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# Detection of and Response to Signals Involved in Host-Microbe Interactions by Plant-Associated Bacteria

Anja Brencic and Stephen C. Winans*

*Department of Microbiology, Cornell University, Ithaca, New York 14853*

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* Corresponding author. Mailing address: Department of Microbiology, 361A Wing Hall, Cornell University, Ithaca, NY 14853. Phone: (607) 255-2413. Fax: (607) 255-3904. E-mail: scw2@cornell.edu.



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## INTRODUCTION

Nutrients are essential to the survival of all organisms; all heterotrophs, by definition, must look to other organisms to meet these fundamental needs. As primary producers, higher plants represent the chief source of carbon and energy for a large number of creatures, ranging from microbes to humans. Some of these organisms have developed diverse and often complicated sensory mechanisms or organs that allow them to identify and locate the appropriate plant-produced nutrients.

An interesting example is found with honeybees, which require pollen and flower nectar as their sole sources of protein, carbon, and energy. Bees are also the most important pollinating insects, and the interdependence between bees and plants makes them an excellent example of an animal-plant symbiosis. Behavioral ecologists have been interested in how various floral characteristics, such as color, shape, and size, enable a bee to choose appropriate flowers that will provide them with sufficient nutrients. These characteristics are detected by color receptors of the animal, and research is being conducted to understand how differences in these characteristics are computed on a neuronal level (54, 457).

Other interesting and well-studied examples of plant detection are found with plant-associated microorganisms. Many types of microbes live in close association with host plants and benefit from these associations by obtaining carbon and other nutrients from their hosts. Events that lead to establishment of these interactions are triggered by bacterial recognition of specific plant-associated signal molecules, which are detected by dedicated bacterial sensory proteins. Similar to what has been observed for honeybees, this recognition may play an important role in the host specificity or the host range of a bacterium. This is the case in the recognition of plant-released flavonoids by rhizobial NodD proteins and also in the recognition of translocated bacterial avirulence proteins by host-encoded resistance proteins (see below).

Over the course of a plant-microbe interaction, bacteria continue to monitor changes in the physiology of their host. These changes are often due to specific activities of the colonizing microbes, which in response continuously make adjustments to their own physiology. Thus, detection and response to various host signals in the plant-microbe interaction is a continuous process. In many cases, in addition to specific regulatory proteins, global regulators play a role in these interactions.

In this review, we describe the process of plant detection as it is known to occur in the best studied plant-microbe systems. These include the symbiosis between rhizobia and legumes and the pathogenesis between *Agrobacterium tumefaciens* and host plants that leads to crown gall tumors. We then turn to other plant pathogens, such as soft rot erwinias, *Pseudomonas syringae*, and biocontrol strains of *Pseudomonas fluorescens*. We describe plant-associated signals, bacterial proteins involved in their detection, and mechanisms and pathways of signal transduction leading to expression of specific bacterial genes that direct these interactions. We also discuss the role of various global regulators involved in the regulation of these processes.

## CHARACTERISTICS OF ENVIRONMENTS INHABITED BY PLANT-ASSOCIATED BACTERIA

Virtually all plants live in intimate association with microorganisms, which can colonize the surfaces of plants (epiphytic colonization) or occupy spaces within plant tissues (endophytic colonization). The surface of the aerial portion of the plant (the phyllosphere) is exposed to rapid and frequent changes in temperature, humidity, UV irradiation, and moisture, the last of which can also influence nutrient concentrations and osmolarity (10, 214, 289). Although this environment has frequently been regarded as hostile to microorganisms (214), microbial inhabitants of the phyllosphere are diverse and include many different genera of bacteria, filamentous fungi, yeasts, algae, and, less frequently, protozoa and nematodes (10). Bacteria, especially *Pseudomonas syringae* and *Erwinia* (*Pantoea*) spp., are by far the most abundant inhabitants of the phyllosphere (10, 214). One of the major determinants of epiphytic colonization of leaves is the availability of carbon-containing nutrients (10). Simple sugars such as glucose, fructose, and sucrose are the dominant carbon sources on leaves or stem surfaces and are thought to simply leach from the plant interiors, mostly from sites of injury or from glandular trichomes (494). These sites are the most heavily populated areas of the plant surface (10, 101, 325, 529).

The interior portions of leaves provide a somewhat more hospitable environment. Importantly, humidity is more carefully controlled within leaf tissues, due to a waxy cuticle on the plant surface that minimizes water loss. Leaf epidermis contains small openings called stomata, which allow the exchange of carbon dioxide and oxygen. Stomata are the main route by which water is lost from the plant; however, they can be closed during periods of dryness to conserve water. When stomata are open, they provide ready access to the intercellular spaces within leaves (the apoplast) and serve as an important entry point for many bacteria (27, 28).

Roots and the zone surrounding them (the rhizosphere) are also readily colonized by microbes (44, 48). The rhizosphere generally provides more protection than the phyllosphere from desiccation, temperature, and light stress. Furthermore, sources of carbon and minerals are more abundant in the rhizosphere (44, 99, 510). Plants exude high levels of nutrients from their roots, often in excess of 20% of all fixed carbon (309). Amino acids, organic acids, sugars, aromatics, and various other secondary metabolites comprise the majority of the low-molecular-weight root exudates, whereas high-molecular-weight exudates primarily include polysaccharides and proteins (309). This complex mixture of organic compounds results in much larger numbers of microbes in the rhizosphere

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than in the nearby bulk soil, where the microbial community is carbon limited. This phenomenon is referred to as the "rhizosphere effect" (44).

A large variety of bacteria, fungi, protozoa, and nematodes colonize the rhizosphere (44, 48). These organisms may exist as free-living organisms in the rhizosphere or may be attached to surfaces of roots. Colonization of root surfaces is characteristically nonuniform; some areas, including the extreme tip of the root, are relatively free of bacteria, whereas other areas can be heavily populated (151, 355). In studies with *Pseudomonas* spp., the heavily populated areas are usually found at junctions between epidermal root cells, indented parts of the epidermal surface, or sites of side root appearance, all of which are presumed sites of exudation (39, 303). Microbes can also gain access to the interior portions of roots through cracks in the epidermis made by the emergence of lateral roots or through wounds caused by various herbivores (18).

In recent years, there has been significant progress in our understanding of the environments that are experienced by the bacteria living in the rhizosphere or the phyllosphere. Much of this knowledge comes from studies that use bioreporter strains, in which an environmentally or metabolically responsive promoter is fused to a suitable reporter such as *lacZ*, *gus*, *lux*, *inaZ*, or *gfp* (286, 454). These studies allow monitoring of the spatial distribution and fluctuations of physicochemical factors that are relevant for the microbes inhabiting the plant-associated environments. The factors studied so far have included UV irradiation, temperature, water potential, and iron availability on surfaces of leaves (17, 251, 268, 499) and carbon, phosphate, nitrogen, iron, and oxygen availability in the soil (217, 241, 271, 275). Bioreporter strains have also been used to detect products of plant metabolism that are released into the surrounding environment and are used by the associated microbes as nutrients (53, 239, 285, 329). In general, these studies showed that there is substantial heterogeneity in the intensities of reporter gene expression in different microenvironments of a leaf or rhizosphere, suggesting that bacteria residing in different parts of these habitats may be exposed to remarkably different environments.

Studies performed with *Pantoea agglomerans* (previously known as *Erwinia herbicola*) harboring a sucrose- and fructose-responsive *scrY* promoter fused to a *gfp* or *inaZ* reporter revealed a high-level heterogeneity of apparent sucrose availability on surfaces of leaves (329). Workers performing a study in which the sucrose- and fructose-responsive *fruB* promoter was fused to a short-half-life variant of *gfp* came to similar conclusions by showing that, within 1 day after inoculation, only 1% of the bacteria expressed this fusion (285). The cells that continued to detect sugars were not randomly dispersed across the leaf surface but instead were localized to sites likely to release these nutrients, including stomata, trichomes, veins, and various crevices that are more likely to retain water (536).

Similar studies were performed to map the availability of sugars and amino acids along roots (239). A strain of *P. agglomerans* harboring an ice nucleation reporter gene, driven by either a sucrose- or tryptophan-responsive promoter, was used as a biosensor. When the strain was introduced into the rhizosphere of an annual grass, both tryptophan and sucrose were detected, but they showed different spatial patterns. Tryptophan was most abundant in soil around roots 12 to 16 cm from the tip, while sucrose was most abundant in soil near the root tip. High sucrose availability at the root tip is thought to be caused by its leakage from the immature, rapidly growing root tissues, while tryptophan loss from older root sections was proposed to result from lateral root perforation of the root epidermis (239). As might be expected, sites having the highest apparent sucrose or tryptophan exudation were the most heavily colonized parts of the root.

## HOST DETECTION BY MEMBERS OF THE *RHIZOBIACEAE* DURING COMMENSAL PLANT COLONIZATION

The detection of and response to host-released signals by members of the *Rhizobiaceae* is being intensively investigated in many laboratories. These α-proteobacteria include various nitrogen-fixing plant symbionts, *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium*, and *Bradyrhizobium*, collectively called rhizobia, and the plant pathogens *Agrobacterium tumefaciens* and *A. rhizogenes*, referred to as agrobacteria, which cause crown gall tumors and other neoplasias on a wide variety of plants. Species of rhizobia and agrobacteria are very closely related, and it was recently proposed that the *Agrobacterium* genus be abolished and that its members be referred to by the genus name *Rhizobium* (132, 528, 543). Major similarities between the two genera include metabolic, transport, and regulatory systems that may promote survival in the competitive rhizosphere, whereas the most striking differences lie in genes specifically required for interaction with a plant host (65, 158). These genes are carried on the tumor-inducing (Ti) plasmid in agrobacteria and on the symbiotic plasmids pSymA and pSymB in *Sinorhizobium meliloti* (158). In *Bradyrhizobium japonicum* and *Mesorhizobium loti*, the symbiosis-related genes are carried on a chromosomally located symbiotic island (258, 259).

Although best known for forming nodules or crown gall tumors, rhizobia and agrobacteria also can colonize plants without causing either of these neoplasias and can utilize plant exudates to support growth and division. These associations are not dependent on the pathogenic or symbiotic determinants of the respective bacteria, and similar phenomena are therefore likely to occur in the various members of this family.

### Chemotaxis toward Plant Root Exudates

An early and essential event in most plant-microbe interactions, at least in water-saturated environments, involves bacterial chemotaxis toward plant root exudates or wound saps. As described above, plants exude high levels of nutrients, and many of these act as chemoattractants for the bacteria. Different strains have been described to be positively chemotactic to sugars; amino acids; various dicarboxylic acids such as succinate, malate, fumarate; and aromatic compounds including shikimate, quinate, protocatechuate, vanillate, acetosyringone, gallate, catechol, and luteolin (15, 34, 62, 201, 371, 372, 414). One protein involved in sugar chemotaxis is ChvE of *A. tumefaciens* (Table 1), which is a chromosomally encoded sugar binding protein located in the periplasmic space (63). A ChvE-like protein is also required for sugar chemotaxis of *Azospirillum brasilense*, a free-living diazotrophic α-proteobacterium. The protein was found in a search for *A. brasilense* plant-

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TABLE 1. Plant signals detected by regulatory systems of plant-associated bacteria, and phenotypes they regulate

| Strain | Plant signal | Receptor | Receptor family | Target operon(s) | Phenotype |
|---|---|---|---|---|---|
| *S. meliloti* | Dicarboxylic acids | DctB-DctD | Two-component system | *dctA* | Uptake of dicarboxylic acids |
| *S. meliloti* | ? | ExoS-ChvI | Two-component system | *exo* | Succinoglycan production (nodule invasion) |
| *S. meliloti* | Phosphate concentration | ? | | *exo*, *exp* | Succinoglycan, EPSII production (nodule invasion) |
| *Rhizobium* spp. | Flavonoids, betaines, ardonic acids, simple phenolics | NodD, SyrM | LysR | *nod* | Nod factor synthesis |
| *B. japonicum* | Flavonoids | NodV-NodW | Two-component system | *nod* | Nod factor synthesis |
| *S. meliloti* | Luteolin | NolR | LysR | *nod* | Nod factor synthesis |
| *S. meliloti* | Stachydrine, trigonelline | ? | | *stc, trc* | Catabolism of *nod* inducers |
| *S. meliloti* | Oxygen tension | FixL-FixJ | Two-component system | *fix*, *nif* | Nitrogen fixation |
| | Oxygen tension | NifA | NtrC | *fix*, *nif*, *moc* | Nitrogen fixation, rhizopine catabolism |
| *A. tumefaciens* | Monosaccharides | GbpR | LysR | *chv* | Sugar chemotaxis and uptake |
| | pH | ChvG-ChvI | Two-component system | *virG*, *katA*, *aopA* | *vir* regulation, catalase, outer membrane protein |
| | Phenolics, monosaccharides, pH | VirA-VirG, ChvE | Two-component system, sugar binding protein | *vir*, *rep* | T-DNA transfer, replication of Ti plasmid, phenolic metabolism |
| *A. tumefaciens* octopine-type Ti plasmid | Octopine | OccR | LysR | *occ*, *traR* | Octopine catabolism, transfer of Ti plasmid |
| *A. tumefaciens* octopine-type Ti plasmid | Mannopine | MocR? | LacI-like repressor | *moc*, *trlR* | Mannopine catabolism, inhibition of TraR, transfer of Ti plasmid |
| *A. tumefaciens* nopaline-type Ti plasmid | Agrocinopine A + B | AccR | LacI-like repressor | *acc*, *traR* | Agrocinopine catabolism, transfer of Ti plasmid |
| *A. tumefaciens* chrysopine-type Ti plasmid | Agrocinopine C + D | AccR$^{Chry}$? | LacI-like repressor | *acc*, *traR* | Agrocinopine catabolism, transfer of Ti plasmid |
| *A. radiobacter*, pAtK84b | Nopaline | ? | ? | *nox*, *traR(noc)* | Nopaline catabolism, transfer of Ti plasmid |
| | Agrocinopine A + B | ? | ? | *acc*, *traR(acc)* | Agrocinopine catabolism, transfer of Ti plasmid |
| *E. carotovora, E. chrysanthemi* | Pectin metabolites (DKI, DKII, KDG) | KdgR | IclR-like repressor | *pel*, *pem*, *out rsmAB* | Pectin catabolism |
| *E. carotovora, E. chrysanthemi* | cAMP | Crp | | *pel*, sugar catabolism | Pectin catabolism, sugar catabolism |
| *E. chrysanthemi* | ? | PecS | MarR-like repressor | *peh*, *pel*, *cel*, *fli* | Pectin catabolism, motility |
| *E. chrysanthemi* | ? | PecT | LysR repressor | *pel*, *cel*, *prt*, *eps*, *fli* | Pectin catabolism, cell wall maceration, EPS production, motility |
| *E. chrysanthemi* | Plant extracts | Pir | IclR-like activator | *pel* | Pectin catabolism |
| *E. chrysanthemi* | Iron status | Fur | | *pel* | Pectin catabolism |
| *E. chrysanthemi* | Iron status | ? | | *fct-cbs*, *acsA* | Siderophore production |
| *E. carotovora* | Plant extracts | AepA | | *pel*, *cel*, *peh*, *prt* | Pectin catabolism, cell wall maceration |
| *E. carotovora* | ? | HexA | LysR repressor | *pel*, *prt*, *fli*, *rpoS*, *rsmB* | Pectin catabolism, cell wall maceration, motility, secondary metabolism |
| *E. carotovora* | ? | RexZ | IclR-like activator | *pel*, *cel*, *prt* | Pectin catabolism, cell wall maceration |
| *E. carotovora* | DNA-damaging agents | RecA/RdgA-RdgB | | *pnlA* | Pectin catabolism |
| *Pseudomonas* spp., *Erwinia* spp., *P. stewartii*, *X. campestris*, *R. solanacearum* | Minimal medium, nutrient limitation? | ? | | *hrp*, *avr*, other TTSS genes | Type III secretion and virulence |
| *R. solanacearum* | Cell wall polysaccharide? | PrhA | Siderophore receptor-like | *hrp*, *pop*, other TTSS genes | Type III secretion and virulence |
| *P. syringae* pv. syringae | Phenolic glycosides, sugars | ? | | *syr* | Syringomycin production |
| *P. syringae* pv. tomato DC3000 | Shikimate, quinate, sugars | ? | | *cor* | Coronatine production |

inducible genes and was designated SbpA (sugar binding protein A) (500). Enteric bacteria use similar sugar binding proteins for sugar chemotaxis, suggesting that sugar chemotaxis may occur by similar mechanisms (1, 204, 205). Rhizobia and agrobacteria also have several flagella located near one pole of the cell (201, 278), and mutations in genes encoding flagellin abolish motility and, in *Agrobacterium*, reduce tumorigenesis (35, 77, 201).

### Binding to Host Surfaces

Binding of rhizobia and agrobacteria to plant surfaces is essential for establishing a long-term interaction of the bacteria with their hosts. Several plant factors have been described that might be involved in this attachment. Plant lectins (proteins that possess at least one noncatalytic domain that binds reversibly to mono- or oligosaccharides) could serve as receptors for bacterial exopolysaccharides (EPS) (215, 411, 433). Several reports showed that lectins are important for establishment of the rhizobium-plant symbiosis and might mediate specificity in the *Rhizobium*-legume symbiosis (215). A gene encoding pea lectin conferred novel bacterial nodulation properties on clover plants; the transgenic clover plants were nodulated by the heterologous strain *R. leguminosarum* bv. viciae, which does not nodulate wild-type clover (110, 411, 503, 505). Two *Arabidopsis* mutants have been described that are defective in their ability to bind *A. tumefaciens* (348, 552). The *rat1* gene (for "resistant to *Arabidopsis* Transformation") encodes an arabinogalactan protein, and the *rat3* gene encodes a putative cell wall protein.

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Binding of rhizobia and agrobacteria to plant surfaces is thought to take place in two steps (315). The first is a rather weak and reversible binding step that may involve a variety of bacterial polysaccharides. The products of the *ndvA* and *ndvB* genes in *Sinorhizobium meliloti* and of the homologous *chvA* and *chvB* genes in *A. tumefaciens* are involved in the synthesis of a cyclic glucan (64, 103, 236, 468), which could act as an adhesin via gelling interactions with host polysaccharides or could interact with plant lectins (215). Mutations in the *chv* genes reduced the binding of the agrobacteria to cultured cells and abolished tumorigenesis (117, 118, 395). However, these mutations are pleiotropic, and so it is difficult to know whether the cyclic glucan is a direct adhesin or whether its loss perturbs some other functions that are important in binding. Mutations in the *ndv* genes caused a moderate decrease in the binding of rhizobia to root hairs and had a strong defect in nodule invasion (123, 124). The invasion defect, however, was not due to the defect in adhesion, since revertants that were fully able to form nitrogen-fixing nodules remained defective in attachment (124).

A 30-kb cluster of *A. tumefaciens att* genes has also been described as being required for attachment and tumorigenesis (316, 317, 319). However, the recently published *A. tumefaciens* genome sequence revealed that the *att* genes are located on the cryptic plasmid pAtC58, which is not essential for virulence (233, 423). There have also been a few reports of a bacterial adhesin called rhicadhesin, although the gene encoding this adhesin has yet to be cloned or disrupted (475–477).

The second binding step requires the synthesis of bacterial cellulose, which causes a tight, irreversible binding and formation of bacterial aggregates on the host surface (314, 413). Mutants with mutations of the *A. tumefaciens celABCDE* operon no longer synthesize cellulose and can be readily dissociated from cultured plant cells by vortexing. However, these mutants are still tumorigenic (317, 318). To our knowledge, mutants with mutations in the orthologous genes of rhizobia have not been tested for binding.

### Alteration of Gene Expression by Plant-Released Sugars

Plant-released sugars are likely to induce the expression of genes required for their uptake and catabolism. The sugar binding protein ChvE of *A. tumefaciens*, in addition to playing a role in chemotaxis, has been implicated in the uptake of glucose and galactose (Table 1). The *chvE* gene is part of an operon encoding an ABC-type uptake system and is transcriptionally induced by these monosaccharides (265). Both glucose and galactose are components of plant-cell wall polysaccharides and are presumably exuded from plants. Induction of the operon is mediated by the product of the divergently transcribed gene *gbpR*, encoding a LysR-type regulator (114).

Another *A. tumefaciens* gene was reported to be induced by plant sugar molecules. The *picA* (for Plant-Inducible Chromosomal) gene was first identified as being induced by the polygalacturonic acid fraction of carrot extracts (419–421). The gene resembles genes encoding a family of polygalacturonidases (also known as pectinases). It lies in a possible operon with a gene encoding a second pectinase and is located adjacent to an operon encoding an ABC-type uptake system for oligogalacturonic acids (176, 535).

