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Rapid and Efficient Methods to Isolate, Type Strains and Determine Species of *Agrobacterium* spp. in Pure Culture and Complex Environments

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1. Introduction

Agrobacterium are Alphaproteobacteria common in most soils that closely interact with plants in two respects. Firstly, and as a general trait of the whole taxon, they are rhizospheric bacteria saprophytically living in the root environment (i.e. rhizosphere) of numerous plants. Rhizospheric interactions are generally considered to be of commensal type with no detrimental effect to the plant, but in most instances they are likely beneficial to plants. For evident agronomic purposes it is worthwhile to explore whether agrobacteria are themselves plant growth-promoting rhizobacteria (PGPR) or not. However, this requires an expert determination of the *Agrobacterium* taxonomy. Indeed our current investigations suggest that only some agrobacterial species from the abundant soil *Agrobacterium* guild are selected in the rhizosphere of a given plant. Secondly, but only when they harbor a dispensable Ti plasmid (i.e. tumor inducing plasmid), agrobacteria are plant pathogens able to cause the crown gall disease to most dicots and gymnosperms and some monocots (Pitzscke & Hirt, 2010). Ti plasmids are conjugative and can easily spread in indigenous soil agrobacteria. As a result transconjugant agrobacteria become in turn pathogenic, contributing both to disease spread and perennial soil contamination. An epidemiological survey of crown gall thus also requires expert determination of the *Agrobacterium* taxonomy. A set of biochemical and molecular methods were thus set up to facilitate the taxonomic assessment of agrobacteria either in pure culture or directly from complex environments such as soils, rhizospheres or tumours. After a presentation of the present state of *Agrobacterium* taxonomy, this work provides efficient methods to : i) isolate agrobacteria from complex environments thanks to elective media; ii) determine the *Agrobacterium* genus status of newly isolated strains on the basis of a minimal set of biochemical tests; iii) determine species and type novel isolates of *Agrobacterium* by sequence analysis of relevant marker genes; iv) determine amplicon content and genome architecture; v) detect the presence of *Agrobacterium* and Ti or Ri plasmid directly in complex environments by PCR using selected primers and metagenomic DNA extracted from whole bacterial communities.

2. Current state of agrobacteria taxonomy

The *Agrobacterium* taxonomy was historically based upon pathogenicity traits that were later found to be determined by dispensable and highly exchangeable plasmids. It is now well known that crown gall and hairy root diseases —that are respectively characterized by tumors and root proliferations on infected plants— are due to the presence of infectious agrobacteria plasmids (i.e. Ti and Ri plasmids for tumor or root inducing plasmids, respectively) (Van Larebeke et al., 1974). However, agrobacteria were isolated and described very early—evidently before the discovery of the plasmidic nature of their pathogenicity. This is why first bacteria isolated from neoformed tissues, which had the ability to reproduce the symptoms, were named *Bacterium tumefaciens* and *Phytomonas rhizogenes*, respectively (Smith & Townsend, 1907; Riker, 1930). For their part, non pathogenic agrobacteria had been isolated earlier from soil and named *Bacillus radiobacter* because they displayed a particular star shape in certain growth conditions (Beijerinck & van Velden, 1902). The genus name *Agrobacterium* was created in 1942 by Conn as a genus very similar but different to the genus *Rhizobium* (Conn, 1942). Hooykaas et al. (1977) and Genetello et al. (1977) showed that Ti plasmid is transferable by conjugation in plasmid free agrobacteria that consecutively become able to incite crown gall. As a result, it was definitively evident that pathogenicity traits led to artificial classification of agrobacteria and could no longer be used to delineate species. In parallel, enzymatic abilities determined with biochemical galleries led to the distinction of several clusters among agrobacteria that were named biovars (Keane et al., 1970; Panagopoulos et al., 1978). Biovars are determined by chromosomal genes and not plasmids, thus tumorigenic, rhizogenic and non-pathogenic strains can be found within the same biovar.

In modern bacterial taxonomy, the criteria to delineate taxa now include genomic information. This has brought considerable modifications to the nomenclature. According to Wayne et al. (1987) and Stackebrandt et al. (2002), homogenous genomospecies is the ultimate criterion to validly delineate a *bona fide* species. Amongst agrobacteria *lato sensu*, genomospecies generally encompass the same set of strains than biovars. Upon this criterion, *Agrobacterium vitis* (i.e. biovar 3), *A. rubi* and *A. larrymoorei* have been validated as *bona fide* species (Ophel & Kerr, 1990; Holmes & Roberts, 1981; Bouzar & Jones, 2001). For its part biovar 1 is heterogeneous, including ten genomospecies (De Ley, 1973, 1974; Popoff et al., 1984) currently called genomovar G1 to G9 (Mougel et al., 2002) and G13 (Portier et al., 2006). Biovar 1 is thus a species complex and not a single bacterial species. Noticeably, G4 contains both type-strains of the formerly described species "*A. tumefaciens*" and "*A. radiobacter*" that should be both renamed *A. radiobacter* for antecedence reasons (Young et al., 2006). However, even though the name "*A. tumefaciens*" is not valid to designate a *bona fide* genomic species, we proposed that the group of closely related species corresponding to biovar 1 should be collectively called "*A. tumefaciens* complex" in order to avoid confusion with the genomic species G4, which must be validly named *A. radiobacter* (Costechareyre et al., 2010). For the ICSP subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium*, this seems to be a good interim solution until genomovars can be formally named (Lindström & Young, 2011). In addition to the epithet *radiobacter* for genomovar G4, *fabrum* and *pusense* have recently been proposed for homogenous species corresponding to genomovar G8 and to genomovar G2, respectively (Lassalle et al., 2011; Panday et al., 2011).

Modifications of the nomenclature also happen at the genus level. Although, biovar 2 is a homogenous genomospecies (Mougel et al., 2002; Popoff et al., 1984), this species clearly appears phylogenetically more related to *Rhizobium* species such as *R. etli* or *R. tropici*, than to other *Agrobacterium* species. Therefore, biovar 2 was proposed to be classified in the genus *Rhizobium*, while all other species were kept in the genus *Agrobacterium* (Costechareyre et al., 2010). This proposal had the great advantage to solve the paraphyletic problem of the old classification pointed out by Young et al. (2001). In agreement with taxonomy rule of name antecedence, biovar 2 should be named *Rhizobium rhizogenes*. The epithet for this species is *rhizogenes* because the type-strain ATCC 113225^T received this epithet at the time of its first description by Riker (1930). However, most members of this taxon are non-pathogenic or induce crown gall but not hairy root symptoms. This is particularly the case for the famous crown gall biocontrol agent K84 (Kerr, 1974). Frequently named "*A. radiobacter*" , it is actually a true member of the species *Rhizobium rhizogenes* in its present definition.

