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CHAPTER 3.1.4

The Genus *Agrobacterium*

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Introduction

The genus *Agrobacterium* is a group of Gram-negative soil bacteria found associated with plants. Many members of this group cause disease on plants. Infections of wound sites by *Agrobacterium tumefaciens* cause crown gall tumors on a wide range of plants including most dicots, some monocots, and some gymnosperms. Infections by *A. rhizogenes* cause hairy root disease. *A. vitis* causes tumors and necrotic lesions on grape vines and is commonly found in the xylem sap of infected plants. Despite the general perception that most of the agrobacteria cause disease, the member of this group most often isolated from soil, *A. radiobacter*, is avirulent.

Phylogeny

Earlier studies using physiological characteristics such as ability to grow on various carbon sources placed the agrobacteria with the rhizobia in the family *Rhizobiaceae*. More recent studies of both 16S rDNA and other chromosomal gene DNA sequence homologies suggest that these two groups of bacteria are indeed closely related (Willems et al., 1993). Both physiological characteristics and 16S rDNA sequence data place these bacteria in the α subgroup of the Proteobacteria. They appear to be closely related to members of the genus *Brucella*.

On the basis of genomic organization the agrobacteria appear to form a unique group within the α2 subgroup of the Proteobacteria (Jumas-Bilak et al., 1998; see also Genetics); Biovar 1 strains and *A. rubi* have both a circular and a linear chromosome. Biovar 2 and 3 strains also have 2 chromosomes, but both appear to be linear. Large plasmids (200–400 kb) are present in most strains. *Rhizobia*, although closely related to agrobacteria, appear to lack the linear chromosome present in biovar 1 agrobacteria.

Genetic experiments suggest that at least some members of the rhizobia are closely related to agrobacteria. The gene order on the circular chromosome appears to be conserved between *Sinorhizobium meliloti* and *A. tumefaciens* (Hooykaas et al., 1982). When plasmids from *Rhizobium phaseoli* were introduced into *A. tumefaciens* strain C58 (a biotype 1 strain; see also Taxonomy), the resulting bacteria were able to form nitrogen-fixing nodules on bean roots. This outcome suggests that all of the chromosomal genes required for the interaction of *R. phaseoli* with plants were present on the *A. tumefaciens* chromosomes (Martinez et al., 1987; see also Genetics, Chromosomal). Along similar lines, the *nodC* gene on the sym plasmid of *S. meliloti* can be induced when this plasmid is present in *A. tumefaciens* but not when the plasmid is transferred to *E. coli*, *Xanthomonas campestris* or *Pseudomonas savastanoi* (Yelton et al., 1987). All of these results suggest that the chromosomal genes of agrobacteria and *rhizobia* are so closely related they can substitute for each other. Sequencing of the genomes of *S. meliloti* and *A. tumefaciens* is currently in progress and should help to elucidate the relationship between these bacteria.

Taxonomy

The genus is divided into species largely based on pathogenic properties, although other physiological characteristics correlate with pathogenic properties. The major species are *A. radiobacter* (nonpathogenic), *A. tumefaciens* (the causative agent of crown gall tumors), *A. rhizogenes* (the causative agent of hairy root disease), and *A. vitis* (the causative agent of tumors and necrotic disease on grapevines). There are also less well studied proposed species such as *A. rubi* isolated from cane galls on *Rubrus* species.

Agrobacteria also have been divided into biotypes (biovars) based on physiological properties. Biovar 1, which includes most strains of *A. tumefaciens*, has no growth factor requirements and will grow in the presence of 2% NaCl. Most strains produce 3-ketolactose. All biovars produce acid from mannitol and adonitol. Biovar 1 bacteria also produce acid from dulcitol,

melizitose, ethanol, and arabitol. Some biovar 1 strains are able to grow at 37°C. However, they may lose the Ti plasmid, which is required for virulence, when grown at this temperature. Biovar 2 includes most strains of *A. rhizogenes*. These bacteria require biotin for growth. They fail to grow in the presence of 0.5% NaCl or at 37°C. Some biovar 2 strains can grow on tartrate, producing alkali. Biovar 3 strains include most *A. vitis* strains. Some authors also include some *A. tumefaciens* strains in this group. Like biovar 1 strains, these bacteria will grow in the presence of 2% NaCl but generally do not grow at 37°C. Both biovar 2 and 3 strains fail to produce 3-ketolactose. Biovar 3 strains can produce alkali from tartrate. Some biovar 3 strains require biotin for growth (Table 2). Selective growth media for various biovars have been reported and are described in the section on isolation of agrobacteria (Table 1). Biovars 1 and 3 contain both strains with wide and others with narrow host ranges (Kerr et al., 1977b).

Habitat

Agrobacteria usually are found in soil in association with roots, tubers, or underground stems. The bacteria also cause tumors from which they can be isolated. Tumors may be prevalent on grafted plants at the graft junction; examples include grapes, roses, poplars, and fruit trees. In some cases, the bacteria can be isolated from the xylem of infected plants. Thus it is often possible to isolate *A. vitis* from the xylem of infected grapevines.

Although agrobacteria are generally isolated from cultivated soils and plants, biovars 1 and 2 can be found in association with roots from uncultivated plants of the natural savanna and tall grass prairie which has never been cultivated (Bouzar et al., 1987). As is the case in most other field studies of agrobacteria, the majority of these isolates were nonpathogenic. Schroth et al. (1971) were able to isolate agrobacteria from almost every soil they tested in California by using selective media and enrichment culture methods. Thus the bacteria appear to be widely distributed regardless of the plants previously grown in the location. However, the number of bacteria pathogenic for a crop grown in a particular location was greater if the same crop had formerly been grown in that location.

Isolation

Agrobacteria can be isolated from soil obtained from the vicinity of infected plants, from galls formed by the bacteria, or, in the case of grapevines, from the xylem sap of infected plants. The bacteria are not numerous in older galls and may be easier to isolate from the surrounding soil than from the tumor tissue.

Agrobacteria grow readily in culture on complex or defined media (Table 1). Nutrient agar (with or without yeast extract [0.5%]) or yeast mannitol agar will support the growth of most strains. Some strains require B vitamins for growth, usually 0.2 mg/liter each of biotin, pantothenic acid and/or nicotinic acid. Many strains, including most *A. rhizogenes* isolates, are sensitive to salt and will not grow on media such as Luria-Bertani agar because this medium contains too much NaCl. The colonies are generally white or slightly cream or pale pink in color. No distinctive pigment is produced. Large amounts of extracellular polysaccharide may be produced on some media, giving the colonies a watery appearance. The bacteria grow at a moderate rate. *A. tumefaciens* will usually require 2 to 4 days to form colonies on complex media. Some strains of *A. rhizogenes* are slow growing and may require as much as 1 week to form colonies on complex media.

Optimal growth temperature for most strains is between 25 and 28°C, although the optimal temperature for plant infection may be lower (22°C).

Selective media may be used to isolate Agrobacteria (Table 1).

Identification

Agrobacteria have been traditionally identified as Gram-negative bacteria that don't produce fluorescent pigment on King's B medium and do produce tumors (or hairy roots) when inoculated onto test plants. The test plants most often used are tomato, sunflower, *Datura* spp., *Kalanchoë daigremontiana* (also called *Bryophyllum*), tobacco, and *Nicotiana glauca* (Figs. 1–4). These plants respond relatively readily and rapidly to inoculation of *Agrobacterium* strains by producing tumors in as few as 10 days. Sugar fermentations and production of ketolactose also have been used in identification of agrobacteria (Table 2). In recent years, lipid and fatty acid profiles have been used to identify both virulent and avirulent agrobacteria (Jarvis et al., 1996; Bouzar et al., 1993a). Polymerase chain reaction (PCR) has also been used in identification and to distinguish pathogenic from nonpathogenic strains. The PCR primers chosen from *vir* genes such as *virD2* (See Genetics) can be used to identify potentially pathogenic strains (Haas et al., 1995). Pathogenic strains have been identified by their ability to grow on different opines, and the

Table 1. Media for growth of agrobacteria.

General Media

Luria Agar (for biovar 1 and some biovar 3 strains)

Tryptone	10g
Yeast extract	5g
NaCl	5g
Water	1 liter
3M NaOH	1ml
Agar	14g

Yeast Mannitol Agar (for all biovars)

Mannitol	10g
Yeast extract	1g
K_2HPO_4	0.5g
$CaCl_2$	0.2g
NaCl	0.2g
$MgSO_4 \cong 7H_2O$	0.2g
$FeCl_3$	0.01g
Water	1 liter
Agar	15g

Adjust to pH 7.0. For biovar 2 add biotin, calcium pantothenate, and nicotinic acid, all at 200µg/liter.

Mannitol Glutamate Agar (for all biovars) (Roberts et al., 1974)

Mannitol	10g
L-Glutamic acid	2g
KH_2PO_4	0.5g
NaCl	0.2g
$MgSO_4 \cdot 7H_2O$	0.2g
Biotin	0.002g
Water	1 liter
Agar	15g

Adjust pH to 7.0 before autoclaving.

H4 Minimal Medium (for biovars 1 and 3; biovar 2 will grow very slowly on this medium) (Matthysse et al., 1976)

NH_4Cl	5g
NH_4NO_3	1g
Na_2SO_4	2g
K_2HPO_4	3g
KH_2PO_4	1g
$MgSO_4 \cdot 7H_2O$	0.1g
Water	1 liter

Dissolve salts in the order given; adjust pH to 7.2; add 10ml of sterile 20% glucose after autoclaving.

AB Minimal Medium

K_2HPO_4	3g
NaH_2PO_4	1g
NH_4Cl	1g
$MgSO_4 \cdot 7H_2O$	0.3g
KCl	0.15g
$CaCl_2$	0.005g
$FeSO_4 \cdot 7H_2O$	0.0025g
Water	1 liter

General Media

Dissolve salts in the order given; adjust pH to 7.2; add after autoclaving 10ml of sterile 20% glucose or sucrose.

Selective Media (Kerr, 1986)*

Selective Medium of Biovar 1*

L(-) Arabitol	3.04g
K_2HPO_4	1.04g
KH_2PO_4	0.54g
NH_4NO_3	0.16g
$MgSO_4 \cong 7H_2O$	0.25g
Sodium taurocholate	0.29g
Water	1 liter
1% Crystal violet	2ml
Agar	15g

Add after autoclaving 10ml of 2% cyclohexamide and 10ml of 1% $Na_2SeO_3 \cong 5H_2O$. On this medium colonies of agrobacteria are white, circular, raised, and glistening. They may become mucoid.