In *S. meliloti*, the *melA* gene is required for utilization of α-galactosides, and its transcription is induced by these substrates (53, 155). An *S. meliloti* strain expressing a *melA-gfp* reporter fusion was used for the detection of α-galactosides around roots of several legumes and grasses. Bacteria expressed high levels of GFP in the areas around zones of lateral root initiation and around roots hairs but not around root tips. Other studies reported that vitamins, choline, stachydrine, trigonelline, and homoserine can also be secreted by plant roots and used by different species of rhizobia (42, 108, 385, 472).

### Detection of Acidity

Environmental acidity is a host-associated signal of central importance in host detection. Apoplastic fluids in leaf tissues, as well as the soil adjacent to plant roots, is generally acidic, with pH values between 5 and 6.5 (181, 309). The *A. tumefaciens* ChvG-ChvI two-component system regulates the expression of several unlinked acid-inducible genes, and ChvG appears to be a global sensory protein that can directly or indirectly sense extracellular acidity (287). This system was found in a search for chromosomally located virulence genes (71). Mutations in either *chvG* or *chvI* abolished tumorigenesis and caused sensitivity to acidic pH (71, 305). Among the genes whose acid-inducible expression is controlled by ChvG-ChvI is *virG*, whose product activates the *vir* regulon (see below) (305); the *aopA* gene, which encodes a surface-exposed outer membrane protein; and the *katA* gene, which encodes a catalase that is important for detoxification of $H_2O_2$ released during plant infection (Table 1) (287).

In *Rhizobium tropici*, the *atvA* gene is transcriptionally upregulated by acid shock and is homologous to the *acvB* gene of *A. tumefaciens* (506). Both *R. tropici atvA* and *A. tumefaciens acvB* mutants are acid sensitive, indicating that the two genes are required for acid tolerance (506). The functions of these genes are unknown, although the AcvB protein (or its Ti-plasmid-encoded homolog, VirJ) is required for tumorigenesis (255, 260, 369, 533).

## HOST DETECTION DURING RHIZOBIUM-PLANT INTERACTIONS

Under appropriate environmental conditions, rhizobia and host plants can initiate a symbiotic interaction, resulting in the development of root nodules, which the bacteria inhabit as nitrogen-fixing endosymbionts. Development of a rhizobium-plant symbiosis is a complex process. It involves a highly coordinated exchange of signals between the plant and the bacteria and leads to a gradual and coordinated differentiation and adjustment of physiology and metabolism in both partners (38, 57, 378, 447, 459).

### Host Detection during Nodule Formation

The key event in nodule formation is the synthesis and release by the bacteria of small molecules that are detected by the plant and that trigger formation of the nodule (95, 119, 163, 188, 302). These molecules are called Nod factors (also known as lipo-chito-oligosaccharides). Detection of Nod factors by a legume host induces major developmental changes in

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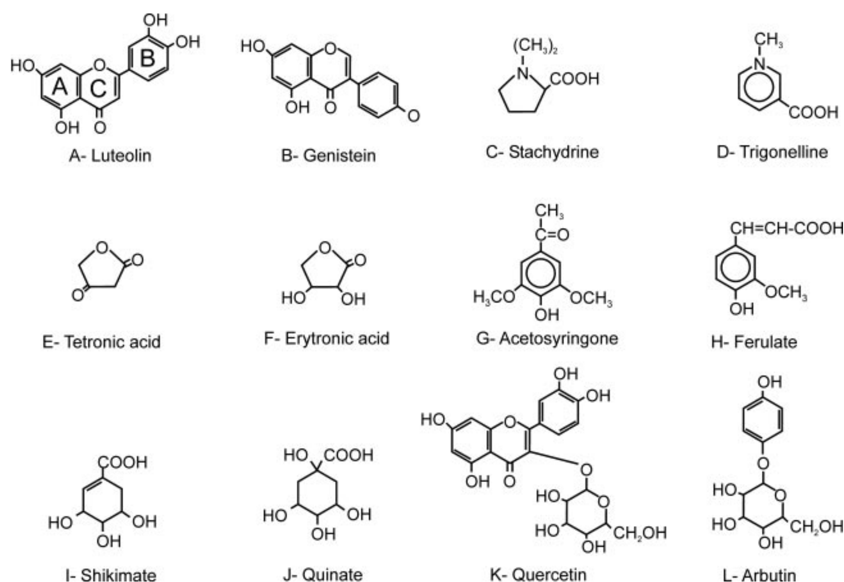


FIG. 1. Examples of plant-released molecules that are recognized as signals for induction of specific responses in various plant-associated bacteria. Luteolin (A) and genistein (B) are flavonoids that induce the transcription of nodulation genes in various rhizobia. Stachydrine (C) and trigonelline (D) are nonflavonoids that induce *nod* genes in *S. meliloti*. Tetronic acid (E) and erytronic acid (F) activate *nod* expression in *S. meliloti*, *M. loti*, and *R. lupini*. Acetosyringone (G) and ferulate (H) are phenolic compounds that induce virulence genes in *A. tumefaciens*. Shikimate (I) and quinate (J) induce coronatine biosynthetic genes in *P. syringae* pv. tomato DC3000. Quercetin (K) and arbutin (L) are phenolic glycosides that activate the production of syringomycin in *P. syringae* pv. syringae.

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the plant, which are required for entry of the rhizobia into the host (120, 163, 188, 470). The tip of a root hair, to which rhizobia are bound, curls back on itself, trapping the bacteria within a pocket, from which they are taken up into a plant-made intracellular-infection thread. Nod factors also induce cell division and gene expression in the root cortex and pericycle, where they initiate development of the nodule (95, 219, 458, 489).

Nod factors consist of a β-1,4-linked *N*-acetyl-D-glucosamine backbone with four or five residues. Of these, the nonreducing-terminal residue is substituted at the C-2 position with an acyl chain, whose structure and saturation vary in different rhizobia. The oligosaccharide can also have acetyl, sulfonyl, or carbamoyl substitutions at defined positions (95, 119, 162, 248, 416). Nod factors are synthesized and exported from the bacteria by the products of *nod* genes (119). The *nodABC* genes are present in all rhizobia and are required for production of the basic Nod factor (162, 248, 416). Interspecific differences in Nod factors are in part due to allelic variations in *nodABC* genes. In addition, each rhizobium species possesses species-specific *nod* genes, which direct species-specific modifications of the basic Nod factor (95, 119). Each *Rhizobium* species therefore produces a different set of Nod factors, which play a critical role in host specificity (95, 119).

**Structure, function, and ecology of flavonoids.** The *nod* genes are coordinately expressed in response to specific polycyclic aromatic compounds called flavonoids, which are released by plants into the rhizosphere. Identification of *nod* gene inducers began with the construction of *nod-lacZ* reporter fusions and the finding that these fusions were induced by factors released from the host plant (3, 21, 24, 238, 261, 341, 367, 381, 382, 545). These factors were identified as 2-phenyl-1,4-benzopyrone derivatives, collectively called flavonoids. Their structure is defined by two aromatic rings, A and B, and a heterocyclic pyran or pyrone ring, the C ring (Fig. 1A and 1B). Specific modifications of this basic structure produce different classes of flavonoids including chalcones, flavanones, flavones, flavonols, isoflavonoids, coumestans, and anthocyanidines (195, 196). Within each of these classes, there are many further variations on the theme, and so far more than 4,000 different flavonoids have been identified in vascular plants (378). Not all of them, however, are active as inducers of the nodulation genes. Comparisons of structures of different *nod*-inducing flavonoids revealed that hydroxylation at the C-7 and C-4 positions are important for *nod*-inducing activity (98, 418). Host legumes are thought to be discriminated from nonhosts partly on the basis of the specific flavonoids that they release (216, 373).

Plants synthesize flavonoids from phenylpropanoids that enter the flavonoid pathway through the enzyme chalcone synthase (467). Each plant produces a distinct mixture of these molecules, and the quantity and spectrum of flavonoids may vary with the age and physiological state of the plant (196, 444). Some flavonoids are pigmented, providing a full spectrum of colors in flowers, fruit, and leaves (195, 196). Based on the observation that nitrogen limitation enhances flavonoid production, it has been proposed that the flavonoid synthesis is used as a disposal mechanism for extra carbon skeletons produced by deamination of phenylalanine under nitrogen-limiting conditions (90). Flavonoids also have been proposed to play a role in plant defenses or to act as intrinsic growth regulators (195, 196). They are often stored and released from plants as glycosides (attached to one or more sugar moieties), which enhances their solubility and diffusion in water. Glycosides, however, are usually less active in inducing *nod* genes (199). Flavonoids are released in their greatest amounts near

root tips (180, 198, 382), and optimal concentrations occur near the emerging root hair zone, which is the most favorable site for rhizobium infection (553).

**Nonflavonoid inducers of *nod* genes.** In addition to flavonoids, several nonflavonoid *nod* inducers have been identified (Fig. 1C to F). Stachydrine (*N*-methylproline methylbetaine) and trigonelline (nicotinic acid *N*-methylbetaine) were identified from exudates of alfalfa seedlings as inducers of *nod* genes in *S. meliloti* (384). These molecules are quaternary ammonium compounds collectively known as betaines. Betaines are found in many plant tissues exposed to osmotic stress, where they serve as osmoprotectants (75). Both trigonelline and stachydrine have been found in seeds, roots, and root exudates of various legumes (431, 432). They are highly soluble in water and especially abundant on dry legume seeds. The concentrations (in the low millimolar range) of betaines required for *nod* induction are much higher than those of flavonoids (low micromolar range), but their abundance on seed coats overcomes this potential problem (383). Two aldonic acids (tetronic acid and erytronic acid) (Fig. 1E and 1F), as well as some simple phenolics (vanillin, coniferyl alcohol, chlorogenic acid, and ferulic acid) were also identified as natural inducers of *nod* genes in certain rhizobial species (24, 156, 261). The concentrations required for their activity are similar to those of betaines (261).

**Transcriptional regulators of *nod* genes.** Plant-released flavonoids are detected by rhizobia through a variety of NodD proteins, which are members of the LysR family of transcriptional regulators (Table 1) (441): NodD proteins are thought to be direct receptors of the plant-released signals and to be flavonoid-dependent transcriptional activators of *nod* promoters (444). Biochemical analyses of NodD proteins have so far been relatively limited, due to difficulties in protein solubility and purification. The correct folding of at least one NodD requires the chaperone GroESL, and the GroES protein copurifies with NodD (363, 542). The NodD proteins of several species are said to be membrane associated, adding further challenges in purification (443). Recently, however, NodD of *R. leguminosarum* was purified to homogeneity in a soluble form, which may lead to further biochemical and structural studies (134).

In general, LysR-type regulators contain a highly conserved N-terminal DNA binding domain and a less highly conserved C-terminal ligand binding domain (441). The structure of the full-length LysR-type regulator CbnR has been solved by X-ray crystallography (Fig. 2) (342), as have the structures of the C-terminal domains of CysB, DntR, and OxyR (79, 453, 496). The N-terminal domain of CbnR consists of three helices and is followed by two β-strands, which are connected to the C-terminal domain by a long helix that mediates protein dimerization (Fig. 2). Helix 3 in the N-terminal domain is thought to lie perpendicular to the major groove of the *nod* box and to mediate sequence-specific DNA binding. The C-terminal domains of all four crystallized proteins are composed of two subdomains that close together on ligand binding and separate on release of the ligand. This domain has a strong structural resemblance to the family of periplasmic binding proteins that are components of ABC-type permeases. It is highly likely that NodD is structurally similar to the structures of these proteins (Fig. 2). Genetic studies have shown that the C-terminal domain of NodD is involved in the binding of flavonoids (460).

NodD proteins bind to highly conserved DNA motifs called *nod* boxes located directly upstream of the promoters of *nod* operons (427, 444). The length of *nod* boxes (approximately 50 nucleotides) is consistent with NodD binding as a tetramer, as has been demonstrated for other LysR-type proteins (441). Binding of NodD to DNA does not require flavonoids and, in at least one case, causes a bend of the *nod* box sequence (139, 141). Since NodD proteins bind to these sequences in the presence or absence of flavonoids but activate these promoters only in the presence of flavonoids, it is highly likely that these signals cause conformational changes in the bound protein that are required for promoter activity (60, 321). This idea was supported by differences in the footprint patterns between flavonoid induced and uninduced protein extracts (272), as well as by the enhanced in vitro NodD-*nod* box binding and increased strength of the NodD-*nod* box complex formation in the presence of flavonoids (172, 272). Chaperonins GroESL were found to be required for DNA binding of NodD1 and NodD3 of *S. meliloti* and might be involved in further interaction of NodD with other components of the transcription apparatus (140, 363, 542).

Many species of rhizobia possess more than one copy of the *nodD* gene, and the properties of different *nodD* genes vary within the same strain as well as from one *Rhizobium* species to another. Some strains possess two to five copies of *nodD* (133, 179, 444, 504) and may in addition possess one or two copies of another LysR-type regulator gene called *syrM* (symbiotic regulator) (193, 327, 328, 340, 474). SyrM is a NodD homolog and also acts as an activator of *nod* genes. Different NodD proteins may differ in their affinity for various *nod* boxes and may also have different flavonoid specificities. The *nodD* genotype therefore in part determines the host range of a given *Rhizobium* strain. Transfer of a *nodD* gene from one *Rhizobium* species to another can in some cases alter the host range of the recipient to that of the *nodD* donor strain (218, 461), whereas point mutations in *nodD* affect the recognition of inducing molecules and cause extension of the host range (60, 321). In *S. meliloti*, NodD3 and SyrM do not require flavonoids for *nod* gene activation and therefore act in a signal-independent fashion (474). *B. japonicum* possesses a two-component system, NodV-NodW, that is responsive to plant-produced isoflavone signals and functions as a positive regulator of *nod* genes (178, 437).

In addition to activating *nod* promoters, certain NodD proteins repress the expression of their own promoters. In several rhizobia, *nodD* genes are transcribed divergently from nearby *nod* operons, and by binding to the operator sequence between the two operons, these NodD proteins act as repressors of their own expression (444, 460). This autorepression occurs in the presence or absence of flavonoid signals.

Several *nod* regulons, in addition to being positively regulated by NodD proteins, are subject to negative regulation by NolR, a 13-kDa protein that contains a DNA binding motif resembling those of other regulators of the LysR family (92, 273). NolR binds as a dimer to conserved sequences found in the promoter regions of target *nod* genes and prevents their expression. Expression of *nolR* is negatively regulated by its own product and by the *nod* gene inducer luteolin (92).

Several environmental factors, such as calcium, ammonium,

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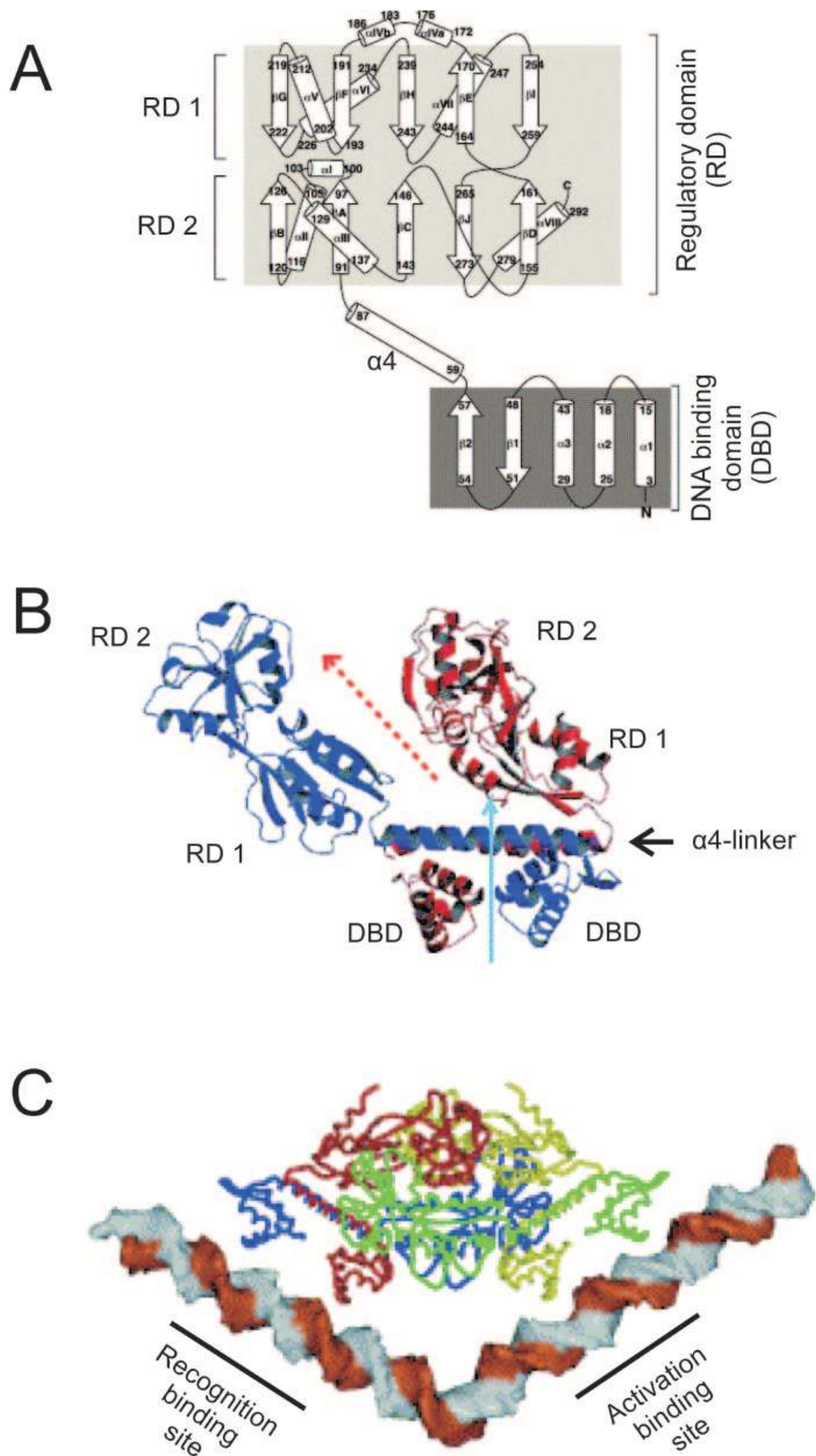


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FIG. 2. Structure of CbnR, a LysR-type regulator homologous to NodD and OccR. (A) Schematic drawing of a subunit of a CbnR tetramer. The subunit can be divided into two domains, the DNA binding domain (DBD) and the regulatory domain (RD), which are connected by the 29 amino acid residues of the linker helix α4. Cylinders and arrows represent α-helices and β-sheets, respectively. (B) Ribbon drawing of a CbnR dimer. The two CbnR subunits, shown in red and blue, are dimerized through the α4 linker helix of each subunit. In the dimer, the two subunits adopt different conformations, such that the two DNA binding domains and the two α4-helices show a two-fold rotational symmetry (blue arrow), while the two regulatory domains are related to each other by a 112° rotation around an axis between them (dotted red arrow). The two axes of

organic acids, and pH, also contribute to *nod* gene expression by unknown mechanisms (408, 409, 444). Low pH has a negative effect on the induction of *nod* genes as well as on rhizobial growth. In addition, plants produce smaller amounts of flavonoids in acidic soil, and flavonoids appear to accumulate in the bacterium in a pH-dependent manner (409, 410). Similarly, elevated levels of combined nitrogen have a negative effect on the production of aromatic compounds in the root, while in *S. meliloti* high levels of ammonia sensed by the Ntr system inhibit the induction of *nod* genes (121, 122). Expression of *nod* genes is also inhibited in the presence of dicarboxylic acids (see below) (544).

**Metabolism of *nod*-inducing signal molecules.** Rhizobia are also able to catabolize *nod* gene inducers. Flavonoid catabolism is initiated by cleavage of the C-ring of the molecule, which yields intermediates that are themselves potential *nod* gene inducers (399). Chalcones are one type of such intermediates and have been reported to be especially potent *nod* gene inducers (399). This feature has been largely attributed to their open C-ring, which offers increased spatial flexibility during interactions with the flavonoid receptor NodD. Genes involved in catabolism of flavonoids have not yet been identified. Trigonelline and stachydrine, on the other hand, are catabolized by products of the *trc* and *stc* genes, respectively, and are also known to act as inducers of these genes. The *trc* and *stc* genes are closely linked to the nodulation and nitrogen fixation genes, and *trc* genes are induced during all stages of symbiosis (173). This suggests that catabolism of trigonelline and stachydrine is important in symbiosis; however, no significant symbiotic defects have been observed in mutants defective in catabolism of trigonelline, whereas mutants with mutations in *stc* genes were delayed in nodulation or were less competitive (43, 61, 385).

