Detection of Activity Responsible for Induction of the *Agrobacterium tumefaciens* Virulence Genes in Bacteriological Agar

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***Agrobacterium tumefaciens* C58 grown on acidic medium containing glucose and solidified with bacteriological agar expressed a *virB::lacZ* fusion. No expression of this fusion was observed on a similar medium which was solidified with purified agarose. The fraction from bacteriological agar which was responsible for *vir* gene induction was extracted with methanol and partially purified by preparative thin-layer chromatography.**

Host plants become susceptible to infection by *Agrobacterium tumefaciens* following wounding, which triggers the release of phenolic signals such as acetosyringone. The capacity to respond to these signals is conferred on *A. tumefaciens* by *virA* and *virG*, two genes encoded by the tumor-inducing (Ti) plasmid and forming part of the *vir* regulon. The products of these genes are responsible for the activation of the entire *vir* regulon, in turn leading to transfer of part of the Ti plasmid to host plant cells and subsequent integration of this transferred DNA of bacterial origin in the chromosomes of the plant cells (for reviews, see references 5 and 17).

The chemical nature of the phenolic *vir* gene inducers varies according to the type of plant host. The first native *vir* gene inducers to be identified were acetophenones originally isolated from tobacco root exudates (15). These compounds, including the potent inducer acetosyringone, also are present in various members of the family *Solanaceae* besides tobacco (14). In addition, acetosyringone was released from the bark of trees of six *Populus* species on treatment with alkali (10), and acetophenone glucoside has been isolated from *Ranzania japonica* (6). The signal compounds in *Pisum sativum* seedlings were identified as sinapyl and coniferyl alcohols (12). Petunia pollen and stigma tissue contain flavonoid glucosides with *vir* gene-inducing activity (20). Another glucoside, coniferin, was purified from Douglas fir needle extracts by Morris and Morris and identified as the major *vir* gene inducer in that species (9). The same authors suggested the possibility of release of the active coniferyl alcohol from its glucoside by a glucosidase of agrobacterial origin (9). Monocotyledonous species, which are only slightly susceptible or not susceptible to *Agrobacterium*-induced transformation, also may produce *vir* gene inducers such as ethyl ferulate, which was extracted from a *Triticum monococcum* suspension culture (8). Various other *vir* gene inducers, such as chalcone derivatives, were recognized by systematically testing a large number of synthetic phenolic compounds (7, 13). Production of *vir* gene inducers by algae of the genera *Porphyria* and *Gracilaria* also has been mentioned (11).

We have previously reported that *vir* gene inducers inhibited the growth of certain strains of *A. tumefaciens* and favored the

massive accumulation of avirulent mutants in bacterial cultures (2, 3). While pursuing these studies, we have accidentally noticed that, under particular conditions, the bacteriological agar which is commonly used to solidify growth media also enhanced the genetic instability of *A. tumefaciens*. This led us to investigate the possible presence of *vir* gene-inducing activity in bacteriological agar.

Organisms and culture conditions. *A. tumefaciens* NT1(pTiC58Tra^c) (1) is a derivative of *A. tumefaciens* C58 containing a functional Ti plasmid. *A. tumefaciens* A136 (16) also is a C58 derivative but does not harbor a Ti plasmid. These agrobacterial strains were kept at 27°C on nutrient agar (Difco).

Induction of *vir* genes. Plasmid pSM243cd, which carries a fusion of *lacZ* with pTiA6 *virB* (18), was used for monitoring the induction of the Ti plasmid-encoded *vir* genes. This plasmid was transferred to strains NT1(pTiC58Tra^c) and A136 as described previously (2). To examine *vir* gene induction, agrobacteria containing pSM243cd were transferred to liquid AT-ammonium sulfate-glucose (ANG) medium adjusted to pH 5.6, as described before (2). In a previous study (2), ANG medium was found to be suitable for expression, by strain C58 derivatives, of the *vir* gene induction response to acetosyringone and other known inducers. The mineral base of ANG medium was modified from the AT medium of Guyon et al. (4) by increasing the concentration of KH₂PO₄, 15; MgSO₄ · 7 H₂O, 0.16; FeSO₄ · 7 H₂O, 0.005; CaCl₂ · 2 H₂O, 0.011; and MnCl₂ · 4 H₂O, 0.002. ANG medium also contained 2 g of glucose and 1 g of ammonium sulfate per liter. When desired, acetosyringone or an extract prepared from Bacto Agar (Difco) was added to liquid ANG medium (see below for details). To prepare solid ANG medium, Bacto Agar (15 g/liter) or purified agarose (Bio-Rad; 12 g/liter) was added. Samples for the measurement of β-galactosidase activity were taken after 24 h of growth for liquid ANG cultures and after 48 h of growth for solid ANG cultures. β-Galactosidase activity was determined as described by Winans et al. (19).

Observation of *vir* gene-inducing activity in bacteriological agar. To verify if Bacto Agar contained a *vir* gene inducer, strain NT1(pTiC58Tra^c, pSM243cd) was grown on ANG medium (pH 5.6) which was solidified with Bacto Agar. This medium did not contain an added *vir* gene inducer. As a negative control, strain A136(pSM243cd) was inoculated in the

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TABLE 1. Analysis of agar for *vir* gene-inducing activity^a

Addition to ANG medium	β-Galactosidase units obtained with strain:	
	NT1(pTiC58Tra ^c , pSM243cd)	A136(pSM243cd)
Solid medium		
Bacto Agar (15 g/liter)	2,427 ± 215	36 ± 4
Agarose (12 g/liter)	26 ± 3	29 ± 5
Liquid medium		
Crude methanolic extract of Bacto Agar (0.2% [vol/vol])	317 ± 36	ND
Methanol (0.2% [vol/vol])	23 ± 5	ND
Acetosyringone (60 μM)	1,135 ± 129	ND

^a Solid and liquid cultures were grown for 48 and 24 h, respectively, and a sample was then taken for determination of β-galactosidase activity. Values reported are the means of results from eight determinations ± the standard deviations. ND, not determined.

same manner. Cells were recovered after 2 days of incubation and resuspended at an optical density at 600 nm of 0.5 in physiological saline (8.5 g of NaCl per liter). The cell suspension prepared from the NT1(pTiC58Tra^c, pSM243cd) solid culture, but not that prepared from the A136(pSM243cd) culture, strongly expressed β-galactosidase (Table 1). To verify that the expression of β-galactosidase activity in strain NT1(pTiC58Tra^c, pSM243cd) was due to impurities from the agar, a slightly modified solid ANG medium was prepared in which Bacto Agar was replaced with purified agarose. Under these conditions, no increase in β-galactosidase activity in strain NT1(pTiC58Tra^c, pSM243cd), as compared to the negative control, A136(pSM243cd), was noted (Table 1). Hence, *vir* gene-inducing activity was associated with Bacto Agar but not with agarose.

Extraction of *vir* gene-inducing activity from Bacto Agar. Bacto Agar was subjected to chemical extraction to confirm that it possessed *vir* gene-inducing activity. A volume of 400 ml of methanol was added to 80 g of Bacto Agar. The resulting slurry was shaken for 24 h at 27°C and subsequently filtered through nitrocellulose (0.45 μm pore size; Millipore). The methanolic extract was dried under vacuum with a rotary evaporator. The residue obtained was resuspended in 20 ml of water and extracted once with ethyl acetate. The ethyl acetate fraction was evaporated under vacuum, and the residue was dissolved in 2 ml of methanol to yield the crude methanolic extract. When added to liquid ANG medium (pH 5.6), this crude methanolic extract of Bacto Agar exhibited *vir* gene-inducing activity (Table 1). