by modulating target cell survival and immunity) and to extend the availability of nutrients. How individual effectors contribute to the development of disease or symbiosis is often not clear, although we have some understanding of their molecular activities and the phenotype of infected cells.

Host colonisation

Some plant-colonising bacteria stimulate the development of specialised organs as a dedicated niche. In petunia, *R. solanacearum* multiplies within lateral root structures that are induced by the T3SS. Legumes undergo symbiosis with rhizobia — soil prokaryotes capable of fixing nitrogen — because atmospheric nitrogen is not accessible to plants. For this association both partners actively form the root nodule, which requires the release of small organic plant molecules that induce bacterial nodulation-initiating Nod factors. Several rhizobia use their T3SS to complement Nod signalling or even to bypass the absence of Nod factors. In fact, a comparison of transcriptional profiles in the symbiosis between soybean and *Bradyrhizobium elkanii* strain USDA61 revealed that Nod factors and T3SS control a partially overlapping set of genes during nodulation.

Pathogens often gain access to deep tissue layers. Several EPEC, EHEC and *Salmonella* effectors, as well as *Y. enterocolitica* YopE, disrupt tight junctions in the intestinal epithelium to invade the underlying mucosa. Upon infection of intestinal epithelial cells, *Salmonella* SopB stimulates paracrine transdifferentiation of neighbouring epithelial cells into M cells, which are specialised for transcytosis of antigen through the cell from the gut lumen and are the preferred port of entry for *Salmonella*.

EPEC, EHEC and *C. rodentium* use the T3SS to attach to the apical side of intestinal epithelial cells (Figure 3A). For example, the EPEC-translocated intimin receptor (Tir), which inserts into the plasma membrane after T3SS injection, contains a domain that recruits Arp2/3. The consequent induction of actin polymerisation by Arp2/3 facilitates attachment of EPEC to the target cell after binding of Tir to intimin on the bacterial surface. *Shigella*, *Salmonella*, *Chlamydia* and, at a low frequency, *V. parahaemolyticus* use the T3SS to

enter normally non-phagocytic cells, such as epithelial cells (Figure 3A). Invasion generally requires a cascade of effectors, which stimulates rearrangements of the actin cytoskeleton and plasma membrane to engulf and internalise bacteria.

After cellular uptake, *Shigella* requires the T3SS to gain access to and move in the cytosol. In contrast, *Salmonella* and *Chlamydia* remain inside vacuoles (Figure 3A) and must therefore protect their niche from becoming a lysosome and must also enlarge the vacuole with new membrane to allow for bacterial replication. Both pathogens divert T3SS-mediated vesicular trafficking to prevent the delivery of hydrolytic enzymes (Figure 3B) and to recruit membranes to allow for enlargement of the vacuole. For example, the *Salmonella* SPI-2 effector SifA sequesters the small GTPase Rab9: this interferes with mannose-6-phosphate receptor-dependent retrograde transport, which alters the sorting of lysosomal enzymes and thereby lysosome maturation. *Salmonella* also infects macrophages and replicates within phagolysosomes by using SPI-2 to divert enzymes that produce reactive oxygen and nitrogen species (Figure 3B).

Several pathogens support the survival of infected cells and deploy effectors to repress apoptotic, pyroptotic or necrotic cell death to maintain their niche and prolong bacterial growth. For example, *X. campestris* XopD regulates the transcription of senescence genes in tomato by targeting a transcription factor (Figure 2). *B. pseudomallei*, which thrives inside macrophages, uses one of its T3SSs to modulate inflammasome activation and pyroptosis (Figure 3B). Likewise, EPEC and EHEC NleH blocks apoptosis in infected epithelial cells.

Immune evasion in plants

In plants, several effectors suppress PTI and ETI to delay the onset of symptoms and enhance bacterial multiplication (Figure 2). Other targets for T3SS-mediated immune avoidance are the protective hormones salicylic acid and jasmonic acid, which are synthesised in a mutually exclusive manner. Because *P. syringae* is only sensitive to salicylic-acid-dependent immunity, it normally uses the toxin coronatine (a jasmonate mimic) to antagonise salicylic acid production. Strains lacking coronatine inject the effector HopX1, a cysteine protease that inactivates the transcriptional repressor of jasmonate-responsive genes.