### Production of Exopolysaccharides during Nodule Invasion

After bacteria enter a root hair, they begin to travel along an infection thread toward a developing nodule. The initiation and extension of the infection thread depends on the production of specific EPS by the bacteria (30, 149). The three known EPS that are important for symbiosis are a cyclic neutral glucan, succinoglycan, and EPS II. The first of these is encoded by the *ndvAB* operon, while succinoglycan is encoded by a 24-kb cluster of *exo* genes and EPS II is encoded by a 32-kb cluster of *exp* genes (30). In *S. meliloti*, the last two clusters are located on the large symbiotic plasmid pSymB (158).

EPS production depends on the concentration of available phosphate, which might be sensed by the bacteria during the process of nodulation (324, 546). Phosphate concentration is very low in the soil (typically 1 to 10 μM) and considerably higher within plant tissues (10 to 20 mM). EPS II is produced preferentially under low-phosphate conditions, whereas succinoglycan synthesis is stimulated at high concentrations of phosphate (324). This suggests that inside the plant, bacteria produce succinoglycan, which is consistent with the observation that although both EPS can mediate nodule invasion, succinoglycan is much more efficient in this process (375). The mechanism by which phosphate concentration controls the production of EPS is unknown. Several *S. meliloti* regulatory proteins have been identified that are involved in the control of EPS synthesis, but most have not been matched with any signal. The ChvI-ExoS two-component system is involved in the control of both succinoglycan production and flagellum biosynthesis (76, 541). The system is homologous to the *A. tumefaciens* ChvI-ChvG system, which senses environmental acidity (Table 1) (287). It remains to be determined whether the ChvI-ExoS system is involved in the regulation of any acid-inducible genes in *S. meliloti* and whether pH plays a role in infection thread extension and nodule invasion.

### Regulation of Symbiotic Nitrogen Fixation

A successful rhizobium-legume interaction results in the establishment of a root nodule filled with nitrogen-fixing bacteroids. Little is known about the molecular basis of events after the bacteria are released from infection threads into the nodule and before nitrogen fixation begins. Host and bacterial genes have been identified that block symbiosis at these stages, although their roles in these events are unknown (169, 235, 356, 366). In contrast, regulation of nitrogen fixation and signals involved in this regulation have been characterized in great detail (136, 137, 257). Nitrogen fixation is the central physiological process within the nodule and is directed by two sets of genes, *nif* genes and *fix* genes (Fig. 3C) (137, 257). *nif* genes encode structural proteins of the nitrogenase enzyme (*nifHDK*), enzymes involved in biosynthesis of the nitrogenase Fe-Mo cofactor (*nifENB*), the regulatory protein NifA, and proteins of unknown functions that are required for full nitrogenase activity (*nifSWX*). The *fixABCX* genes might, based on their sequence, code for an electron transport chain to nitrogenase. The function of *fixGHIS* is unknown, while *fixNOQP* encode the membrane-bound cytochrome oxidase that is required for respiration of the rhizobia in low-oxygen environments (106, 392). The *fixL*, *fixJ*, and *fixK* genes encode regulatory proteins (see below) (Fig. 3C).

**Regulation of *nif* and *fix* genes by intracellular oxygen tensions.** Oxygen concentration is the major signal controlling the expression of *nif* and *fix* genes (456). Inside a nodule, the concentration of oxygen has to be very low, due to the extreme oxygen sensitivity of nitrogenase (279, 280). However, the colonizing rhizobia need oxygen to generate ATP, which is required in large amounts for the energy-costly nitrogen fixation. Adjacent to the surface of the nodule, an oxygen diffusion barrier made up of a layer of tightly packed plant cortical cells protects the nitrogen-fixing bacteroids in the central zones of

the rotation lie at approximately a 50° angle to each other. (C) Model structure of the CbnR tetramer in complex with bent DNA. The tetramer, which is thought to be the DNA-bound form of CbnR, can be regarded as a dimer of dimers, which are related to each other by a two-fold axis but are each composed of two subunits in different conformations. This unique subunit composition of the tetramer enables the arrangement of the DNA binding domains in a linear fashion, which is required to interact with the long DNA binding sites that are bound by LysR-type proteins. DNA bending seems to be caused by the curved arrangement of the four DNA binding domains in the tetramer. Adapted from reference 342 with permission of the publisher.

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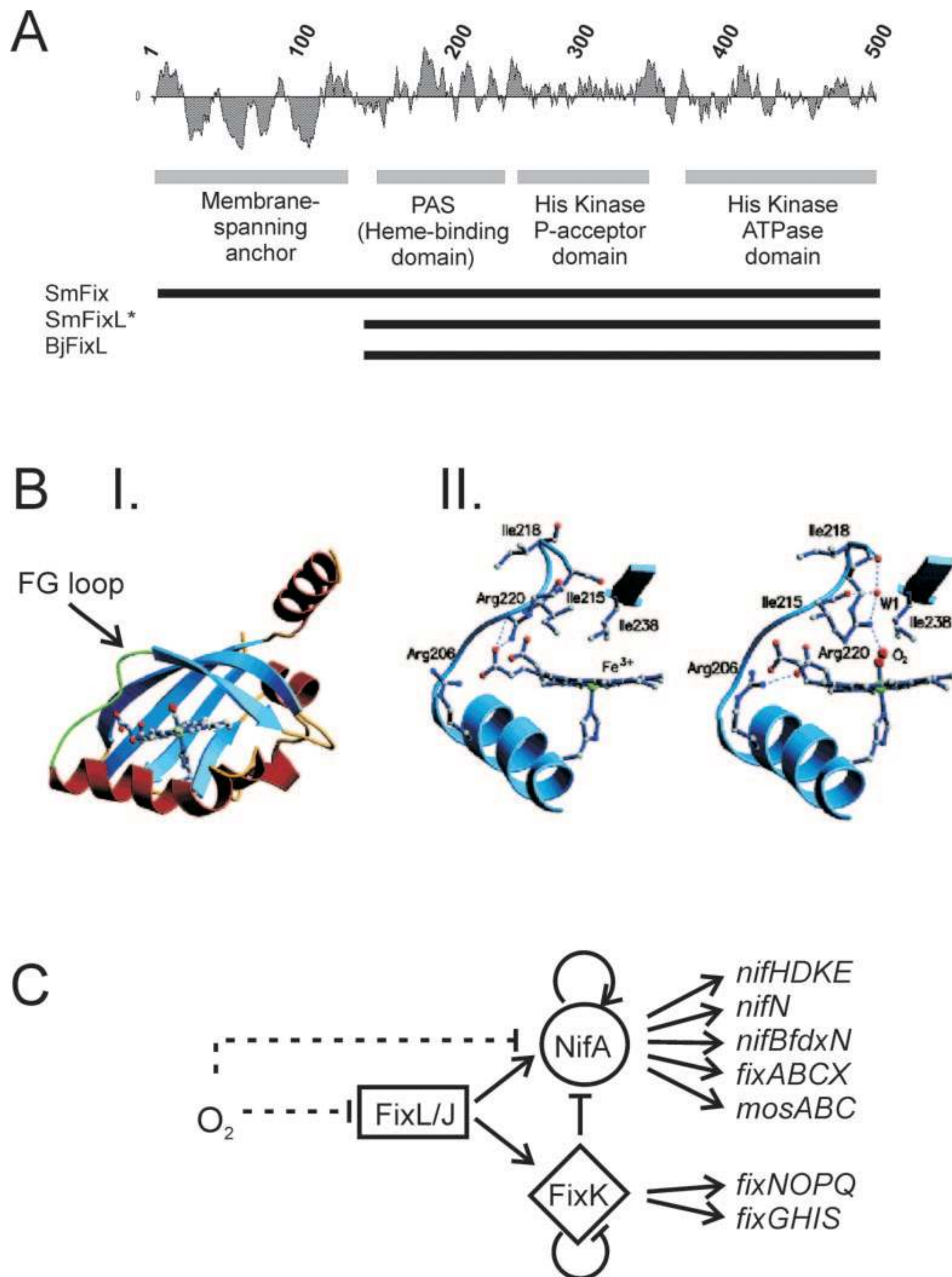


FIG. 3. Structure of the oxygen-sensing two-component kinase FixL, and regulation of rhizobial nitrogen fixation genes. (A) Hydropathy profile of FixL of *S. meliloti*. The hydropathy plot was constructed with the amino acid sequence of the complete FixL protein (SmFixL), which contains four hydrophobic stretches that serve to anchor the protein to the membrane. The periplasmic and membrane-spanning residues are not required for the function of this protein, since a truncated FixL lacking this region is fully functional (SmFixL*). FixL of *B. japonicum* (BjFixL) lacks the membrane-spanning domain. (B) Detection of oxygen by the PAS domain of BjFixL. (Reprinted in part from reference 194 with permission of the publisher). Panel I shows a ribbon diagram of the BjFixL PAS domain complexed with carbon monoxide (CO-BjFixLH). The PAS domain has a glove-like fold consisting of a central β-sheet with flanking α-helices. The heme cofactor lies in the palm of the glove. Panel II shows the BjFixL heme-binding pocket for deoxy-BjFixLH and oxy-BjFixLH. In the unliganded (on) state, the FG loop (residues Thr209 to Arg220) moves close to the heme pocket, while in the oxygen-bound state, the loop is shifted away from the heme pocket. This FG loop shift presumably induces a global conformational change in the full-length protein, which in turn inhibits the kinase activity of the protein. (C) Regulation of nitrogen fixation genes of *S. meliloti* in response to oxygen. Dashed blocked lines represent inhibition of FixL and NifA activity under aerobic conditions. Solid arrows represent transcriptional activation, and blocked solid lines represent transcriptional repression, under microaerobic conditions.

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nodules (484). Plant cells in the nodule also synthesize large amounts of leghemoglobin, which accumulates in their cytoplasm and binds oxygen with an extremely high affinity (12). Its role is to buffer the oxygen concentration and control the diffusion of oxygen to the actively respiring bacteroids. The combination of leghemoglobin and the oxygen diffusion barrier leads to an extremely low concentration of free oxygen around the bacteroids. This concentration was measured to be approximately 25 nM, which is four orders of magnitude lower than normal aerobic conditions (534). The actual signal, therefore, that triggers transcription of nitrogen fixation genes is the drop in levels of oxygen that bacteria experience within the nodule. A gradient of oxygen was shown to exist inside nodules, and the expression pattern of the nitrogen-fixation genes corresponded to the distribution of oxygen along the nodule (456). Oxygen concentrations are sensed by the bacteria through two proteins, FixL and NifA (Table 1). At low oxygen concentrations, these proteins are active and are responsible for induction of genes involved in the fixation of nitrogen.

**Oxygen-controlled regulatory systems. (i) FixL-FixJ.** In *S. meliloti*, the FixL-FixJ two-component system is the master regulator of all *nif* and *fix* genes (2). FixL is a membrane-bound histidine kinase, which in response to low levels of oxygen autophosphorylates and then transfers the phosphoryl group to FixJ (165, 166, 299). Phosphorylated FixJ in turn activates transcription of the regulatory *fixK* and *nifA* genes, whose products regulate transcription of the rest of the nitrogen fixation genes (Fig. 3C) (257).

FixL is directly responsible for detecting intracellular oxygen tensions. It is composed of a membrane-anchoring domain; as well as sensor and kinase domains, both of which are located in the cytoplasm (Fig. 3A) (298). The protein has only a few periplasmic residues (Fig. 3A), and a FixL fragment lacking these and the membrane-spanning residues is fully functional (165, 298, 299). FixL of *B. japonicum* is composed only of sensor and kinase domains and is thus fully cytoplasmic (168). In general, the most common physiological strategy for detecting gases occurs via heme-based sensors (415). Different structures are possible for the heme binding domains in these sensors, and of these, PAS domains are most commonly encountered. The sensor domain of FixL is a prototypical heme-binding PAS domain and is responsible for sensing oxygen. It is approximately 130 residues long and has a predicted $\alpha/\beta$ fold (Fig. 3B) (167). The heme-bound PAS domains can accomplish a ligand-dependent switching of a neighboring transmitter domain, which in the case of FixL is the histidine kinase domain.

The heme binding PAS domain of *B. japonicum* FixL has been crystallized in the presence and absence of oxygen (Fig. 3B) (175, 194). Oxygen binding is thought to cause the movement of a loop away from the heme center, accompanied by a switch in the H bonding of the heme with protein residues (167, 174, 175). It is not yet known whether the movement of the heme causes the conformational change or vice versa, and because of a lack of the three-dimensional structure of the full-length protein, the entire regulatory mechanism remains unknown.

The FixL-FixJ system is one of the few two-component systems whose signal-responsive autophosphorylation and phosphotransfer have been reconstituted in vitro (165, 299). Studies of this system showed that anoxic conditions enhance FixL autophosphorylation whereas phosphorylation of FixJ is independent of oxygen status. More recently, the rate and oxygen sensitivity of FixL autophosphorylation were reported to be greatly enhanced by the presence of the response regulator FixJ, and a model was proposed in which FixL forms a sensing complex with FixJ and ATP. Detection of oxygen and the consequent phosphorylation reactions occur within this complex, after which the phospho-FixJ and ADP are released (492, 493). FixL also possesses a phosphatase activity which is repressed under anoxic conditions (299). Therefore, the antagonistic effect of oxygen on kinase and phosphatase activity of FixL regulates transcriptional activity of FixJ.

FixJ is composed of an N-terminal receiver domain and a C-terminal DNA binding domain. A truncated FixJ containing just the C-terminal domain exhibits high-affinity binding to the *nifA* promoter, whereas unphosphorylated full-length FixJ is inactive (2, 159). Therefore, it seems that under high oxygen concentrations, the unphosphorylated receiver domain inhibits the C-terminal domain, which prevents the protein from binding to DNA. Phosphorylation of the FixJ receiver relieves this inhibition, resulting in the activation of the inherent DNA binding and the activation capacity of the C-terminal domain. At the *fixK* promoter, however, the N-terminal domain of FixJ was shown to contribute positively to transcriptional activation. The domain was required for the recruitment of RNA polymerase to the *fixK* promoter by phosphorylated FixJ (486). Apparently, the mechanism of action of FixJ can vary from promoter to promoter.

**(ii) FixK.** FixK is a regulatory protein whose expression is activated by FixJ in response to low concentrations of oxygen (Fig. 3C) (213, 257). It is homologous to the oxygen-sensing regulator Fnr, which senses oxygen through the Fe-S cluster bound by essential cysteine residues in its N-terminal domain (25, 274). These cysteine residues are absent from FixK, and indeed it has been shown that FixK activity is not subject to direct oxygen control (456). Similar to Fnr, FixK can act either as an activator or as a repressor, depending on the position of its binding site within the target promoter. In *S. meliloti*, FixK activates the transcription of *fixNOQP* and *fixGHIS* operons and negatively regulates its own expression as well as the expression of *nifA* (Fig. 3C) (145, 509).

**(iii) NifA.** Nitrogen fixation genes are also controlled by oxygen at the level of NifA (Fig. 3C). NifA is a transcriptional regulator whose expression and activity are inhibited by high oxygen concentrations (36, 277, 334). The protein is an NtrC homolog and thus works in conjunction with sigma 54 and requires hydrolysis of an ATP molecule to activate transcription. Unlike NtrC, NifA does not contain a receiver domain and therefore does not belong to the family of two-component systems (136, 137). A cysteine-rich motif is located between the central and C-terminal domain of NifA and has been proposed to be involved in the detection of oxygen, possibly via a bound iron atom (138). In the absence of oxygen, NifA activates the expression of its own gene as well as that of the *nifHDKE* and *fixABCX* operons (Fig. 3C) (111, 136, 137). In addition, it induces transcription of genes involved in the synthesis of rhizopines (see below).

**Detection and metabolism of dicarboxylic acids.** In nitrogen-fixing bacteroids, dicarboxylic acids are the major source of

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carbon and energy and are necessary for the generation of ATP and of the reducing power needed for nitrogenase activity (254, 257). In addition, several dicarboxylic acids also inhibit the expression of *nod* genes in *B. japonicum* and may therefore be responsible, at least in part, for the observed reduction in *nod* expression in the bacteroids (442, 544). The negative regulation of *nod* genes seems to be important, since artificial constitutive expression of these genes led to a Fix$^-$ phenotype (270).

Dicarboxylic acids are imported into bacteroids through two transporters. One is of plant origin and is located in the so-called peribacteroid membrane, which encloses the bacteroids within the nodule (102, 497). The other is encoded by the *dctA* gene of rhizobia and is embedded in the inner membrane of the bacteroid (250, 422). In free-living rhizobia, transcription of *dctA* is activated by dicarboxylic acids via the DctB-DctD two-component system (164, 517). DctB was proposed to sense the presence of dicarboxylic acids in the periplasm and to phosphorylate DctD in a signal-dependent manner. DctD is an NtrC-like transcriptional regulator of the *dctA* promoter (164). The DctB-DctD system is, however, not required under symbiotic conditions, which suggests the existence of an alternative regulatory system operating in the bacteroids (257).

### Opine-Like Molecules in *Sinorhizobium*-Plant Interactions

The bacteroids of certain strains of rhizobia produce nutritive compounds called rhizopines, which are consumed by free-living bacteria as sources of carbon, nitrogen, and energy. The role of these compounds is analogous to that of opines made by *Agrobacterium* spp. (see below). So far, only *R. meliloti* and *R. leguminosarum* have been found to produce rhizopines, and within these species, only 11% of *S. meliloti* and 12% of *R. leguminosarum* bv. viciae strains synthesize them. In all but one of these strains, the structure of the rhizopine was identified as 3-*O*-methyl-*scyllo*-inosamine (3-*O*-MSI), while the remaining strain was found to produce a closely related compound, *scyllo*-inosamine (SI) (Fig. 4A and 4B) (109, 346).

Genes involved in rhizopine synthesis (*mos* genes) and those involved in rhizopine catabolism (*moc* genes) are closely linked and in *S. meliloti* are located on the symbiotic megaplasmid pSymA, which also harbors the nitrogen fixation genes (344, 435). Since rhizopines are produced in bacteroids, they are most probably synthesized from plant-derived precursors. Importantly, the *mos* locus is regulated by the symbiotic nitrogen fixation regulator NifA (Table 1), which ensures that the locus is coordinately regulated with nitrogen fixation and controlled by low oxygen levels (345). Rhizopine catabolic genes are expressed in free-living bacteria, with no evidence that they are also expressed in the bacteroid (435). Rhizopine catabolism is highly specific to the strains producing the rhizopines, and these compounds were indeed shown to affect intraspecies competition for nodulation (109, 177, 346). It remains puzzling that relatively few rhizobia are known to synthesize rhizopines. However, it is quite plausible that new classes of rhizopines await discovery, and this phenomenon may be more universal among rhizobia than is currently appreciated.

## HOST DETECTION DURING *AGROBACTERIUM*-PLANT INTERACTIONS

*A. tumefaciens* causes crown gall tumors in a wide variety of dicotyledonous plants by transferring the oncogenic DNA fragments (T-DNA) from the tumor-inducing (Ti) plasmid to individual plant cells (252, 555). Genes located on T-DNA are expressed inside the infected plant cells, and some of them direct the overproduction of plant growth hormones (37, 91). This causes rapid neoplastic proliferation of the infected plant cells, ultimately resulting in the formation of a gall. Other T-DNA-located genes direct the production of amino acid and/or sugar derivatives called opines, which are released from the galls and serve as a source of nutrients for the colonizing bacteria (109). Opines are also recognized by the bacteria as signals that turn on conjugal transfer of the Ti plasmid (see below) (131, 532).

Proteins responsible for T-DNA processing and transfer are encoded in the *vir* region of the Ti plasmid (104, 249, 425, 549). Twenty-one genes in this region are essential for wild-type levels of pathogenesis and are expressed in six operons, *virA*, *virB*, *virC*, *virD*, *virE*, and *virG*. The proteins required for cleavage of the T-DNA borders are encoded by *virD1* and *virD2* (540). VirC1 and VirC2 bind to a site adjacent to T-DNA borders, called overdrive, and are required for efficient T-strand processing (487). The *virB* operon encodes the T-DNA transfer apparatus, which delivers the T-DNA strand with the VirD2 protein bound to its 5′ terminus into the plant cell cytoplasm (66). The transfer process is very similar to bacterial conjugation, and the VirB channel closely resembles a type IV secretion system (80). Once in the plant cytoplasm, the VirE2 protein, which is also transferred to the host cell cytoplasm through the VirB pore, binds tightly and cooperatively to the single-stranded T-strand. VirE1 is required for transfer of VirE2 into the plant cytoplasm and probably acts as an export chaperone for VirE2. Both VirD2 and VirE2 contain nuclear localization sites that mediate transport of the T-strand from the cytoplasm to the nucleus, where the T-DNA is integrated into the plant genome. Other members of the *vir* regulon are not essential for tumorigenesis on all hosts and may be required only in specific hosts or may play other roles in pathogenesis. These include *virD5*, *virE3*, *virF*, *virH*, *virJ*, *virK*, *virL*, *virM*, *virP*, and *virR*. In addition to T-DNA and *vir* genes, the Ti plasmid harbors genes that are involved in the uptake and catabolism of opines, others that are required for replication of the Ti plasmid, and still others that direct the conjugal transfer of the plasmid (549).