Selective Medium for Biovar 2*

Erythritol	3.05g
K_2HPO_4	1.04g
KH_2PO_4	0.54g
NH_4NO_3	0.16g
$MgSO_4 \cdot 7H_2O$	0.25g
Sodium taurocholate	0.29g
Yeast extract	0.01g
Malachite green	0.005g
Water	1 liter
Agar	15g

Add after autoclaving 10ml of 2% cyclohexamide and 10ml of 1% $Na_2SeO_3 \cong 5H_2O$. On this medium colonies of agrobacteria are white, circular, raised, and glistening. They may turn brown a they age.

Selective Medium for Biovar 3*

Adonitol	4.0g
K_2HPO_4	0.9g
KH_2PO_4	0.7g
NaCl	0.2g
$MgSO_4$	0.2g
Yeast extract	0.14g
Boric acid	1.0g
Water	1 liter
Agar	15g

Adjust pH to 7.2 before autoclaving. After autoclaving add 10ml of 2.5% cyclohexamide, 1ml of 8% triphenyltetrazolium chloride, 1ml of 2% D-cycloserine, and 1ml of 2% trimethoprin. On this medium colonies of agrobacteria have dark red centers with white edges.

*Note that these media are only semi-selective. Other organisms may grow. Additional tests are necessary to positively identify an isolate as *Agrobacterium*.

Table 2. Traits used for identification of biovars of *Agrobacterium*.

Characteristic	Biovar 1	Biovar 2	Biovar 3	*A. rubi*
Growth on selective medium 1A[a]	Yes			
2E[b]		Yes		
RS[c]				Yes
Growth factor requirements	None	Biotin	Biotin, some strains	Biotin, pantothenic acid, nicotinic acid
3-Ketolactose production	Most strains	No	No	No
Growth on 2% NaCl	Yes	No	Yes	Yes
Growth at 37°C	Yes	No	No	Yes
Acid production from mannitol	Yes	Yes	Yes	Yes
Adonitol	Yes	Yes	Yes	Yes
Erythritol	No	Yes	No	No
Dulcitol	Yes	Yes	No	No
Melizitose	Yes	No	No	No
Ethanol	Yes	No	No	No
Arabitol	Yes	No	No	No
Alkali production from tartrate	No	Yes	Yes	No

Data from Kerr (1986).

formation of particular opines by tumors caused by various strains has been used to group these strains. In general, grouping by sugar fermentations, fatty acid profiles, PCR, opine production and utilization, and genome organization all reach similar conclusions so that no one method of identification of agrobacterial species or biovars is preferable.

Preservation

The bacteria can be stored as stabs into vials of nutrient agar (all biovars) or Luria agar (biovars 1 and 3) at room temperature essentially indefinitely (more than 10 years). They can also be stored frozen in 25% glycerol at –70°C. Liquid cultures of biovars 1 and 3 can be spun down, resuspended in phosphate buffered saline containing 1 mM $MgSO_4$, and stored in the refrigerator for approximately 10 weeks.

Physiology

General

Agrobacteria are Gram-negative, nonspore–forming, short rods. They can use glucose as a carbon source, growing aerobically. Table 1 lists agrobacterial growth media formulations and Table 2 lists characteristics of different biovars.

Fig. 1. The stem of a tobacco plant wounded at two places and inoculated with *A. tumefaciens*. The tumors are shown at 6 weeks after inoculation.

Opines: Production and Utilization

Crown gall tumors produce specific substances (often substituted L-amino acids) called opines. The production of opines is catalyzed by enzymes encoded by genes introduced into crown gall tumor cells on the T DNA. These genes are usually expressed constitutively in the tumor tissue. Typical opines result from condensation reactions between compounds already

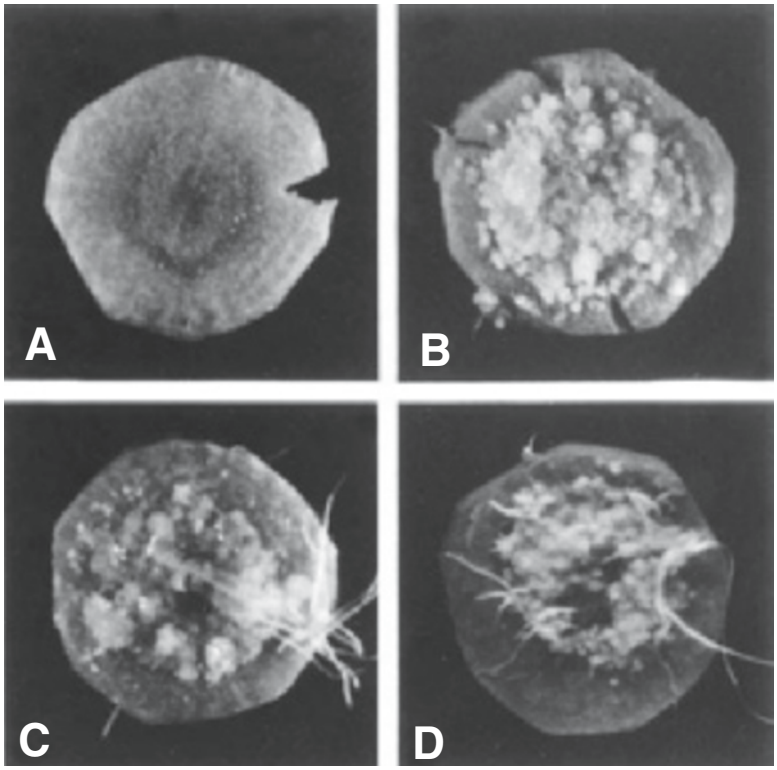

Fig. 2. Carrot root discs (A) uninoculated, (B) inoculated with *A. tumefaciens*, and (C) and (D) inoculated with *A. rhizogenes*. The discs are shown after 5 weeks incubation.

Fig. 3. A leaf of *Bryophyllum daigremontiana* (also called *Kalanchoë daigremontiana*) inoculated with *A. tumefaciens*. The site on the back right was inoculated with a strain lacking the Ti plasmid. Tumors are shown after 4 weeks growth.

Fig. 4. A leaf of *Bryophyllum daigremontiana* (also called *Kalanchoë daigremontiana*) inoculated with *A. rhizogenes*. Note that the roots formed at the wound sites are branching and ageotropic. The leaf is shown 5 weeks after inoculation.

present in plant cells. For example, octopine results from the reaction of the *a*-amino group of arginine and the keto group of pyruvate to form octopine [N_2-(1-D-carboxyethyl)-L-arginine]. The resulting compound (the opine) accumulates in the tumor tissue and can be used by the inciting bacteria as a carbon and nitrogen source. The genes encoding the enzymes for opine utilization are located on the Ti plasmid (pTi) outside the T DNA (the bacterial DNA which is transferred to the host cell) and on the chromosome. In the case of octopine, nitrogen appears to be recovered by the arginase-urease pathway. The genes for these enzymes are chromosomal. The carbon from octopine may be utilized by conversion of arginine to ornithine and then to proline via ornithine cyclase. The gene for this enzyme is located on pTi and, in some strains, a second copy is found in the chromosome (Dessaux et al., 1986). Other opines and their constituent compounds include lysopine (lysine and pyruvate), octopinic acid (ornithine and pyruvate), histopine (histidine and pyruvate), nopaline (arginine and *a*-ketoglutaric acid), nopalinic acid (ornithine and *a*-ketoglutaric acid), agrocinopine A (sucrose-4′-phosphate and arabinose-2-phosphate), agrocinopine C (glucose phosphate and sucrose phosphate), agropine (mannitol and glutamine), cucumopine [4-carboxyl-4-(2-carboxyl)spinacine], leucinopine (leucine and *a*-ketoglutaric acid), succinamopine (asparagine and a-ketoglutaric acid) and vitopine. Ti plasmids generally encode the enzymes for the synthesis of one or more opines in the T DNA and the enzymes for the utilization of the same opine elsewhere on the plasmid. Many Ti plasmids are named and grouped by the opine(s) that the plasmid-induced tumors produce (Dessaux et al., 1998).

Motility and Flagella

A. tumefaciens is motile by means of circumthecal flagella (Fig. 5). Some investigators have also observed polar and lateral flagella. There are four flagellar genes (*flaA*, *flaB*, *flaC* and *flaD*; Deakin et al., 1999). These genes are closely related to each other and to the *flaA* and *flaB* genes of *Sinorhizobium meliloti*. Deletion of the *flaA*, *flaB*, and *flaC* genes results in nonmotile bacteria that are slightly attenuated (tumors are about 70% the size of those induced by wild type bacteria) when inoculated directly into a wound site (Chesnikova et al., 1997). Bacteria, when inoculated into soil surrounding a wounded plant, must be able to move to wound sites on the plant to form tumors and thus must have flagella in order to be virulent. *A. tumefaciens* is chemotactic to a number of substances released by roots including sugars, amino acids and dicarboxylic acids. Genes involved in chemotaxis and motility have been sequenced (Wright et al., 1998; Deakin et al., 1997). These genes have a high level of homology with genes from *S. meliloti* and *Rhodobacter sphearoides*.

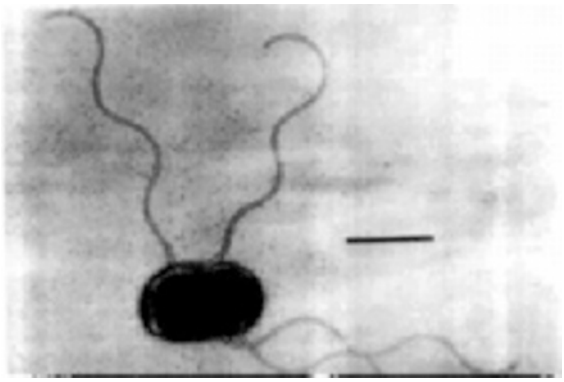

Fig. 5. *A. tumefaciens* as seen in the transmission electron microscope. Note the flagellae. The bar is 1 micron. From Brisbane et al. (1983).

Unique Aspects of *A. vitis*

Tartrate is found in grape sap. Most strains of *A. vitis* are able to degrade tartrate and use it as a carbon source. The genes for the metabolism of tartaric acid often are located on a plasmid, pTAR or pTr. A homologue of the *Pseudomonas ttuC* gene (encoding tartrate dehydrogenase) has been identified on these plasmids. Many of the tartrate plasmids are conjugative in *planta*, allowing their movement between strains (Otten et al., 1995; Salomone et al., 1996).

