THE ROLE OF SECRETION SYSTEMS AND SMALL MOLECULES IN SOFT-ROT *ENTEROBACTERIACEAE* PATHOGENICITY

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■ **Abstract** Soft-rot *Enterobacteriaceae* (SRE), which includes the genera *Pectobacterium* and *Dickeya*, consist mainly of broad host-range pathogens that cause wilt, rot, and blackleg diseases on a wide range of angiosperm plants. They are found in plants, insects, soil, and water in agricultural regions worldwide. SRE encode all six known protein secretion systems present in gram-negative bacteria, and these systems are involved in attacking host plants and competing bacteria. They also produce and detect multiple types of small molecules to coordinate pathogenesis, modify the plant environment, attack competing microbes, and perhaps to attract insect vectors. This review integrates new information about the role protein secretion and detection and production of ions and small molecules play in soft-rot pathogenicity.

KEYWORDS

Type II secretion system
Type III secretion system
Plant cell wall degrading enzymes
Pectobacterium
Dickeya
Quorum-sensing

INTRODUCTION

Soft-rot *Enterobacteriaceae* (SRE) belong primarily to the genera *Pectobacterium* and *Dickeya*, which consist mainly of broad host-range pathogens that cause wilt, rot, and

blackleg diseases of plants. The SRE are found in agricultural regions worldwide and have been isolated from plants in over half of angiosperm families as well as from soil, clouds, sea water, fresh surface water, ground water, insects, and mollusks (96, 124). They are prolific gene exchangers (80) and, perhaps because of this, detangling the phylogeny of this group has proved difficult and remains contentious. SRE encode all six known protein secretion systems present in gram-negative bacteria, and these systems are involved in attacking host plants and competing bacteria. They also produce and detect multiple types of small molecules to coordinate pathogenesis, modify the plant environment, attack competing microbes, and perhaps to attract insect vectors. As is common for pathogens, a complex network of regulators,which allow the bacteria to sense small molecule signals produced by bacteria or plants, controls the expression of virulence genes in SRE (18, 88)). Despite knowledge on SRE generated over the past 100 years (70, 71), control methods have changed little in recent decades. This review highlights some of our more recent knowledge on SRE and focuses on the roles of small molecules and ions in the ecology and pathogenesis of development of the disease. For this review, we define small molecules as compounds that are produced and detected by bacteria or plants and that are not polymers.

SOFT-ROT *ENTEROBACTERIACEAE* GENOMICS AND PHYLOGENETICS

Complete or draft genome sequences are now available for numerous SRE phytopathogens, including five *Pectobacterium* and four *Dickeya* (11, 46, 47) , with many more in draft formats. A typical member of this group has a single circular chromosome of just under 5 Mb and no large plasmids, although small plasmids may be present. The genomic backbone remains conserved across these genera, allowing complete genome sequences to be used to different extents as scaffolds for draft genome sequences (46). The majority of the variation among these genomes occurs in horizontally acquired islands (HAIs), where multiple forms of horizontal gene transfer have inserted into the bacterial chromosome. These include genes related to bacteriophages, insertion elements, and plasmid fragments, which often carry **this should have been deleted** genes involved in virulence and plant association, as well as those genes of unknown function. Several of these HAIs encode biosynthetic pathways for small molecules important for virulence.

Comparison of these genomes using OrthoMCL revealed a total of 2,307 sets of orthologous genes conserved among all of these phytopathogens. There are an additional 134 sets found in all *Pectobacterium* and 144 sets found in all *Dickeya* isolates sequenced to date. Each genome also includes a substantial organism-specific repertoire (11, 46, 47). This was highlighted in the work of Pritchard et al. (128) , who showed that HAIs within SRE genomes are highly variable between closely related species and that these HAI exhibit major differences in their gene contents that are related to environmental survival and plant disease. When the genome sequence of *Pectobacterium atrosepticum* strain 1043 was compared with other plant-associated bacteria and animal pathogenic enterobacteria, using a comparative visualization tool called GenomeDiagram,**[**AU: AR House Style**]** its genome was shown to be similar in size to the animal-infecting enterobacteria and to share a backbone of common enterobacterial genes, with numerous common regulators that appear to have been redirected for the control of genes associated with disease on plants (129). In addition, many of the HAIs within its genome, including a type III secretion system (T3SS), phytotoxins, plant cell wall--degrading enzymes (PCWDE), and adhesions shown to be involved in the plant association and disease, may have been transferred from other plant-associated bacteria.

The complexity of SRE phylogenetics is reflected in the many nomenclature changes these species have undergone. Recently, *Erwinia carotovora* was divided into multiple *Pectobacterium* species and *Erwinia chrysanthemi* was divided into multiple *Dickeya* species (56, 140) . Similarly, an orchid-infecting species, *Pectobacterium cypripedii*, previously included within *Pectobacterium*, was recently transferred to the related genus *Pantoea* (13). Both *Dickeya* and *Pectobacterium* encompass diverse taxa (76, 96), and multiple SRE taxa may be present in a field or even an individual plant (76, 147, 162), which adds complexity to understanding how soft-rot disease progresses under natural conditions.

New *Pectobacterium* and *Dickeya* species certainly remain to be described. For example, a *Pectobacterium* clade that was recently isolated from monocot hosts may represent a new species, as may *Dickeya* species (including that tentatively described as *Dickeya solani*) that are causing considerable economic losses to potato production in Europe (96, 145, 165).

SOFT-ROT *ENTEROBACTERIACEAE* ECOLOGY

We know much about how SRE interact with their plant hosts during disease, but considering that SRE are widespread in nature and that soft-rot disease is rare under natural conditions, we understand little about how the SRE spend the majority of their life in the environment. Along with the clear association that SRE have with a wide range of plants (96), SRE are also found in water, soil, and invertebrates (123, 124) . They have also been identified on leaf surfaces, possibly via the vascular system, in bacterial splash from the soil or neighboring plants, in rain water, and in insects, although they lack the pigments typically produced by leaf-dwelling microbes (123, 124). Although these organisms may behave as leaf epiphytes, they are also able to macerate leaves. Several features of ecological interactions of *Pectobacterium* and *Dickeya* with plants remain unknown. For example, there are suggestions that nematode feeding wounds on roots, the relative ability of *Pectobacterium* and *Dickeya* species to survive in different soil types, and shedding of SRE from plant roots all impact SRE ecology, but much work remains in these areas. In addition, very little is known about genes required for spread and persistence in the environment, or competition or cooperation with other microbes. Most of the information on SRE concerns their interaction with plant hosts during disease development, and surprisingly little is known about how SRE spend the majority of their life in the environment.

INSECTS AS VECTORS AND ALTERNATE HOSTS

Insects have long been suspected of transmitting members of **SRE** (83, 84), but association of these bacteria with insects as vectors or alternate hosts has not been studied in detail. *Pectobacterium* has been found in dipterous insects collected from potato and lettuce fields, potato cull piles, dumps, and settling ponds but not in insects of other orders (79). Adult *Drosophila melanogaster* artificially infected with *Pectobacterium* can transmit the bacteria to injured potato stems, and transmission by insects of *Pectobacterium* from rotting tubers to artificially injured field plants has been described (78). The recent widespread use of insect resistance transgenic corn has essentially eliminated stalk rot caused by SRE in maize (31), supporting the role of insects in transmission of SRE.