### Host Detection and Expression of Genes Required for Infection

***vir* gene inducers.** One key process in *Agrobacterium*-plant interaction involves the induction of virulence genes, which are not expressed in saprophytic bacteria. *vir* genes are induced by plant-released signals, which include specific phenolic compounds and monosaccharides in combination with acidic pH and temperatures below 30°C (Table 1).

Studies of the induction of *vir* genes began with the demonstration that cocultivation of strains carrying a *vir-lacZ* fusion with cultured plant cells or cultured roots caused elevated

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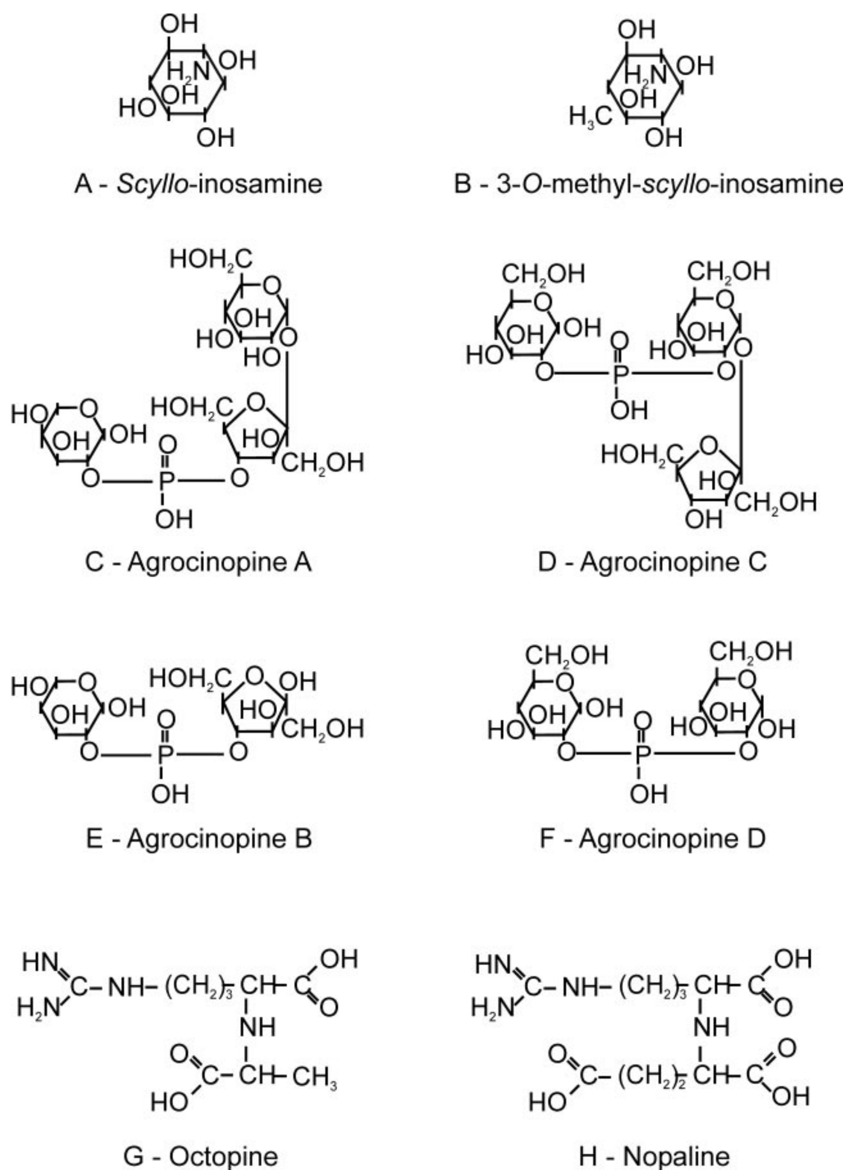


FIG. 4. Structures of rhizopines produced and released by bacteroids of *S. meliloti* and *R. leguminosarum* and of conjugal opines released from tumors induced by *A. tumefaciens*. (A and B) Rhizopines 3-O-MSI (B) and SI (A) are released from nodules infected by *S. meliloti* and *R. leguminosarum*. (C to G) Agrocinopines A and B, agrocinopines C and D, and octopine are the conjugal opines of nopaline-, chrysopine-, and octopine-type *A. tumefaciens* strains. (H) Nopaline and agrocinopines A and B serve as conjugal opines in *A. rhizogenes*.

expression of β-galactosidase (463, 465). Two phenolic compounds, acetosyringone (Fig. 1G) and α-hydroxyacetosyringone, isolated from tobacco root cultures, were the first two compounds identified as specific *vir* gene inducers (464). Comprehensive analyses of many chemical derivatives of acetosyringone showed that *A. tumefaciens* detects numerous related compounds, many of which are ubiquitous or at least widespread among host plants (322, 462). The essential structural features required for *vir*-inducing activity of a compound are a benzene ring with a hydroxyl group at position 4 and a methoxy group at position 3. The presence of another methoxy group at position 5 enhances the activity of the inducer, while a wide variety of substituents at position 1 are tolerated (Fig. 1G and 1H) (322, 464). Relatively high concentrations of the inducing phenolic (5 to 500 μM, depending on the compound) are required for full activation, which may help to account for the relatively low signal specificity of this pathogen (322, 462, 464).

Phenolic-induced expression of *vir* genes is greatly enhanced by specific monosaccharides including arabinose, galactose, galacturonic acid, glucose, glucuronic acid, mannose, fucose, cellobiose, and xylose (11). Most of these sugars are monomers of plant cell wall polysaccharides or are otherwise involved in plant metabolism. Their effect on *vir* induction is especially pronounced at low concentrations of the inducing phenolic. Galacturonic and glucuronic acid were reported to have the strongest activity, having an effect at concentrations as low as 100 μM (11). Acidity and temperature are also important for *vir* induction; activation of *vir* gene transcription occurs only in

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acidic environments in the pH range of 5.2 to 5.7 (465). Similarly, tumor formation on several host species is optimal at 22°C and does not occur at temperatures above 29°C (49). The temperature sensitivity of tumor formation was correlated with that of *vir* gene expression, which does not occur at temperatures above 32°C (9, 322).

The combination of phenolics, monosaccharides, and acidity that is required for induction of the *vir* regulon is thought to reflect the chemical components of wound sap. It is thought that these metabolites are released in largest amounts from plant wound sites, specifically from cells that are undergoing lignin synthesis or cell wall repair. The *vir*-inducing phenolics accumulate at wound sites as precursors of lignin biosynthesis, which is required for wound healing (112, 116). In addition, they may play a role in protection against potential pathogens (112). The inducing sugars are present at a wound site as degradation products of plant cell wall polysaccharides and may be generated by both mechanical means and the enzymatic activity of the cell wall glycosidases. Wound sap also tends to be acidic due to acidic compounds (e.g., phenolic acids and acidic monosaccharides) that are released from plant cell vacuoles (116). Wounding may be caused by foreign agents, such as herbivores or frost, or may be the result of tissue damage occurring during normal plant growth, for example the cracks produced at the site of emergence of side roots.

The idea that release of *vir* gene inducers requires wounding is appealing, since wounds are generally thought to be required for tumorigenesis (253). However, this idea may be flawed in several ways. First, acetosyringone and α-hydroxyacetosyringone were first isolated not from plant wounds but, rather, from cultured cells and cultured, unwounded roots (464). Their concentration was reported to increase on wounding (464). There are few if any other studies demonstrating that phenolics are released preferentially from wound sites, and these same compounds have been detected from unwounded tobacco seedlings (unpublished data). Second, monosaccharides are, as described above, released from unwounded roots (99, 309). Finally, the pH of apoplastic fluids is acidic and roots generally acidify the adjacent soil, possibly in order to increase the solubility of phosphate (309). It may therefore be necessary to question whether these signals are really "wound released" and to think of them instead as being "plant released." Furthermore, wounding may not be essential for tumorigenesis after all. A study in which *vir*-induced bacteria were sprayed onto tobacco plantlets demonstrated that cells in unwounded plants could also be efficiently transformed (129), suggesting that the proposed plant responses evoked by wounding (enhanced cell division and DNA replication) are not essential for transformation.

**VirA-VirG-ChvE regulators of *vir* genes.** VirA and VirG proteins compose a two-component system that is required for the induction of *A. tumefaciens vir* genes in response to the plant-released signals (Fig. 5) (466). VirA is a membrane-spanning histidine kinase (284), and in the presence of signal it phosphorylates the response regulator VirG, which in turn activates the transcription of *vir* genes (208, 469, 530, 531).

VirA is a dimer in both the presence and absence of inducing stimuli (368); it is composed of four domains: the periplasmic domain and the cytoplasmically located linker, kinase, and receiver domains (Fig. 5) (70, 284). Based on the nuclear

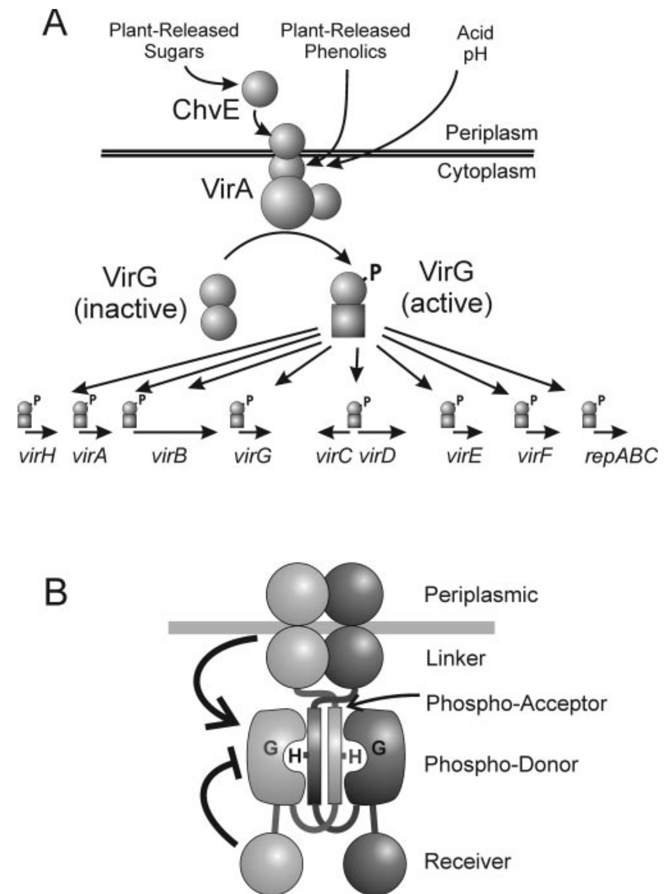


FIG. 5. Regulation of *A. tumefaciens* virulence genes by plant-released signals. (A) The *vir*-gene regulatory cascade involving the VirA-VirG two-component system and ChvE sugar binding protein. VirA is a transmembrane histidine kinase that detects plant-released phenolics, sugars, and acidity and phosphorylates the DNA binding response regulator VirG. Acidic pH and phenolics are detected by the linker domain of VirA, while sugars are detected by the chromosomally encoded ChvE protein. ChvE is a periplasmic sugar binding protein that makes contacts with the VirA periplasmic domain, enhancing the phenolic-induced activity of VirA. The phosphorylated VirG activates the transcription of *vir* genes and of the *repABC* operon, which is required for replication of the pTi plasmid. (B) Model for regulation of kinase activity of a VirA dimer. VirA histidine kinase is a dimer in its native form (368). Each of its subunits is composed of the periplasmic, linker, kinase, and receiver domains. Each kinase domain is further divided into the phospho-donor subdomain, which contains the ATP binding site, and the phospho-acceptor subdomain, which contains the conserved His residue that is the site of autophosphorylation. Within a VirA dimer, the phospho-donor subdomain of each VirA subunit interacts with the phospho-acceptor domain of the opposite subunit. Environmental signals are transmitted from the periplasmic and linker domains of one subunit to a complex containing the phospho-donor subdomain of the same subunit and the phospho-acceptor domain of the opposite subunit. Similarly, the receiver domain of each subunit inhibits the complex composed of the phospho-donor subdomain of the same subunit and the phospho-acceptor domain of the opposite subunit. Adapted from reference 51 with permission of the publisher.

magnetic resonance spectroscopy structure and biochemical studies of the homologous EnvZ protein, the kinase domain of VirA is thought to be composed of two subdomains (480, 485). The C-terminal phosphodonor subdomain contains the conserved G-box that forms an ATP-binding pocket, whereas the

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N-terminal phosphoacceptor subdomain contains the conserved histidine residue that serves as the site of autophosphorylation (Fig. 5B). The phosphoacceptor subdomain is composed of two antiparallel α-helices and also serves as the dimerization domain of the protein (485). The two kinase domains of the VirA dimer, long thought to undergo autophosphorylation, have recently been shown to undergo intradimer transphosphorylation (Fig. 5B) (51). This was demonstrated by constructing merodiploid strains containing two mutant *virA* alleles. One allele was defective at the site of autophosphorylation, while the other was defective in the ATP binding site. This strain expressed functional VirA, presumably due to the formation of functional heterodimers (51).

The activity of the VirA kinase domain is influenced by the inducing stimuli, which are detected by the periplasmic and linker domains. The periplasmic domain is responsible for detecting monosaccharides. Mutant VirA proteins whose periplasmic and membrane-spanning regions have been removed are unable to detect the monosaccharide signal but can still detect the phenolic signal and acidity (70). As described above, ChvE is a periplasmic sugar binding protein required for chemotaxis toward and uptake of monosaccharides (208, 220). Mutants with mutations in this protein fail to detect the monosaccharide stimulus but remain responsive to phenolic compounds. A genetic analysis using suppressor mutations has demonstrated that ChvE directly interacts with the VirA periplasmic domain (450), suggesting that sugars are detected by VirA indirectly through the ChvE protein. *A. tumefaciens* strains containing a mutation in *chvE* or in the VirA periplasmic domain are defective in *vir* gene induction and have limited host ranges compared with those of wild-type strains (115). Since the sugar stimulus is detected by ChvE and the periplasmic domain of VirA, this signal must be transduced across the cytoplasmic membrane to reach the kinase domain. The membrane-spanning region between the periplasmic and linker domains (TM2) is predicted to have a helical structure and has been implicated in transmitting the sugar signal from the periplasmic domain across the membrane (516).

Phenolics and acidity are detected by the VirA linker domain. This is based on the observation that a truncated VirA protein containing just the linker and kinase domains (and lacking the periplasmic domain, the transmembrane regions, and the receiver domain) is able to detect phenolics and acidity in vivo (70). Sites required for detection of the phenolics are scattered over the entire linker domain, suggesting that a topological feature formed by the linker may be crucial for detection of and response to these compounds (488). The same study also showed that only one functional linker per VirA dimer is required for detection of the phenolic signal and that detection of this signal by the linker domain of one subunit of a VirA dimer activates the kinase domain of the opposite subunit (Fig. 5B) (488).

There is some controversy about whether VirA detects the phenolic signal directly or whether a separate phenolic binding protein detects phenolics and then communicates the signal to VirA. In one study, a radiolabeled phenolic compound failed to bind VirA but instead bound two small proteins (281). However, genetic evidence suggests that VirA is the direct receptor. VirA proteins of different *A. tumefaciens* strains, when expressed in identical genetic backgrounds, recognized different phenolic compounds (282, 283). In another study, phenolic-responsive *vir* gene expression was reconstituted in an *Escherichia coli* strain containing just *virA*, *virG*, *chvE*, and the RNA polymerase α-subunit gene *rpoA* (296, 297), indicating either that VirA is the direct receptor or that a separate phenolic binding protein is conserved between *E. coli* and *A. tumefaciens*. Conclusive proof that VirA is the direct phenolic receptor will require biochemical reconstitution of this system using purified proteins.

VirA appears also to be at least in part responsible for the thermosensitivity of tumor formation. At temperatures of 32°C and higher, this molecule undergoes a reversible inactivation whereas the VirG protein is not affected (245). Furthermore, *vir* gene induction was temperature sensitive in a constitutive *virA* mutant but not in a constitutive *virG* mutant. This signaling system showed the same temperature optimum when reconstituted in *E. coli* (297).

As described above, the activity of the VirA kinase domain is influenced in response to environmental signals; however, it is still not clear which step in the VirA-mediated VirG phosphorylation is enhanced by the inducing stimuli. Possible steps include ATP binding, autophosphorylation, and phosphotransfer to VirG. In one study, autophosphorylation of a purified VirA fragment occurred in the absence of phenolics, although this fragment lacked intact periplasmic and linker domains (243). In a later study, this same truncated VirA was able to phosphorylate VirG independently of phenolics and failed to dephosphorylate phospho-VirG (242). However, a recent genetic study has shown that in the absence of phenolics, wild-type VirA strongly inhibits the activity of several constitutive VirA alleles (51), suggesting that VirA is a potent phospho-VirG phosphatase. This inhibitory activity appeared to be modulated by plant-released stimuli and could also be the target of the phenolic regulation of VirA. The conserved autophosphorylation and ATP binding sites were dispensable for this activity, while the receiver domain was required (51).

VirA mutants lacking the C-terminal receiver domain are hyperactive, indicating that the receiver plays an inhibitory role, possibly by competing against VirG for access to the kinase active site (70). Recently, the two receivers of a VirA dimer were shown to act independently, each inhibiting the phosphoacceptor subdomain of the opposite VirA subunit (Fig. 5B) (51).

Phosphorylation of VirG at the conserved residue Asp52 converts this protein to a form capable of activating transcription of *vir* genes (208, 242). VirG binds to *vir* boxes, which are found upstream of each target promoter, generally centered approximately 40 to 80 nucleotides upstream of the transcription start sites (244). The DNA binding helix-turn-helix motif is located in the C-terminal domain of VirG, and this domain can bind to a *vir* box in the absence of the N-terminal receiver domain (388). It is not known whether the C-terminal domain can also activate transcription.

A constitutive VirG mutant, VirG(N54D) (191, 192, 246, 374, 439), binds to *vir* box DNA approximately 10-fold more tightly than does wild-type VirG (192). Since the mutation introduces a new negative charge adjacent to the site of phosphorylation, it is thought that this mutation mimics the phosphorylated form of the protein. If so, this result indicates that phosphorylation increases the affinity of VirG for *vir* box DNA, possibly by promoting the formation of VirG multimers or by

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causing a conformational change in the C-terminal domain. It is not clear, however, whether the increased DNA binding affinity and dimerization of VirG are sufficient to activate transcription, since phosphorylation might also cause changes in the ability of this protein to interact with RNA polymerase.

**Metabolism of phenolic compounds.** Phenolic compounds, the major signal required for induction of virulence genes, are metabolized by the product of one of the *vir* genes, namely, VirH2 (50, 256). VirH2 is not required for tumorigenesis. It belongs to the family of P-450-type cytochromes and catalyzes the O demethylation of phenolic inducers. The products of these reactions are completely inactive as *vir* gene inducers. They are also far less toxic than the substrates. Furthermore, some of them can be catabolized via the β-ketoadipate pathway and serve as sole sources of carbon and energy. VirH2 therefore plays a role in quenching of the *vir*-inducing signals, in detoxification of the infection site, and in utilization of phenolic compounds as nutrients for growth. All these traits might in be important for survival of the bacteria at the site of infection.

**Other regulators of *vir* genes.** Although all *vir* genes are activated by phospho-VirG, some *vir* promoters are also regulated by other proteins. The *virG* gene is one example. It is expressed from two promoters, and only the upstream promoter is activated by phospho-VirG in response to phenolics. However, the same promoter is also activated rather strongly by phosphate starvation, and sequence analysis suggests that this regulation might occur via orthologs of the PhoR-PhoB proteins of enteric bacteria (530). The downstream *virG* promoter is activated by acidic pH, acting via the ChvG-ChvI two-component system (305). These latter stimuli are thought to "prime the pump," that is, to increase the pool size of VirG sufficiently that positive autoregulation can occur. The divergent *virC* and *virD* promoters are also affected by additional regulators. They are repressed by the Ros repressor (82, 83, 478), which is orthologous to the MucR regulator of *S. meliloti* (264). The *virC* and *virD* promoters are partially constitutive in Ros mutants, even in the absence of *vir* gene induction stimuli. The significance of this dual control is unclear.

### Detection of Opines Released by Crown Gall Tumors

After the establishment of a crown gall tumor, the transformed plant cells produce and release amino acid and sugar derivatives called opines, which serve the colonizing bacteria as sources of carbon and energy and, in some cases, as sources of nitrogen and phosphorous (109, 549). Most or possibly all *A. tumefaciens* strains cause their hosts to synthesize more than one opine, and their Ti plasmids direct the uptake and catabolism of the cognate opines (109, 549). Moreover, these bacteria are chemotactic toward opines and chemotaxis is dependent on periplasmic binding proteins that are associated with opine uptake systems (267). Therefore, each strain is attracted only to opines that are released from the plant infected by these bacteria. The opine uptake systems are members of the ABC-type high-affinity permeases (549).