Genetics

General

Initial research on the agrobacteria suggested the presence of a circular chromosome and several large plasmids. More recent work has identified two chromosomes in *A. tumefaciens*. One is a roughly 3 Mbp circular chromosome that carries most of the known auxotrophic markers, and the other is a linear 2.1 Mbp chromosome to which some auxotrophic transposon mutants requiring adenine, threonine, serine, and pantothenic acid map (Jumas-Bilak et al., 1998; Goodner et al., 1999). Thus both DNA molecules can be considered chromosomes because they appear to contain genes required for normal cell functioning and metabolism. Most strains also have at least one large (approximately 300 kbp) cryptic plasmid, and virulent

strains have a Ti plasmid (approximately 200 kbp). Both the circular and linear chromosomes can be mobilized for conjugation by R68.41, a broad host-range conjugative plasmid (Goodner et al., 1999).

Chromosomal

A. tumefaciens Agrobacteria and *rhizobia* are closely related. The genetic maps of the circular chromosomes in *R. meliloti* and *A. tumefaciens* are very similar (Hooykaas et al., 1982). After *R. leguminosarum* plasmids were introduced into *A. tumefaciens*, the resulting bacteria could induce the formation of nitrogen-fixing nodules on the roots of beans. This finding suggested that all of the chromosomal genes required for the interaction of rhizobia with legumes are already present in the *A. tumefaciens* genome (Martinez et al., 1987). A variety of chromosomal genes from *A. tumefaciens* have been studied. Most of them are involved in the interaction of the bacterium with the plant. A summary of some of these genes is given below. They are grouped as follows: genes involved in exopolysaccharide synthesis, genes involved in attachment to plants, other genes required for virulence, and heat shock genes.

Genes Involved in Exopolysaccharide Synthesis
A. tumefaciens like *S. meliloti* makes a succinoglycan exopolysaccharide, which appears to have the same structure in both species. The pathway and the genes involved (named *exo* genes) for succinoglycan biosynthesis have been determined in *S. meliloti* (Reuber et al., 1993) and appear to be similar in *A. tumefaciens*. Mutations in the genes required for the synthesis of succinoglycan affect nodule formation in *S. meliloti*. However, similar mutations have been reported not to affect the virulence of *A. tumefaciens* (Cangelosi et al., 1987). Succinoglycan is also not required for colonization of tomato roots by *A. tumefaciens*, but mutations in *exo* genes did affect the ability of the bacteria to colonize legume roots (Matthysse, 2000).

In addition to succinoglycan, *A. tumefaciens* makes cellulose (Figs. 6–8). Genes required for cellulose synthesis (*cel* genes) are located on the bacterial chromosome near, but not adjacent to, the *att* genes (Robertson et al., 1988). Five genes organized into 3 operons (*celAB*, *celC* and *celDE*) have been identified. The *celD* and *celE* genes encode putative cytoplasmic proteins. The *celA*, *celB*, and *celC* genes encode putative membrane proteins. The CelA protein has homology to cellulose synthases from *Acetobacter xylinum*, *R. leguminosarum*, *P. fluorescens*, and plants. The C-terminal region of the CelC protein, which may be a glucosyl-transferase, has homology to endoglucanases from *E. chrysanthemi* and *A. xylinum*. The CelB, CelD, and CelE proteins have no significant homology with proteins in the databases (Matthysse et al., 1995). Although *A. tumefaciens* synthesizes some cellulose when grown in culture, the quantity increases markedly when the bacteria are grown with plant cells or roots. The mechanism by which the amount of cellulose synthesized is regulated is unknown. Mutants in *cel* genes are markedly reduced in virulence.

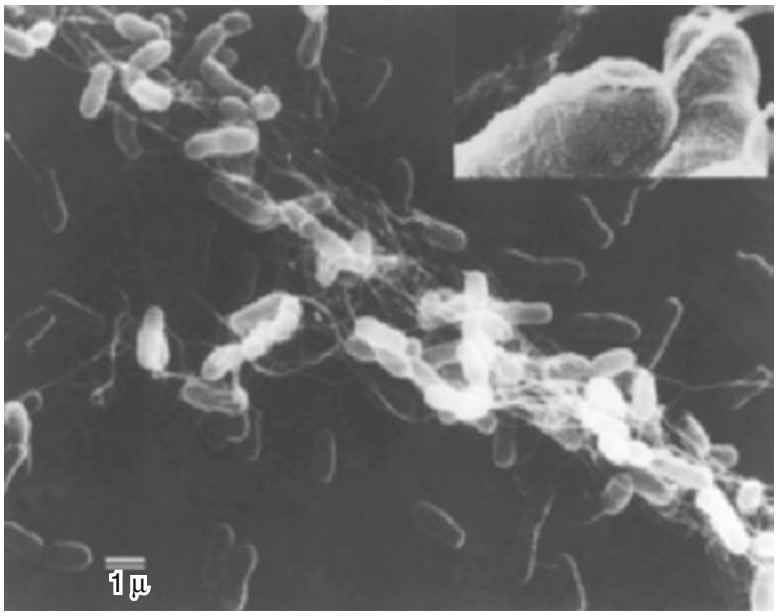

Fig. 6. Scanning electron micrograph showing *A. tumefaciens* grown with plant extract (soytone). The fibrils are cellulose made by the bacterium.

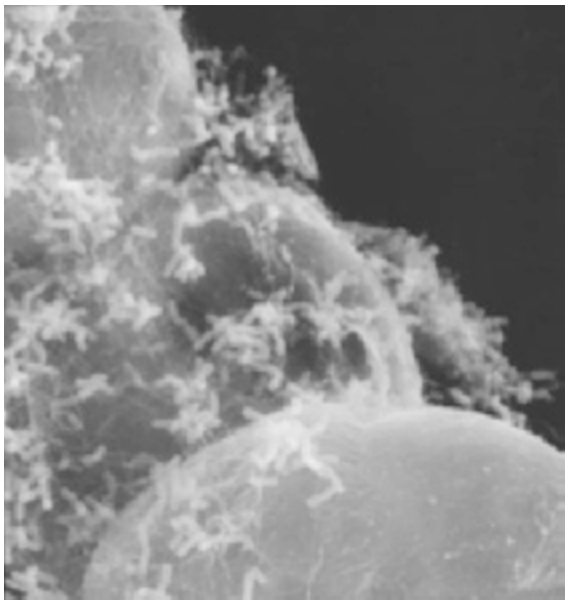

Fig. 7. Scanning electron micrograph of carrot suspension culture cells incubated with *A. tumefaciens* for 18 hours. Note the presence of both individually attached bacteria and clusters of bacteria on the surface of the plant cells. The fibrillar meshwork is cellulose fibrils made by the bacteria. From Matthysse et al. (1981).

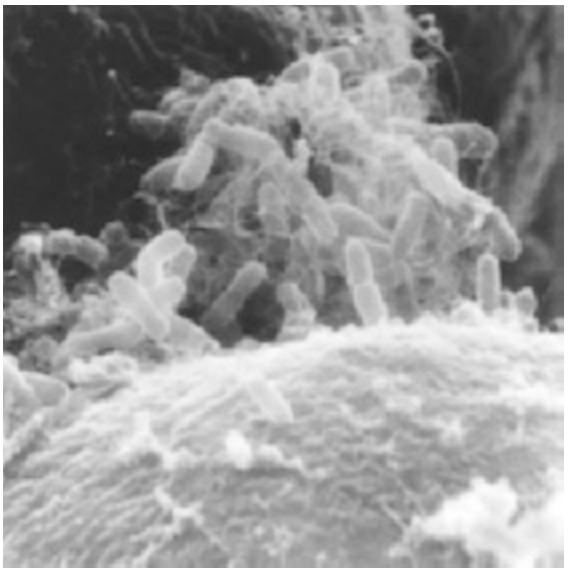

Fig. 8. Scanning electron micrograph of a carrot suspension culture cell incubated with *A. tumefaciens* for 18 hours. Note the presence of both individually attached bacteria and clusters of bacteria on the surface of the plant cells. The large cluster appears to be held together by cellulose fibrils produced by the bacteria. The majority of the bacteria adhering to the plant cell surface appear to be indirectly bound to the plant cell in large aggregates. From Matthysse et al. (1981).

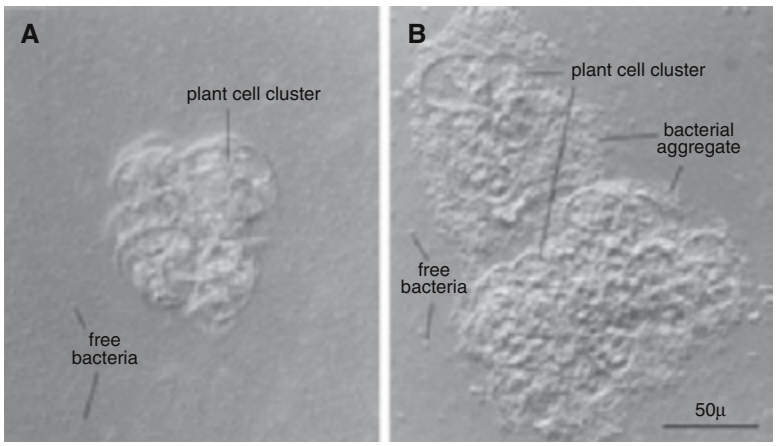

Fig. 9. Light micrographs showing bacteria (with and without attachment genes) incubated with carrot suspension culture cells for 24 hours. (A) A strain of *A. tumefaciens* lacking attachment genes. (B) Wild type *A. tumefaciens*.

Genes Involved in Initial Interactions with the Plant The region of the bacterial chromosome (*att*) in which transposon insertions result in an inability of the bacteria to attach to plants and thus in a lack of virulence is large (more than 35 kb). This region contains more than 32 open-reading frames (ORFs) organized in a minimum of 10 operons (based on the directions of the ORFs; Matthysse et al., 2000; Figs. 9–14). Many of these ORFs have no significant homology to any proteins in the databases. *Att* genes appear to fall into at least two categories: those that can be complemented by conditioned medium (CM) are presumably involved in signaling, and those that are not complemented by CM are presumably involved in the synthesis of the substance(s) that bind the bacteria to the plant surface. The *attA1–E* genes have homology to ABC transporters from a number of bacteria. Conditioned medium complemented the mutations in these genes. The genes are believed to be involved in signaling between the bacteria and the plant

(Matthysse et al., 1996). Conditioned medium also complemented several other *att* mutations. These genes are presumably also required for initial signaling. Conditioned medium did not complement mutations in *attJ*, *attK*, *attN*, *attO*, *attR*, or *attV-Z* genes. *AttR* mutants fail to make an acetylated capsular polysaccharide, which may be involved in binding of the bacteria to the plant surface (Reuhs et al., 1997). Three *att* ORFs products have homology to genes encoding DNA-binding proteins and may be involved in the regulation of gene expression. These ORFs are atrA, attJ and attO (Table 3).