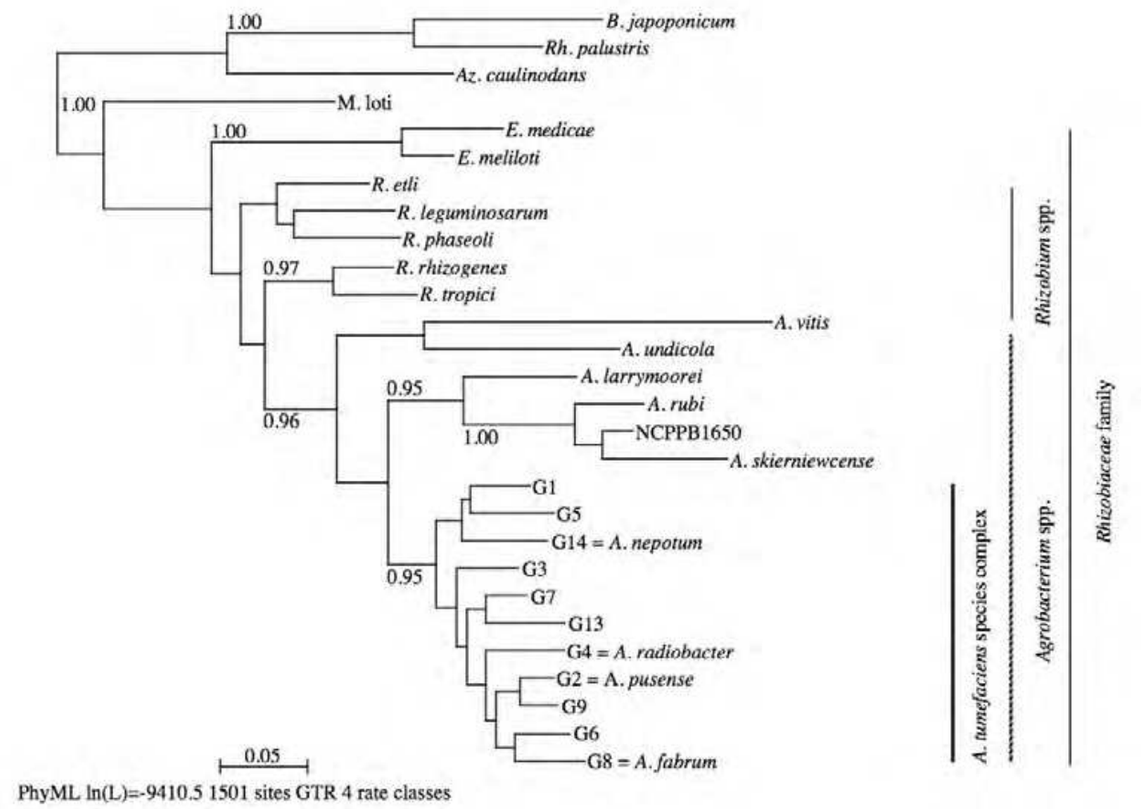

Fig. 1. Phylogeny of the *recA* gene of type-strains of all *bona fide* genomic species of *Agrobacterium* spp. known to date using the revised nomenclature proposed by Costechareyre et al. (2010). The maximum likelihood method was used. Only significant ML values (>0.95) are given. The branch length unit is the number of substitutions per nucleotidic site. *B. Bradyrhizobium, Rh. Rhodopseudomonas, Az. Azorhizobium, M. Mesorhizobium, E. Ensifer, R. Rhizobium, A. Agrobacterium.*

There are likely numerous other species of *Agrobacterium* either within or out the *A. tumefaciens* complex, such as the unnamed species that includes strain NCPPB 1650, or the novel species that got the epithet *skierniewicense* (Puławska et al., 2011) and—at least provisionally— *nepotum* for the novel *A. tumefaciens* genomovar G14 found by Puławska et al. (unpublished). In addition, it is also likely that bacteria able to induce the formation of nodules with some plants are true members of the genus *Agrobacterium* in its novel definition. It could be the case of the nitrogen fixing bacteria initially called *Allorhizobium undicola* (Costechareyre et al., 2010). A reasonably good view of the phylogeny of the *Agrobacterium* genus is given by the *recA* gene phylogeny (Figure 1) adapted from Costechareyre et al. (2010), completed with sequences from type-strains of novel species *A. skierniewicense* and *A. nepotum* (i.e. genomovar G14). The recent denomination *A. fabrum* and *A. pusense* are used for genomovars G8 and G2, respectively. Table 1 lists type-strains and *recA* accession numbers used to construct the phylogeny.

Genomic species	Type strain	LMG code	*recA*	Allele nb	Genome nb
Rhizobium rhizogenes	CFBP 2408^{T}	150^{T}	AM182126	nd	1
Agrobacterium vitis	K309^{T}	8750^{T}	AB253194	nd	1
A. undicola	ORS 992^{T}	11875^{T}	EF457952	nd	0
A. larrymoorei	AF3.10^{T}	21410^{T}	FN432355	4	0
A. rubi	TR3^{T}	17935^{T}	AM182122	2	0
A. sp.	NCPPB 1650^{PT}	230	FN813466	1	0
A. skierniewcense	CH11^{T}	2161^{T}	HE610311	1	0
A. tumefaciens genomovar G1	TT111^{PT}	196	FM164286	11	4
A. tumefaciens genomovar G2 = ***A. pusense***	NRCPB10^{T}	25623^{T}	HQ166059	5	1
A. tumefaciens genomovar G3	CFBP 6623^{PT}	nd	FM164304	2	1
A. tumefaciens genomovar G4 = ***A. radiobacter***	ATCC 19358^{T}	140^{T}	FM164311	6	3
A. tumefaciens genomovar G5	CFBP 6626^{PT}	nk	FM164318	3	2
A. tumefaciens genomovar G6	NCPPB 925^{PT}	225	FM164319	1	1
A. tumefaciens genomovar G7	Zutra 3/1^{PT}	198	FM164323	12	3
A. tumefaciens genomovar G8 = ***A. fabrum***	C58^{T}	287	FM164330	6	3
A. tumefaciens genomovar G9	Hayward 0363^{PT}	27	FM164332	2	1
A. tumefaciens genomovar G13	CFBP 6927^{PT}	nd	FM164333	1	1
A. tumefaciens genomovar G14 = ***A. nepotum***	39/7^{T}	26435^{T}	(HE610312)	4	0

Table 1. Representative strains and infraspecific diversity of *Agrobacterium* species and related species. ^{T}, type-strains. ^{PT}, proposed type-strain. LMG, Laboratorium voor Microbiologie, Ghent University, Belgium. *recA*, accession number of *recA* allele of representative strain. (HE610312) is the *recA* accession number of strain C3.4.1. Allele nb, number of strains displaying different MLSA patterns. Genome nb, number of completely sequenced genome. nd, not deposited.

3. Isolation of agrobacteria from complex environments

Agrobacteria usually form ecological guilds in soils consisting of several genomovars with several strains within genomovars. There are also several strains even within a single tumor (Nesme et al., 1987; Vogel et al., 2003; Costechareyre et al., 2010). As a consequence, it is necessary to take extreme care during strain isolation to be sure of the genotypic purity of isolates. Efficient purification of agrobacteria requires both selective media and the strict respect of the procedure described below.

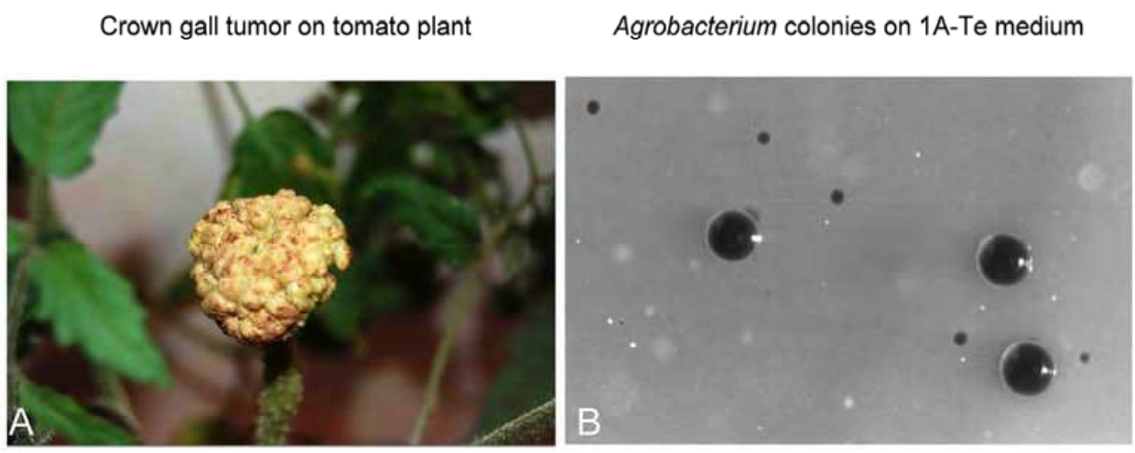

Fig. 2. Typical crown gall symptom (A) and *Agrobacterium* colony morphology on tellurite amended medium (B).