However, immune avoidance is necessary to establish not only disease, but also symbiosis. The association of rhizobia and legumes is highly specific. In a somewhat similar manner to the interaction between pathogens and resistant hosts, detection of rhizobial effectors in plants carrying resistance genes restricts nodulation. Hence, rhizobia actively escape immune recognition upon infection of matching hosts.

Immune evasion in animals

In animals, T3SS-mediated immune evasion is extremely multifaceted. Inhibition of ‘alert’ responses in infected cells to delay immune recognition is a common strategy (Figure 3B). Several *Yersinia* effectors and *P. aeruginosa* ExoU dampen inflammasome activation and the ensuing cytokine release. Besides the Arp2/3-binding domain, EPEC Tir contains an immunoreceptor tyrosine-based inhibition motif (ITIM), which blocks PRR-dependent signalling and thereby cytokine production in epithelial cells. Many *Salmonella*, *Shigella*, EPEC, EHEC, and *C. rodentium* effectors interfere with NF-κB and MAP kinase signalling, the master regulators of pro-inflammatory cytokines. Furthermore, upon infection with *S. flexneri*, *S. enterica* or EPEC, intestinal epithelial cells release the endogenous danger signal ATP across connexin hemichannels, a swift alert response mediating strong inflammation upstream of cytokine production. *Shigella* escapes this reaction by injecting IpgD, which hydrolyses phosphatidylinositol (4,5)-bisphosphate to phosphatidylinositol 5-phosphate, thereby closing the hemichannels and stopping ATP release.

After pathogens have been detected, the T3SS helps them to escape elimination mostly by targeting innate immunity, but interference with adaptive immune mechanisms has also been described. When pathogens are confronted with immune effector cells, T3SS is used to block or kill the latter (Figure 3B). For example, several *Yersinia*, EPEC and *P. aeruginosa* effectors inhibit phagocytosis. During experimental *in vivo* infections *Y. pestis* predominantly injects macrophages, neutrophils and dendritic cells and, to a lesser extent, T and B cells. *S. flexneri* blocks migration in activated T cells in an IpgD-dependent manner and kills B cells via the translocator protein IpaD, which might contribute to the poor adaptive immune response and frequent re-infections observed in patients.

Intracellular cytosol-dwelling pathogens have to face autophagy, a process that contains intruding microbes within membranous compartments that are subject to lysosomal degradation (Figure 3). To stop autophagosome assembly mechanisms, the GAP activity of *Shigella* VirA inactivates the Rab1 GTPase, whilst *Shigella* IcsB (and possibly the *B. pseudomallei* homologue BopA) prevents the bacterial surface protein that triggers autophagy, VirG, from binding to the autophagy protein Atg5.

Acquisition of nutrients

Nutrients are not freely available: they are out of reach, stored within a cell, for example, and therefore not available to extracellular bacteria, or found in the host cell cytoplasm and therefore not available to vacuole-dwelling bacteria. Because of this segregation of nutrients, bacteria use the T3SS to increase nutrient availability. For example, in rice the *X. oryzae* pv. *oryzae* effector PthXo1 stimulates sugar efflux from host cells by activating transcription of *OsSWEET11*, which encodes a glucose transporter.

Intracellular vacuole-dwelling pathogens quickly consume nutrients enclosed in their compartment and do not have access to cytosolic substances. Hence, *Chlamydia* and *Salmonella* use the T3SS to alter vesicular trafficking to acquire nourishment. For example, *Salmonella* SseF and SseG stimulate the formation of filaments that extend from the vacuole to the plasma membrane to capture Rab9/Rab11-containing, nutrient-transporting vesicles.