Other known genes (aside from *att* and *cel*, which are required for the initial binding of the bacteria to the plant) appear to be involved in specifying for the general surface structure of the bacteria. These include the *chvAB* genes, which are required for the synthesis of β-1,2-D-glucan and the bacterial response to low external osmotic pressure, and the *pcsA* (*exoC*) gene, which encodes a phosphoglucomutase (Zorreguita et al., 1988; Thomashow et al., 1987; Leigh et al., 1992). Mutations in these genes, which are chromosomal, result in pleiotropic alterations in the bacterial surface. The *chvAB* mutants are avirulent on most but not all host plants. On some plants, they are virulent only at lower temperatures (19° but not 28°C; Banta et al., 1998). No Ti plasmid genes are known to be involved in the initial interactions of the bacteria with the plant.

Other Chromosomal Genes Involved in Pathogenesis Chromosomal genes involved in pathogenesis described elsewhere in this section include *att* genes, which are required for virulence; *cel* genes, which when mutated result in severe attenuation of virulence; *chvAB* genes, which are required for virulence on most hosts at normal (as opposed to cooler) temperatures; the *pscA* gene, which is required for virulence; and the *chvE* gene, which increases the induction

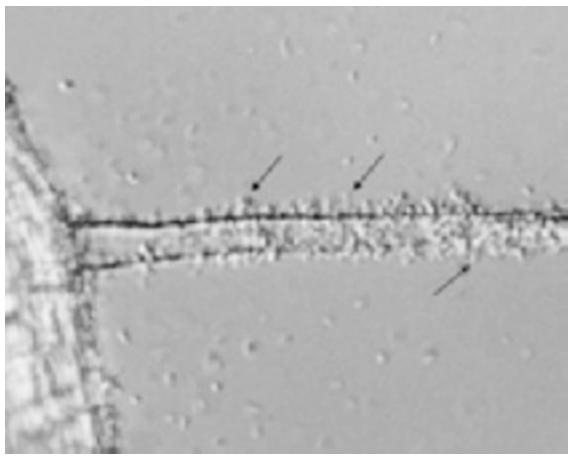

Fig. 10. Light micrograph showing *A. tumefaciens* strain C58 incubated with *A. thaliana* roots. Note the bacteria bound to the root hair surface preferentially in a vertical orientation (arrows). The orientation of bacterial binding varies with the strain examined.

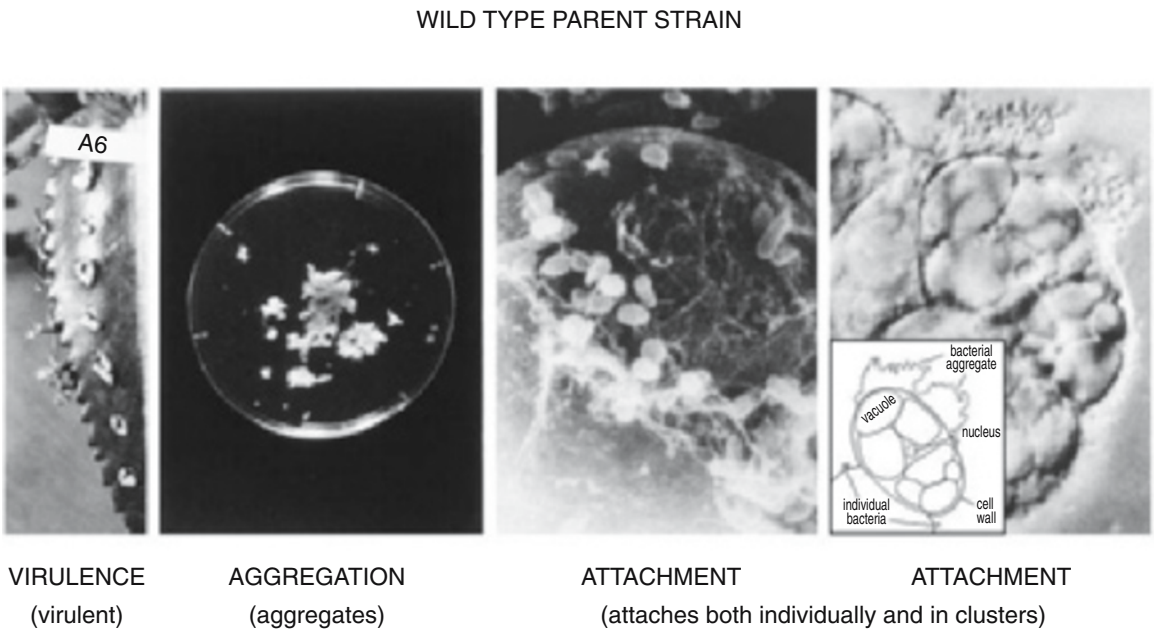

Fig. 11. Interactions of wild type *A. tumefaciens* with plants. From left to right: tumors on a leaf of *Bryophyllum daigremontiana*; aggregation of carrot suspension culture cells; scanning electron micrograph showing attachment to carrot cells; light micrograph showing attachment to carrot cells.

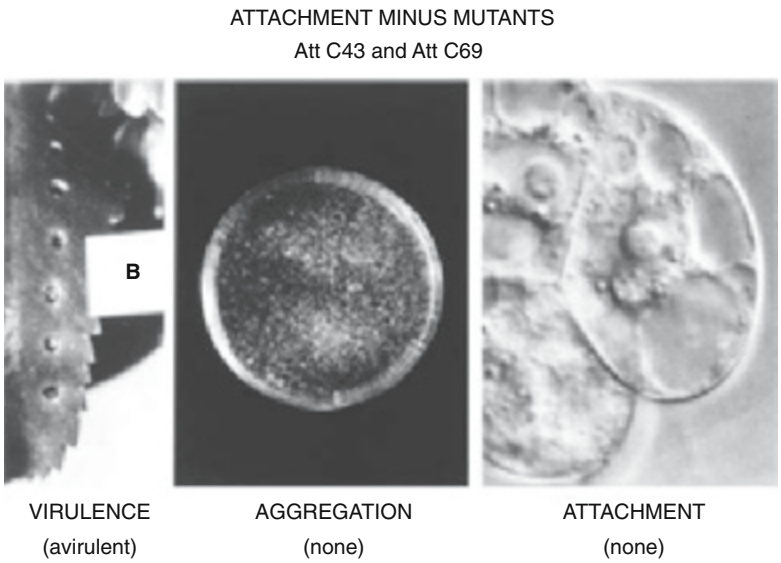

Fig. 12. Interactions of attachment-minus mutant *A. tumefaciens* with plants. From left to right: lack of tumor formation on a leaf of *Bryophyllum daigremontiana*; failure to aggregate carrot suspension culture cells; light micrograph showing lack of attachment to carrot cells. The concentrations of bacteria and plant cells in these incubations were the same as those used in Fig. 11 showing similar incubations of wild type bacteria.

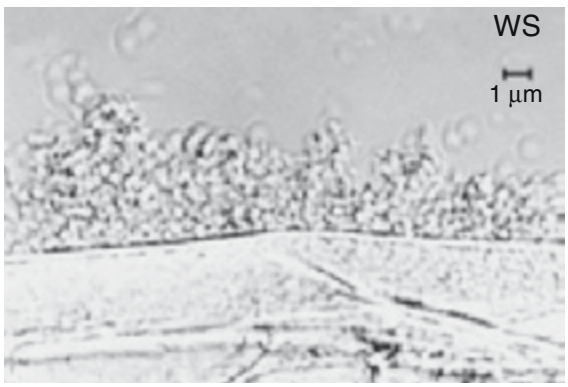

Fig. 13. Light micrograph showing *A. tumefaciens* bound to the surface of a root of *Arabidopsis thaliana*.

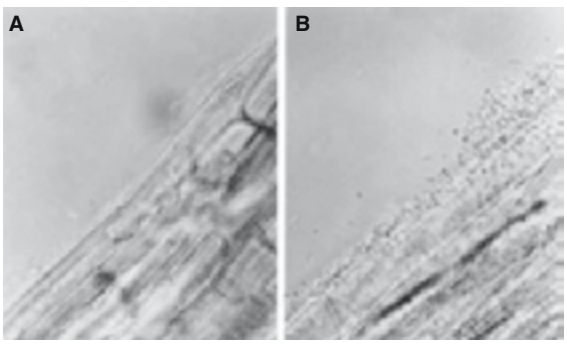

Fig. 14. Light micrographs showing *A. tumefaciens* wild type (B) and nonattaching mutant (A) incubated with tomato roots.

of *vir* genes by acetosyringone. Other chromosomal genes may affect the induction of *vir* genes. The *Ivr* (induction of *vir* genes) mutants are deficient in *vir* gene induction, although the mechanism is not understood (Metts et al., 1991). Some chromosomal genes are involved in *vir* gene induction on specific hosts. Thus some bacterial strains which are virulent on conifers carry a gene (*cbg*) that converts coniferin (which is not a *vir* gene inducer) to coniferyl alcohol (which is a *vir* gene inducer; Castle et al., 1992; Morris et al., 1990). Expression of *virC* and *virD* is regulated by a chromosomal gene *ros*, which encodes a repressor of these genes (Table 3). Ros mutants do not make succinoglycan, suggesting that *ros* is also involved in regulating the expression of *exo* genes. However, Ros mutants are still virulent on all hosts tested (Cooley et al., 1991a; Cooley et al., 1991b; Tiburtius et al., 1996; Hussain et al., 1997).

A two component system, *chvG*/*chvI*, is required for virulence and *vir* gene induction (Table 3). Mutations in these genes have pleiotropic effects; the mutant bacteria are sensitive to detergents and do not grow on complex medium. Because of the severely limited growth of these mutants, it is difficult to assess whether the requirement for *chvG*/*chvI* for virulence is due to a direct requirement for the gene products or to an indirect effect. The signal to which this system responds is unknown (Charles et al., 1993; Mantis et al., 1993).

Mutations in another chromosomal gene, *chvD*, reduce the induction of *virG* by acidic pH and phosphate starvation. This gene encodes a periplasmic ATPase (Winans et al., 1988). One gene, *acvB*, present only on the chromosome in nopaline strains, is required for virulence in these strains. In octopine strains, a second copy of this gene (*virJ*) is located on the Ti plasmid

Table 3. Regulatory genes in *A. tumefaciens* which affect expression of genes involved in the interaction with the host.