Recent data suggest that the association of SRE with insects is more than an occasional and temporary step in the life cycle of bacteria. *Pectobacterium* and *Dickeya,* like many bacteria, encode the butanediol pathway, which results in the production of the potent insect attractant acetoin, suggesting that these bacteria may attract insect vectors to infected plant material through this route (37, 100). Once associated with an insect, some isolates of *Pectobacterium carotovorum* can infect and persist in *D. melanogaster* and activate an immune response (8, 9). The protein Evf (*Erwinia* virulence factor), present only in insect-associated strains, promotes the persistence of bacteria in the insect midgut. Evf synthesis is regulated by SlyA (Hor), which also regulates plant virulence genes (1, 9). As yet, no Evf-containing *Pectobacterium* genomes have been sequenced, suggesting that different, as yet unknown, genes may play a role in insect-infection by *Pectobacterium*.

The genome of *Dickeya dadantii* contains four *cyt* genes, which are not present in *Pectobacterium*, that encode proteins homologous to *Bacillus thuringiensis* Cyt toxins. *D. dadantii* has a limited host range in insects, effectively killing only a small number of species, including the pea aphid *Acyrthosiphon pisum* (24, 50). Infection of this highly susceptible host insect may help in dispersion becauase dead aphids can contain up to 10^7 CFU. However, infection of tolerant hosts may be more important for *Dickeya* survival and spread. Cyt toxins are not required to kill aphids, although their deletion significantly delays insect death. The exact mechanism for this is unclear but, because the reduced virulence of a deletion mutant is visible in ingestion tests and not in injection tests, it may act in a similar way to the *B. thuringiensis* toxins (14), which puncture holes in gut epithelial cells and allow sepsis. Bacterial virulence in aphids is controlled by the same global regulators as plant virulence genes. For example, the regulators H-NS, PecS, and VfmE control the Cyt genes but in an opposite way compared with virulence genes required for bacterial attack on plants (25). Mutations in other global regulators of virulence on plants that do not directly control *cyt* gene expression, e.g., GacA, OmpR, PhoP, also result in reduced virulence in insects, suggesting that other *D. dadantii* factors involved in the attack on insects are integrated into the global network that controls virulence (25).

Together, this information suggests that integrated pest management used for insect control may also aid in control of soft-rot diseases. Only recently have we appreciated that numerous bacterial plant pathogens are vectored by insects (110), and this suggests that the

search for methods that interfere with insect attraction to diseased plants or bacterial uptake will be fruitful in the search for bacterial disease-control strategies.

SOFT-ROT *ENTEROBACTERIACEAE* PROTEIN SECRETION SYSTEMS

Few microbes digest their hosts more dramatically than the soft-rot pathogens, which can reduce a mountain of vegetables stored in a warehouse to slime in just a few weeks. Protein secretion is key to virulence in soft rot pathogens and SRE encode all six of the known protein secretion systems found in the *Enterobacteriaceae* (11, 46, 47). Of these secretion systems, types I, II, and III play significant roles in the pathogenicity of multiple species of Pectobacteriun and Dickeya . The type IV secretion syste is sporadically distributed within the SRE and its role remains cryptic. Its role in SRE pathogenicity is untested, except in *P. atrosepticum,* where it makes a small contribution to virulence (11), and secreted proteins remain unidentified. The type V (two partner) system and type VI system act in adherence and/or competition with other microbes.

Type I Secretion System: Metalloproteases and Adhesins

The type I secretion system (T1SS) consists of only three proteins, and multiple T1SSs are encoded by SRE genomes. Metalloproteases secreted through the Prt T1SS contribute to SRE virulence (59). In *Pectobacterium*, the T1SS is upregulated by plant extracts and acyl-homoserine lactone (AHL), and controlled by GacAS (97, 98). In *Dickeya*, this system is controlled by PecS (59, 104) cand GacAS (85). Metalloproteases may play two roles in virulence; they may attack plant cell wall proteins or may degrade enzymes secreted by the pathogen to affect their activity. Plant cell wall targets remain unknown, but *Dickeya* proteases are known to cleave the N-terminal residues of PelI pectate lyase after its secretion. The resulting protein is not greatly modified in activity, but it is smaller, more basic, and the modified form acts as a defense elicitor in plants (144). Recently, Pérez-Mendoza et al. (122) showed that a second T1SS, which is regulated by a diguanylate cyclase and secretes a multi-repeat adhesin, is also important for *Pectobacterium* virulence.