Elective media 1A and 2E, proposed by Brisbane and Kerr (1983) to isolate different biovars, are based on the particular ability of agrobacteria to use certain compounds, such as carbon and energy sources. In addition, these media contain selenite salt (Na_2SeO_3) because agrobacteria *lato sensu* has the general ability to reduce this salt. However, as the ability to reduce selenite also determines a general ability to resist to toxic amounts of tellurite (K_2TeO_3), this latter compound was substituted for selenite to improve the medium selectivity, especially when isolating bacteria from complex environments such as soil and decaying tumors (Mougel et al., 2001). Members of the *A. tumefaciens* species complex (i.e. biovar 1) and *A. rubi* can be isolated with 1A-Te containing arabitol as an elective carbon source and 80 mg/l K_2TeO_3. *R. Rhizogenes* (i.e. biovar 2) can be isolated using 2E-Te containing erythritol and 320 mg/l K_2TeO_3. Agrobacteria *lato sensu* (i.e. *Agrobacterium* spp. and *Rhizobium rhizogenes*) as well as most *Rhizobiaceae* can be isolated using MG-Te containing mannitol and 200 mg/l K_2TeO_3 (Table 2). On tellurite-amended media, colonies have the typical circular glistening morphologies of agrobacteria plus a characteristic black color with a metallic shine (Figure 2B).

Agrobacterium vitis (i.e. biovar 3) and *Agrobacterium larrymoorei* can be isolated on 3DG medium (Brisbane and Kerr, 1983) or Roy and Sasser medium (Roy and Sasser, 1983). On this latter medium, colonies have a typical pinkish-white (red centre) color. Both media are based on the utilization of sodium L-tartrate as the carbon source. It is likely that the selectivity of these media could be improved by addition of tellurite. This is because *A. vitis* members are resistant to this compound as well as most *Rhizobiaceae* (unpublished results).

For the first purification step, ca. 0.5 g of soil, root, shoot or tumor tissues are crushed with a micropestle in microtube containing 500 µl of sterile distilled water and then indispensably let to macerate for at least 30 min. This maceration is thought to be necessary to allow efficient cell separation from polysaccharides produced by agrobacteria. Next, ca. 25 µl of macerate (100 µl for soil samples) are streaked on appropriate selective media and then incubated at 28°C. Agrobacteria usually require between 3 and 4 days to form colonies on tellurite amended media because this compound tends to slow down bacterial growth. For the second purification step, clear individual colonies with the typical morphology (Figure 2B) are picked and suspended separately in 100 µl of sterile distilled water. These

1A-Te		2E-Te		MG-Te	
L-arabitol	3.04 g	Erythritol	3.05 g	D-Mannitol	5.0 g
NH_4NO_3	0.16 g	NH_4NO_3	0.16 g	L-glutamic acid	0.2 g
KH_2PO_4	0.54 g	KH_2PO_4	0.54 g	KH_2PO_4	0.5 g
K_2HPO_4	1.04 g	K_2HPO_4	1.04 g	NaCl	0.2 g
$MgSO_4$, $7H_2O$	0.25 g	$MgSO_4$, $7H_2O$	0.25 g	$MgSO_4$, $7H_2O$	0.2 g
Sodium taurocholate	0.29 g	Sodium taurocholate	0.29 g	Yeast Extract	0.5 g
0.1% Crystal violet	2.0 ml	1 % Yeast Extract	1 ml	Agar	15 g
Agar	15 g	0.1 % Malachite green	5.0 ml	Adjust volume to 1 l with H_2O	
Adjust volume to 1 l with H_2O		Agar	15 g	Adjust to pH 7.2	
Sterilize by autoclaving		Adjust volume to 1 l with H_2O		Sterilize by autoclaving	
		Sterilize by autoclaving			
After autoclaving:		After autoclaving:		After autoclaving:	
K_2TeO_3	0.08 g	K_2TeO_3	0.32 g	K_2TeO_3	0.2 g
2 % cycloheximide	1 ml	2 % cycloheximide	1 ml	2 % cycloheximide	1 ml

Table 2. Improved selective media for agrobacteria members (adapted from Mougel et al., 2001).

individual colony suspensions are left to rehydrate overnight with shaking at 28°C before they are streaked onto appropriate media that must NOT contain tellurite. A third purification step following the same procedure is absolutely necessary before introduction of the novel isolate into the lab collection.

4. Presumptive genus determination by minimal biochemical tests

The INCO-DC European program ERBIC18CT970198, "Integrated Control of Crown Gall in Mediterranean Countries" has delivered a simple and efficient identification scheme for agrobacteria. By a simple and fast urease and/or esculinase biochemical test, any strains isolated from soil, tumor, or root that posseses the typical circulate glistening colony morphology on tellurite amended media can be conviently and confidently classified as part of the genus *Agrobacterium* (Table 3). To test the presence of these activities, fresh cells are suspended in urease solution or esculin solution (Table 3). After incubation at 28°C for 1 h, a colorimetric change is observed for agrobacteria strains: pink and black coloration, respectively, for urease and esculinase tests.

The *A. tumefaciens* species complex (biovar 1) has the enzymatic ability to aerobically convert lactose to 3-ketolactose. This is tested by streaking on medium containing lactose (Bernaerts and De Ley, 1963). After 2 days of growth at 28°C, the plates are flooded with a layer of Benedict's reagents. The presence of 3-ketolactose in the medium is indicated by the formation of a yellow ring around the growth of a positive strain (Table 3 and Figure 3).

A. fabrum (G8 genomovar of *A. tumefaciens* species complex), has the biovar 1 characteristics, plus a specific ability to degrade ferulic acid and caffeic acid. These properties are due to a gene cluster present only in the *A. fabrum* strains (Lassalle et al., 2011). A minimal medium supplemented with caffeic acid turns the color brown in five days. As caffeic acid degradation by *A. fabrum* inhibits this browning, inoculation of an isolate of agrobacteria in a minimal medium supplemented with caffeic acid is an easy test to detect if a strain belong to the *A. fabrum* species (Figure 3).

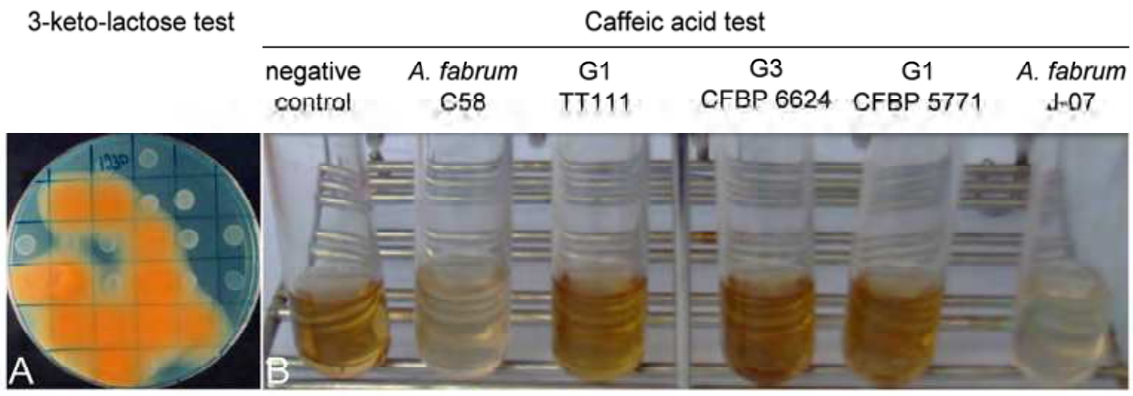

Fig. 3. Positive and negative reactions to the 3-keto-lactose test of Bernaert and De Ley (1963) of various *A. tumefaciens* complex and *R. rhizogenes* members (A) and identification of *A. fabrum* by caffeic acid test (B). AT minimal medium (Petit et al., 1978) supplemented with caffeic acid 0.1 mg.ml^{-1} is browning after five days of incubation except when the medium is inoculated with *A. fabrum* (here *A. fabrum* C58 or J07).