Gene	Location	Signals to which gene responds	Mutant phenotype	References
virG[a]	pTi	Acetosyringone and monosaccharides, acid pH, low phosphate	Avirulent, does not express *vir* genes	Winans et al., 1988 Roitsch et al., 1994 Stachel et al., 1985
occR	pTi	Octopine	Can not catabolize octopine, does not express *occ*, *ocd*, *oph*, *msh*, or *traR* genes	Cho et al., 1997
traR	pTi	Homoserine lactone (AAI autoinducer)	Can not conjugate pTi between bacteria, does not express *tra* or *trb* genes	Alt-Morbe et al., 1996 Fuqua et al., 1994 Hwang et al., 1995 Hwang et al., 1994
ros	Chromosomal	Iron?	Succinoglycan (EPS) minus, *virD* and *virC* constitutive	Cooley et al., 1991a Cooley et al., 1991b Tiburtius et al., 1996 Hussain et al., 1997
*chvI**	Chromosomal	? Unknown	Does not grow in complex media, acid and detergent sensitive, avirulent, *vir* gene expression attenuated	Charles et al., 1993 Mantis et al., 1993
atrA	Chromosomal	? Unknown	Avirulent, does not bind to plant	Matthysse et al., 2000
attJ	Chromosomal	? Unknown	Avirulent, does not bind to plant	Matthysse et al., 2000
attO	Chromosomal	? Unknown	Avirulent, does not bind to plant	Matthysse et al., 2000

[a]Member of a two component system. The sensor genes are *virA* and *chvG*, respectively.

(Kalogeraki et al., 1995). In octopine strains, both genes must be mutated to observe a requirement for the gene for virulence.

Heat Shock Genes Two heat shock operons, *groESL* and *dnaKJ*, have been cloned from *A. tumefaciens* (Segal et al., 1995a; Segal et al., 1995b). The *groESL* operon contains an inverted repeat (IR) that appears to be involved in regulation of this operon's expression (Segal et al., 1995a). This inverted repeat is conserved in many bacteria (Segal et al., 1999). It acts as a transcription inhibitor, probably binding a protein repressor (Segal et al., 1996). Expression of the *groESL* operon is regulated not only at the level of transcription, but also at the level of translation by two different systems: the inverted repeat controls the stability of the transcript (Segal et al., 1996), and a temperature-activated RNA processing system specifically cleaves the mRNA of the operon, resulting in differential transcript stability (Segal et al., 1995c). In addition, the *rpoD* gene which encodes the vegetative transcription activator σ70 has been cloned (Segal et al., 1995d). This gene is a heat shock gene in *E. coli*, but it is not part of the heat shock regulon in *A. tumefaciens* (Segal et al., 1995d). A new heat shock promoter that is common to many α-purple bacteria, including *A. tumefaciens*, *Rhizobium meliloti* and *Bradyrhizobium*, has been identified. This promoter is recognized by a heat shock transcriptional activator, a homologue of σ32, which has been cloned from *A. tumefaciens* (Nakahigashi et al., 1995; Segal et al., 1995b).

A. rhizogenes There is relatively little work on the chromosomal genes of *A. rhizogenes*. They are generally believed to be similar to those of the other agrobacteria. However, differences in the binding of biotype 2 *A. rhizogenes* strains to plant cells suggest that at least some of the *att* genes must differ between biotype 1 *A. tumefaciens* and biotype 2 *A. rhizogenes* (Sykes et al., 1988). Differences of biotype 1 and 2 strains in their sensitivity to salt and in the sugars that they are able to metabolize suggest that there may be many differences in chromosomal genes between these strains.

A. vitis Genes are involved in the breakdown of pectin are important virulence factors in *A. vitis*. The genes affecting synthesis of polygalacturonase and endoglucanase are both chromosomal (McGuire et al., 1991). The *pehA* gene has been cloned and sequenced and appears to be closely related to *peh* from *Ralstonia solanacearum* and *Erwinia carotovora* (Herlache et al., 1997). Polygalacturonase mutants are reduced in virulence on grape (Rodriguez et al., 1991).

Ti Plasmid Genes

The *A. tumefaciens* Ti plasmid is about 200 kb. The complete sequences of 3 Ti plasmids are

available. Major groups of genes located on pTi include: the T DNA (the DNA transferred to the host cell), *vir* genes (genes required for, or associated with, virulence), and genes involved in quorum sensing, Ti plasmid conjugation, and opine metabolism.

T DNA The T DNA is the DNA fragment transferred to the host cell whose expression results in tumor formation. Located on the Ti plasmid, T DNA is bounded by 24 base-pair direct repeats referred to as the border sequences. Some Ti plasmids have two segments of T DNA (called TR and TL) with four border sequences. The segments bounded by the border sequences may be transferred individually or as an entire piece carrying both TR and TL. The T DNA carries genes for enzymes for plant growth hormone biosynthesis, for the modification of plant responses to growth hormones, and for the synthesis and secretion of opines. (See Physiology of Opines in this Chapter.)

Two enzymes encoded in the T DNA (tryptophan monooxygenase and indole-3-acetamide hydrolase) carry out the biosynthesis of auxin (indole-3-acetic acid, IAA). Tryptophan monooxygenase (encoded by *iaaM*) converts tryptophan into indole-3-acetamide, and indole-3-acetamide hydrolyase (encoded by *iaaH*) converts indole-3-acetamide into auxin. This pathway of auxin biosynthesis from tryptophan differs from that used by most plants and is the same as that used by some other plant pathogenic bacteria, notably *Pseudomonas savastanoi*, which infects olive and oleander, producing tumors. No DNA transfer is involved in this infection. The *P. savastanoi* genes are expressed in the bacterium, whereas the *A. tumefaciens* genes for auxin biosynthesis, which have eukaryotic promoters, are expressed in the plant host. Host biosynthesis of cytokinins is increased by the expression of the T DNA gene *ipt*, which encodes an isopentyl transferase. These genes encoding enzymes involved in plant hormone biosynthesis have constitutive eukaryotic promoters. Their expression and the resulting overprodution of auxin and cytokinins cause tumor formation. Two other genes carried on the T DNA appear to regulate the response of the plant to hormones, although their mechanism of action is not understood. These are gene 6b in which mutations give rise to large tumors and gene 5 in which mutations have a phenotype only in *iaa*$^-$ mutants. In this background gene 5 mutants produce an increased shoot-like tumor morphology. *Gene 5* encodes an indole lactate synthase. However, the role of indole-3-lactic acid in the plant is unknown (Binns et al., 1998).

The T DNA also encodes enzymes for the synthesis and secretion of opines, peculiar compounds made by the plant only in crown gall tumors. These genes are also preceded by constitutive eukaryotic promoters. It is thought that it is opine production by the tumor cells which results in the advantage to the bacteria in causing tumor formation. The bacteria possess genes for enzymes to metabolize these opines, which they can use as carbon and nitrogen sources. These genes are located outside the T DNA but on the Ti plasmid. Different Ti plasmids encode the enzymes for the production of different opines and their utilization. Ti plasmids and bacterial strains are often referred to by the opine(s) that they produce and use, e.g., octopine or nopaline strains.

The Ri plasmid of *A. rhizogenes* also contains a T DNA. Agropine Ri plasmids carry *iaaH* and *iaaM* genes in their T DNA, but hairy root formation by *A. rhizogenes* is not due to excess auxin production. Other Ri plasmids such as the mannopine and cucumopine plasmids don't contain *iaa* genes. Instead, the *rol* genes, particularly *rolB*, appear to be responsible for the growth of hairy roots (branched, ageotropic roots). There are four *rol* genes, *rolA*, *rolB*, *rolC*, and *rolD*. The mechanism by which they cause root formation and their biochemical activities are not understood, although their sequences are known (the whole of the T_L region of pRi has been sequenced). The *rol* genes appear to modify the hormone receptors or action of growth hormones in the plant. RolB alone is necessary and sufficient to cause full hairy root growth. Fertile plants can easily be regenerated from hairy roots on most species of plants. However, the regenerated plants have an altered morphology due to *rolA*, *rolB*, *rolC*, *rolD*, and ORF13. Ri plasmids contain genes for the synthesis of opines similar to those found on Ti plasmids (Binns et al., 1998).

vir Genes The transfer of T DNA to the host plant is dependent on *vir* genes and the presence of 24 bp direct repeats at the ends of the T DNA (called the border sequences). The particular sequences contained between the border sequences do not influence the DNA transfer. The *vir* genes include *virA* and *virG*, which are a two component system including a sensor for acetosyringone (VirA, an inner membrane protein) and a regulator (VirG, a DNA-binding protein that can be phosphorylated by VirA) which activates the expression of the other *vir* genes (Leroux et al., 1987). In addition to acetosyringone, expression of genes in the *vir* regulon is increased by acid pH and by the presence of monosaccharides. The latter response involves the binding of the sugar by the product of a chromosomal gene, *chvE* (Huang et al., 1990).

ChvE is a periplasmic galactose-binding protein and is required for chemotaxis to several sugars as well as influencing *vir* gene induction. ChvE mutants are avirulent on some but not all plants (Cangelosi et al., 1990; Huang et al., 1990). *VirD1* and *VirD2* encode an endonuclease that cuts the Ti plasmid DNA at the T DNA border sequences (Yanofsky et al., 1986). The *virE2* gene encodes a single-stranded DNA-binding protein (Christie et al., 1988). The *virB* operon encodes a DNA transfer system including a pilus analogous to that used by some conjugative plasmids including pRSF1010, pRP4, and pKM101, as well as to that used by the transport system for *Bordetella pertussis* toxin (Pohlman et al., 1994; Kado, 1994; Zupan et al., 1995; Shirasu et al., 1994; Lessi et al., 1994).

Of the *vir* genes, only *virA* and *virG* are expressed in the absence of inducers. These genes make up a two-component system. VirA, the sensor, is a plasma membrane protein that responds to phenolic compounds such as acetosyringone released by the plant. These inducers are most effective at low pH (5.3–6.0) and in the presence of monosaccharides. The sugars are bound by ChvE, which then interacts with VirA, increasing the response of VirA to the phenolic inducers. Low phosphate concentrations also increase the induction of vir genes. In response to the inducers, VirA autophosphorylates at a histidine residue and then transfers the phosphate to an aspartic acid in VirG. Phosphorylated VirG binds to specific DNA sequences referred to as *vir* boxes located 24 to 72 bp upstream of the *vir* operons and activates the *vir* genes' expression. VirG also binds to *vir* boxes located upstream of *virG* and thus increases its own synthesis (Johnson et al., 1998).

The *virB* and *virD* operons show considerable homology to genes required for conjugation between bacteria and they can mediate the conjugative transfer of other plasmids such as RSF1010. The *virD* operon contains five ORFs. VirD1 and VirD2 bind to the T DNA border sequences and nick the DNA between the third and fourth base of the repeat sequence on the bottom strand. VirD2 is covalently linked via a phosphotyrosine linkage to the 5′ end of the nicked DNA. DNA homology suggests that the border sequences and *virD2* belong to the P-family of conjugal transfer systems, which includes RP4, RK6, and R64. In some Ti plasmids, a sequence is located near the right border outside the T DNA, referred to as overdrive. VirC2 binds to this sequence and increases the efficiency of the formation of single-stranded nicks by VirD2. VirC1 (the other protein endcoded by the *virC* operon) may aid in this process. VirD4 is an integral membrane protein required for transfer of T DNA to host cells. It belongs to the class of TraG-like proteins, which are required for pilus formation. The *virB* operon contains 11 ORFs; nine of these encode membrane proteins. All except *virB1* are required for T DNA transfer. Mutants in *virB1* retain the ability to transfer T DNA but at a hundred-fold reduced efficiency. VirB1 is a secreted protein with an N-terminal region that resembles lysozyme. The remainder of the *virB* genes appears to be involved in the elaboration of a pilin and of a pore for the transfer of T DNA. This structure is formed best at cool temperatures (19–22°C). Sequence and structural studies suggest that VirB2 is the pilin. It may be processed after synthesis to a circular peptide. VirB7 is a lipoprotein and forms a tight complex with VirB4, VirB9, VirB10 and VirB11. VirB4 and VirB11 have ATP-binding sites and possess ATPase activity. The conjugation system encoded by *virD4* gene and the *virB* operon belongs to the group of bacterial conjugation systems that have their highest transfer efficiency on surfaces rather than in liquid suspension. This system is capable of transferring other DNAs as well as T DNA. Using this system, *A. tumefaciens* can transfer pRSF1010 to other bacteria or to plant cells (Johnson et al., 1998).

The *virE* operon contains two ORFs: *virE1* and *virE2*. VirE2 is a single-stranded DNA-binding protein. VirE2 is not required for T DNA transfer, although it is required for efficient tumor formation. VirE2 mutants can be complemented in trans by another *A. tumefaciens* strain, which does not have to contain T DNA, but must contain the *virB* and *virE* operons and *virD4*. Transgenic plants expressing *virE2* can also complement VirE2 mutants. It appears that the VirE2 (and VirF) proteins can be transferred to the host cell independently of the transfer of T DNA. This transfer requires the *virB* operon and *virD4* gene products as well as VirE1. VirE2, a single-stranded DNA-binding protein, may serve to protect the T DNA from degradation in the plant cell. Both VirE2 and VirD2 contain sequences that are similar to plant nuclear localization signals (NLS) and may aid targeting of the T DNA to the plant nucleus. Integration of the T DNA into the plant chromosomal DNA appears to proceed via illegitimate recombination into nonhomologous DNA at random and to involve proteins supplied by the plant rather than the bacterium (Johnson et al., 1998; de la Cruz et al., 1998; Rossi et al., 1998).

VirG regulates the expression of several other Ti plasmid operons. However, the role of these operons in tumorigenesis is unclear. These include *virF*, which is present in octopine Ti plasmids only. *VirF* mutants fail to cause tumors when a low number of bacteria are used in the

inoculation. They also fail to form tumors on some plants, including *Nicotiana glauca*. VirF appears to be transferred to the host cell along with VirE2. The *virH* operon contains four ORFs. Two of these have homology to cytochrome P450 enzymes. This operon is present in octopine and nopaline Ti plasmids and in some Ri plasmids (Deng et al., 1998).

The *virJ* gene is present on the Ti plasmid and in some strains also on the chromosome. The chromosomal copy of the gene is called *acvB*. VirJ may be associated with the T strand DNA (Kalogeraki et al., 1995). The *tzs* gene encodes a cytokinin prenyltransferase, which forms transzeatin. It is present in nopaline, agropine, and mannopine Ti plasmids but not in octopine and succinamopine Ti plasmids. It also is present in some Ri plasmids.

There is evidence to suggest that *virA*, *virC*, *virE*, *virF*, and *tzs* affect the host range of *A. tumefaciens*.

Genes Involved in Quorum Sensing *A. tumefaciens* like many other Gram-negative bacteria has a quorum-sensing system. The genes involved in this system are located on the Ti plasmid and are involved with the bacterial response to opines and with the conjugation of pTi between bacteria. They include *traR* and *traI*, which are homologues of the *luxI* and *luxR* genes of *Vibrio fisherii*. These genes have similar functions to the *V. fisherii* genes; *traI* encodes the enzyme for the synthesis of a homoserine lactone [N-(3-oxo-octanoyl)-L-homoserine lactone], which is the *Agrobacterium* autoinducer (AAI), and *traR* encodes a regulatory protein which binds AAI and DNA. The enzymatic activity of TraI has been demonstrated in vitro; it catalyzes the synthesis of AAI from 3-oxo-octanoyl-ACP (Acyl Carrier Protein) and S-adenosyl-methionine. The TraR protein plus AAI activates several promoters including the *traA* and *traC* promoters (to induce conjugation of bacteria which results in the transfer of pTi between bacteria), the *traI* promoter (a promoter upstream of an operon that includes *traR*) and the *traM* promoter. These observations suggest that the level of TraR in the cell is regulated by positive regulatory feedback loops involving the activation of TraI synthesis to make more AAI and the activation of *traR* expression. There is also apparently a negative feedback loop since TraM is an antagonist of TraR (Winans, 1998; Fuqua et al., 1996).

The expression of *traR* is controlled by *occR* in octopine and by *accR* in nopaline/agrocinopine Ti plasmids (see also Plasmid Conjugation) in response to the presence of octopine or agrocinopine. Thus the genes involved in quorum sensing are only expressed in the presence of the opine made by a crown gall tumor (or by plant cells which have received the opine synthesis genes from pTi). Why quorum sensing should only be necessary in the presence of transformed host cells is unclear (Winans, 1998; Fuqua et al., 1996).

Genes Involved in Plasmid Conjugation to Other Bacteria In addition to transferring a segment of DNA to the host cell (the T DNA), the Ti plasmid can be transferred from one bacterium to another. The genes for this conjugation are distinct from the *vir* genes involved in the transfer of T DNA, although both sets of genes are located on pTi. The *tra* and *trb* genes are required for this process. These genes show homology to the conjugation systems of other plasmids including the 11 *trb* genes of RP4, an IncP plasmid. The *tra* regulon of octopine Ti plasmids is positively regulated in a complicated manner. The *occR* gene product activates the transcription of *traR* in the presence of octopine. The *traR* gene product is homologous to *luxR* and activates the transcription of the *tra* genes in the presence of the *Agrobacterium* autoinducer (AAI), a homoserine lactone which is produced by *traI* (a *luxI* homologue). In nopaline Ti plasmids, AccR regulates the expression of *traR* by acting as a repressor. The inducer is agrocinopine. The result of this regulatory cascade is that conjugation of the Ti plasmid occurs only in the presence of transformed plant cells required to produce opines and a high bacterial population density required to produce a sufficient concentration of the autoinducer. These facts explain the early observations of Kerr et al. (1977a) that strains of *Agrobacterium* could only be induced to transfer the Ti plasmid by conjugation when the donor and recipient strains were inoculated together into a wound site on a susceptible host plant. The advantage to the bacterium in regulating the conjugation of pTi in this manner is unknown (Farrand, 1998).

A. vitis

The genetics and diversity of the Ti plasmids found in strains of *A. vitis* have been studied extensively. Three types of pTi have been characterized: plasmids with one large T DNA that encodes the enzymes for the synthesis of the opines octopine and cucumopine, plasmids with small T DNA that lead to octopine and cucumopine synthesis, and plasmids of the nopaline type Ti plasmids. Some of these Ti plasmids contain two T DNAs. The *vir* regions are conserved among all three types of pTi. One pTi from a vitopine strain (S4) belongs to a different incompatibility group than the other Ti

plasmids belong to (Szegedi et al., 1996). The limited host range of some *A. vitis* strains appears to be due to the *virA* gene since the host range can be extended by replacing this gene with *virA* from *A. tumefaciens* strain A6 (Yanofsky et al., 1985).

The genes of the T region of *A. vitis* include *iaaM*, whose product catalyzes the formation of indoleacetamide (IAM) from tryptophan, and *iaaH*, whose product catalyzes the conversion of IAM into the plant hormone indoleacetic acid (IAA). Wide host range strains also contain the *ipt* gene, which encodes an isopentenyl transferase required for the synthesis of cytokinins (Hoekema et al., 1984). Some limited host-range strains lack the *ipt* gene. Strong expression of *ipt* in grape is toxic to the cells unless *ipt* expression is accompanied by strong expression of the *iaa* genes, particularly *iaaM* (Huss et al., 1990). At least one pTi from *A. vitis* also carries the genes for the metabolism of tartaric acid. In other strains, these genes are carried on a separate plasmid. (See Physiology in this Chapter.)

Ecology

A. tumefaciens

Agrobacteria generally persist for long periods of time in the soil, particularly in the rhizosphere of susceptible host plants. When inoculated onto cherry, the bacteria established populations of about 10^5 CFU per gram of plant tissue and persisted for 2 years (the duration of the experiment). The biocontrol strain of *Agrobacterium* K84 (see Applications) also showed similar population densities and long persistence when inoculated onto several plants. However, bacterial persistence in fallow soil was much reduced; bacterial populations declined from 10^5 per gram soil to 10 in as short a time as 16 weeks. The bacteria could apparently move out from the inoculated rhizosphere and were recovered as far as 40 cm from the original plants (Stockwell et al., 1993). In other studies, agrobacteria were recovered from a fallow soil. The isolates were roughly evenly split between biotypes 1 and 2. About 1/3 of the isolates were pathogenic. All of these pathogenic strains belonged to biotype 1 (Bouzar et al., 1993b). It is often difficult to recover virulent *A. tumefaciens* from galls. In some cases this appears to be due to loss of virulence of bacteria growing with host plants. Belanger et al. (1995) found that when virulent strains isolated from apple were reintroduced onto to axenic apple plants, most of the bacteria recovered were avirulent. This was due to alterations in the pTi *vir* genes. The mutant strains grew faster in the presence of the plant than their virulent parents did and thus came to dominate the bacterial population.

Opines are thought to provide nutrition for *A. tumefaciens* and *A. rhizogenes* located near the transformed plant cells (Dessaux et al., 1993; Guyon et al., 1993). In at least some cases, opines can be transported from their site of synthesis to the rest of the plant and may be found in root exudates. Thus the production of opines at the site of infection may well influence the composition of the rhizosphere community at distant sites (Savka et al., 1996). Utilization of opines is not limited to agrobacteria. Several isolates of soil pseudomonads and coryneform bacteria have been shown to be able to use opines as carbon and energy sources (Beaulieu et al., 1988; Tremblay et al., 1987).

A. vitis

The fate of the bacteria on grapes colonized with *A. vitis* has been followed for 2 years after planting. The bacteria were still present and virulent although the grapes did not have galls or other obvious symptoms of disease (Burr et al., 1995). When the maintenance of bacterial populations was compared in infested soils planted with oats and those planted with grapes, higher population levels were maintained in the presence of grapes (Bishop et al., 1988).