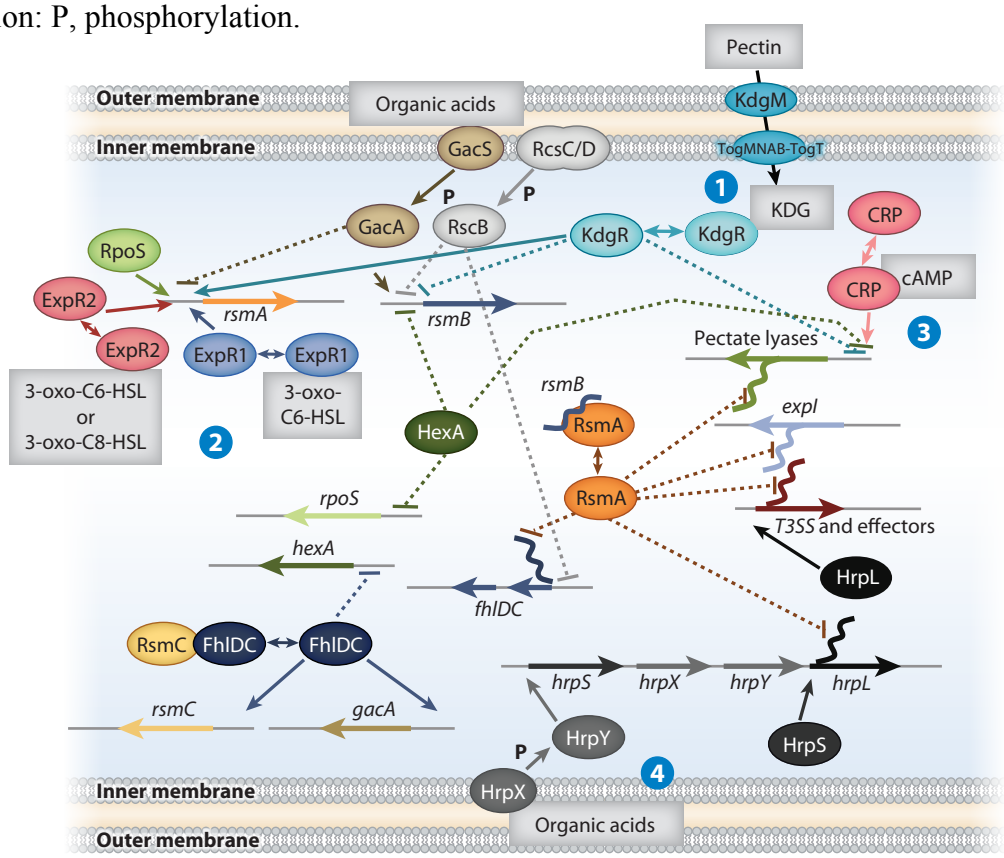
AHL: acyl-homoserine lactone

Type II Secretion System: Digestion of Plant Cell Walls

Most SRE **PCWDE** involved in pathogenesis are secreted through the type II secretion system (T2SS), which is also known as the Out system. The plant cell wall is a complex and dynamic mesh of polymers, and digestion of plant cell walls by SRE causes the rotting symptoms characteristic of these pathogens. T2SS are found in many pathogenic gram-negative bacteria and allow secretion through the outer medium of proteins, following translocation into the periplasm by the Sec or Tat translocons (67). The T2SS are composed of 12 core proteins: C, E, F, L, and M constitute a platform in the inner membrane. The secretin D forms a pore in the outer membrane. The pseudopilins G, H, I, J, and K, processed by the prepilin peptidase O, form a short pilus in the periplasm. Accessory proteins are present in some strains. Expression of the T2SS and the enzymes it secretes are controlled by small molecules produced by both bacterial cells and plant host cells, such as pectic fragments, plant organic acids, and AHL (**Figure 1**). A subset of the proteins making up the T2SS machinery also contributes to iron homeostasis, thereby controlling its acquisition, which is critical for SRE cell function (36). This observation leads to interesting and as yet unanswered questions about evolution of the T2SS and iron acquisition systems.

Unfortunately, the secretion signal targeting proteins to the T2SS has remained elusive despite decades of research, although some clues as to its nature are available. Despite our lack of understanding of the secretion signal(s), the T2SS and its secreted proteins have been thoroughly studied because of their role in pathogenesis. Type II--secreted proteins have been identified during individual enzyme studies and enzyme or global secretome analyses of *D. dadantii* or *P. atrosepticum* (26, 73). Multiple pectinases are present in the supernatant of *Dickeya* and *Pectobacterium*, even when bacteria are grown in rich media, although some T2SS-secreted proteins are found in the supernatant only in the presence of specific inducers. For example, the rhamnogalacturonate lyase RhiE is secreted when bacteria are grown in the presence of rhamnose (81), and the feruloyl esterase FaeD is secreted in the presence of ferulic acid (55). Other proteins, such as Svx (also called AvrL), are constitutively secreted (73). Svx is homologous to a large family of little-characterized proteins from gram-negative and gram-positive bacteria and fungi. One Svx family member

Figure 1: Pectobacterium. Integration of small-molecule signals into regulation of exoprotein production. Regulatory proteins are shown as ovals. Upregulation is indicated by an arrow and downregulation by a dashed line. mRNA is shown as a curved line. Small molecules are indicated by labeled gray boxes. 1. Pectin is catabolized and 2-keto-3-deoxygluconate (KDG) is produced: KdgR repression of pectate lyases and rsmB is released, whereas rsmA is no longer activated, resulting in exoprotein expression. 2. AHL is detected: ExpR binding of AHL (3-oxo-C6-HSL or 3-oxo-C8-HSL) ends ExpR-mediated induction of rsmA, resulting in exoprotein production. 3.3 Glucose levels are low: The cAMP-CRP complex activates pectate lyase production. 4. Organic acids are detected: GacAS represses rsmA and induces rsmB. The HrpXY two-component system ma also detect organic acids, activating the HrpXY-HrpS-HrpL cascade and inducing T3SS genes. Expression tied to the motility regulator FhlDC: gacA and rsmC are induced, whereas hexA is repressed. This results in repression of hexA and rsmA and induction of rsmB, which is followed by expression of exoproteins. This suggests that exoproteins are secreted by motile cells.Whether motility and the T3SS are co-regulated remains unknown. Control by RscBCD: This system represses rsmB, thereby reducing exoprotein production. Abbreviation: P, phosphorylation.



encoded by *Xanthomonas campestris* confers an avirulence phenotype during interaction with *Arabidopsis thaliana* (23).

A second T2SS, called Stt, is present in *D. dadantii* 3937 and *Dickeya* sp. 1591 (41). Despite extensive mutagenesis of *D. dadantii* and a search for pectinases that spans decades, this gene cluster was not found until the genome of *D. dadantii* 3937 was sequenced. The Stt system secretes the pectin lyase homolog PnlH. PnlH is not found in the external medium but anchored at the outer face of the outer membrane. It has a Tat sequence signal and crosses the inner membrane via the Tat translocon. However, this sequence signal is not cleaved and anchors the protein in the outer membrane.

Type III Secretion System: Disease Through Elicitation of Plant Cell Death?

The type III secretion system (T3SS) has been more closely examined in hemibiotrophic phytopathogenic bacteria, such as *Pseudomonas syringae*, than SRE, but is required for pathogenesis in both bacterial groups. Unlike *P. syringae* which can have up to 30 potential type III--secreted effector proteins in individual strains, SRE appear to have relatively few (77), including a small number of harpins or helper proteins and the single known effector, DspA/E. The DspE allele in SRE is smaller than homologs found in other phytopathogenic bacteria, such as *Erwinia amylovora*, and unlike other DspE alleles, it is unable to inhibit callose formation in leaves (77). Deletion of the T3SS from *Pectobacterium* or *Dickeya* has only subtle effects on virulence in some pathosystems (58, 157). An exception to this occurs when *Pectobacterium* is infiltrated into the leaves of solanaceous plants, where it causes a cell death response that is dependent upon DspE. This plant cell death can progress to disease, suggesting that elicitation of programmed cell death in plant leaves promotes virulence of this necrotrophic pathogen (77). Similar phenomena have been seen with other necrotrophs (48, 146, 152).

Strains from both *Pectobacterium* and *Dickeya* lacking a T3SS have been isolated, suggesting that the T3SS is not required for survival (76, 126). *Pectobacterium* strains naturally lacking a T3SS are not virulent on leaves, although they can macerate potato stems and tubers. Together with the strong phenotype on leaves resulting from a T3SS deletion, these data suggest that the T3SS extends the tissue type that *Pectobacterium* can initially infect leaves. Whether SRE naturally lacking a T3SS use other genes to compensate during attack of plant stems or tubers remains unknown. Genomic tools have allowed us to better

differentiate among SRE strains than we could in the past, and an emerging theme is that although the genera *Dickeya* and *Pectobacterium* have wide host ranges, strains within each genus are likely to be more fit on particular species or tissues.