Esculin		Urease		3-ketolactose	
Peptone	10 g	L-tryptophan	3 g	Lactose medium:	
Esculin	1 g	Urea	20 g	Lactose	10 g
Ferric ammonium citrate	20 g	KH_2PO_4	1 g	Yeast Extract	1 g
Adjust to 1 l with H_2O		K_2HPO_4	1 g	Agar	15 g
		NaCl	5 g	Adjust volume to 1 l with H_2O	
		Ethanol 95%	10 ml		
		Phenol Red	25 mg	Benedict's reagent solution A:	
		Adjust to 1 l with H_2O		Sodium citrate	173 g
				Na_2CO_3	100 g
				Adjust to 850 ml with H_2O	
				Benedict's reagent solution B:	
				$CuSO_4$	18 g
				Adjust to 150 m with H_2O	
				Add solution B to solution A	

Table 3. Recipe for esculin, urease, and 3-ketolactose tests (adapted from Bernaerts and De Ley, 1963).

5. Species determination and strain typing using marker genes

5.1 Infraspecific diversity of *Agrobacterium* spp. at the genome level

Multi-locus sequence analysis (MLSA) performed with housekeeping or ecologically relevant genes *recA, mutS, gyrB, glgC, chvA, ampC,* reveals the occurrence of several strains with different alleles in most species (Table 1). As already showed with *chvA* and *recA* (Costechareyre et al., 2009, 2010), these genes allow the identification of novel isolates of *Agrobacterium* spp. at both species and strain levels. Nevertheless, even if MLSA is better than single gene analysis, we found that *recA* alone is definitively a good proxy for the identification of *Agrobacterium* species, since we have so far not found any indication that this gene has been laterally transferred between different species. Identification can be easily

done by a nucleotide Blast in databases. Thus, in order to facilitate the identification of *A. tumefaciens* genomovars, we have clearly indicated the genomovar and allele variant status of sequences deposited at EMBL in the description section, as shown below.

Accession#: FM164286
Description: Agrobacterium tumefaciens partial recA gene for recombinase A, genomovar G1, strain TT111, allele recA-G1-1

Extensive analysis of the infraspecific diversity is now conducted at the whole genome level by comparative genomics. The first analyses were done with DNA microarrays containing probes covering the whole genome of C58. This procedure permitted us to find species specific genes of *A. tumefaciens* genomovar G8 that were used to find typical phenotypic traits of this species (such as the ability to degrade the caffeic acid, Fig. 3). It was then possible to propose this genomovar as a novel species: *A. fabrum* (Lassalle et al., 2011). Comparative genomics of *Agrobacterium* spp. is now done with 22 complete genomes using the Agrobacterscope plateform at Genoscope:

https://www.genoscope.cns.fr/agc/microscope/about/collabprojects.php?P_id=51

However, while MLSA and genome sequencing are suited to type a limited number of strains for taxonomy purposes, these methods are not easily applicable to analyze large agrobacterial populations, as is required for epidemiological investigations. For this reason, we developed a PCR-RFLP approach consisting of the restriction of PCR products obtained from selected genome regions (Figure 4). The intergenic region spanning between the 16S and 23S rRNA genes (16S+ITS) was found to allow the distinction of strains within both the *R. rhizogenes* and *A. tumefaciens* complex (Ponsonnet & Nesme, 1994), with an accuracy we now know to be equivalent to those of *recA* (unpublished results).

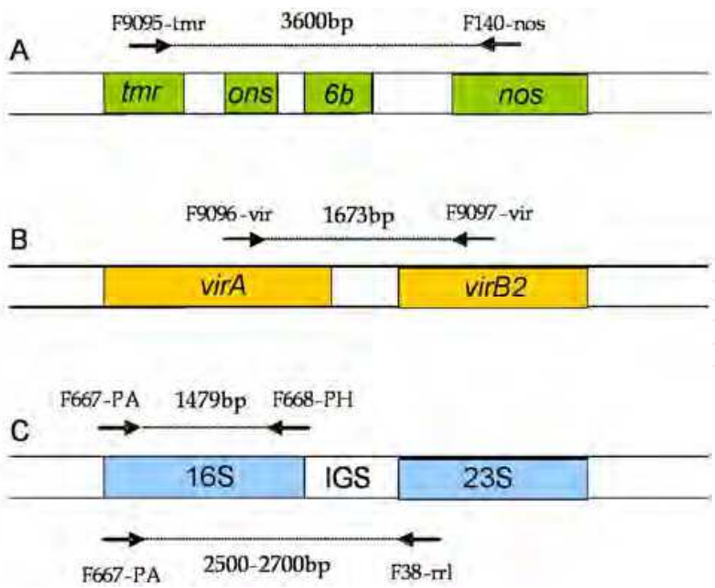

Fig. 4. Location of primers used for PCR-RLFP typing of Ti plasmid and chromosome. A, B and C indicate primer locations in the T-DNA region (regions between the *tmr* and *nos* genes), the *virA* and *virB*$_2$ genes, and the ribosomal operon (16S and 23S rRNA genes), respectively.

5.2 Ti plasmid diversity

Although agrobacteria are primarily natural soil inhabitants and also commensal rhizospheric bacteria, they become a plant pathogen when they acquire a Ti (Tumor inducing) plasmid (size ~200 kb). Thus it becomes important to characterize agrobacterium plasmid content. Generally agrobacteria of the *A. tumefaciens* complex harbor, besides their chromosome and chromid, another large plasmid called the At plasmid (~400 kb). Large Ti and At plasmids can be visualized on an agarose gel using a method modified from Eckhardt (1978) by Wheatcroft et al. (1990), which is based on the action of gentle bacterium lysis directly in agarose gel wells in order to avoid DNA breakages. Briefly, agrobacterial strains are grown in standard medium to an optical density of 0.2 at 600 nm. After which a total of 1 ml of cell culture is centrifuged at 13,000g for 1 min at 4°C. The pellet is then washed once in 1 ml Na N-lauroyl sarcosinate 0.3% and resuspended in Ficoll buffer (10 mM Tris, pH 7.5, and 0.1 mM EDTA, pH 8.0, 20% Ficoll® 400; Sigma) and incubated on ice for 15 min. Then lysis solution consisting of 10 mM Tris, 10 mM EDTA, RNase A (0.4 $mg.ml^{-1}$), bromophenol red (1$mg.ml^{-1}$; Sigma), and lyzosyme (1$mg.ml^{-1}$; Sigma) is added to the cell suspension. Immediately after addition of the lysis solution to cells, 25 µl of this solution is loaded on to a 0.75% agarose gel. Migration is run for 3 h at 100V. Characteristic plasmid size of different *Agrobacterium* and *Rhizobium* strains are given figure 5. In addition, the presence of a linear chromosome (i.e. chromid) can be easily visualized in the *A. tumefaciens* complex, *A. rubi* and *A. larrymoorei* by pulse field electrophoresis (Ramirez-Bahena et al., 2011).