Noncultivated grapes (*Vitis* spp.) have been examined for the presence of *A. vitis*. The bacteria were detected in association with more than half the plants examined. Interestingly the bacterial isolates, which appeared to be diverse as judged by DNA fingerprinting, were largely nonpathogenic (Burr et al., 1999). *A. vitis* is also routinely found in healthy grapes where it can cause gall formation at sites of injury. The bacteria may be found in the phloem or in the tissue just below the bark. Higher bacterial populations are usually recovered from susceptible as compared to resistant cultivars. Freezing injury may facilitate the spread of the bacteria in the plant (Burr et al., 1999).

Disease

General

Infections of wound sites in dicotyledonous plants by agrobacteria lead to the development of crown gall tumors or hairy roots. These abnormal growths result from the transfer of DNA sequences (the T DNA) from the bacterial tumor-inducing (pTi) or root-inducing (pRi) plasmid to the host plant cell. The T DNA sequences become incorporated into plant chromosomes and are maintained and expressed in the plant cell. They code for enzymes involved in

the synthesis of the growth hormones, auxin and cytokinin. It is the overproduction of these hormones that results in the growth of a tumor.

A. tumefaciens

The steps identified in tumor formation by *A. tumefaciens* include bacterial chemotaxis to wound sites in plants (Loake et al., 1988; Ashby et al., 1987); an exchange of signals between the plant and the bacteria (Matthysse, 1994); initial bacterial attachment to the surface of plant cells (Matthysse, 1986); induction by phenolics (including acetosyringone) released from the wounded plant of bacterial *vir* genes encoded on the Ti plasmid (Stachel et al., 1985); the formation of a pilus encoded by the *virB* operon (Fullner et al., 1996); the transfer of the T DNA sequences from the bacterium to the plant cell mediated by *vir* gene products (and possibly the products of other genes; Klee et al., 1983); the integration of the T DNA into plant chromosomes (Chilton et al., 1977); and the expression of the T DNA in the plant cell, leading to increased auxin and cytokinin production, resulting in uncontrolled growth of the cell to form a crown gall tumor (Binns et al., 1988; Akiyoski et al., 1982; Figs. 15–19). Other genes expressed in the plant cell are included in the T DNA, most notably the genes encoding the opine biosynthetic enzymes (Tempe et al., 1984). The bacterial Ti plasmid contains the genes required for the ability to use these opines as carbon and nitrogen sources and thereby support bacterial growth. The benefit of tumor formation to the bacterium is thought to be explained by crown gall-opine production and the subsequent use of opines as substrates for bacterial growth.

Because a wound is required for the interaction of the bacteria with the host, the sites most often infected are the sites of graft junctions. Thus crown gall occurs most often on fruit trees, grapes, roses, and other plants that are routinely grafted. However, disease can be observed on plants that are not grafted. Agrobacteria are found in the soil and in the rhizosphere and easily infect wounds on the roots or at the base of the stem (the crown of the plant).

A. rhizogenes

Two other less well-known species of agrobacteria have a similar pathogenic mechanism. *A. rhizogenes* causes the formation of hairy roots on a wide variety of dicots. These roots are caused by the transfer of DNA from the bacterium to the host, which contains genes (*aux* and *rol*) that lead to the formation of ageotropic branched roots rather than tumors (White et al., 1985; Amselm et al., 1992). Aside from the difference in the T-DNA of the plasmid, this bacterium differs from *A. tumefaciens* in its ability to grow on various carbon sources and in its low tolerance to NaCl (Kerr et al., 1977b). These traits presumably reflect differences in the bacterial chromosomes. Insofar as is known, the mechanism of pathogenesis of *A. rhizogenes* is similar to that of *A. tumefaciens*. The Ri plasmid contains vir genes that are analogous to those of pTi, and the steps in DNA transfer described above for *A. tumefaciens* appear to be similar for *A. rhizogenes* (White et al., 1985).

A. vitis

Agrobacterium vitis causes the formation of tumors on stems of grapes. On grape roots it causes necrotic lesions (Burr et al., 1987). This necrosis results in part from the enzyme polygalacturonase, which is secreted by the bacteria. Other agrobacteria appear to lack this enzyme (McGuire et al., 1991; Herlache et al., 1997). Although some strains of *A. vitis* have a broad host range in the laboratory, in nature *A. vitis* appears to be largely a pathogen of grapes. The bacteria are capable of invading the vascular system of the plant and multiplying in the xylem without any apparent symptoms; indeed, apparently most grape plants have *A. vitis* resident in the xylem (Burr et al., 1995). Unlike *A. tumefaciens*, these bacteria are able to use tartrate (which is found in grape xylem in higher concentrations than in most other plants) as a carbon source (Crouzet et al., 1995). When an infected grape stem is wounded, tumors develop at the wound site. The mechanism of DNA transfer appears to be similar to that of *A. tumefaciens*. The Ti plasmid of *A. vitis* carries *vir* genes which are similar to those of *A. tumefaciens* except that some limited host-range strains lack *virC*. The T DNA is also similar to that of *A. tumefaciens* except for the lack of cytokinin biosynthesis genes in some limited host-range strains (Bonnard et al., 1989; Otten et al., 1994; Canaday et al., 1992; Paulus et al., 1991; Otten et al., 1993). There is little information about the interaction of either *A. rhizogenes* or *A. vitis* with prospective hosts prior to the induction of *vir* genes and the initiation of T-DNA transfer.

Control of Disease Caused by Agrobacteria

Biocontrol of Crown Gall

Biological control of some strains of *A. tumefaciens* can be achieved by dipping the planting material in a suspension of *Agrobacterium* strain

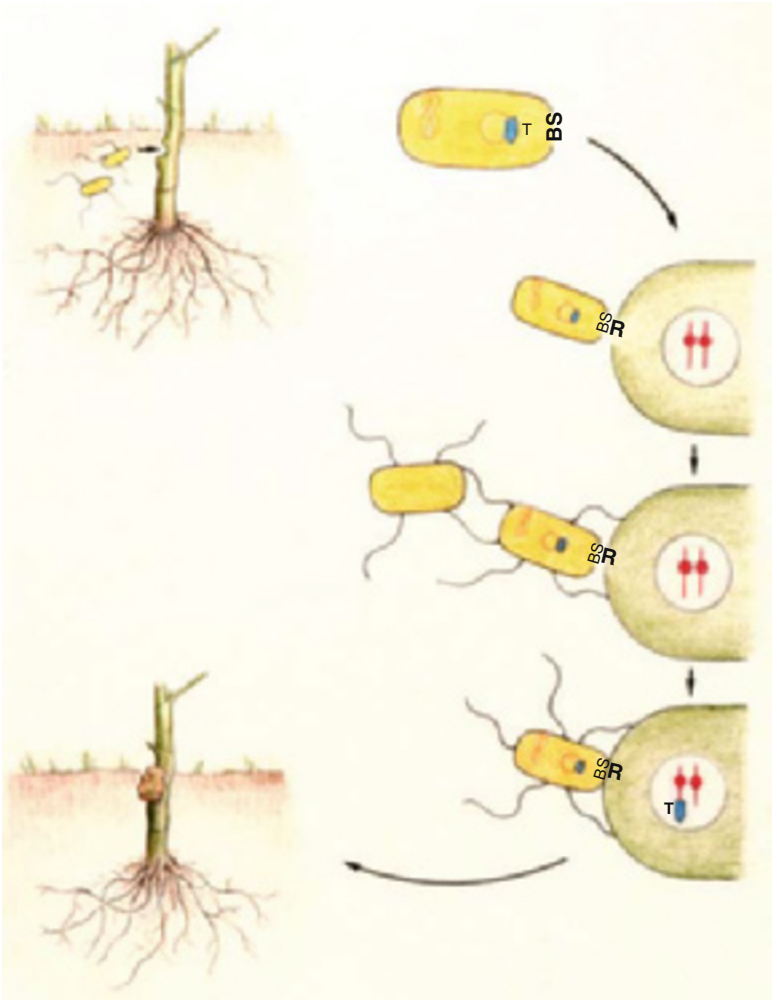

Fig. 15. Drawing showing the steps in the interaction of *A. tumefaciens* with a wounded plant leading to tumor formation. *A. tumefaciens* can be found in the upper layers of the soil. The bacteria are chemotactic to substances released from plant roots and wound sites. The bacteria (yellow) migrate to the wound site and bind to the surface of a plant cell (green). Substances from the plant induce bacterial cellulose synthesis, and the bacteria form aggregates on the plant surface held together with cellulose fibrils (only 2 bacteria are shown in the drawing for simplicity). The *vir* genes are induced and the bacteria transfer a piece of plasmid DNA, the T DNA (blue), to the host cell, where it becomes integrated into the host cell chromosomes. The expression of genes contained in the T DNA (which have eukaryotic promoters) leads to the overproduction of growth hormones in the plant cell and the resulting growth of a tumor. The tumor cells also produce opines which the bacteria use as a source of carbon and energy.

K84. These bacteria can also be applied to graft junctions during the grafting process. Strain K84 makes antibiotic-like compounds called agracins, which kill many strains of *A. tumefaciens*. A genetically engineered derivative of K84, K1026, is often used because it is unable to transfer resistance/immunity to agracins to the target pathogens. Strain K84 produces two agracins (K84 and K434). Agracin K84 only inhibits the growth of *Agrobacterium* strains carrying nopaline/agrocinopine type Ti plasmids. It is not effective against *A. vitis*. Agracin 434 is effective against biotype 2 strains. Strain K84 contains three plasmids: pAgK84 which encodes agracin K84 production, pAgK434 which encodes agracin K434 production and immunity to agracin K434, and pAtK84b which is a deleted pTi and carries nopaline catabolism and resistance to agracin K84. This last plasmid is self-transmissable (Clare et al., 1990). The structures and mechanisms of action of agracins K84 and K434 have been determined. There is some evidence that strain K84 may possess some biological control activity in addition to the production of the two agracins. However, currently this evidence is incomplete.