The Flagellar Type III Secretion System: Attraction to and Repulsion from Small Molecules

SRE are motile via peritrichous flagella, and the flagellar apparatus is categorized as a subtype of the T3SS. Unlike the model bacterial plant pathogens, *P. syringae*, SRE are motile during infection (99), but whether they secrete proteases or plant cell wall--degrading enzymes while motile remains unknown. Motility itself, but not the presence of flagella, is required for the virulence of some *Dickeya* and *Pectobacterium*, suggesting that the agitation provided by the motile cells may assist in disease development (60). The flagellar secretion system is also tied to microbe-microbe interactions; it is the secretion system used to deliver colicin, a microbial toxin that kills closely related bacterial species (17). Flagellar and virulence gene regulation is closely tied together through the action of the master regulatory FhlDC. Other than these data, and what can be inferred from experiments with related genera, little is known about regulation of motility and chemotaxis in SRE.

One of the most striking differences between plant pathogens and closely related animal pathogens is the relatively high number of methyl-accepting chemotaxis proteins (MCP) in the plant pathogens. Both SRE genera encode flagellar genes homologous to those found in many other *Enterobacteriaceae* and, as in *Yersinia*, nearly all of SRE flagellar genes are encoded in one locus (65). Both genera encode more than 30 MCP and multiple aerotaxis (Aer)-like proteins. In comparison, *Escherichia coli* typically encodes only five MCP and one Aer protein. The high number of MCP suggests that SRE must contend with and can thrive in a fluctuating environment, which is consistent with their widespread presence. It also suggests that detection of small molecules helps SRE navigate these complex and dynamic environments.

Chemotaxis enables bacterial cells to move toward certain stimuli and away from others via sensing by MCP arrays located on the bacterial membrane (95). These MCP are present as trimers of dimers, with up to three different MCP present in each complex. Considering that more than 30 MCP are encoded by each SRE cell, more than 27,000 combinations are possible. However, not all 30 may be able to complex with each other, and all of the MCP

are unlikely to be produced at the same time and in equal amounts. Once a signal is sensed, a signal transduction pathway communicates with the flagellar motor to alter swimming behavior.

The role of chemotaxis in the pathogenicity of *D. dadantii* has been studied by systematic mutation of chemotactic signal transduction system genes and a flagellar motor gene (3). The swimming ability of the mutant strains was reduced in distance with respect to the wild type: motA (94%), cheY (80%), cheW (74%), cheB (54%), and cheZ (48%). All these mutants showed a significant decrease of virulence in multiple plant hosts, but the degree of virulence reduction varied depending on the virulence assay. The ability to penetrate *Arabidopsis* leaves was impaired in all the mutants, whereas the capacity to colonize potato tubers after artificial inoculation was affected in only two mutant strains. In general, the virulence of the mutants could be ranked as *motA>cheY>cheB>cheW>cheZ*, which correlated with the degree to which swimming was affected. These results clearly indicate that chemotaxis and motility play an important role in the pathogenicity of this bacterium (3).

Bacterial entry is a critical question in plant pathology because phytopathogenic bacteria lack specific structures to force entry into plants and must therefore enter through natural openings, such as stomata, lenticels, orwounds. Jasmonic acid is a key signaling compound in plant defense, and it is produced by wounded tissue. Antúnez-Lamas et al. (4) hypothesized that bacterial chemotaxis toward jasmonic acid may enable the bacterial cells to move toward plant wounds. *D. dadantii* 3937 has a strong chemotactic response toward jasmonic acid during in vitro assays, unlike the related *Escherichia coli* or the plant pathogen *P. syringae* (4). This suggests that jasmonic acid plays a dual role in pathogenesis, acting as both a plant defense signal and a bacterial attractant. Furthermore, jasmonic acid induced the expression of bacterial genes possibly involved in virulence and survival in the plant apoplast, and bacterial cells pretreated with jasmonic acid showed increased virulence in chicory and *Saintpaulia* leaves. The *A. thaliana aos1* mutant, which has reduced jasmonate production is more resistant to bacterial invasion by *D. dadantii* 3937, but once the bacteria have invaded, jasmonate mutants are increased in susceptibility to SRE (39).

Flagella of many plant pathogenic bacteria elicit defenses in plant cells (22, 40, 57). When FliC preparations of *D. dadantii* and *P. carotovorum* subsp. *carotovorum* are

infiltrated into the leaves of tobacco, a hypersensitive response appears 24 hours after infiltration. Both flagellins contain motifs similar to Flg22, the flagellin region implicated in plant recognition. However, when tobacco BY2 suspension cultures are treated with these two flagellins, only $FliC_{Pcc}$ caused cell death, and a synthesized $Flg22_{Pcc}$ peptide elicited an oxidative burst, but the $Flg22_{Dd}$ peptide did not. Experiments with a deletion series of $FliC_{Pcc}$ and synthesized peptides showed cell death was elicited not only by the Flg22 region, but also by flagellin residues 51--70. Although both $FliC_{Pcc}$ and $FliC_{Dd}$ are glycosylated to different extents, glycosylation was not responsible for the differential activity of $FliC_{Pcc}$ and $FliC_{Dd}$ on tobacco suspension cultures. Thus, at least three *Pectobacterium* proteins can cause plant cell death, DspE, FliC, and the toxic protein Nip (102, 117, 119, 120). Together, these results suggest that *P. carotovorum* uses the conserved toxin Nip, the T3SS-secreted DspE, flagellin, and perhaps other cell death--inducing proteins to promote disease through elicitation of plant cell defenses or direct toxic effects on plant cells.

Type V Secrection System: Adherence to Host Cells and Defense Against Microbes

T5SS are simple secretion systems comprising only one or two proteins. The latter are called two-partner secretion systems (Tps) and consist of an outer membrane TpsB protein that facilitates secretion of a larger TpsA protein comprising an N-terminal transport domain and a large hemagglutinin repeat region that likely forms a fiber-like structure. Tps systems are encoded within some *Dickeya* T3SS gene clusters and Rojas et al. (138) found that this gene cluster contributed to bacterial adherence to leaves. Tps systems in *Dickeya*, *E. coli*, and *Burkholderia* spp. have been shown to function in contact-dependent growth inhibition (CDI) (5). CDI is a phenomenon in which the TpsA protein, designated CdiA, binds to target bacterial cells and inhibits their growth by delivery of a C-terminal toxin domain, the CdiA-CT. CDI systems also encode CdiI immunity proteins that prevent autoinhibition. Two CDI systems are present in *D. dadantii* 3937, each expressing a different CdiA-CT toxin. The $CdiA_{3937\text{-}1}$ toxin is a tRNase, and the $CdiA_{3937\text{-}2}$ toxin has DNase activity (5). Notably, *D. dadantii* mutants lacking the $CDI_{3937\text{-}1}$ system were outcompeted by CDI^{+} wild-type bacteria on chicory, whereas deletion of the $CDI_{3937\text{-}2}$ system did not affect competition. These results indicate that CDI plays a role in growth competition between *D. dadantii* strains and may explain previous work that identified the

D. dadantii EC16 *virA* gene as a virulence factor (139). The *virA* gene encodes the CdiI immunity protein for the EC16 CDI system, and therefore the virulence defect of *virA* mutants could be due to autoinhibition caused by the induction of CDI expression on plant hosts.