At present, over 20 different opines have been described (Fig. 4) (78, 109). They can be divided into distinct opine families; opines of the octopine family are synthesized in a reductive condensation of pyruvate with arginine, ornithine, lysine, or histidine. Opines of the nopaline family are synthesized in a condensation of α-ketoglutarate and either arginine or ornithine. Mannityl opines are made by condensation of glucose with glutamine or glutamic acid followed by reduction of the sugar to form mannopine or mannopinic acid, respectively. Mannopine is enzymatically lactonized to make agropine and also undergoes spontaneous lactonization to form agropinic acid. A family of Amadori opines is very similar to the mannityl opines and consists of chrysopine, santhopine, and isochrysopine. They are derived from the condensation of glucose with glutamine followed by Amadori rearrangement. Agrocinopines are sugar phosphodiesters; agrocinopine A and B are made by condensation of arabinose with sucrose or fructose, whereas agrocinopine C and D are made by condensation of glucose and sucrose or fructose.

Each type of opine is detected by a different regulatory protein and induces the transcription of genes required for its uptake and catabolism (Table 1). In addition, a subset of opines, known as conjugal opines, also induce the transcription of genes required for conjugal transfer of Ti plasmid (109, 131). The fact that these opines induce more than just their own catabolism genes means that they ought properly to be considered host-released signal molecules. Although most Ti plasmids code for the production and utilization of two or more opine types, usually only one serves as the conjugal signal (109, 131).

Opine catabolism is directly controlled by opine-responsive transcription factors, whereas conjugation is regulated in a more indirect manner (131). Certain opine-binding transcription factors regulate operons that contain opine uptake and catabolic genes, as well as a gene called *traR* (Fig. 6B). TraR is a LuxR-type protein and is the direct regulator of genes involved in conjugation of the Ti plasmid (549, 551). TraR activity requires the diffusible ligand 3-oxooctanoyl-L-homoserine lactone, which is synthesized by TraI, also encoded on Ti plasmids (153). The target *tra* and *trb* genes are induced in a cell density-dependent manner, a phenomenon referred to as autoinduction or sometimes referred to as quorum sensing (QS) (154). As a consequence, conjugal transfer is regulated by two stimuli: host-released opines and the population density of the donor population. Although the overall strategy by which opines control their catabolism and pTi conjugal transfer is conserved among Ti plasmids, the nature of the regulatory proteins and genetic organization of target operons are completely different among different Ti plasmids (Fig. 6B) (386). This indicates that control of conjugation via opines must have arisen multiple times and must be very important to Ti plasmid biology and ecology.

T-DNA of octopine-type Ti plasmids directs the synthesis of at least eight different opines, belonging to four different opine families (549). On release from a tumor, each of these opines is recognized by *A. tumefaciens* through a different type of a regulatory protein that induces transcription of the cognate catabolic genes. Octopine-type opines, which also serve as conjugal opines of octopine-type strains, are recognized through a LysR-type regulator, OccR, resulting in the induction of *occ* genes (152, 187, 548). Mannopine and agropine induce the expression of *moc* genes, probably via the MocR protein, which resembles the LacI repressor of *E. coli*. Expression of the *aga*

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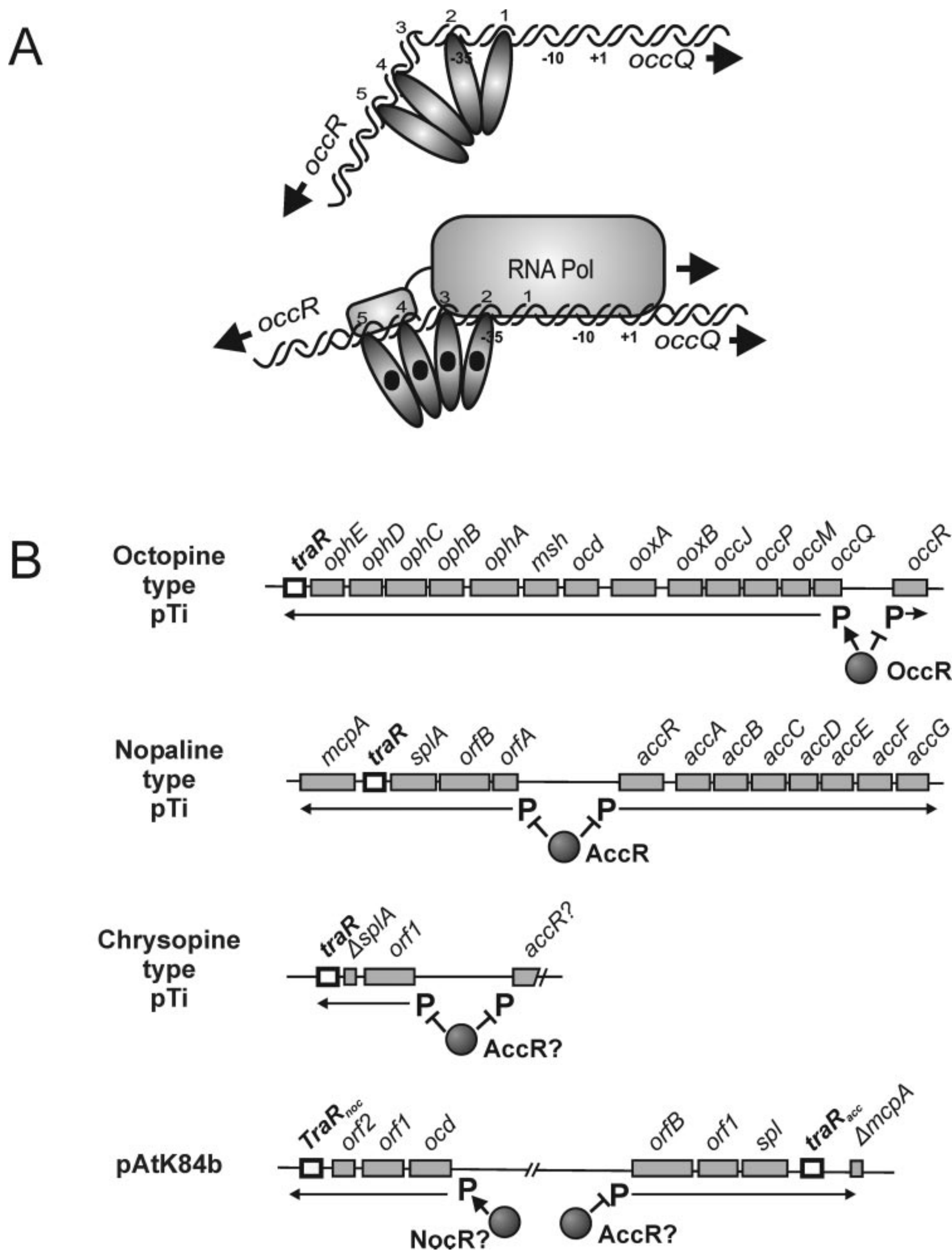


FIG. 6. Regulation and organization of conjugal opine-regulated operons in different types of Ti plasmids. (A) Model for OccR activation of the *occQ* promoter. In the absence of octopine, OccR binds four nonconsecutive major grooves (sites 1, 2, 4, and 5), resulting in a 60-nucleotide footprint and a high-angle DNA bend. Octopine (represented by black dots) causes one dimer to shift position by one helical turn (from sites 1 and 2 to sites 2 and 3), shortening the footprint and relaxing the DNA bend. This conformation allows stimulatory contacts with RNA polymerase that are not permitted by the elongated conformation. OccR binds at all times to the high-affinity subsite (sites 4 and 5). (B) Location of *traR* in the opine-regulated operons on different *Agrobacterium* Ti plasmids. For octopine-type Ti plasmids, the *occ* operons of pTiR10, pTiA6, pAch5, and pTi15955 are regulated by octopine via a LysR-type regulator OccR. *traR* is the last gene of this 14-gene operon. Gene organization and designations are as described in reference 152. For nopaline-type Ti plasmids, the *acc* and *arc* operons of pTiC58 are regulated by agrocinopines A and B via the repressor AccR. *traR* is the fourth gene of the *arc* operon (386). For chrysopine-type Ti plasmids, the *arc* operon and a putative *acc* region of pTiChry5 are presumably regulated by the product of $accR_{Chry5}$, which, based on the incomplete sequence, could code for a protein related to AccR of pTiC58. $traR_{Chry5}$ is part of a two-gene *arc* operon (364). For plasmid pAtK84, the *nox* and *arc* operons of pAtK84b are regulated by nopaline and agrocinopines A and B, respectively. $traR_{noc}$ and $traR_{acc}$ lie within the *nox* and *arc* operons, respectively (365).

and *moa* genes is induced by agropinic and mannopinic acid, respectively, and is mediated by the MoaR repressor (304).

The OccR protein is thought to bind octopine through its C-terminal domain, whereas the N-terminal domain has a DNA binding motif and is probably involved in transcriptional regulation of target genes. As in the case for NodD, the structure of OccR is likely to resemble that of the homologous CbnR, whose full-length structure has been determined by

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X-ray crystallography (Fig. 2) (342). In the presence or absence of signal, OccR binds as a tetramer to an operator that lies between the *occR* gene and the divergently transcribed *occQ* operon (4, 513). The latter contains 14 genes, the last of which is *traR* (Fig. 6B) (152). In the absence of octopine, the protein binds five helical turns of DNA (the region from −80 to −28 upstream of the *occQ* transcription start site) and causes a high-angle bend at the center of this binding site (Fig. 6A) (513). In the presence of octopine, the conformation of OccR changes such that the protected DNA region shrinks to four helical turns (an interval from −80 to −38) and the angle of the bend is dramatically decreased (514). This conformation induces transcription of the *occQ* operon, while both conformations of OccR autorepress the divergent *occR* gene (Fig. 6A) (513, 514).

Tumors induced by strains harboring nopaline-type Ti plasmids produce nopaline, nopalinic acid, and agrocinopines A and B. Nopaline and nopalinic acid resemble octopine; the nopaline synthase, nopaline detection protein (NocR), and nopaline uptake and catabolic proteins strongly resemble their octopine counterparts (109, 307, 308). NocR has not been studied as extensively as OccR but appears to function by a similar mechanism (307, 308).

Agrocinopines A and B serve as the conjugal opines of nopaline-type *Agrobacterium* strains (Fig. 4C and E). They are detected by AccR, which is related to the LacI repressor of *E. coli* (31). AccR is encoded by the first gene of the *acc* operon (Fig. 6B) and represses this operon in the absence of the cognate opine (266). Purified AccR binds to DNA upstream of *accR*, and the addition of agrocinopines abolishes binding (131). AccR also regulates the expression of the adjacent, divergently oriented *arc* operon, of which *traR* is a member (386). Thus, in the absence of the conjugal opine, AccR represses the expression of *arc*, and therefore neither *traR* nor the *tra* regulon is expressed (Fig. 6B) (131).

Tumors induced by *A. tumefaciens* strains harboring a chrysopine-type Ti plasmid, pTiChry5, produce agrocinopines C and D, succinamopine, and a set of Amadori-type opines (78). Agrocinopines C and D induce the conjugation of pTiChry5 and are thus considered to be the conjugal opines of this system (Fig. 4D and F) (364). The two opines also induce the expression of the *traR* gene, which has been identified as a part of a two-gene operon called *arc* (Fig. 6B) (364). The mechanism by which agrocinopines C and D control *traR* is unknown. An open reading frame has been identified that could code for a protein that is very similar to AccR of a nopaline-type Ti plasmid and thus is likely to function as a regulator of the *arc* operon (Fig. 6B) (364). Agrocinopines C and D are also known to induce the transfer of an agropine-type Ti plasmid pTiBo542; however, at present, no information is available about the regulation and organization of genes involved in this process (125).

Plasmid pAtK84b is a catabolic plasmid found in the nonpathogenic *A. radiobacter* isolate K84. It encodes the catabolism of nopaline and agrocinopines A and B (Fig. 4C, E, and H), thus resembling nopaline-type Ti plasmids (81, 202, 203). However, pAtK84b lacks the *vir* regulon and the T-DNA region and therefore does not confer tumorigenicity on its host. However, it enables its host to utilize opines produced by tumors induced by other agrobacteria (81, 109). pAtK84b contains two copies of *traR* (Fig. 6B) (365). One copy, $traR_{noc}$, is the last gene of the *nox* operon and is induced by nopaline but not by agrocinopines A and B (Fig. 6B). The second copy, $traR_{acc}$, is located in an operon of four genes and is induced by agrocinopines A and B but not by nopaline (Fig. 6B). Conjugal transfer of pAtK84b can be induced by nopaline as well as by agrocinopines A and B, and each of the two *traR* genes is required for induction of transfer by the cognate opine (365). It is likely that both TraR paralogs activate the single *tra* regulon.

The octopine-type Ti plasmids pTi15955 and pTiR10 also contain two independently regulated *traR*-type genes (549). However, one of these genes, called *trlR*, codes for a mutant protein that lacks the C-terminal DNA binding domain and consequently fails to activate transcription. The product of this gene is induced by mannopine, a member of the mannityl opine family, and strongly inhibits the activity of TraR by forming inactive heterodimers (69, 550).

## PRODUCTION OF PECTINOLYTIC ENZYMES BY SOFT ROT ERWINIAS

The enterobacteria *Erwinia chrysanthemi* and *E. carotovora* belong to the soft rot group of *Erwinia* species, which cause tissue-macerating or soft-rotting diseases in susceptible plants (85, 377). The maceration process involves depolymerization of plant cell walls, which requires a combination of extracellular enzymes: pectate lyases (Pel), pectin methylesterases (Pem), a pectin lyase (Pnl), polygalacturonases (Peh), a cellulase (Cel), and a protease (Prt). All classes of these enzymes except proteases are secreted into the external medium via a common (type II) secretion system, which is essential for pathogenicity and is encoded in the out operon (436). Proteases are secreted via a type I pathway resembling that of the alpha hemolysin of *E. coli* (23, 200). Pectate lyases play the major role in pectinolysis and hence also in the soft-rot symptoms. *E. chrysanthemi* strain 3937 has five major pectate lyase isoenzymes, encoded by the *pelA*, *pelB*, *pelC*, *pelD*, and *pelE* genes. These genes are organized in two clusters, *pelADE* and *pelBC*. PelA, PelD, and PelE are closely related, as are PelB and PelC, suggesting that recent gene duplications have occurred (225, 479). Each *pel* gene, however, is transcribed from its own promoter, and no single *pel* gene is essential for virulence (29, 412). In addition to PelA, PelB, PelC, PelD, and PelE, *E. chrysanthemi* produces another set of pectate lyases that are capable of macerating plant tissues. They were found when deletion of the major *pel* genes of *E. chrysanthemi* failed to eliminate tissue maceration (29, 263, 412). Three of these genes, *pelL*, *pelI*, and *pelZ*, belong to three different families of pectate lyases (189, 240, 301, 449).

### Detection of Pectin and Pectin Catabolic Products

Pectin oligomers are the main signal required for induction of the genes involved in pectinolysis. Expression of these genes is also inducible by demethylated derivatives, of pectin, polygalactoronate (PGA), or the monomer galacturonate. The analysis of mutants that are deficient in each step of the pectinolytic pathway allowed the identification of the true intracellular inducers, namely, KDG (2-keto-3-deoxygluconate), DKI (5-keto-4-deoxyuronate), and DKII (2,5-diketo-3-deoxygluconate

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(74, 84, 88, 226, 405). These compounds are the breakdown products of the long-chain pectate polymers, presumably generated by the basal level of pectate lyases (490).

**KdgR (RexZ).** Expression of genes required for the degradation of pectate polymers is repressed by three independent repressors, KdgR, considered here, and by the PecS and PecT (described below) (Fig. 7A; Table 1). A triple *kdgR-pecS-pecT* mutant synthesizes more pectate lyase than do any of the single or double mutants, suggesting that the three proteins regulate *pel* expression via independent regulatory networks (473). KdgR is responsible for the induction of pectinase genes by pectin and its metabolites (89, 223, 351, 352), while signals recognized by PecS or PecT have not yet been identified (Fig. 7A).

KdgR is a global regulator of genes involved in pectin catabolism and of the out system required for secretion of the extracellular enzymes (87). It resembles other regulatory proteins controlling catabolic pathways, such as GlyR of *Streptomyces coelicolor*, IclR of enterics, and PobR of *Acinetobacter calcoaceticus* (403). In the absence of the inducing signal, KdgR acts as a repressor (351, 352). The protein binds as a dimer to 25-bp DNA regions that overlap or reside close to the −35 or −10 regions of the putative promoters and thus competes with RNA polymerase for binding to these sequences. Purified KdgR-DNA complexes can be dissociated by KDG (352). A number of KDG analogs were tested as inducers, and all the inducing molecules contained the motif COOH-CO-$CH_2$-CHOH-C-C in a pyranic cycle (349). This motif is also found in DKI and DKII, but a direct interaction of these two compounds with KdgR has not been verified.

Studies of KdgR have been done mostly with *E. chrysanthemi*, but *E. carotovora* KdgR has been shown to act similarly (313, 483). A KdgR homolog, RexZ, has also been identified and characterized in *E. carotovora*. Unlike KdgR, RexZ is an activator of exoenzyme production (483). RexZ orthologs were also identified in all species of *Erwinia* tested, including *E. chysanthemi* EC16 and 3937, *E. carotovora* subsp. *atroseptica*, *E. amylovora*, and *E. herbicola*.

**PecS and PecM.** Inactivation of the *E. chrysanthemi* 3937 *pecS* gene results in derepressed synthesis of genes required for pectate catabolism (*pel*, *cel*, and *out*) and of genes involved in the production of a blue pigment, indigoidine (*ind*) (404). Mutants impaired in indigoidine production are unable to cause systemic invasion of a host plant, and indigoidine production conferred an increased resistance to oxidative stress, indicating that this compound may protect the bacteria against the reactive oxygen species generated during the plant defense response (406).

The PecS repressor is a member of a family of small (about 20-kDa) regulatory proteins that includes EmrR, SlyA, MarR, and HpcR (389). PecS acts as a dimer and binds the promoter regions of its target genes (*pelA*, *pelE*, *pelL*, *celZ*, *outC*, *indA*, and *indC*) (389, 406). The binding affinity, however, is relatively low, suggesting that either a cofactor or postranslational modifications might be required to increase PecS affinity and specificity. In general, the PecS affinity for different regulatory sites reflects the efficiency of the PecS regulation in vivo (389). PecS binding sites either overlap with (for example, *pelE*) or are located downstream of (*pelA* and *celZ*) the promoters of the controlled genes (389). Scanning of the *E. chrysanthemi*

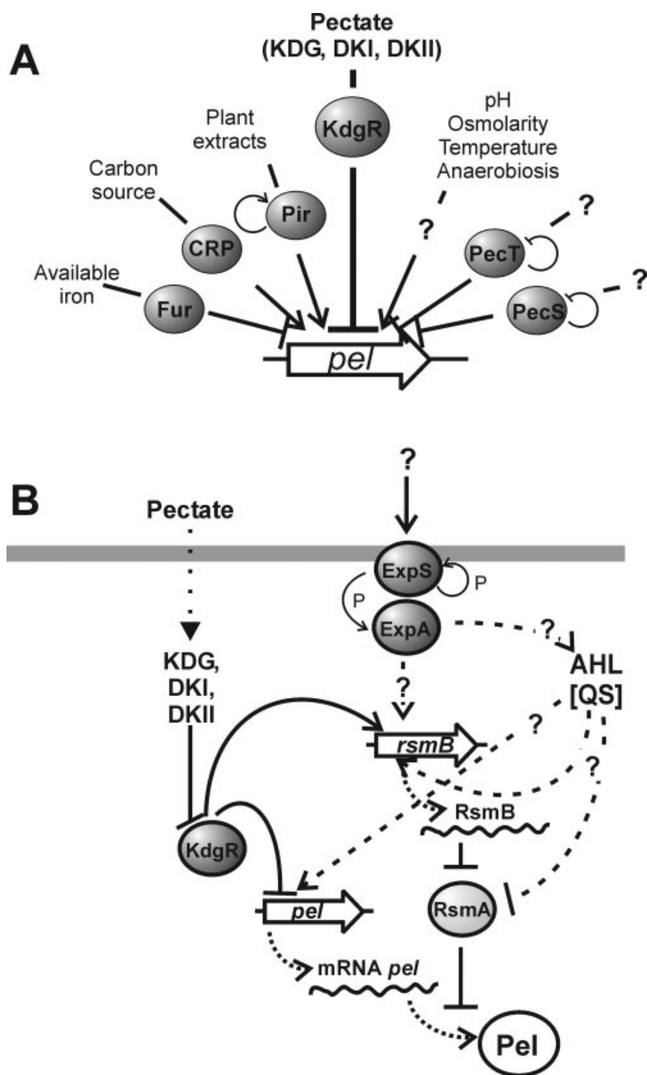


FIG. 7. Regulation of the production of pectinolytic (Pel) exoenzymes in soft rot erwinias. (A) Signals and regulatory proteins involved in regulation of expression of pectinolytic enzymes in *E. chrysanthemi*. Several environmental signals such as pH, temperature, and osmolarity affect the expression of the exoenzyme genes; however, it is unknown how these signals are recognized and integrated. PecT and PecS are transcriptional regulators of exoenzyme genes, but the signals to which they respond are unknown. See the text for details. (B) Interaction between KdgR repressor and global regulatory system ExpS-ExpA in regulation of exoenzyme production in *E. carotovora*. Synthesis of extracellular enzymes in *E. carotovora* is under the control of a global regulatory two-component system, ExpS-ExpA, which is homologous to the GacS-GacA systems found in various proteobacteria. Based on the current model (211, 247), activation of the sensor kinase ExpS by unknown signals triggers a phosphorelay, activating the response regulator ExpA. By an unknown mechanism, phosphorylated ExpA positively controls the transcription of regulatory RNA species RsmB, which sequester the translational repressor RsmA and thereby render the mRNAs of pectinolytic exoenzymes accessible for translation. The ExpS-ExpA system also induces production of AHLs, and there is evidence that AHLs control the production of *E. carotovora* virulence factors through RsmA and RsmB (72). Expression of extracellular enzymes is also controlled by KdgR, which, in the absence of pectate, represses the transcription of exoenzyme genes. Repression is relieved by pectate and its intracellular metabolites KDG, DKI, and DKII. KdgR also regulates the expression of *rsmA* and *rsmB*; it induces the transcription of *rsmA* and represses the expression of *rsmB* (234, 292). *E. carotovora pel* genes are also regulated by several other regulators, which are described in the text.