While pathogenic agrobacteria differ from the non-pathogenic strains by the presence of Ti or Ri plasmids, there is also a large diversity within this plasmid class that must be considered in epidemiological investigations. Briefly, the eco-pathology of crown gall and hairy root diseases is centered upon the fact that Ti and Ri plasmids both incite the diseased plant to produce a particular class of compounds called 'opines', which give the agrobacteria a unique ability to use these particular compounds for their growth. There are several kind of opines produced in tumors and consumed by agrobacteria. The most common types of Ti plasmids produce nopaline, octopine or agropine and mannopine (for a review about opines and opine concept see Dessaux et al., 1992). While genes encoding the biosynthesis and the consumption of opines are very different according to opine types, genes involved in the pathogenic process are conserved amongst Ti and Ri plasmids and can be used to define the primers to amplify Ti-plasmid specific regions. Conserved regions are the region of virulence or *vir* region involved in the processing of the DNA transferred from agrobacterium to plant, (i.e. the T-DNA) and T-DNA genes determining the tumoral morphology such as *tmr* or *tms*. Thus, the PCR-RFLP approach is also applicable to study the Ti-plasmid diversity using T-DNA and *vir* regions (Figure 4 and table 4).

There are however a diversity of Ti plasmid conserved genes that can be used to rapidly assess the opine-type of pathogenic agrobacteria. With this idea we developed a set of nopaline-type specific primers in one of the pioneering works using PCR to detect plant pathogenic bacteria (Nesme et al., 1989). Primers F14 and F44 (Table 4) allowed the amplification of a 247 bp-long fragment only with nopaline-type Ti plasmids such as pTiC58 or pTiT37, while primer set F14-F750 permits us to specifically amplify a 217 bp-long amplicon of octopine-type Ti plasmids, such as pTiB6 or pTi15955. The 'universal' primer set F14-F749 was designed based on alignments of *vir* regions of the most common Ti-plasmid opine type (Figure 6). As these primers match different genes separated by intergenic regions of different lengths, amplicon sizes can be used to determine Ti-plasmid opine types.

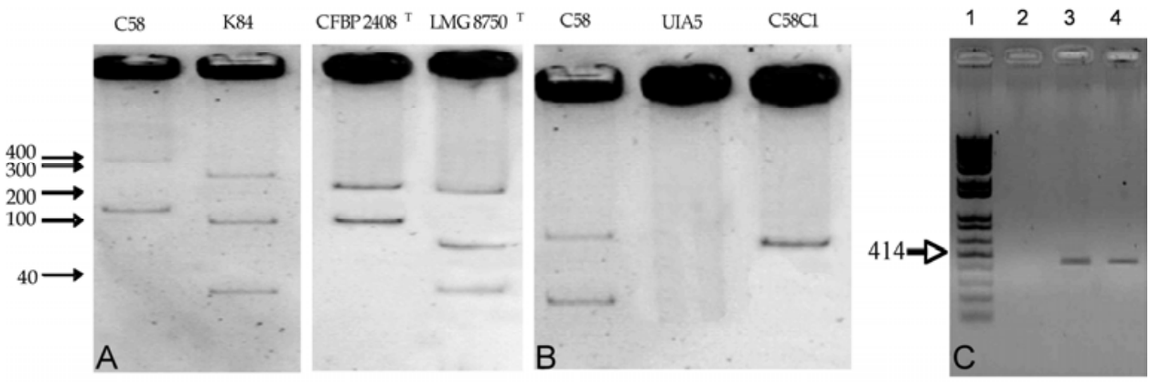

Fig. 5. Plasmids in *Agrobacterium sp.* and *Rhizobium rhizogenes.* A and B plasmid content determined by the Wheatcroft's method using gentle in well lysis of bacteria. A , *A. fabrum* C58^T, *Rhizobium rhizogenes* K84 and CFBP2408^T, *Agrobacterium vitis* LMG8750^T. B, *A. fabrum* C58 derivatives showing At and Ti plasmids of ca. 210 and 450 kbp, respectively. Molecular weight in kb. C, PCR detection of pathogenic *Agrobacterium* in environmental DNAs with primers VCF3 and VCR3. Lane 1, marker 1kb+; 2, negative control with DNA extracted from healthy plant tissue; 3 and 4, positive detections of a *virC1-virC2* region using DNAs extracted from tumors of tomatoes infected three weeks earlier with strain C58.

Primer set				Amplified regions
PCR-RFLP typing				
F667-PA	AGAGTTTGATCCTGGCTCAG	F668-PH	AAGGAGGTGATCCAGCCGCA	*rrs* (16S rRNA)
F667-PA	AGAGTTTGATCCTGGCTCAG	F38-rrl	CCGGGTTTCCCCATTCGG	16S+ITS
F9095-tmr	CCATGTTGTTTGCTAGCCAG	F140-nos	CACCATCTCGTCCTTATTGA	T-DNA
F9096-vir	TCAAAAGGCAAGCAAGCAGATCTGG	F9097-vir	TCAGTGCCGCCACCTGCAGATTG	*virA-virB*$_2$
PCR detection of Ti plasmids				
F14-vir	GAACGTGTTTCAACGGTTCA	F44-vir	TGCCGCATGGCGCGTTGTAG	*virB-G* (nopaline)
F14-vir	GAACGTGTTTCAACGGTTCA	F750-vir	GTAACCTCGAAGCGTTTCAC	*virB-G* (octopine)
F14-vir	GAACGTGTTTCAACGGTTCA	F749-vir	GCTAGCTTGGAAGATCGCAC	*virB-G* (oct. + nop.)
F8521-VCF3	GGCGGGCGYGCYGAAAGRAARACYT	F8522-VCR3	CGAGATTGCGTGCTTGTAGA	*virC1-C2* (pTi & pRi)

Table 4. PCR primers used to type agrobacteria by PCR-RLFP or for the detection of Ti and Ri plasmids (adapted from Bruce et al., 1992; Ponsonnet et al., 1994 and Kawaguchi et al., 2005).

5.3 Strain and Ti plasmid diversities in crown gall outbreaks

In the case study presented below, the PCR-RFLP method was used to type agrobacteria at both chromosomal and Ti plasmid levels. Agrobacteria were isolated from tumors of hybrid poplars (*Populus alba* x *P. termuloides*) in different tree nurseries (Orléans, Loiret, France and Peyrat-le-Château, Haute-Vienne, France) supposedly affected by the same crown gall epidemic. However, results summarized in figure 7 show that the pathogenic isolates belonged to different strains (as indicated by chromosomal ribotypes) of *Rhizobium rhizogenes* (28%) or *Agrobacterium tumefaciens* (72%). For the latter taxon, strains essentially belonged to genomovar G1 (96%). Results also showed a notable diversity of Ti-plasmids that were all of the nopaline-type (data not shown), despite the fact that they were dispatched in four different PCR-RFLP patterns: pTi2516 (50%), pTiM80 (8%), pTi1903 (23%), pTi2177 (9%) and pTi292 (10%).

Fig. 6. Aligment of *vir*G15-*vir*B11 regions amplifiable with Ti-plasmid-universal primers F14-F749 in Ti plasmids of different opine types. The first box corresponds to F14. The second box corresponds to reverse complement of F749. oct, octopine-type Ti plasmids such as pTi15955 or pTiB6. agr, agropine-mannopine-type Ti plasmid pTiBo542. nop, nopaline-type Ti plasmids such as pTiC58 or pTiT37. Amplicon lengths are 384 bp, 387 bp and 372 bp for octopine-, agropine-mannopine- and nopaline-type Ti plasmids, respectively.

Remarkably, a given Ti plasmid type could be found in different strains while a given strains could harbor different Ti plasmids, likely as the results of Ti plasmid conjugal transfers. In addition, none of the indigenous agrobacteria isolated from a non-contaminated plot harbored a Ti plasmid. Indigenous agrobacteria were found to be different of pathogenic strains isolated from tumors, except for some isolates of ribotype 2520 that harbored a pTi2516. This strongly suggests that indigenous soil agrobacteria —that were initially Ti plasmid free and consequently not pathogenic, such as 2520— were able to receive a Ti plasmid from introduced pathogenic strains, and then in turn become pathogenic. This may facilitate the establishment of Ti plasmids in indigenous soil agrobacteria and could be at the basis of perennial soil contaminations recorded for decades in several instances (Krimi et al., 2002; Costechareyre et al., 2010).