More recently, Poole et al. (127) identified a new class of growth inhibition systems called Rhs (rearrangement hotspot system), which are present in all *Dickeya* and *Pectobacterium* species as well as many other bacteria. Rhs proteins have YD peptide repeats analogous to the hemagglutinin repeats of CdiA and C-terminal toxin domains, which are inactivated by RhsI immunity proteins. Several Rhs and CDI systems contain additional toxin-immunity modules that are arranged in tandem arrays downstream of the the main *rhs-rhsI* (and *cdiAI*) gene clusters. These orphan toxin-immunity pairs appear to be horizontally transferred between bacteria and may contribute to toxin diversity. It seems likely that Rhs systems, like CDI, play roles in intrastrain growth competition, but this hypothesis remains to be tested.

SMALL MOLECULES AND REGULATION OF SOFT-ROT *ENTEROBACTERIACEAE* VIRULENCE PROTEINS

Gene regulation is usually represented with a web of arrows controlling target genes. These models lack the dynamic and quantitative characteristics of gene expression. They are also typically built from data obtained from cell populations grown in culture and not individual cells nor bacteria grown in plants. A further complication is that it is fairly simple to identify compounds or conditions that induce genes and to identify regulators that control gene expression, but tying the compound or condition to specific sensors is challenging. Finally, even though core regulators are conserved among genera, their function and the networks in which they participate can vary (131). Despite this, there has been significant progress in modeling gene regulation in SRE, including mathematical models of virulence (75) and examination of gene expression at the single cell level (87, 158, 159, 166),).

It has been clear for many years that *Dickeya* and *Pectobacterium* differ from each other in sensing small molecules that regulate key virulence genes (**Figures 1** and **2**). There is also variation among strains within these genera in how core regulators function. At least some of the variation appears to be from lateral acquisition of genes, such as PecS/M, and

possible degradation of pathways, such as the **AHL** and oxygen-sensing pathways in *D. dadantii*.

Intracellular Signal Molecules

Bacterial cells use intracellular signal molecules to sense their physiological condition, to induce virulence genes, and to develop into new states. These signals may be small molecules, such as cyclic diguanylate (c-di-GMP), which is enzymatically modified in response to cell physiology or environmental cues (66), or they may be tied directly to metabolic pathways, such as levels of glucose inside cells or glucans in the periplasm. Their effects may be both at the level of transcription and posttranscriptional. These intracellular signal molecules have been studied individually; much remains to be learned about how they work together to coordinate gene expression and the resulting cell behavior and development.

c-di-GMP acts as an intracellular signal molecule, and that it is often involved in switching cells from one lifestyle, such as motile, to another, such as a sessile biofilm cell (66). *Dickeya* and *Pectobacterium* encode numerous putative diguanylate cyclases (GGDEF domain proteins) and phosphodiestreases (EAL domain proteins) that may act on c-di-GMP. Seven of the 18 proteins in these classes in *D. dadantii* were mutated by Yi et al. (164), and two had significant effects on multiple phenotypes. Deletion of *ecpB* and *ecpC* enhanced biofilm formation and reduced virulence, motility, pectate lyase production, and T3SS gene expression.

Homologs of *ecpB* (Eca3270) and *ecp*C (Eca3271) are also present in *P. atrosepticum* and contribute to virulence in this bacterium as well. These genes are encoded adjacent to a T1SS, which is regulated by EcpB and EcpC and which secretes a repetitive adhesin. The T1SS-encoding and adhesin-encoding genes are present in other *Pectobacterium* as well, and only the genes coding for the T1SS are located in the vicinity of the *ecpB* and *ecpC* loci in the *Dickeya* genomes. Overexpression of the *P. atrosepticum* EcpC homolog increases motility, whereas overexpression of EcpB slightly reduced it. The effects of the *Pectobacterium* EcpB and EcpC on **PCWDE** or the T3SS remain unknown.

The role that central metabolism plays in virulence gene regulation is becoming more evident. Crp, which represses the expresion of genes when cells are grown in media containing glucose, has long been known to repress *Dickeya* virulence genes, linking

metabolism to virulence. Moreover, the virulence regulator KdgR controls the expression of genes encoding key gluconeogenic steps (137). Thus, KdgR could participate in the coordination of central carbon metabolism by modulating the direction of carbon flow. More recently, (p)ppGpp, which is produced at high levels when cells are starved, also affects PCWDE production, and it does so independently of quorum sensing (151). Osmo-regulated periplasmic glucans (OPGs) are required for sensing the environment, but a mutation resulting in a lack of OPG production can be compensated for by mutation of the RcsCD-RcsB phosphorelay (12), thus linking a glucose polymer to regulation. More surprisingly, gluconate metabolism affects *Pectobacterium* virulence. Mutation of gluconate metabolic genes causes hypermaceration and lack of motility, and regulators controlling these functions (KdgR and FlhD) are misregulated in gluconate mutants (105).

Metabolomic analyses have recently been used to further examine how **SRE** degrade plants, and this work may lead to new insights into ties between metabolism and regulation. Hugouvieux-Cotte-Pattat et al. (61) found that the sugars glucose, fructose, and sucrose are rapidly consumed by SRE during disease. Despite a high growth rate observed in plants, the relative importance of the different sugar catabolic pathways is unknown. The degradation of plant cell wall constituents is essential for soft-rot symptoms and their assimilation probably greatly influences bacterial growth during disease.

Intercellular Bacterial Signal Molecules

Bacterial cells use small molecules to communicate with each other and with plant cells and insect vectors as well as to modify their environment. Of these signals, AHL-mediated quorum sensing is the most widely studied. There are many original discoveries in SRE research, one of these being the first demonstration of a role for AHL in carbapenem antibiotic production (6) and later in pathogenicity (72, 125). Later, Liu et al. (88) showed that AHL-mediated quorum sensing regulates one quarter of *Pectobacterium* genes, including many virulence genes. AHL is unstable at alkaline pH (15) and SRE raise the pH of their environment during infection (100, 109, 118), so the role AHL plays during disease may be transient.