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genome sequence revealed the presence of strong PecS binding sites in the intergenic region between two *fli* operons that encode proteins involved in the synthesis of the bacterial flagellum (430). PecS also bound to this region and was thus proposed to directly repress *fliE* gene expression (430). These results are supported by the observation that a PecS null mutant is hypermotile (430). PecS is also an activator of the synthesis of polygalacturonase enzymes (*pehN*, *pehV*, *pehW*, and *pehX*) (227, 353). These genes are negatively regulated by KdgR, and PecS appears to act as an antirepressor of KdgR by competing for overlapping DNA binding sites (227, 353). The signal to which PecS responds is not yet known.

The activity of PecS requires a second protein, PecM, which is encoded by a gene that is adjacent to and divergent from *pecS* (404). PecM is a membrane-anchored protein and has been proposed to be the sensor controlling PecS activity, although it displays no similarities to any known signal-transducing receptors (404, 428). PecM is required for PecS activity in vivo (390) and is involved in modulating the PecS DNA binding ability (391). By binding to a site between *pecS* and *pecM* genes, PecS negatively autoregulates both genes (390).

**PecT.** Although pectate lyase expression is high in a *kdgR-pecS* double mutant, it remains somewhat inducible in the presence of PGA (404). This suggested that additional regulatory genes control *pel* expression in response to pectate and led to the isolation of the *pecT* gene (473). Mutation of *pecT* derepresses the expression of some of the pectate catabolic genes (*pelCDE*, *pelL*, and *kdgC*) and activates the expression of *pelB* (473). PecT also represses genes involved in motility and genes required for EPS synthesis (Table 1) (68, 86). An *eps* mutant is less efficient than the wild-type strain in initiating a maceration symptom, suggesting that production of EPS is required for the full virulence (86).

PecT is a LysR-type regulator and is a dimer in solution (67). Electrophoretic mobility shift assays demonstrated that two PecT dimers bind cooperatively to the regulatory regions of pectate lyase genes, although the binding affinity varies for different promoters (67). PecT also specifically interacts with the *pecT* regulatory region and represses its own synthesis (67, 68). The nucleoid-associated protein H-NS plays a role in the negative control of *pecT* and also represses the expression of *pel* genes and of the QS system *expI-expR* (350). An ortholog of the *E. chrysanthemi pecT* was found in two subspecies of *E. carotovora* and was named *hexA* (for "hyperproduction of exoenzymes") (197). Mutations in *hexA* resemble mutations in *pecT*, although in addition, *hexA* mutants show increased expression of the *fliA* and *fliC* genes and are hypermotile (197). *hexA* was also shown to negatively regulate the production of RpoS and of a global regulator of exoenzyme production, *rsmB* (see below) (339).

**Catabolite repression and CRP.** Early physiological studies suggested that pectinase production in *Erwinia* species is subject to catabolite repression (221). Catabolite repression was observed during growth in the presence of glucose and also in the presence of pectin catabolic products (84, 491). The cyclic AMP (cAMP) receptor protein (CRP) is essential for this regulation (Fig. 7A; Table 1). *E. chrysanthemi crp* mutants have greatly decreased maceration capacity in potato tubers, chicory leaves, and celery petioles as well as highly diminished virulence (402). These mutants are also unable to grow on pectin or PGA as the sole carbon sources (402). Expression of pectinase genes (*pemA*, *pelB*, *pelC*, *pelD*, and *pelE*) and of genes of the intracellular part of the pectin degradation pathway (*ogl*, *kduI*, and *kdgT*) is dramatically reduced in *crp* mutants (84). Similarly, an *E. carotovora cya* mutant (defective in cAMP production) is defective in Pel synthesis (336). Purified *E. chrysanthemi* and *E. carotovora* CRP proteins bind specifically to the promoter regions of the pectinolytic genes, whose expression is positively regulated in vivo by CRP (313, 429). Therefore, CRP was proposed to act as a global activator of the pectinolytic genes (402). In *E. chrysanthemi*, CRP and KdgR directly compete for the occupation of a common DNA region on the target genes (429).

**Regulation of the secondary or minor *pel* genes.** Deletion of the five major pectate lyases does not totally eliminate the ability of *E. chrysanthemi* to macerate plant tissues. Analysis of tissues macerated by such mutants revealed the presence of a new set of pectolytic enzymes, which, due to their low activity in synthetic medium, were first described as secondary or minor pectate lyases. They were detected only when bacteria were grown on plant material or in synthetic medium with macerated plant extracts (29, 263, 412). Three genes, *pelL*, *pelZ*; and *pelI*, encoding three of these secondary pectate lyases, were cloned and characterized from a derivative of *E. chrysanthemi* strain 3937 (301, 387, 449). Transcription of all three genes was inducible by plant extracts as well as by pectate catabolic products. In the case of *pelL*, this induction was independent of KdgR but required PecS and PecT (301, 473). Induction of *pelZ* seemed to be partially mediated by the KdgR protein but did not result from a direct interaction of KdgR with the *pelZ* 5′ region. The transcription of *pelZ* occurs from multiple promoters, including one upstream of *pelC*, which is controlled by KdgR, leading to a bicistronic mRNA (387). Expression of *pelZ* is also controlled by *pecT* but is independent of *PecS* (387). In contrast, *pelI* expression is controlled by KdgR, PecS, and PecT, and a KdgR binding site is located adjacent to the putative *pelI* promoter (449).

### Induction of Pectinolytic Enzymes by Plant Extracts

*E. chrysanthemi* strain EC16 differs from strain 3937 in producing only four instead of five major pectate lyases (479). Similar to strain 3937, a mutant of EC16 missing the four major *pel* genes still produced significant pectate lyase activity in chrysanthemum tissue and in minimal media containing chrysanthemum extracts (263, 412). However, these isozymes were not expressed in minimal medium containing pectate, and the mutant was inactive in a standard pectate agar medium used to detect pectolytic activity in bacteria (263). Thus, expression of the new set of pectate lyases in EC16 appears to require the presence of the plant tissue and is regulated independently of the major *pel* genes. One of the minor EC16 *pel* genes, *pelL* was shown to be regulated differently from the *pelL* of strain 3937 in that it was not induced by PGA in culture (189). In addition, it was speculated to be less highly regulated by the *pecS* and *pecT* genes.

Plant extracts also influence the expression of the major *pel* genes in both *E. chrysanthemi* and *E. carotovora*. In *E. chrysanthemi* EC16, transcription of the major *pel* genes is induced by pectin metabolites and is further stimulated by the presence

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of plant extracts, but only in synergy with PGA or galacturonate (47). Moreover, in an *ogl* mutant, which lacks the capacity to produce the direct inducers from PGA (KDG, DKI, and DKII), plant extracts do not exhibit any inducing activity (47). Different *pel* genes exhibit different sensitivity to signals in the plant extracts. The expression of *pelE* and *pelA* is weakly sensitive, while *pelB* and *pelC* are moderately induced and the expression of *pelD* is very sensitive to these signals (47). The chemical nature of the inducing factors in plant extracts is not known. Preliminary characterization of one inducing factor from carrot roots showed that this compound is thermoresistant, has a low molecular mass, and is hydrophilic, which is consistent with its being an oligosaccharide (47).

The regulator responsible for the induction by plant extracts is encoded by the *pir* (for "Plant-Inducible Regulator") gene (Fig. 7A; Table 1) (359). A mutation in *pir* resulted in the loss of *pel* hyperinduction by plant extracts and caused reduced virulence of the mutant. The Pir protein is a 30-kDa DNA binding protein that belongs to the IclR family. It functions as a dimer and was shown to bind to a 35-bp DNA sequence in the promoter region of *pelE* (359). The Pir binding site overlaps with that of KdgR, and gel shift experiments showed that Pir and KdgR competed for binding to overlapping sites (359). By binding to the *pir* promoter, Pir also positively regulates its own expression (360). The regulation of Pir synthesis seems to be independent of KdgR, PecS, or CRP, based on the failure of these proteins to bind the *pir* promoter (360).

The pectinolytic enzymes of *E. carotovora* are also induced by factors present in plant extracts. Extracellular levels of Pel, Cel, Peh, and Prt enzymes increase strongly in medium supplemented with celery extract, which also caused considerably stronger induction than pectate (343). This induction requires the *aepA* gene (for "Activator of Extracellular protein Production") (293, 343), which codes for a 51-kDa protein (AepA) (Table 1). The AepA protein possesses several hydrophobic domains, suggesting that it is membrane localized. It lacks a typical DNA binding domain and thus might act as a sensor of environmental signals, possibly transmitting them to another regulatory protein (293). The *aepA-lacZ* fusion was itself inducible by pectate and by celery extracts. This induction did not require a functional *aepA*, indicating that it is regulated by yet another system.

### Induction of Pectinolytic Enzymes by Iron Limitation

Intercellular fluids of most plants are, like those of animals, very poor in available iron (127). In response to iron deprivation, *E. chrysanthemi* 3937 synthesizes two siderophores, chrysobactin and achromobactin, of which chrysobactin is a stronger iron ligand and its synthesis requires a more severe iron limitation (130). A mutant with a mutation in the chrysobactin-dependent iron transport (encoded in a single chromosomal locus named *fct-cbsCEBA*) fails to incite a systemic disease in a host plant (126). Similarly, mutants affected in the achromobactin synthetic genes *acsA* or *acsC* cause only localized symptoms (147). Chrysobactin was also detected in plant tissues infected by *E. chrysanthemi* (354), and a *fct-lacZ* fusion was strongly expressed in planta during infection (310). These data show that siderophores are important for *Erwinia* pathogenicity and that iron availability thus plays a major role in regulation of *E. chrysanthemi* virulence.

Iron deprivation induces the synthesis of *pelB*, *pelC*, *pelD*, *pelE*, and *pelL*, although induction of *pelD* requires more severe iron starvation than does induction of the other four genes (311, 438). Iron sensing seems to be mediated by the ferric uptake regulator Fur (Fig. 7A; Table 1), which is found in many proteobacteria and acts as a transcriptional repressor, using ferrous iron as a corepressor (128, 370). Fur negatively regulates the *pelD* and *pelE* genes (148), but does not regulate *pelA* (148). It binds to the regulatory regions of the *pelD* and *pelE* promoters and was proposed to function by competing against the activator CRP for overlapping binding sites (147).

### Effect of pH on the Expression of Pectinolytic Enzymes

When *Erwinia* spp. infect plants, they generally colonize intercellular apoplastic fluid, whose pH lies between 5.0 and 6.5 (181, 347). On colonization, bacteria induce cell lysis, which results in a change in the pH of the intercellular environment. Chicory leaves showed a significant change in pH from acidic to basic in plant tissues during infection (347). Expression of pectinolytic enzymes is strongly affected by the pH, which seems to play an important role in *pel* regulation during infection (347). *pelA* and *pelD* are expressed only when bacteria are grown in an acidic medium, while *pelE* is transcribed only in basic medium. This suggests that PelA and PelD might be important in the early steps of infection, when the medium surrounding *E. chrysanthemi* is acidic. Conversely, PelE could act in the later stages of the infection, when the environment turns alkaline. KdgR, PecS, and Fur are not involved in the pH modulation of the exoenzyme expression (347), and the mechanism of this regulation is unknown.

The proposal that different Pel enzymes act sequentially in response to changes in pH implies a role for PelA and provides a rationale for understanding the ecological advantage of the *pel* redundancy. PelA exhibits a very poor macerating ability and seems always to be produced at very low levels (22, 224). Nevertheless, a mutation in the *pelA* gene results in a delay of symptoms (29). PelA activity produces oligomers with a high degree of polymerization (394), which induce a rapid $H^+$ influx in plant cells (20). This results in an increase in the pH of the apoplastic fluid (19, 20), which induces the synthesis of PelE and possibly of other Pel enzymes. These enzymes have stronger maceration capacities and generate oligomers with a low degree of polymerization (394), which are a more efficient carbon source. Hence, production of PelA as soon as the bacteria invade the plant might constitute a key event in the initial steps of infection by generating an inducing signal.

### Other Environmental Signals That Affect the Expression of Pectinolytic Enzymes

Many other environmental conditions, such as temperature, anaerobiosis, osmolarity, and nitrogen starvation, are also known to influence the expression of genes involved in pectinolysis (223, 224), although, for the most part, it is not yet known how these signals are detected and integrated to modulate the activity of the pectinolysis genes (Fig. 7A). Similar to the above-described *pel* regulation by plant extracts, iron lim-

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itation, or pH levels, different *pel* genes tend to be affected by these environmental factors in different ways. For example, transcription of *pelA*, *pelD*, and *pelE* is increased by anaerobic conditions whereas *pelC* and *pelB* are not affected (224). High osmolarity increases the expression of *pelE* but decreases the expression of *pelD* and *pelL* (224, 301).

Different *pel* genes are also expressed at different levels during infection and appear to be differentially regulated depending on the host plant and even on the types of tissues (113, 300, 311). It is well established that each Pel enzyme has particular properties (301, 387, 449) and may play specialized roles during infection. It has been shown that the role of each pectate lyase depends on the host plant (29, 41), suggesting that the variety of Pel isoenzymes may result from specific adaptations of the bacteria to macerate different plant tissues. Multiple regulatory proteins (in combination with several global regulatory systems described below) allow different signals to activate these virulence factors independently and permit the bacteria to adapt to continuous modifications of their physicochemical environment.

Some pectinolytic enzymes are subject to specific regulation. The PehR-PehS two-component system of *E. carotovora* responds to the extracytoplasmic levels of $Ca^{2+}$ and $Mg^{2+}$ and controls the production of an endopolygalacturonase encoded by *pehA* (143, 144, 434). Another example is the *pnlA* gene of *E. carotovora*, whose expression is induced by DNA-damaging agents such as mitomycin C, nalidixic acid, or UV light but not by PGA (290). Transcription of *pnlA* is dependent on the *recA*, *rdgA*, and *rdgB* gene products (290, 291, 294). In the proposed model, in the presence of DNA-damaging agents, the activated proteolytic form of RecA processes RdgA to a form that activates *rdgB* expression, whose product in turn activates *pnlA* expression.

## REGULATION OF TYPE III SECRETION SYSTEMS AND ASSOCIATED EFFECTORS IN VARIOUS PLANT-PATHOGENIC BACTERIA

Type III secretion systems (TTSS) are key virulence determinants used by proteobacteria to deliver effector proteins directly into the host cell cytoplasm (8, 157, 206, 222). *Erwinia*, *Pseudomonas*, *Pantoea*, *Xanthomonas*, and *Ralstonia* cause diverse diseases in many different plants, but they all colonize intercellular spaces of susceptible plants and are capable of killing plant cells. The ability of these bacteria to multiply inside their hosts and produce necrotic symptoms is dependent on their *hrp* genes, which encode components of the TTSS, and presumably on the virulence proteins that are secreted via this system. The functional redundancy of the latter, however, often makes it difficult to demonstrate a role for these proteins in a successful infection. While Hrp-dependent protein secretion is required for bacterial pathogenicity on host plants by compatible pathogens, it is also required for elicitation of the so-called hypersensitive response (HR) in nonhost plants (7, 46, 262). An HR is a programmed death of the plant cells at the site of pathogen invasion and is associated with plant defense (100, 209). Many bacterial proteins, called Avr (avirulence) proteins, are delivered to the interior of the plant cell via the Hrp secretion system, where they are recognized by plant R (resistance) proteins, resulting in elicitation of the HR (7, 45, 501). Avr proteins are thought to play roles in virulence on host plants, while nonhost plants are thought to have coevolved the appropriate R gene product to recognize a specific virulence protein, which then acts as an Avr protein (7, 262). Type III secretion is also required for the secretion of structural components of the Hrp pilus and of helper proteins, such as harpins. Harpins are glycine-rich cysteine-lacking proteins that possess heat-stable HR elicitor activity when infiltrated at relatively high concentrations into the intercellular leaf spaces of many plants (8).

### Induction of Expression of Type III Secretion Systems by Host-Released Chemical Cues

The expression patterns of *hrp* genes in the five genera (*Erwinia*, *Pseudomonas*, *Pantoea*, *Ralstonia*, and *Xanthomonas*) are very similar. The *hrp* genes are repressed when bacteria are cultured in complex media (with the possible exception of a necrotroph, *E. chrysanthemi*) but are induced to high levels when bacteria grow in the plant apoplast or are in close contact with plant cells (13, 232, 398, 446, 522, 539). Induction of *hrp* genes in bacteria occurs early after contact with a plant. In *P. syringae*, in planta expression of *hrp* genes was detected as early as 1 h after infection and continued to increase for at least 6 h (398). Induction of various genes in the *hrp* cluster ranged from 5- to 70-fold (398, 539).

No specific plant inducers of *hrp* gene expression have thus far been characterized, and the nature of the signals inducing *hrp* gene expression in planta is not clearly defined. The *hrp* genes, however, are also expressed when bacteria are growing in minimal medium (13, 232, 398, 446, 522, 523, 539). Factors that affect *hrp* gene expression in minimal medium are different in different species and pathovars, and thus the composition of the so called *hrp*-inducing minimal medium is somewhat variable for each organism. In each case, high osmolarity and complex nitrogen sources, such as peptone or Casamino Acids, have a strong repressive effect on *hrp* gene expression (13, 232, 398, 446, 522, 539). The nature of the carbon source also plays an important role; however, the activities of particular substrates are very different in different organisms (13, 232, 398, 446, 522, 539). For example, pyruvate causes optimal induction of *hrp* genes in *R. solanacearum* but has no stimulatory activity in *P. syringae* pv. glycinea, and is actually inhibitory in *X. campestris* pv. vesicatoria. Another example is fructose, which stimulates *hrp* gene expression in *P. syringae* pv. glycinea but has very little activity in *R. solanacearum*. Mannitol is the best substrate for *hrp* induction in *E. amylovora*, but is inactive in *X. campestris* pv. vesicatoria. In contrast, sucrose, which is also the most abundant sugar in leaf tissue, appears to be a good inducer in all these organisms. In general, growth substrates that enter glycolysis at the pyruvate step or earlier (mannitol, fructose, and sucrose) seem to have a positive effect on *hrp* expression while succinate, citrate, and glutamate are inhibitory (232, 398, 446). The latter substrates support higher growth rates, suggesting that *hrp* gene induction is inhibited by the favored carbon sources and stimulated by the less favored ones (232, 446). Expression of *hrp* genes is also affected by pH. The *hrp* genes are best transcribed at pH around 5.5, with an exception of *X. campestris* pv. vesicatora, where a pH between 6.5 and 7.5 gives the best induction (446). In *E. amylovora*, *hrp*

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expression is repressed by the presence of nicotinic acid and is also modulated by temperature, in that expression is 2- to 10-fold higher at 18 than 30° C (522). In *X. campestris* pv. vesicatoria, *hrp* induction requires sulfur-containing amino acids (446, 523).