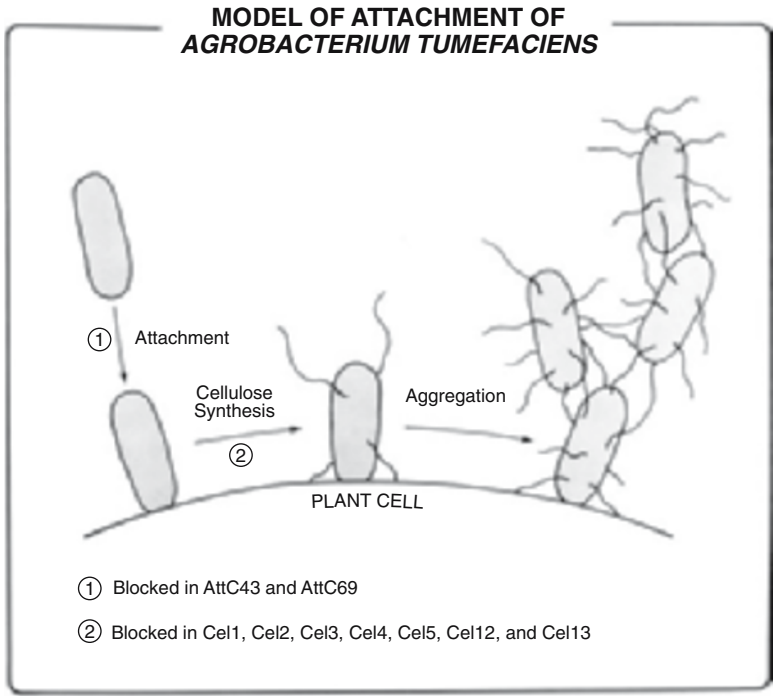

Fig. 16. Drawing showing the steps in bacterial aggregate formation on the surface of a plant cell. The bacteria are chemotactic to substances released by the plant cell. In response to substances released by the plant, the bacteria make and secrete a substance required for bacterial attachment to the plant surface. This step is blocked in *att* mutants, which can be complemented by conditioned medium. The bacteria then bind to the plant surface in a loose fashion and can be removed by shear forces such as water washing or vortexing of cultures. This step requires an acetylated capsular polysaccharide made by the bacteria. The bacteria elaborate cellulose fibrils. These fibrils bind the bacteria tightly to the plant surface. Shear forces can no longer remove the bacteria. The fibrils also trap other bacteria and these new bacteria are induced to form cellulose fibrils that in turn entrap additional bacteria, resulting in the formation of a large bacterial aggregate on the plant cell surface. Strong shear forces can remove the outer portions of these aggregates but the bacteria at the base of the aggregate are tightly bound to the host cell. Aggregate formation is blocked in *cel* mutants.

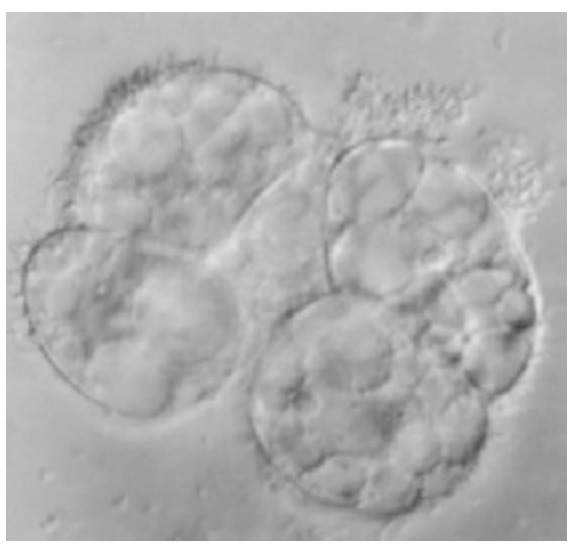

Fig. 17. Light micrograph showing *A. tumefaciens* bound to the surface of carrot cells. Note the presence of both individually attached bacteria and bacteria aggregates on the plant cell surface. From Matthysse (1987).

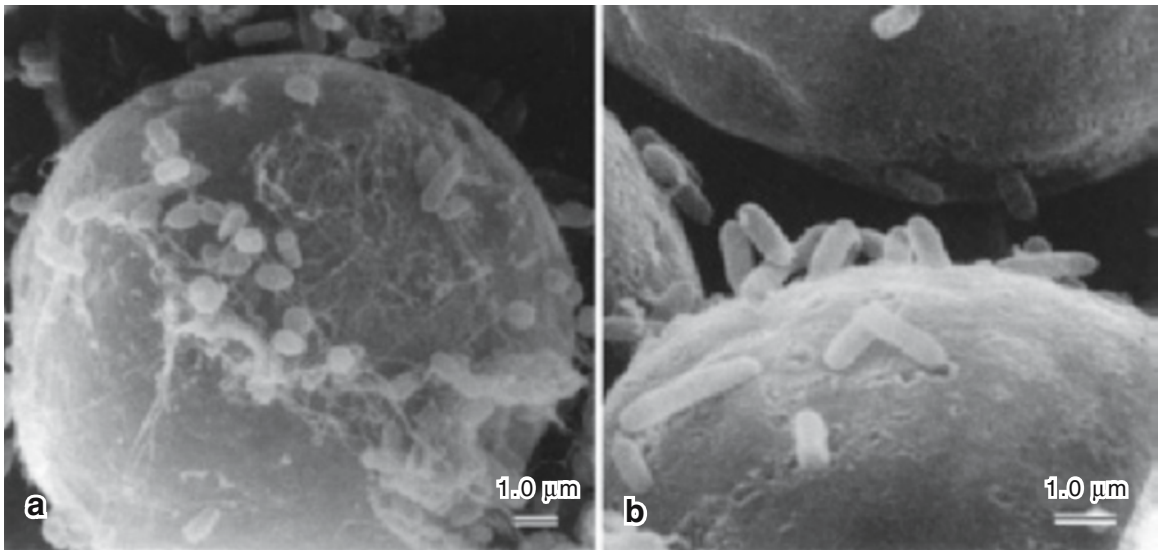

Fig. 18. Scanning electron micrographs showing *A. tumefaciens* bound to the surface of carrot cell protoplasts. A: wild type bacteria. The fibrils are composed of cellulose made by the bacteria. B: cellulose-minus bacteria. Note the absence of fibrils. The bacteria are still able to bind to the plant cells. From Matthysse (1983).

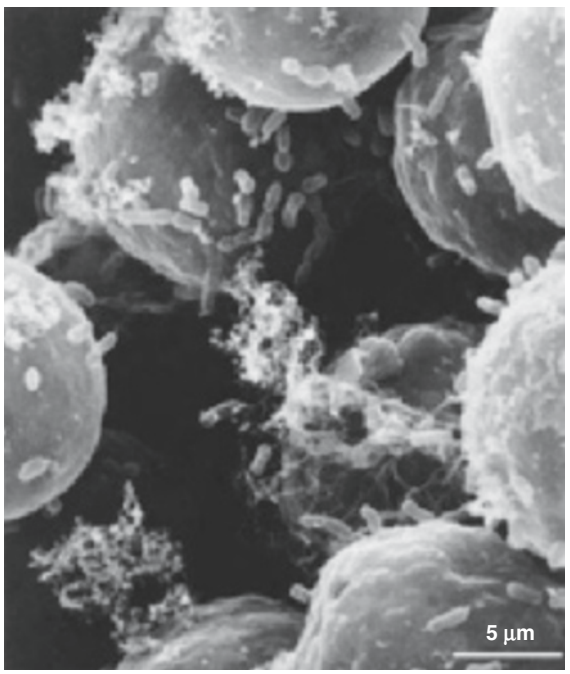

Fig. 19. Scanning electron micrograph showing *A. tumefaciens* on the surface of carrot cells. Both individually attached bacteria and bacterial aggregates held together by cellulose fibrils can be seen.

CONTROL OF *A. VITIS* Crown gall disease on grape has proven to be difficult to control. This is largely because of the persistence of the bacteria in plants that show no symptoms of disease. Management of the disease on grape combines the selection where possible of relatively resistant cultivars with cultivation practices designed to limit the growth of the bacteria (Burr et al., 1998). Cuttings also can be indexed for the presence of the bacteria by examining callus tissue formed at their base. Liquid can be forced through the vascular tissue and plated on semi-selective medium to detect *A. vitis*. In addition, PCR can be used to detect the presence of various bacterial virulence genes in DNA extracted from cuttings (see Identification). Propagation of vines from cultured shoot tips will often give rise to plants that are bacteria free.

Numbers of bacteria carried by grapevines can be reduced by submerging the vines in hot water prior to planting. However, this treatment does have the potential to injure the buds on the vine.

Biological control of *A. vitis* on grape is currently being studied. If *A. vitis* is found largely in the vascular tissue of asymptomatic grapes, then treatment of cuttings with external bacteria as biocontrol agents may fail to kill the internal bacteria. *A. vitis* is not susceptible to biocontrol by *A. radiobacter* K84 unlike *A. tumefaciens*. This may be due in part to the fact that the bacteria are not killed by the agracins produced by K84 and its derivatives (Burr et al., 1998).

Applications

Genetic Engineering

The ability of *A. tumefaciens* and *A. rhizogenes* to transfer genes to plant cells, where they are

stably integrated into the host chromosome(s) and expressed, has made these bacteria extremely useful in genetic engineering of plants. Transfer of T DNA to plants is not dependent on the sequences contained within it. Instead it is dependent on the border sequences which surround it. In addition to the border sequences, which must be located adjacent to the DNA to be transferred, other Ti plasmid and chromosomal genes are required. On the Ti plasmid these are the vir genes. The chromosomal genes required are numerous and not all of them are identified or characterized. Thus the general strategy used to transform plant cells is to clone the genes that are to be introduced along with a selectable marker, such as the *npt2* gene (which gives resistance to kanamycin and G418) or a hygromycin resistance gene, in a plasmid vector (which is a shuttle vector and will replicate in both *E. coli* [for ease of cloning] and agrobacteria). If it is important to be certain that the cloned genes are expressed only in the plant host, an intron can be included in the genes to prevent expression in the bacteria. Eukaryotic promoters and stop signals are placed before and after the gene(s) to be introduced. Once the plasmid carrying border sequences and the genes to be introduced into the plant has been constructed, it is transferred from *E. coli* into *A. tumefaciens* or *A. rhizogenes*. The *Agrobacterium* strain must contain the chromosomal genes required for virulence and a modified Ti plasmid that contains the *vir* region but has the border sequences and the T DNA deleted.

The resulting *Agrobacterium* strain is then used to infect plant tissue, often tissue culture cells or organs such as leaves or shoot meristems. The plant tissue and the bacteria are incubated together long enough for the cloned DNA to be transferred, expressed and integrated into host cell chromosomes (usually a few days). The bacteria are then killed with an antibiotic (such as cefotoxime) to which they are susceptible and the plant cells resistant, and the transformed plant material is treated to select for the marker contained in the transferred DNA. Success rates vary with the plant material used, the particular plasmid construct, the *Agrobacterium* strain, and the protocol employed. Some species of plants, notably legumes and many monocots including grasses, are more difficult to transform than are other plants such as tomatoes. This technique has been used commercially to construct many new lines of crop plants (Birch, 1997; van Wordragen et al., 1992).

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