AHLs produced by *Pectobacterium*, or by other bacteria, can bind to the LuxR homologs ExpR1 and ExpR2 (also denoted VirR), and may stabilize the ExpR (**Figure 1**) (150). These ExpR homologs differ in specificity: ExpR1 binds 3-oxo-C6-HSL; ExpR2 binds both 3-

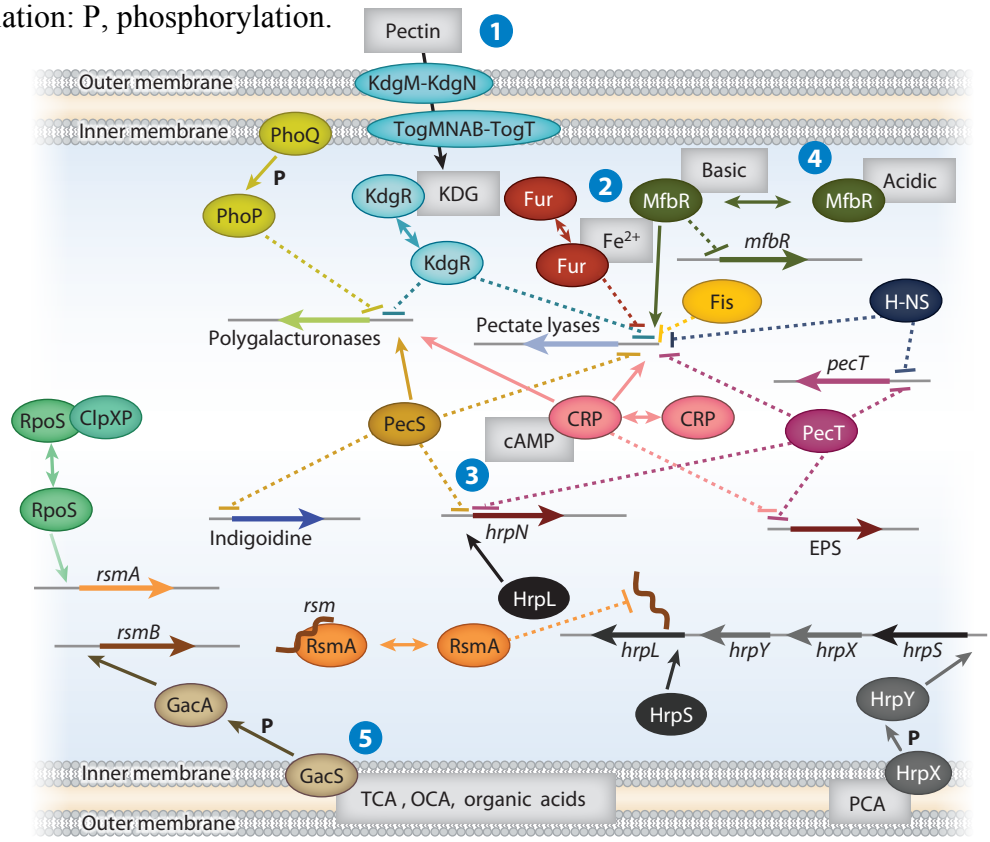
oxo-C6-HSL and 3-oxo-C8-HSL (28). In *Pectobacterium*, interaction with AHL reduces the affinity of ExpR for its target, the *rsmA* promoter, resulting in decreased RsmA production (29). Given that RsmA targets virulence protein-encoding mRNAs for degradation, less RsmA results in more PCWDE production (20, 106) . PecT (HexA) and the RsmA/*rsmB* system are transcriptional and posttranscriptional regulators, respectively, that directly control PCWDE expression. The *pecT* and *rsmAB* genes are controlled by a complex network of transcription factors that sense aspects of the bacterial environment or cell state, including the two-component regulatory systems GacAS (27) and RcsABC (2), the IcIR-like regulator KdgR (89), the LysR homolog HexA(PecT) (107), the sigma factor RpoS (108), the master regulator FhlDC (30), and the core regulator H-NS (112).

AHL-mediated quorum sensing plays, at most, a minor role in virulence of most *Dickeya* strains, although there are some exceptions (63, 104) (**Figure 2**). Although *D. dadantii* is not dependent on AHL, it does require bacterial auxin production for virulence (161). Auxin is used as a bacterial intercellular signal by many species, and auxin biosynthsis genes are required by *D. dadantii* 3937 for expression of key virulence genes, suggesting that auxin acts analogously to AHL. Like AHL in *Pectobacterium*, auxin acts via the RsmA/*rsmB* pathway in *Dickeya* (161). Curiously, plant roots respond to AHL by upregulating auxin-related genes, but how *D. dadantii* senses auxin remains a mystery (101, 116). Given that both auxin and AHL can be key regulators of bacterial virulence genes, with striking similarities on root development, albeit mediated by different pathways (116), these molecules may play convergent roles in both soft-rot virulence and plant development.

Plant-Derived Signal Molecules

SRE also respond to small molecules produced by plants. Most studies on plant-derived signal molecules are with *Dickeya*, with the most closely studied example being induction of PCWDE by pectate fragments, specifically KDG, which is produced by pectate metabolism (62, 113). An early dramatic example of plant-induced genes was reported independently by two research groups in 1993 (10, 74), who found a second set of plant-inducible pectate lyases after laboriously deleting all of the known pectate-inducted pectate lyases in *Dickeya*. There has been much recent progress in identifying plant-produced molecules that induce SRE virulence genes, and this progress has been aided by gene comparisons across bacterial genera and by genome sequences that allow larger scale

Figure 2: Dickeya. Integration of small-molecule signals into regulation of exoprotein production. Dickeya exoprotein differs from Pectobacterium in that it does not rely upon AHL-mediated quorum sensing and that the horizontally acquired PecS, which is not present in Pectobacterium, is tightly integrated into virulence gene regulation. 1. Pectin is catabolized, fragments are imported through KdgMN and TogMNAG or TogT, and 2-keto-3-deoxygluconate (KDG) is produced: KdgR repression of pectate lyases is released, resulting in exoprotein expression. 2. Low magnesium levels are detected by the PhoPQ system and pectate lyase expression is repressed. 3. Iron levels are low in apoplastic fluids: The Fur-Fe2+ complex is dissociated and Fur repression of pectate lyases is released. 4. Glucose levels are low: The cAMP-CRP complex activates pectate lyase production. 5. Apoplast pH increases during infection: MfbR becomes active and activates pectate lyase production. 6. Organic acids are detected: GacAS represses rsmA and induces rsmB. The rsmA gene is also activated by the stationary phase sigma factor RpoS; RpoS is controlled in part by the protease ClpXP. The HrpXY twocomponent system activates the HrpXY-HrpS-HrpL cascade and induces T3SS genes. Plant-derived organic acids that affect these regulatory systems include o-coumaric acid (OCA), p-couramic acid (PCA), and t-cinnamic acid (TCA). Additional regulators directly control pectate lyase genes by interacting with their regulatory regions: The nucleoid-associated protein FIS represses plant cell wall–degrading enzyme (PCWDE) production during the early exponential phase. Disappearance of FIS in stationary phase results in induction of PCWDE genes. PecT and PecS are two pleiotropic repressors that control PCWDE and HrpN production as well as motility. In addition, PecS also controls indigoidine and other oxidative stress response genes, whereas PecT controls exopolysaccharide (EPS) production. PecT is itself under the tight control of the associated nucleoid protein H-NS. Abbreviation: P, phosphorylation.



mutagenesis and transcriptomic studies within SRE. For example, Van Gijsegem et al. (149) examined 7 of the 18 *lacI* homologs in *D. dadantii* 3937 that lacked known or predicted function and found that four of these seven are expressed during plant infection and that two are induced by plant extracts.