In several cases, the level of induction found in plants is comparable to that observed in the synthetic medium. Moreover, several species, when preincubated in the *hrp*-inducing medium prior to inoculation, could induce an HR even in the presence of transcription or translation inhibitors (232, 522). This shows that protein synthesis is not required at the early stages of infection if bacteria are incubated in minimal medium prior to plant inoculation, indicating that no specific plant signals are required for transcriptional activation of the *hrp* regulons. The *hrp*-inducing minimal medium therefore seems to mimic the conditions that bacteria experience inside the plant. The absence of complex nitrogen sources, the low pH, the presence of sucrose, and the presence of low temperature correspond to the environment the bacteria might encounter in the apoplast. It has thus been proposed that the induction of *hrp* gene expression after contact with plant tissue results from an alteration in nutritional conditions.

However, several recent reports provide evidence that full induction of *hrp* genes in *R. solanacearum* requires a specific plant factor(s). On coculture with *Arabidopsis* or tomato cell suspensions, expression of *hrpB* gene was induced up to 20-fold more than in minimal medium and was detected only when the bacteria directly contacted the plant cells (5, 107). The use of a medium conditioned by cocultivation of plant cells and bacteria did not increase the level of *hrp* induction compared with a medium conditioned only by a plant (6), and it has therefore been proposed that bacterium-plant cell contact is required for the induction. Expression of *hrp* genes was also enhanced by contact with cell wall fragments, although to a slightly lower level than by intact cells. Because the cell wall fragments were treated to remove lipids and proteins, these results suggest that the signal might be a part of the cell wall polysaccharide matrix (5). The chemical identity of the plant cell wall signal is still unknown.

*hrp* genes are also induced when bacteria are infiltrated into nonhost plants, where they are involved in elicitation of the HR. Several studies have reported that levels of expression of the *hrp* loci are different in host and nonhost plants. *E. amylovora hrp* genes were induced earlier after infection and much more strongly in tobacco (a nonhost plant) than in pear (a host plant) (522). In *R. solanacearum*, a mutation in a PrhA protein, which is thought to be involved in detection of the *hrp*-inducing signal and is a part of the *hrp* regulatory cascade (see below), had a differential effect on *hrp* expression in coculture with tomato and *Arabidopsis* cells (306). The *prhA* mutation abolished induction with *Arabidosis* but not with tomato cells. Both these examples suggest that the environments and signals detected by the bacteria during plant interaction may differ based on the plant and possibly on the type of interaction. Overall, it seems that although *hrp* gene induction can be detected in minimal medium, full induction of at least some *hrp* regulons may require specific plant factors. This seems advantageous, since the expression of TTSS is a highly energy-consuming process, and it would therefore benefit the bacteria to fully induce the *hrp* genes only when they are in close contact with host cells.

### Regulatory Cascades Controlling the Expression of Type III Secretion System Genes

Regulation of *hrp* expression downstream of signal detection is understood in some detail (8, 206, 222). Transcription of TTSS genes is controlled by multicomponent regulatory networks that integrate diverse sets of environmental cues. These regulatory networks, however, differ significantly among different species (Fig. 8). Based on differences in regulation, *hrp* genes can be divided into two groups. Group I *hrp* clusters are found in *P. syringae* and *Erwinia* and *Pantoea* spp., where *hrp* genes are activated by a member of the extracytoplasmic function (ECF) subfamily of sigma factors, called HrpL (150, 520, 537, 538). Group II *hrp* operons are found in *X. campestris* and *R. solanacearum*, where transcription of TTSS-associated genes is regulated by members of the AraC family of proteins (161, 523).

***Pseudomonas syringae*.** In *P. syringae*, the ECF sigma factor HrpL is required for transcription of the *hrp* genes as well as of genes encoding potential type III secreted proteins, such as *hrmA* and *avr*, which are located outside the *hrp* cluster (537). In addition, HrpL regulates the transcription of several other virulence-implicated factors, which probably act independently of the Hrp system (146). These include genes involved in the biosynthesis of the phytotoxins syringomycin and coronatine (see below) and the phytohormone indole acetic acid. Transciptional activation of HrpL-regulated genes presumably involves interaction of HrpL with the so-called *hrp* box sequences, whose consensus sequence is (5′-GGAACCNA-N13-14-CCACNNA-3′) (146, 237, 448, 538). Similar sequences are found in the promoters of all HrpL-regulated genes, although direct interaction of HrpL with the *hrp* box sequences and determination of essential nucleotides within these sequences have not been reported.

HrpL is encoded in a monocistronic operon located at the left end of the *hrp* cluster (537). The *hrpL* gene is transcribed from a $\sigma^{54}$-dependent promoter in an RpoN-dependent manner (212). Transcription also requires the *hrpRS* operon, which is located at the right end of the *hrp* cluster (182, 229, 537). The products of this operon, HrpR and HrpS, are 60% identical and belong to the NtrC family of two-component response regulators. A sequence motif that, in NtrC-like proteins, functions in interaction with $\sigma^{54}$ RNA polymerase holoenzyme is conserved in both HrpR and HrpS. Both HrpR and HrpS are required for HrpL expression (Fig. 8A); however, there is some controversy regarding the mechanism by which these two proteins regulate the *hrpL* promoter. The *hrpR* gene is dispensable for hypersensitive response in tobacco leaves if *hrpS* is expressed from a strong promoter (182). An apparent *hrpS* transcript was detected that appeared to initiate near a minimal $\sigma^{54}$ promoter consensus sequence internal to the *hrpR* coding sequence, and HrpR was shown to bind to a DNA fragment containing this region. It has thus been proposed that HrpR functions as a transcriptional activator of *hrpS* expression and that HrpS acts independently of HrpR to activate the expression of *hrpL* (182). Results of another study contended, however, that *hrpR* and *hrpS* are expressed as a single operon

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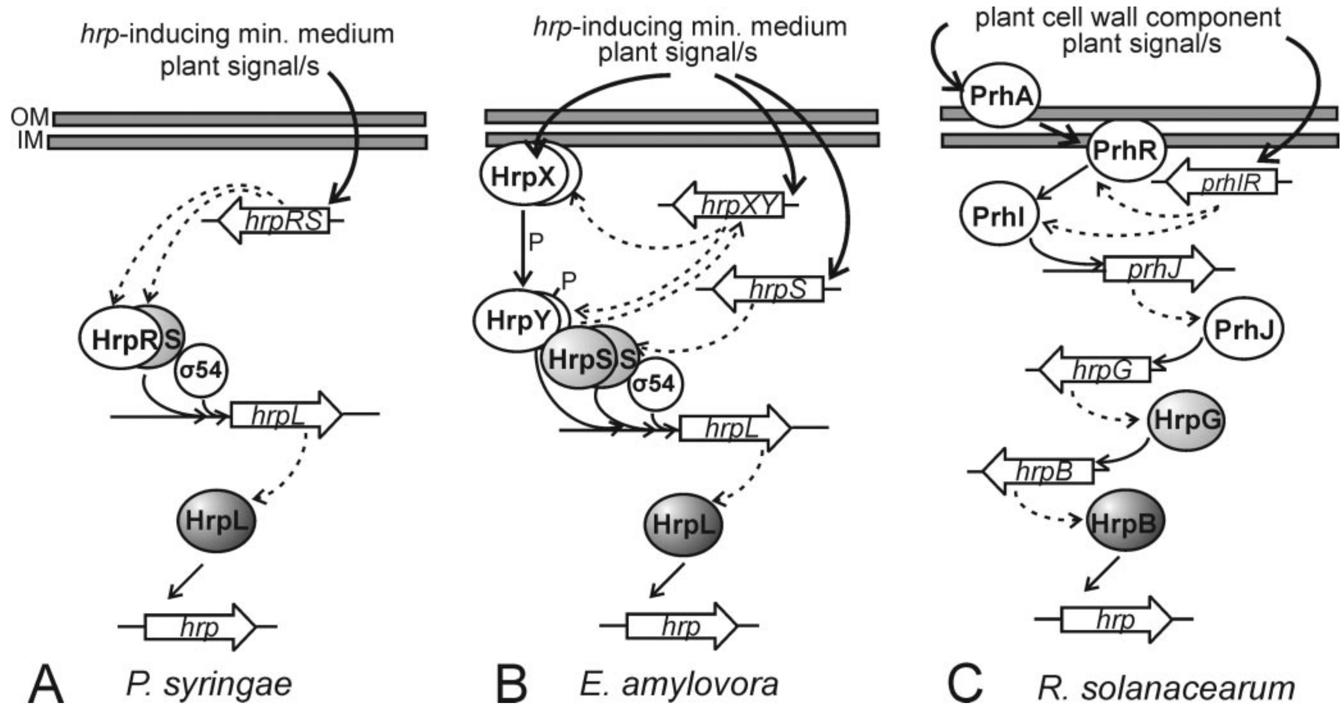


FIG. 8. Models of the *hrp* gene regulatory cascades in *P. syringae*, *E. amylovora*, and *R. solanacearum*. Based on differences in regulation, *hrp* genes can be divided into two groups. Group I *hrp* clusters are found in *P. syringae*, *Erwinia*, and *Pantoea* spp., where *hrp* genes are activated by a member of the ECF subfamily of sigma factors, called HrpL (150, 520, 537, 538). (A) In *P. syringae*, expression of *hrpL* requires the products of the *hrpRS* operon, which are 60% identical to each other and belong to the NtrC family of two-component response regulators. There is some controversy regarding the mechanism by which HrpR and HrpS regulate the *hrpL* promoter. It has been proposed by one group that *hrpL* transcription is induced by an HrpR-HrpS heterodimer (229). Another group reported that HrpR functions as a transcriptional activator of *hrpS* expression and that HrpS acts independently of HrpR to activate expression of *hrpL* (data not shown) (182). (B) In *E. amylovora*, expression of HrpL is activated by HrpS, which is highly similar to HrpS of *P. syringae* (520). In *E. amylovora*, a two-component system, HrpX-HrpY, is also involved in the *hrpL* regulation. Transcription of *hrpRS* in *P. syringae* and *hrpS* in *E. amylovora* is upregulated in the plant apoplast and in *hrp*-inducing minimal medium. HrpL and HrpS are conserved in all members of this group and are shaded in gray in panels A and B. (C) Group II *hrp* operons are found in *X. campestris* and *R. solanacearum*, where an OmpR-like transcriptional regulator HrpG induces the transcription of *hrpB*, whose product is a member of the AraC family of regulators and induces transcription of the rest of the *hrp* and *hrc* genes. HrpG and HrpB are conserved in all members of this group and are shaded in gray. In *R. solanacearum*, an outer membrane protein, PrhA, is involved in detection of a plant signal, which appears to be a nondiffusible component of the plant cell wall and whose detection was proposed to require a physical bacterium-plant cell contact. Based on a proposed model (55), the signal detected by PrhA is transmitted to the membrane protein PrhR, which activates an ECF sigma factor, PrhI. PrhI induces the transcription of a gene encoding a transcriptional regulator, PrhJ, leading to expression of *hrpG*. HrpG activates the expression of *hrpB*, resulting in induction of the remaining *hrp* genes. OM, outer membrane; IM, inner membrane.

from a promoter upstream of *hrpR* gene (229). Moreover, HrpR and HrpS were shown to physically interact, suggesting that the two proteins may form a heterodimeric complex to regulate the *hrpL* promoter (Fig. 8A) (229).

Neither HrpR- or HrpS carries an N-terminal regulatory domain commonly found in other members of this family, implying that the activity of HrpR or HrpS may not be controlled by any signal. Thus, the pool sizes of HrpR and HrpS might be key in regulating *hrp* expression. Expression of the *hrpRS* operon is low during growth in rich medium and is induced in *hrp*-inducing minimal medium (398). The *hrpRS* operon is also induced during plant infection; however, there is some controversy regarding whether the *hrpRS* expression is higher in planta than in culture under *hrp*-inducing conditions (398, 539). It has also been suggested that different mechanisms are involved in regulation of these genes in planta versus those in vitro. In one study, the expression of *hrpRS* was highly induced in planta (more than 1,000-fold higher than in minimal medium) and was not dependent on the integrity of the *hrpRS* genes themselves (398). However, a functional *hrpRS* operon was required for induction of these genes in the *hrp*-inducing minimal medium, indicating positive autoregulation (398).

Several regulatory proteins have been identified that affect expression of the *hrpRS* operon. One of them is HrpV, which acts as a negative regulator of the HrpRS-HrpL cascade (393). In *hrp*-inducing minimal medium, overexpression of the *hrpV* gene downregulates *hrp* gene expression, while *hrp* genes are expressed at elevated levels in a *hrpV* mutant. The *hrpV* gene has no known homologs in the current protein sequence databases and appears to be found only in group I *hrp* clusters. Although HrpV seems to act upstream of HrpRS, it is not yet known whether it directly controls the expression of the *hrpR* promoter. It has been proposed that HrpV-mediated repression might be alleviated by its translocation from the bacterium via the TTSS (393).

Another protein that affects expression of the *hrpRS* operon is HrpA, the major subunit of the Hrp pilus (518). In *P. syringae* DC3000, a mutation in *hrpA* affects the full expression of the *hrp* regulon by causing a downregulation in the *hrpRS* operon (518). Interestingly, a functional HrpA protein was also

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required for secretion of HrpW and AvrPto into the culture medium, although the mechanism by which this Hrp pilus structural gene regulates and possibly coordinates *hrp* transcription and protein secretion is unknown.

In addition to transcriptional control of the *hrpRS* operon, HrpR is subject to posttranslational regulation via the Lon protease (52). In an *hrp*-repressing rich medium, the half-life of HrpR, but not HrpS, was significantly shorter in wild-type bacteria than in a *lon* mutant. Under *hrp*-inducing conditions, however, HrpR was largely resistant to Lon, resulting in increased expression of the downstream *hrp* genes (52).

***Erwinia amylovora* and *Pantoea* spp.** Like *P. syringae*, *E. amylovora* contains an ECF-type sigma factor, HrpL (Fig. 8B), which is required for transcription of *hrp* and *dsp* (for "Disease-Specific Protein") genes that produce the secretion machinery and virulence proteins that interact with plant cells (520). Expression of HrpL is activated by HrpS, a transcription factor highly similar to HrpS from *P. syringae* (520). Unlike *P. syringae*, *Erwinia* spp. appear not to encode a protein corresponding to HrpR.

Transcription of *hrpL* in this organism also requires the products of the *hrpXY* operon (Fig. 8B) (519). This operon was required for virulence in pear fruit and for elicitation of HR in tobacco (519). HrpX is predicted to be a histidine kinase similar to BvgS, while HrpY is the cognate response regulator. The N-terminal input domain of HrpX shows similarities to PAS domains. Expression of *hrpXY* is autoregulated and is low in rich medium but increases threefold when bacteria grow in minimal medium. Enhanced levels of *hrpXY* expression were also observed during infection of tobacco leaves and of immature pears. Transcription of *hrpS* is also induced in minimal medium but is not autoregulated (519), suggesting that there may be upstream regulatory components involved in the regulation of this gene. HrpX and HrpY, however, do not affect the expression of *hrpS*, indicating that environmental signals go to *hrpS* through a different pathway (519).

Based on the model proposed by Wei et al. (519), when the bacteria enter the plant apoplast, HrpX detects environmental signals and phosphorylates HrpY, while expression of *hrpS* is also induced by an uncharacterized regulatory system. Phospho-HrpY and HrpS bind to the *hrpL* promoter and interact with $\sigma^{54}$-containing RNA polymerase to drive transcription of *hrpL* (Fig. 8B). Thus, environmental signals that are independently detected by *hrpXY* and *hrpS* converge at *hrpL*, leading to transcription of the TTSS genes. Similar to *E. amylovora*, *P. stewartii* and *P. agglomerans* possess an *hrpS* gene and an HrpXY two-component system, but do not possess HrpR. In *P. stewartii* and *P. agglomerans*, activated HrpY acts as a positive regulator of *hrpS* transcription (326, 357).

***Xanthomonas campestris* and *Ralstonia solanacearum*.** In *X. campestris* pv. vesicatoria and *R. solanacearum*, the TTSS-associated genes are regulated by HrpX and HrpB (Fig. 8C), respectively, which both belong to the AraC-type family of regulators and are 40% identical to each other (161, 523). In *R. solanacearum*, the HrpB protein activates the transcription of *hrp* genes and of the *popABC* operon, which is located outside the *hrp* cluster and codes for proteins that are secreted via the Hrp machinery (14, 161, 184). HrpB is thought to bind a 25-bp sequence that is defined as (TTCG-$N_{16}$-TTCG) and was named the $hrp_{II}$ box. This sequence was required for the *hrpB*-dependent activation of *hrpY* and *popABC* promoters and is found in all HrpB-dependent promoters known so far (96, 97). Genome-wide searches for new members of the Hrp regulon identified numerous genes that contain the $hrp_{II}$ box and are HrpB regulated (97). Most of them have no homologs in other bacterial species, and their functions are still unknown. In *X. campestris* pv. vesicatoria, HrpX controls the expression of *hrp* operons and the avirulence gene *avrXv3*, which is located outside the *hrp* gene cluster (16, 523). A conserved DNA motif corresponding to the $hrp_{II}$ box has also been identified in several *X. campestris* pv. vesicatoria *hrp* promoters. The element was named the PIP (for "plant-inducible promoter") box and was proposed to be important for HrpX-dependent regulation (135). No binding of HrpB to the $hrp_{II}$ boxes or of HrpX to PIP-box-containing promoters could thus far be detected.

Transcription of *hrpX* in *X. campestris* pv. vesicatoria and of *hrpB* in *R. solanacearum* is activated by growth in *hrp*-inducing minimal medium and is regulated by the product of *hrpG* (Fig. 8C) (56, 524). The *hrpG* gene resembles members of the OmpR subfamily of two-component response regulators. A genome-wide analysis of the *hrpG* regulon showed that HrpG regulates genes encoding transcriptional regulators, degradative enzymes, an adhesin, and type III effectors from other plant pathogens and that expression of most these genes is dependent on *hrpX* (358). Expression of *hrpG* in *X. campestris* is low in complex medium, increases in *hrp*-inducing minimal medium by a factor of 4, and is independent of *hrpG* and of other *hrp* loci (524). No upstream factors affecting *hrpG* expression or HrpG activity have so far been identified in this organism.

On the other hand, several factors involved in the *hrp*-regulatory cascade acting upstream of *hrpG* have been identified and characterized in *R. solanacearum*, (Fig. 8C) (5, 55, 56, 306). As described above, full induction of *hrp* genes in this organism appears to require direct contact between bacteria and host plant cells (5, 55). PrhA is a putative outer membrane receptor protein that resembles siderophore receptors, although its expression is not modulated by iron availability (306). It is encoded by the *prhA* gene, which is located outside of the *hrp* cluster and is not regulated by HrpB (306). PrhA was required for plant cell contact-dependent activation of *hrp* genes and is thought to be responsible for detection of the plant signal (5, 55). In addition to HrpG and HrpB, transduction of the plant signal from PrhA to promoters of *hrp* genes involves a membrane protein PrhR, an ECF sigma factor PrhI, and the transcription factor PrhJ (55, 56). Based on a proposed model (55), the signal detected by PrhA is transmitted to PrhR, which in turn activates PrhI. PrhI induces transcription of *prhJ*, leading to expression of *hrpG*. The HrpG protein activates the expression of *hrpB* gene, resulting in induction of the remaining *hrp* genes.

Interestingly, the induction of *hrp* genes by coculture with *Arabidopsis* cells is abolished by mutations in *prhA* or *prhJ*, while these mutations only partially reduce induction by coculture with tomato cells, and do not affect induction by minimal medium (306). *prhA* and *prhJ* mutants are also nonpathogenic on *Arabidopsis*, while virulence on tomato plants is not affected. In contrast, HrpG and HrpB are required in all hosts (306). These observations are consistent with the integration of

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multiple input signals at different levels of the regulatory hierarchy.

### Secretion of Effectors by the Type III Secretion System

The final outcome of turning on the TTSS is delivery of virulence proteins into the host cell cytoplasm. In the animal pathogens *Shigella flexneri* and *Yersinia* spp., translocation of effector proteins to animal cells occurs in a one-step process that is activated on contact with the host cell (222, 323, 379, 424, 455). A similar situation appears to be true for the effector proteins of plant pathogens, which, based on indirect evidence, are thought to be delivered by the TTSS inside plant cells (8, 157). These proteins are detected in culture supernatants in very small amounts and only under appropriate culture conditions, while under most conditions in the absence of host cells, the bulk of these proteins are retained within the bacteria (8, 337, 426, 502). This supports the hypothesis that secretion and translocation of the effector proteins are coordinately regulated and require contact with a plant cell; however, this has not yet been shown directly. In contrast, harpins and proteins that function as components of the extracellular secretion apparatus seem to be readily secreted in culture media and can be detected in the supernatants in large amounts (14, 207, 417, 521). The significance and the mechanism of the differential regulation of secretion of effector and helper proteins is unknown.