From an epidemiological point of view, the present chromosome and Ti plasmid diversities were found even in single tumors, illustrating the fact that a single isolate is not enough to characterize pathogenic populations involved in crown gall epidemics. However, by studying numerous isolates, we found that pathogenic agrobacteria were almost the same in Orléans and Peyrat-le-Château (data not shown), demonstrating that the crown gall outbreak of the latter nursery was caused by the introduction of apparently healthy (i.e. tumor free) —but in fact contaminated— plant material from Orléans. The same procedure involving both chromosome and Ti plasmid typing of numerous isolates was used to discover the source of crown gall contamination of rose bushes (Pionnat et al., 1999) and is currently used for routine epidemiological investigations.

6. Detection in complex environments

The detection of pathogenic *Agrobacterium* in complex environments without prior strain isolation can be done easily by PCR using primers designed in the conserved *vir* region (Table 4). Kawaguchi et al. (2005) also reported primers VCF3 and VCR3 amplifying DNA fragments of 414 bp in the *virC1-virC2* regions of both Ti and Ri plasmids. Ti plasmid detection can be conducted with metagenomic DNAs extracted from complex environments such as bulk soil, rhizosphere and tumors (Figure 5C). To date, in terms of accuracy and reproducibility the best results are obtained with DNA extracted with the PowerSoil® DNA isolation kit (http://www.mobio.com/soil-dna-isolation/powersoil-dna-isolation-kit.html).

In a similar manner, it is also possible to detect *Agrobacterium* and *Rhizobium* spp. in complex environments by using PCR probes designed in the 16S rDNA gene (Mougel et al., 2001). However, the 16S is not diverse enough to allow the distinction of closely related species such as *A. tumefaciens* genomovars. Instead, Nucleotide sequences of protein encoding genes have a much more resolutive power at both species and infraspecies levels. Nevertheless, the present challenge is to find gene regions well conserved within a species but with marked differences between species in order to design species specific primers. Only a small number of genes fulfill this requirement in *A. tumefaciens*, because genomovars are very closely related but also have a huge infraspecies diversity. Primary results suggest however that *recA* could be a convenient gene to find species specific primers suited for in situ detection of *Agrobacterium* species and genomovars (unpublished results).

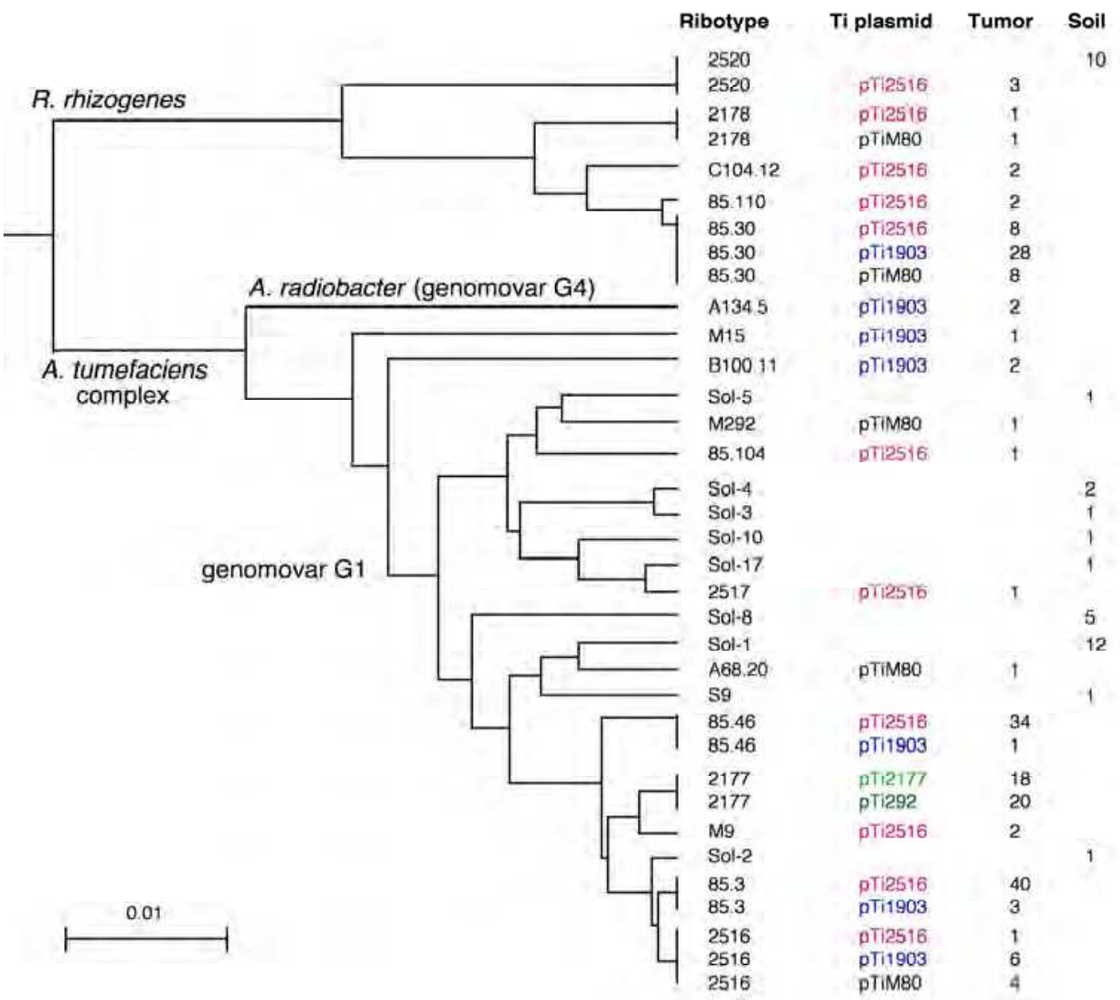

Fig. 7. Chromosome and Ti-plasmid diversity of agrobacteria isolated from poplar tumors in a single crown gall epidemic involving two distant nurseries. Ribotypes and Ti-plasmid types were determined by PCR-RFLP of the 16S+ITS region using primers F667-PA and F38 (Table 4), and digestion with *Cfo*I, *Hae*III, *Nde*II and *Taq*I; and primers F9095 and F140, and F9096 and F9097 (Table 4) and digestion with *Taq*I, *Dde*I, *Msp*I and *Rsa*I, respectively as described in Ponsonnet & Nesme (1994). 2520 and 2178 are ribotypes identical to those of *R. rhizogenes* strains CFBP 2520 and CFBP 2178, respectively. 2517, 2177 and 2516: ribotypes identical to those of *A. tumefaciens* genomovar-G1 strains CFBP 2517, CFBP 2177 and CFBP 2516, respectively. pTi2516, pTiM80, pTi1903, pTi2177: nopaline-type Ti-plasmids with PCR-RFLP patterns identical to those of strains M80, CFBP 1903 (i.e. C58) and CFBP 2177, respectively. pTi292: pTi2177 with the insertion element IS292 (Ponsonnet et al., 1995). Tumor indicates total numbers of isolates for each conjugated chromosome and Ti-plasmid patterns obtained in four different plots in Orléans and Peyrat-le-Château. Soil indicates agrobacteria isolated directly from a non-contaminated soil plot in the vicinity of

contaminated plots planted with poplars at Peyrat-le-Château; none of which harboring a Ti plasmid. The dendrogram was built with UPGMA using number of substitutions per nucleotidic sites calculated from RFLP patterns as genetic distance.