In SRE, the T3SS is regulated by a dedicated signal transduction chain that includes a two-component system (HrpXY), a sigma 54 enhancer binding protein (HrpS), and an alternative sigma factor, HrpL (19, 51, 163)). T3SS regulation differs subtly between *Pectobacterium* and *Dickeya* and also differs among *Dickeya* strains. For example, expression of the *Dickeya* T3SS is induced in acidic minimal medium only in some strains, whereas in others it is expressed in both rich and minimal media (51).

Expression of the *D. dadantii* T3SS is enhanced by two plant phenolic compounds, *o*-coumaric acid (OCA) and *t*-cinnamic acid (TCA), both of which are intermediates in plant phenylpropanoid biosynthesis pathways, which includes important defense compounds such as salicylic acid (158). These two compounds upregulate the expression of the small regulatory RNA *rsmB* and the T3SS regulator *hrpL*, suggesting that OCA and TCA signaling occurs via the GacA/S two component system (159). This finding led to the discovery that another plant phenolic compound, *p*-coumaric acid (PCA) represses the expression of T3SS genes (87). PCA reduces expression of *hrpS* and *hrpL*, suggesting that it acts through the HrpX/Y-HrpS-HrpL pathway. Thus, phenylpropanoid biosynthesis intermediates can both induce and repress the expression of *D. dadantii* T3SS genes. Phenylpropanoids are a group of secondary metabolites produced by plants that stem from L-phenylalanine. The phenylpropanoid biosynthesis pathway can give rise to a variety of secondary compounds, such as flavonoids, isoflavonoids, stilbenes, and lignin, that are involved in resistance to a broad spectrum of pathogens (49, 103). Expression of the *Dickeya* PCWDE is enhanced by another plant phenolic compound, ferulic acid, and this induction occurs independently of GacA (55). In *E. coli*, the physiological stimuli of the GacA/S homologs (BarA/UvrY) are formate and acetate (21). These compounds are end products of sugar degradation, and they are produced by SRE during disease (61). Thus, the molecular signals of the GacA/S regulation in SRE remain to be clarified.

OCA: *o*-coumaric acid
TCA: *t*-cinnamic acid
PCA: *p*-coumaric acid

Many *D. dadantii* virulence genes are repressed by PecS (59). In *Agrobacterium tumefaciens*, this repression is released when PecS senses urate, xanthine, or salicylate (121). Xanthine and urate are products of purine nucleotide degradation and may be present in high concentrations in bacterial cells during stationary phase (136). Xanthine and urate are also produced as by-products of the reactive oxygen burst produced by plants in response to pathogen attack, and salicylate is a well-known plant defense signal molecule and has antimicrobial activities. It remains unknown if any of these molecules affect *Dickeya* PecS. PecS also affects gene expression when the bacteria are in insects, and the most important PecS-sensed signal in insects may differ from the key signal sensed in plants. The *Dickeya* PecS is a member of the MarR family; *Pectobacterium* also encodes MarR homologs. Identifying which, if any, of the *Pectobacterium* MarR homologs respond to this family of signal molecules and determining the targets of this regulator would provide useful information on SRE pathogenicity.

Ions, ranging from iron, which is discussed below, to hydrogen, also affect SRE pathogenicity. However, despite the importance of ions in virulence, relatively little is known about regulation in response to most of these signals. As with related genera, SRE use the PhoPQ two-component system and SlyA, which responds to magnesium levels, to monitor their environment and control expression of virulence genes (42, 52, 53, 90, 92) . In addition to regulation, ions also affect the efficacy of virulence proteins, and this has been most clearly demonstrated with SRE pectate lyases. Pectate lyases require an ion cofactor, generally calcium, to function. In addition, most pectate lyases have a pH optimum of 8, but the intercellular pH of plants is acidic. Thus, to macerate plant cell walls, the bacteria must raise the pH of their environment, and the pectate lyases must acquire calcium (109, 118)). pH is also an important signal in the regulation of *D. dadantii* virulence genes via the global regulator MfbR, whose activity is modulated in vivo by acidic pH, a stress encountered by pathogens during the early stages of infection (135).

SMALL MOLECULES PRODUCED BY SOFT-ROT *ENTEROBACTERIACEAE* THAT CONTRIBUTE TO VIRULENCE.

SRE not only respond to small molecules in their environment, they also produce small molecules to acquire metal ions, such as iron, from their host. Iron is a necessary cofactor for enzymes involved in important cellular functions. SRE also regulate genes in reponse to iron, and low iron conditions trigger SRE virulence gene expression (44, 45). Given that SRE thrive in diverse environments, they must carry versatile iron acquisition tools. SRE, like many other bacteria, possess high-affinity iron transport systems that are mediated by low molecular weight iron chelators called siderophores. Iron acquisition has mainly been studied with *D. dadantii* 3937, which synthesizes and secretes two siderophores, achromobactin and chrysobactin, both of which contribute to virulence (38, 43). The structures of *Dickeya* chysobactins were only recently described (141). These siderophores are produced in a sequential manner in culture supernatants of bacterial cells grown under iron limitation (43). In addition to these siderophores, SRE possess the corresponding transport systems that enable them to internalize the ferrisiderophore via a specific outer membrane receptor and an ABC permease. Acquisition of iron by *D. dadantii* siderophores affects plant iron homeostasis, thus iron acquisition not only affects SRE gene expression but also has significant effects on plant responses (32, 33, 143).

Analysis of *Dickeya* and *Pectobacterium* genomes revealed multiple TonB-dependent outer membrane receptors and TonB homologs, suggesting that the capacity of use of diverse exogenous siderophores is common among SRE and may confer fitness in complex environments (142). Genome analyses also revealed several other iron acquisition systems, including a heme uptake system that may be used after plant cell lysis by PCWDE. The induction of *D. dadantii hmuSTUV* genes was indeed observed in planta by Okinaka et al. (115) and Yang et al. (160).

One striking difference between *Dickeya* and *Pectobacterium* is the presence of two ferrous iron transport systems in *Dickeya*, FeoAB, which is likely to be active under anaerobic-microaerophilic conditions, and the EfeUOB system, which, in *E. coli*, is a low-pH iron transporter (16). A second difference is the presence in some *Pectobacterium* of a transport system for ferric citrate (*fecABCDE*), as an exogenous siderophore. Expression of

the Fec system by *Pectobacterium*, which invades stem xylem vessels where citrate is used to transport ferric iron, could be beneficial.

SRE also produce pigments and small phytotoxins that affect virulence, although the exact functions of these molecules remain unclear. *Dickeya* and *Pectobacterium* differ in which pigments they produce and variation also occurs among strains within these genera. Pigment production is repressed under most growth conditions and overproduction of pigment reduces bacterial growth rates or, in the case of the orange pigment produced by *Pectobacterium*, is toxic.