## PRODUCTION OF PHYTOTOXINS BY *PSEUDOMONAS SYRINGAE*

Various strains of *P. syringae* possess virulence determinants that do not require TTSS. Examples are phytotoxins, which are not required for pathogenicity but enhance the aggressiveness of these strains (32). For two such phytotoxins, coronatine and syringomycin, specific plant-released signals have been identified that are detected by the toxin-producing bacteria and are responsible for expression of the toxin biosynthetic genes.

Syringomycin is a pore-forming toxin that inserts into the plant plasma membrane and induces ion fluxes, resulting in cytolysis and necrosis of plant tissues (230, 231). Biosynthesis of this phytotoxin is encoded by the chromosomally located cluster of *syr* genes (32, 185). Induction of the syringomycin biosynthesis genes was detected during infection of immature cherry fruits (333, 396). The primary signals responsible for this induction are specific phenolic β-glycosides (Fig. 1K and L), which are abundant in the leaves, bark, and flowers of many plant species parasitized by *P. syringae* pv. syringae (332, 333). The aglycone derivatives of these compounds are inactive as inducers, suggesting that the beta-glycosidic linkage is necessary for signal activity. Specific sugars common to plant tissues, including fructose, mannose, and sucrose, enhance the expression of *syr* genes up to fivefold (332, 333). The effect of sugars was most noticeable at low concentrations of phenolic glycosides (i.e., 1 to 10 μM), suggesting that sugars increase the sensitivity of *P. syringae* pv. syringae to the phenolic signal. This is highly reminiscent of the phenolic detection system of *A. tumefaciens*, although the sugar specificities are quite different. A common predisposing factor to infection by *P. syringae* pv. syringae is frost injury (183), which would lead to a sudden release of plant cell wall-associated sugars, as well as of phenolic glycoside signals, the bulk of which are compartmentalized in vacuoles (332). It is not known how these phenolics and sugars are detected and how they increase the expression of the *syr* operon.

Biosynthesis of syringomycin is influenced by the GacS-GacA system (see below), which in *P. syringae* pv. syringae regulates toxigenesis and the ability to cause necrotic lesions in plants (211). The environmental signals that activate the *gacA-gacS* regulon in this organism have not been identified, although they do not appear to be phenolic plant signal molecules (32, 407). Overexpression of a probable transcription factor called *salA* restores syringomycin production in a *gacS* mutant, suggesting that *salA* is a member of the GacS-GacA regulon (269). One gene within the *syr* cluster, *syrP*, also affects transcription of the syringomycin biosynthesis genes (32, 547). SyrP is two-component histidine kinase, and the corresponding response regulator has not been identified.

Coronatine is a non-host-specific chlorosis-inducing phytotoxin produced by various strains of *P. syringae* (170, 171, 330, 331). The coronatine biosynthetic genes were strongly induced by crude extract and intercellular fluid of tomato leaf, and the active components responsible for this induction were identified as malic and citric acids, with minor contributions coming from shikimic and quinic acids (288). Shikimic and quinic acids are intermediates in the shikimic acid pathway, which is the primary route for the synthesis of phenylalanine and phenols in plants. Several other compounds including glucose and inositol also activated the toxin genes when tested at high concentrations (3 to 5 mM); however, shikimic and quinic acids (Fig. 1I and J) were the only two that exhibited activity at concentrations below 0.1 mM. In addition, neither acid could be used as a sole carbon source by this bacterium, and the signal activity of shikimic acid was enhanced 10-fold by the presence of low levels of glucose. All these data suggest that the two acids are the true *cor*-inducing signals, but it is not clear how these environmental signals influence the transcription of toxin biosynthetic genes.

Coronatine biosynthesis is also affected by temperature (32, 451). The toxin is produced at highest levels at 18°C, while at 28°C, the optimal growth temperature for *P. syringae*, its biosynthesis is undetectable (58). The thermoregulation occurs at the level of transcription of the biosynthetic genes and is mediated by products of three regulatory genes, *corS*, *corR*, and *corP*. These genes are located in a 32.8-kb plasmid-borne gene cluster, which also includes genes involved in coronatine biosynthesis (33, 498). The *corS*, *corR*, and *corP* genes encode an unconventional two-component system consisting of a transmembrane histidine kinase, CorS, and two response regulators, CorR and CorP (498). CorP lacks a typical helix-turn-helix motif but possibly functions as a modulator of CorR or CorS activity (512). CorR binds to its target DNA sequences in a thermoresponsive manner, and its DNA-binding activity is controlled by CorS. A recent report suggested that at elevated temperatures, one of the six transmembrane domains of CorS might flip into the periplasm, causing the conserved H-box-containing region to become sequestered by insertion into the cytoplasmic membrane (452). The ecological rationale for the thermoinduction of this toxin is not understood. The phenomenon could be explained by the fact that *P. syringae* often lives

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on the surfaces of host plants and thus requires water films for efficient infection. Such water films might occur predominantly when the air temperature is low (451). Coronatine production is also controlled by the GacS-GacA system (see below) (73).

## ANTIBIOTIC PRODUCTION BY ROOT-COLONIZING *PSEUDOMONAS FLUORESCENS*

Antibiosis is an important mechanism used by plant-beneficial microorganisms to overcome the effects of soil-borne pathogens (397, 511). Production of the polyketide antimicrobial metabolite 2,4-diacetyl-phloroglucinol (DAPG) is a key factor in the biocontrol activity of *P. fluorescens* CHA0 (186). It is effective against bacteria, fungi, and helminths. In *P. fluorescens* CHA0, the four DAPG biosynthesis genes, *phlACBD*, are organized as an operon, which is followed by a gene coding for a putative efflux protein (*phlE*). Upstream of the *phlACBD* operon is a divergently transcribed *phlF* gene, which encodes a TetR-type repressor of DAPG synthesis (105). The dimeric PhlF protein binds to the *phlA* promoter region, and this binding is prevented by the addition of DAPG (105, 445). Thus, DAPG positively controls its own biosynthesis, and the fact that the signal is made by bacteria and is diffusible is reminiscent of the autoinduction systems of various bacteria (154). A second TetR-like regulator, PhlH, has been identified as a regulator of DAPG synthesis. The *phlH* gene lies downstream of *phlF*. Inactivation of *phlH* causes low expression of the *phl* operon, suggesting that PhlH works as an activator or antirepressor. An effector molecule binding to PhlH remains to be discovered (445).

In the rhizosphere, the resident microflora as well as the plant can strongly influence the expression of these antibiotic biosynthetic genes. A DAPG-responsive *phlA-lacZ* fusion was expressed more strongly on roots of maize or wheat than on those of bean or cucumber (362). This differential regulation is likely to be caused by differences in exudate composition between monocots and dicots. The host genotype and cultivar, as well as plant age, were also found to have an effect on expression of the reporter (362). In addition, root infection by *Fusarium* spp. can significantly alter *phlA* expression (361). The effect is due to the fungal production of the toxin fusaric acid, which is a potent inhibitor of DAPG synthesis in *P. fluorescens* CHA0 (361). Fusaric acid is produced by many *Fusarium* spp. and is toxic to various plants, fungi, and bacteria. In the *P. fluorescens* CHA0 *phlF* mutant, *phlA* expression was not altered by the presence of *F. oxysporum*, suggesting that the inhibitory effect of fusaric acid on DAPG production is mediated through the PhlF repressor (361). It is thought that fusaric acid antagonizes the derepressing effect of DAPG, resulting in PhlF-mediated repression of the *phlACBD* operon.

In addition to DAPG, *P. fluorescens* strain CHA0 produces the antibiotics pyoluteorin and pyrrolnitrin, as well as the biocide hydrogen cyanide (186). The relative importance of all of these compounds in disease control depends on the plant host, suggesting that specific plant factors might be involved in regulation of their production. For example, pyoluteorin production by *P. fluorescens* is involved in the suppression of *Pythium* damping-off of cress but not of cucumber (276, 320). The regulation of *P. fluorescens* exoproducts synthesis is also positively controlled by the GacS-GacA system (see below) (59).

## GLOBAL REGULATION OF PLANT-ASSOCIATED PHENOTYPES

As described above, expression of bacterial genes involved in plant-microbe interactions is controlled by various specific plant-produced signal molecules, as well as by nutritional and environmental factors specific for the plant-associated habitats (e.g., apoplastic fluids). Regulatory pathways responding to these signals are generally integrated into much larger control networks with many positive and negative inputs, and the ability of bacteria to respond to these signals often depends on various additional regulatory elements, including QS systems and global environmental and physiological regulators such as GacS-GacA, RpoS, and CRP.

### Role of Quorum Sensing in Regulation of Plant-Associated Phenotypes

In many plant-associated bacteria, various phenotypes that are induced in response to specific plant signals are also controlled by QS systems. These phenotypes include various pathogenicity determinants, conjugation, rhizosphere competence, and the production of antifungal metabolites (295, 508, 526). QS systems are based on the production of diffusible signal molecules referred to as autoinducers. These molecules accumulate in the environment and trigger specific bacterial responses when they exceed a critical concentration (154). QS is thought to allow bacteria to act in a coordinated manner and ensures that certain traits are expressed only when the population reaches a high density (154). Among gram-negative bacteria, the best studied and possibly most common group of autoinducer signals are *N*-acylhomoserine lactones (AHLs). Other signals are found in *R. solanacearum*, where 3-hydroxy palmitic acid methyl ester (3-OH PAME) serves as an autoinducer controlling the production of a major virulence factor EPS (142), and in *X. campestris*, where exoenzymes and EPS production are regulated in a cell-density-dependent manner by an α,β unsaturated fatty acid (*cis*-11-methyl-2-dodecenoic acid) (507, 515). In *E. carotovora*, for example, the QS model proposes that placing pathogenicity-associated genes under density-dependent control provides a mechanism for avoiding the host plant's defense systems (527). According to this model, *Erwinia* uses AHLs to initiate a pathogenic attack only when its population density is above a critical level, which ensures a high probability of overcoming host resistance. Recently, however, it has been proposed that for many bacterial species, the primary role of secreted autoinducers is not in measuring the population size but, rather, in sensing local diffusion rates in the microenvironment surrounding the cell (400). 3-OH PAME in *R. solancearum* is thought to be used by the bacteria to measure the level of bacterial confinement, which is maximal when cells are growing inside the plant vasculature (440, 508).

Because QS systems are often required for pathogenesis, they provide excellent targets for plant defense mechanisms (26). Several host plants, including pea, rice, tomato, soybean, and *Medicago truncatula*, secrete various compounds that mimic bacterial AHL signals and can either inhibit bacterial responses to an added autoinducer or stimulate autoinducer-induced behaviors (160, 482). The chemical identities of these

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compounds have not yet been determined, but they appear to be different from any of the known bacterial autoinducers (481). Results of a recent proteomic analysis of responses of *M. truncatula* to autoinducers of *S. meliloti* and *P. fluorescens* suggest that plants might also use bacterially released autoinducers as signals for induction of their defenses. Autoinducers caused significant changes in the accumulation of over 150 different plant proteins, of which approximately 23% had functions related to plant defenses or stress response (312). This suggests that these bacterial signals might have the unintended side effect of alerting the host to impending invasion and triggering defense responses. Any such responses, however, would need to be very specific, in order to avoid adverse effects on mutualistic symbionts, which often use QS to regulate behaviors that may benefit the plant. Nevertheless, plant interference with QS, as well as the diverse effects of autoinducers on the plant host, suggests that these signals might play an important role in many pathogenic and symbiotic plant-microbe associations.

### Role of GacS-GacA in Regulation of Plant-Associated Phenotypes

The GacS-GacA system is a two-component system that has been found in many proteobacteria, including *Pseudomonas*, *Vibrio*, *Salmonella*, *Legionella*, and *Erwinia* spp. (190, 211), where it controls a variety of phenotypes. Most commonly, GacS-GacA-controlled phenotypes involve the synthesis of extracellular enzymes and secondary metabolites that can be required for virulence or, in the case of plant symbionts, can protect the host from pathogenic fungi. GacS-GacA controls antibiotics, QS signals, toxins, EPS, and extracellular enzymes, as well as motility, biofilm formation, and protein secretion (211).

GacS is a hybrid transmembrane histidine kinase, containing a periplasmic domain and cytoplasmic kinase, receiver, and output domains (211). In response to environmental signals, GacS is thought to autophosphorylate and then transfer the phosphoryl group to the response regulator GacA in a four-step His-Asp-His-Asp phosphorelay (376, 554). The nature of the signal perceived by GacS is unknown. When bacterial cells are growing in batch culture, the GacS-GacA-dependent phenotypes are expressed mostly when the culture is in the transition from exponential to stationary phase (40, 211, 401). This indicates that activation of the GacS-GacA system does not require the presence of host cells and suggests that the regulation of this system may involve a QS-like mechanism. Indeed, the biocontrol strain *P. fluorescens* CHA0 synthesizes an extracellular signal that activates the GacS-GacA-dependent production of extracellular products (210, 554). The activator is extracted by organic solvents, has a low molecular mass, and is not an AHL. Mutational inactivation of either GacS or GacA abolished the response of the strain to the added signal (554). The poorly conserved GacS periplasmic domain was nonessential for GacS activity and for extracellular signal-dependent activation of the GacS-GacA pathway. In contrast, deletion of the GacS linker domain caused signal-independent, strongly elevated expression of exoproduct genes at low cell densities. Both the *gacS* linker mutant and a *gacS* null mutant were, unlike the wild-type strain, unable to protect tomato plants from crown and root rot, indicating that detection of the extracellular signal is important for the regulatory function of the GacS-GacA system in this bacterium (554). It is not known whether all bacteria that use GacS-GacA respond to the same signal molecule or if, like AHL signaling, different bacteria produce different but related molecules.

*gacA* expression in *P. syringae* was found to be affected by growth phase and medium composition, with high levels of *gacA* transcripts occurring at high cell densities or in the *hrp*-inducing minimal medium (see above) (73). To better define the inventory of GacA-controlled genes, a *gacA* mutant of *P. syringae* was screened for effects on the level of transcripts of a number of genes (73). The mutant produced much-reduced levels of transcripts of the *rpoN* gene and the *hrpRS* operon, which are required for expression of *hrpL*, which in turn is required for expression of genes encoding the TTSS. GacA deficiency also reduced the expression of *rpoS*, encoding an alternative sigma factor required for stress responses and secondary-metabolite production; of *ahlR* and *ahlI* genes encoding a QS system; of *salA*, a regulatory gene that controls virulence; and of *corS* and *corR*, which control the expression of genes involved in the biosynthesis of a phytotoxin coronatine. These results clearly established that GacA is located at the top of the regulatory hierarchy controlling an assortment of regulatory factors. However, the primary target genes of GacA are still unknown.

RsmA-*rsmB* is a global regulatory system that has been implicated in the downstream GacS-GacA regulatory cascade. The system is conserved throughout the proteobacteria (380, 525, 526), where it is proposed to act as a global post-transcriptional regulator (247). RsmA is an RNA binding protein, which promotes the decay of the mRNA transcripts of target genes, while *rsmB* encodes a regulatory RNA that inactivates RsmA, presumably by stoichiometric binding.

RsmA was first defined in *E. carotovora*, where it was shown that *rsmA* mutants were hypervirulent (94). The RsmA mutants overproduced pectinases, cellulases, proteases, and the HrpN (harpin) protein, while exoenzyme synthesis was inhibited by multicopy *rsmA* expressed in *trans* (94). Furthermore, *rsmA* can downregulate a physiologically diverse spectrum of phenotypes, including flagellum synthesis, antibiotic production, pigment synthesis, and EPS production in various *Erwinia* species (338).

In *E. carotovora*, expression of *rsmB* is under the positive control of the ExpS-ExpA two-component system, which is orthologous to GacS-GacA. It is through this regulation that the ExpS-ExpA system controls the production of extracellular enzymes and virulence in this bacterium (Fig. 7B) (93). The GacS-GacA system also positively regulates the expression of an *rsmB*-like gene in *P. fluorescens* CHA0 and in *P. syringae* DC3000 (73, 210). Thus, in the proposed pathway of signal transduction involving the GacS-GacA system, activation of the sensor kinase GacS by unknown signals triggers a phosphorelay, activating the response regulator GacA. Phosphorylated GacA positively controls the transcription of regulatory RNAs such as RsmB, which sequesters the translational repressor RsmA, rendering the target mRNAs accessible for translation (Fig. 7B) (40, 186, 210). It is not known, however, whether the induction of *rsmB* transcription by GacA is direct or indirect.

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The interactions and cross talk between various cascades that form the complex regulatory network controlling virulence genes in various plant-pathogenic bacteria are largely unknown. In *E. carotovora*, the ExpS-ExpA system positively regulates the production of AHLs, and there is evidence that AHLs control the production of *E. carotovora* virulence factors through the RsmA-*rsmB* system (72), while RsmA was shown to have a negative effect on expression of the gene encoding the AHL synthase (94). Expression of *rsmA* and *rsmB* is also controlled by the KdgR regulator, which induces the transcription of *rsmA* and represses the transcription of *rsmB* (234, 292). Therefore, it seems that in *E. carotovora*, KdgR and ExpS-ExpA interact by controlling virulence gene expression through the RsmA-*rsmB* system and that AHL production might be involved in the ExpS-ExpA control of RsmA-*rsmB* (Fig. 7B). The ExpS-ExpA system was shown to positively regulate the synthesis of AHLs in several other organisms, including *P. aeruginosa*, *P. syringae*, and *P. aureofaciens* (211).

The complex regulation of virulence suggests that numerous conditions must be met before pathogenicity factors are produced in planta and that each regulatory system or signal serves as only one switch among many. These complexities make it extremely challenging to obtain a comprehensive picture of the functioning of these systems in natural environments, especially because many of the signals involved in the regulation of these pathways remain unidentified.

## CONCLUSIONS

In the past 20 years, numerous studies have shown that plant-associated bacteria can detect various host-released chemicals, to which they respond in ways that allow them to colonize their host. In this review, we described such plant-released signals, the way they are recognized, and the way they trigger the appropriate responses in the bacteria. Many aspects of these processes are understood in some detail. However, as each new insight opens new questions, a lot of exciting work is waiting to be done.

*A. tumefaciens*-plant pathosystem and rhizobium-legume symbiosis are among the best-characterized plant-microbe systems available. Signals, as well as receptors responsible for their detection, have been characterized for several stages of these plant-microbe interactions, but many questions still remain unanswered. It is still not known for certain whether VirA or some other protein in *A. tumefaciens* contains a binding site for phenolic compounds, and we still do not know how these inducing compounds alter the ability of VirA to phosphorylate VirG. While the OccR protein has been characterized in great detail by genetic and biochemical methods, crystal structures of this protein in complex with DNA with and without bound ligand will provide conclusive evidence for its proposed mechanism of action. The process of nodule invasion in rhizobia remains a rich area for future studies. NodD also remains rather poorly characterized biochemically, and the functions of multiple NodD and SyrM proteins are underdeveloped. The recently reported purification of *R. leguminosarum* NodD should provide new possibilities to study mechanistic details of flavonoid detection by this protein. For both the rhizobia and in *A. tumefaciens*, more studies must be done to address gene expression in the phylosphere and rhizosphere rather than in broth cultures.

A related challenge awaits those studying host detection by other plant pathogens. The ability to alter *hrp* gene expression and TTSS-mediated protein secretion through genetic manipulation and use of the appropriate media has provided many new insights into this process. However, our knowledge of in planta Hrp regulation and the presumed contact-dependent activation of TTSS-dependent protein translocation remain incomplete. Also incomplete is the inventory of regulatory components and our understanding of how these factors are modulated by signals and how they are integrated into regulatory cascades.

In various plant pathogens, many environmental conditions, such as pH, temperature, osmolarity, or nitrogen limitation play important roles in the regulation of genes that are involved in plant-microbe interactions, and although in some cases the relevant regulatory proteins have been identified, in most cases the mechanisms of how the signals are detected and integrated to regulate their target genes are completely unknown. Furthermore, many signals that regulate the expression of plant-associated phenotypes through well-characterized regulatory proteins, such as PecS, PecT, Pir, AepA, and the GacS-GacA system, have not yet been identified.

While a lot of work still remains to be done on the model systems described above, many other plant-microbe interactions remain completely unexplored. Genetic and molecular approaches have just recently been developed to investigate host detection by nonpathogenic epiphytic bacteria and between mycorrhizal fungi and plant roots (289, 471, 495). In addition, nodulation of legumes by members of the β-proteobacteria such as *Burkholderia* are just now coming to light (335), while *Azoarcus* has long been known to fix nitrogen during its interaction with grasses and has recently been shown to contribute nitrogen to its host (228). Interesting and underexplored areas also include interactions between plants and *Agrobacterium rhizogenes* or *A. vitis*. Thus, there are many challenges to the development of new model systems. By meeting these challenges, we will gain new insights into general properties of plant-microbe interactions and at the same time help answer old questions about these phenomena.

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