Perspectives

The T3SS has proven to be a crucial instrument for manipulating host cells at all stages of infection. Even though T3SS injectisomes and effectors are major virulence factors of many

pathogens, so far they have been insufficiently exploited as targets of antibacterial therapy. Nevertheless, inhibitors of secretion and molecules blocking the expression of T3SS genes have been identified *in vitro*. Furthermore, vaccines comprising injectisome-derived antigens are under evaluation. Unlike antibiotics, T3SS-targeting anti-virulence drugs do not put pressure for survival on microorganisms (as bacteria are viable without T3SS) and are potentially more specific, which reduces the risk of widespread resistance. However, since our knowledge of the T3SS in symbiotic, commensal and environmental prokaryotes is poor, the possible impact of T3SS-targeting drugs on microbial communities is difficult to evaluate.

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Fish choose appropriately when and with whom to collaborate

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Collaborative abilities are integral to human society [1] and their evolutionary origins are of great interest. Chimpanzees are capable of determining appropriately when and with whom to collaborate in a rope-pull experiment [2] — the only non-human species known to possess both abilities. Chimpanzees are thought to share these abilities with humans as a result of common ancestry [2]. Here, we show that a fish — the coral trout *Plectropomus leopardus* — has partner-choice abilities comparable to those of chimpanzees in the context of its collaborative hunting relationship with moray eels [3]. Using experiments analogous to those performed on chimpanzees [2], but modified to be ecologically relevant to trout, we showed that trout recruit a moray collaborator more often when the situation requires it and quickly learn to choose the more effective individual collaborator. Thus, these collaborative abilities are not specific to apes and may be more closely linked to ecological need [4] than brain size or relatedness to humans.

Humans frequently decide on whether and with whom to collaborate [1]. Of the collaborative abilities experimentally demonstrated in chimpanzees, amongst the most sophisticated are the abilities to choose appropriately when and with whom to collaborate. In a collaborative rope-pulling paradigm chimpanzees recruit a partner more often when collaboration is necessary to retrieve a baited food platform than when it can be retrieved alone. They also choose the more effective of two collaborative partners after a few trials [2]. The abilities of chimpanzees are consistent with a close link between a species' ecology and its cognitive abilities [4]. Chimpanzees hunt in groups more often in dense forest where solo hunting may be less effective, and make alliances for territorial defense potentially based on previous experience with various partners [2]. Other large-brained species have since been tested for their ability to determine when cooperation is possible (albeit without the necessity to recruit a partner), with elephants succeeding [5] and rooks failing [6]. Here, we test for the abilities to determine when and with whom to collaborate in a species for which these abilities should be ecologically relevant.

We studied the coral trout (hereafter 'trout'): a fish of the genus *Plectropomus*, which use gestural communication to initiate collaborative hunts with moray eels on coral reefs [3, 7]. This relationship relies on naturally complementary hunting tactics, that, when combined, reduce the prey's escape options and benefit both partners. Plectropomids are fast to chase prey fish above the reef, while morays have a sinuous body to access prey hidden in crevices. Therefore, collaboration with a moray should only be useful to the plectropomid if the prey is inaccessible in a crevice. Regarding partner-choice, field observations suggest that individual morays differ consistently in their willingness to collaborate, and trout should benefit from preferentially recruiting these individuals.

To determine if trout can determine when to collaborate, we presented them with a situation (experiment 1) where prey was either in a crevice (collaborative condition) or in the open (solo condition; Figure 1A). The correct choices (respectively) were to recruit a nearby model moray that would flush the prey out into the open or attack alone, after which the trout was fed a reward to simulate a successful hunt (Supplemental information; Movie S1). For both experiments, the number of trials and subjects was designed to match that for chimpanzees [2]. In experiment 1, eight trout participated in up to four trials per condition per day for six days (one testing period per day), with solo and collaborative trials alternated within testing periods. Chimpanzees undertook the same maximum number of trials (48 total) but these were divided between two sessions of unspecified duration [2], making days 1–3 and 4–6 for trout equivalent to sessions 1 and 2 for chimpanzees.

Trout were similarly proficient to chimpanzees at determining when to collaborate. Trout recruited the moray significantly more often in the