The pigment that has been most closely examined is the blue indigoidine, produced by *Dickeya*. This pigment may help *Dickeya* combat reactive oxygen produced by plant defense responses (134). Indigoidine is insoluble in water but can be dissolved in organic solvents, such as DMSO. It accumulates in culture as beads larger than the bacterial cells themselves (64), but whether indigoidine beads form during pathogenesis remains unknown. Indigoidine production is controlled by a gene island present in *Dickeya* but not *Pectobacterium*, and is repressed by PecS, which is adjacent to the indigoidine biosynthesis gene cluster (133). *P. carotovorum* produces an orange pigment of unknown structure and function. Only remnants of the pigment biosynthesis genes are present in the narrow host-range pathogen *P. atrosepticum*, suggesting that this pigment may contribute to the broad host range of *P. carotovorum* (153).

Both *Dickeya* and *Pectobacterium* genomes encode large polyketide synthetase genes and the functions of these genes are just now being explored. Of these, the zeamine phytotoxin gene has been most closely examined in recent years. Zeamine and zeamine II, both of which require *zmsA* for production inhibit rice seed germination and growth of other bacterial species (154, 167).

EXPORT OF SMALL MOLECULES IS REQUIRED FOR SOFT-ROT *ENTEROBACTERIACEAE* PATHOGENICITY

Plants produce many secondary metabolites, such as phytoalexins, peptides, and alkaloids, that play a role in protecting against SRE (34, 93, 94). To successfully colonize a host, SRE must counteract the presence of these antimicrobial compounds. Because SRE also must survive in the soil, water, and invertebrates, and defend against secondary invaders during

pathogenesis, they must contend with fungal and bacterial toxins. In the 1970s, a phytoalexin produced by maize was associated with resistance to *Dickeya* stem rot (54, 82), but little additional work was done with SRE in this area until the past decade.

Multidrug resistance (MDR) transport proteins export a wide range of antimicrobial compounds and are very important for bacterial survival in hostile host environments (130). Numerous MDR systems are encoded in SRE genomes, and several of these contribute to virulence or allow bacteria to successfully compete with secondary microbial invaders (7, 91, 148). As with many other SRE virulence genes, genes encoding efflux pumps are induced by plant-produced phenolic acids, such as salicylic acid (132). In addition to exporting toxic molecules produced by host cells, these transporters may also transport toxic sugars that are produced by the bacterial cell as by-products of metabolism and also help the cell control osmotic pressure within the cell through transport of solutes out of the cytoplasm (21, 68, 69).

CONTROL OF SOFT-ROT *ENTEROBACTERIACEAE* THROUGH INHIBITION OF VIRULENCE PROTEINS WITH SMALL MOLECULES

We still know little about how even simple small molecules affect SRE; only recently was the basis for the inhibition of some salts on *Pectobacterium* growth described (155). Inhibition of regulator or structural proteins or RNAs involved in virulence with small molecules is an attractive approach for control of SRE. Ideally, these virulence blockers would inhibit pathogenesis without placing severe selective pressure on the survival of the target pathogen (86). The T3SS is a major virulence mechanism of many gram-negative pathogens and because the T3SS is not required for bacterial growth outside of plants, antimicrobials that inhibit T3SS might limit the development of bacterial resistance toward such antivirulence therapies (114).

Given that the *Dickeya* T3SS is controlled by plant-produced phenolics, development of T3SS inhibitors for soft-rot bacteria was initiated by exploring natural phenolic products in plants. Based on the structure-activity relationship analysis of known T3SS inhibitors and inducers, analogs were synthesized and assayed (156). Novel compounds were identified in a compound inventory that inhibit the T3SS of different phytopathogens, including *P. syringae, E. amylovora,* and *D. dadantii*, and the human pathogen *Pseudomonas aeruginosa*

(156). Knowledge of induction of the PCWDE by AHL-mediated quorum sensing has led to the development of SRE-resistant plants (35), but this strategy has not been accepted for commercial use. Attempts to use plant cell wall fragment analogs to inhibit production of PCWDE have been unsuccessful (111).

Development of inhibitors is challenging because tracking the molecule uptake and metabolism by the plant is challenging, and additional drawbacks may limit the use of inhibitors. For example, although bacteria with virulence gene mutations may be reduced in virulence, we do not know if an inhibitor could reverse infection once it has started. In addition, inhibitors may be strain- or species-specific.

CONCLUSION

Recent studies focusing on the role small molecules play in SRE pathogenicity has resulted in a better understanding of SRE interactions with plants and insect vectors, but many crucial questions remain unanswered. Because much recent work has shown that plants can detect bacterial molecules, such as AHL and acetoin, and that bacteria respond to plant-produced molecules, such as auxin, salicylic acid, and jasmonic acid, it is important to consider that phenotypes observed may be due to the direct action of the molecule on regulatory proteins in both partners. Progress on these interesting, but challenging, questions requires work by multidisciplinary research groups with expertise in disciplines as broad as chemistry, genomics, entomology, and bacterial ecology. The end result may be novel and effective controls for SRE and other bacterial pathogens based on interference with how these pathogens sense and respond to the small molecules that surround them.

SUMMARY POINTS

1. **Production of SRE virulence proteins is controlled by integration of small molecule signals produced by both pathogen and host plant, and regulators responding to these signals control production of virulence proteins [**AU: production of what?**] at both transcriptional and posttranscriptional stages.**
2. **Control mechanisms for SRE in agriculture have changed little over the past decades, but our increased knowledge of how these pathogens sense small molecules may lead to new control methods centered on interference on these sensing pathways.**

3. **Although *Pectobacterium* and *Dickeya* cause similar symptoms, there are important differences in the virulence proteins they produce and how they regulate production of these proteins.**

FUTURE ISSUES

1. **As with most other bacterial pathogens, researchers have studied regulatory proteins in SRE in isolation, often only looking at a few genes and not including quantitative or temporal data. SRE researchers are moving toward more sophisticated mathematical models of virulence gene expression that may someday lead to predictions of the effects of mutations or environmental changes on the degree and timing of virulence protein production.**
2. **SRE appear to have intimate interactions with insects, which may serve as both vectors and hosts, but much work remains to be done on the mechanisms of interactions, their ecological significance, and the role that insect-SRE interactions play in both insect and SRE evolution.**
3. **T3SS gene expression appears to be bi-stable in SRE, with only half of cells expressing T3SS genes in culture. In comparison, other virulence genes, such as those encoding pectate lyases, are expressed in a majority of cells. The regulatory pathways that cause this effect and whether this phenomenon plays an important role in disease remain unknown.**

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RELATED RESOURCES

1. ASAP (a systematic annotation package for community analysis of genomes) https://asap.ahabs.wisc.edu/asap/ASAP1.htm (accessed 4 Apr 2012)

2. Time lapse movie of a potato infected with *Pectobacterium.* http://www.plantpath.cornell.edu/PhotoLab/TimeLapse2/TimeLapse_MainGallery.html (accessed 4 Apr 2012)

ACRONYMS AND DEFINITIONS

AHL: acyl-homoserine lactone

OCA: *o*-coumaric acid

PCA: *p*-coumaric acid

PCWDE: plant cell wall--degrading enzymes

SRE: soft rot *Enterobacteriaceae*

TCA: *t*-cinnamic acid