7. Conclusion

The genus *Agrobacterium* encompasses several species and several strains within species that are now clearly named using a novel nomenclature. A set of efficient procedures is available to study either pure culture isolates or agrobacteria directly in soil microbiomes. These methodologies are necessary to unmask the ecologies of *Agrobacterium* species that usually live sympatrically in soils. By this knowledge we expect to differentially manage agrobacterial species directly in soils to improve the crown gall as well as to stimulate their putative PGPR effects.

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9. References

Beijerinck, M.W. & van Delden, A. (1902). Ueber die Assimilation des freien Stickstoffs durch Bakterien. *Zentralbl Bakteriol Parasitenk Infektionskr HygAbt*, Vol. 9, pp. 3-43

Bernaerts, M.J. & de Ley, J. (1963). A biochemical test for crown gall bacteria. *Nature*, Vol. 197, pp. 406-407, ISSN 0028-0836

Bouzar, H. & Jones, J.B. (2001). *Agrobacterium larrymoorei* sp. nov., a pathogen isolated from aerial tumours of *Ficus benjamina*. *International Journal of Systematic and Evolutionary Microbiolology*, Vol. 51, No. 3, pp. 1023–1026, ISSN 1466-5026

Bruce, K.D.; Hiorns, W.D.; Hobman, J.L.; Osborn, A.M.; Strike, P. & Ritchie, D.A. (1992). Amplification of DNA from native populations of soil bacteria by using the polymerase chain reaction. *Applied and Environmental Microbiology*, Vol. 58, No. 10, pp. 3413-3416, ISSN 0099-2240

Brisbane, P.G. & Kerr, A. (1983). Selective media for the three biovars of *Agrobacterium*. *Journal of Applied Bacteriology*, Vol. 54, No. 3, pp. 425-431, ISSN 1364-5072

Conn, H.J. (1942). Validity of the genus *Alcaligenes*. *Journal of Bacteriology*, Vol. 44, No. 3, pp. 353-360, ISSN 0021-9193

Costechareyre, D.; Bertolla, F. & Nesme, X. (2009). Homologous recombination in *Agrobacterium*: potential implications for the genomic species concept in bacteria, *Molecular Biology and Evolution*, Vol. 26, No. 1, pp. 167-176, ISSN 0737-4038

Costechareyre, D.; Rhouma, A.; Lavire, C.; Portier, P.; Chapulliot, D.; Bertolla, F.; Boubaker, A.; Dessaux, Y. & Nesme, X. (2010). Rapid and efficient identification of

Agrobacterium species by *recA* allele analysis, *Microbial Ecology*, Vol. 60, No. 4, pp. 862-872, ISSN 0095-3628

De Ley, J. (1974). Phylogeny of prokaryotes, *Taxon*, Vol. 23, pp. 291-300, ISSN 0040-0262

De Ley, J.; Tijtgat, R.; De Smedt, J. & Michiels, M. (1973). Thermal stability of DNA: DNA Hybrids within the genus *Agrobacterium*. *Journal of General Microbiology*, Vol. 78, No. 2, pp. 241-252, ISSN 0022-1287

Dessaux, Y.; Petit, A. & Tempé, J. (1992). Opines in *Agrobacterium* biology, In : *Molecular Signals in Plant-Microbe Communications*. D. P. S. Verma, (Ed.), 109-136, CRC Press, Boca Raton

Eckhardt, T. (1978). A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. *Plasmid*, Vol. 1, No. 4, pp. 584-588, ISSN 0147-619X

Genetello, C.; Van Larebeke, N.; Holsters, M.; De Picker, A.; Van Montagu, M. & Schell, J. (1977). Ti plasmids of *Agrobacterium* as conjugative plasmids. *Nature*, Vol. 265, pp. 561-563, ISSN 0028-0836

Holmes, B. & Roberts, P. (1981). The classification, identification and nomenclature of agrobacteria. *Journal of Applied Bacteriology*, Vol. 50, pp. 443-467

Hooykaas, P.J.; Klapwijk, P.M.; Nuti, M.P.; Schilperoort, R.A. & Rörsch, A. (1977). Transfer of the *Agrobacterium tumefaciens* Ti plasmid to avirulent agrobacteria and to *Rhizobium ex planta*. *Journal of General Microbiology*, Vol. 98, No. 2, pp. 477-484, ISSN 0022-1287

Kawaguchi, A.; Inoue, K. & Nasu, H. (2005). Inhibition of crown gall formation by *Agrobacterium radiobacter* biovar 3 strains isolated from grapevine. *Journal of General Plant Pathology*, Vol. 71, No. 6, pp. 422–430, ISSN 1345-2630

Keane, P.J.; Kerr, A. & New, P.B. (1970). Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. *Australian Journal of Biological Sciences*, Vol. 23, No. 3, pp. 585-595, ISSN 0004-9417

Kerr, A. (1974). Soil microbiological studies on *Agrobacterium radiobacter* and biological-control of crown gall. *Soil Science,* Vol. 118, pp. 168-172, ISSN 0038-075X

Krimi, Z.; Petit, A.; Mougel, C.; Dessaux, Y. & Nesme, X. (2002). Seasonal fluctuations and long-term persistence of pathogenic populations of *Agrobacterium* spp. in soils. *Applied and Environmental Microbiology*, Vol. 68, No. 7, pp. 3358-3365, ISSN 0099-2240

Lassalle, F.; Campillo, T.; Vial, L.; Baude, J.; Costechareyre, D.; Chapulliot, D.; Shams, M.; Abrouk, D.; Lavire, C.; Oger-Desfeux, C.; Hommais, F.; Guéguen, L.; Daubin, V.; Muller, D. & Nesme, X. (2011). Genomic species are ecological species as revealed by comparative genomics in *Agrobacterium tumefaciens*. *Genome Biology and Evolution*, Vol. 3, pp. 762-781, ISSN 1759-6653

Lindström, K. &Young, J.P.W. (2011). International Committee on Systematics of Prokaryotes. Subcommittee on the taxonomy of Agrobacterium and Rhizobium: Minutes of the meeting, 7 September 2010, Geneva, Switzerland. *International Journal of Systematic and Evolutionary Microbiolology*, Vol. 61, No. 12, pp. 3089-309, ISSN: 1466-5034

Mougel, C.; Cournoyer, B. & Nesme, X. (2001). Novel tellurite-amended media and specific chromosomal and Ti plasmid probes for direct analysis of soil populations of *Agrobacterium* biovars 1 and 2. *Applied and Environmental Microbiology*, Vol. 67, No. 1, pp. 65-74, ISSN 0099-2240

Mougel, C.; Thioulouse, J.; Perrière, G. & Nesme, X. (2002). A mathematical method for determining genome divergence and species delineation using AFLP. *International Journal of Systematic and Evolutionary Microbiolology*, Vol. 52, No. 2, pp. 573-586, ISSN 1466-5026

Nesme, X.; Michel, M.-F. & Digat, B. (1987). Population heterogeneity of *Agrobacterium tumefaciens* in galls of *Populus* L. from a single nursery. *Applied and Environmental Microbiology*, Vol. 53, No. 4, pp. 655-659, ISSN 0099-2240

Nesme, X.; Leclerc, M. C. & Bardin R. (1989). PCR detection of an original endosymbiont: the Ti plasmid of *Agrobacterium tumefaciens*. In *Endocytobiology IV*, Nardon, P.; Gianinazzi-Peason, V.; Greines, A. M.; Margulis, L. & Smith, D. C. pp. 47-50, Institut National de la Recherche Agronomique, Paris

Ophel, K. & Kerr, A. (1990). *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. *International Journal of Systematic Bacteriology*, Vol. 40, No. 3, pp. 236-241, ISSN 0020-7713

Panday, D.; Schumann, P. & Das, S.K. (2011). *Rhizobium pusense* sp. nov., isolated from the rhizosphere of chickpea (*Cicer arietinum L.*). *International Journal of Systematic and Evolutionary Microbiolology*, Vol. 61, pp. 2632 -2639, ISSN 1466-5026

Panagopoulos, C.G.; Psallidas, P.G. & Alivizatos, A.S. (1978). Studies on biotype 3 of *Agrobacterium radiobacter* var. *tumefaciens*. In Station de Pathologie Végétale et Phytobactériologie, ed., Plant Pathogenic Bacteria Proc 4th Internat Conf Plant Path Bact, Angers, France, pp 221-228

Petit, A.; Tempe, J.; Kerr, A.; Holsters, M.; Van Montagu, M. & Schell, L. (1978). Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti plasmids. *Nature*, Vol. 271, pp. 570-572, ISSN 0028-0836

Pionnat, S.; Keller, H.; Héricher, D.; Bettachini, A.; Dessaux, Y.; Nesme, X. & Poncet, C. (1999). Ti plasmids from *Agrobacterium* characterize rootstock clones that initiated a spread of crown gall disease in Mediterranean countries. *Applied Environmental Microbiology*, Vol. 65, No. 9, pp. 4197-4206, ISSN 0099-2240

Pitzscke, A. & Hirt, H. (2010). New insights into an old story: *Agrobacterium* induced tumour formation in plants by plant transformation. *EMBO Journal*, Vol. 29, No. 6, pp. 1021-1032, ISSN 0261-4189

Ponsonnet, C. & Nesme, X. (1994). Identification of *Agrobacterium* strains by PCR-RFLP analysis of pTi and chromosomal regions. *Archives of Microbiology*, Vol. 161, No. 4, pp. 300-309, ISSN 0302-8933

Ponsonnet, C.; Normand, P.; Pilate, G. & Nesme, X. (1995). IS292 : a novel insertion element from *Agrobacterium*. *Microbiology*, Vol. 141, No. 4, pp. 853-861, ISSN 1350-0872

Popoff, M.Y.; Kersters, K.; Kiredjian, M.; Miras, I. & Coynault. C. (1984). Position systématique de souches de *Agrobacterium* d'origine hospitalière. *Annals of Microbiology*, Vol. 135, No. 3, pp. 427-442, ISSN 1590-4261

Portier, P.; Fisher-Le Saux, M.; Mougel, C.; Lerondelle, C.; Chapulliot, D.; Thioulouse, J. & Nesme, X. (2006). Identification of genomic species in *Agrobacterium* biovar 1 by AFLP genomic markers. *Applied and Environmental Microbiology*, Vol. 72, No. 11, pp. 7123-7131, ISSN 0099-2240

Puławska, J.; Willems, A. & Sobiczewski, P. (2012) *Rhizobium skierniewicense* sp. nov. isolated from tumors on chrysanthemum and *Prunus* in Poland, *International Journal of Systematic and Evolutionary Microbiology* doi: 10.1099/ijs.0.032532-0 (in press), ISSN 1466-5026

Puławska, J.; Willems, A.; De Meyer, S. & Süle, S. *Rhizobium nepotum* sp nov. isolated from tumors on different plant species, unpublished paper

Ramirez-Bahena, M.; Nesme, X. & Muller, D. (2012). Rapid and simultaneous detection of linear chromosome and large plasmids in Proteobacteria. *Journal of basic microbiology* , Vol. 52, No. 1, pp. 1-4 ISSN 1521-4028

Riker, A.J.; Banfield, W.M.; Wright, W.H.; Keitt, G.W. & Sagen, H.E. (1930). Studies on infectious hairy root of nursery trees of apples. *Journal of Agricultural Research*, Vol. 41, pp. 507-540

Roy, M.A. & Sasser, M. (1983). A medium selective for *Agrobacterium tumefaciens* biotype 3. *Phytopathology*, Vol. 73, No. 1, pp. 810, ISSN 0031-949X

Smith, E.F. & Townsend, C.O. (1907). A plant-tumor of bacterial origin. *Science*, Vol. 25, pp. 671-673, ISSN 0036-8075

Stackebrandt, E.; Frederiksen, W.; Garrity, G.M., Grimont, P.A.; Kampfer, P.; Maiden, M.C.; Nesme, X.; Rossello-Mora, R.; Swings, J.; Truper, H.G.; Vauterin, L.; Ward, AC. & Whitman, W.B. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology*, Vol. 52, No. 3, pp. 1043-1047, ISSN 1466-5026

Van Larebeke, N.; Engler, G.; Holsters, M.; Van den Elsacker, S.; Zaenen, I.; Schilperoort, R.A. & Schell, J. (1974). Large plasmid in *Agrobacterium tumefaciens* essential for Crown Gall-inducing ability. *Nature*, Vol. 252, pp. 169-170, ISSN 0028-0836

Vogel, J.; Normand, P.; Thioulouse, J.; Nesme, X. & Grundmann, G. (2003). Relationship between spatial and genetic distance in *Agrobacterium* spp. in 1 cubic centimeter of soil. *Applied and Environmental Microbiology*, Vol. 69, No. 3, pp. 1482-1487, ISSN 0099-2240

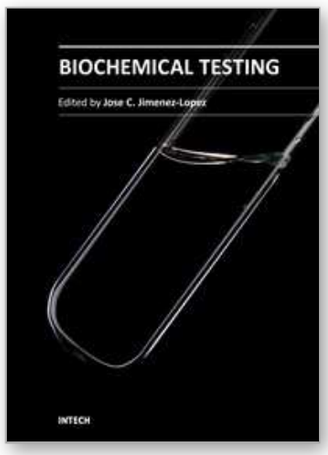
Waine, L.G.; Brenner, D.J.; Colwell, R.R.; Grimont, P.A.D.; Kandler, O.; Krichevsky, M.I.; Moore, L.H.; Moore, W.E.C.; Murray, R.G.E.; Stackebrandt, E.; Starr, M.P. & Trüper, H.G. (1987). Report of the ad hoc committee on the reconciliation of approaches to bacterial systematics. *International Journal of Systematic and Evolutionary Microbiology*, Vol. 37, No. 4, pp. 463-464, ISSN 1466-5026

Wheatcroft, R.; McRae, D.G. & Miller, R. W. (1990). Changes in the *Rhizobium meliloti* genome and the ability to detect supercoiled plasmids during bacteroid development. *Molecular Plant-Microbe Interactions*, Vol. 3, No. 1, pp. 9-17, ISSN 0894-0282

Young, J.M.; Kuykendall, L.D.; Martinez-Romero, E.; Kerr, A. & Sawada, H. (2001). A revision of *Rhizobium* Franck 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola*

de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. *International Journal of Systematic and Evolutionary Microbiology*, Vol. 51, No. 1, pp. 89-103, ISSN 1466-5026

Young, J.M.; Pennycook, S.R. & Watson, D.R.W. (2006). Proposal that *Agrobacterium radiobacter* has priority over *Agrobacterium tumefaciens*. Request for an Opinion. *International Journal of Systematic and Evolutionary Microbiology*, Vol. 56, No. 2, pp. 491-493, ISSN 1466-5026



Biochemical Testing

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Biochemical testing necessitates the determination of different parameters, and the identification of the main biological chemical compounds, by using molecular and biochemical tools. The purpose of this book is to introduce a variety of methods and tools to isolate and identify unknown bacteria through biochemical and molecular differences, based on characteristic gene sequences. Furthermore, molecular tools involving DNA sequencing, and biochemical tools based in enzymatic reactions and proteins reactivity, will serve to identify genetically modified organisms in agriculture, as well as for food preservation and healthcare, and improvement through natural products utilization, vaccination and prophylactic treatments, and drugs testing in medical trials.

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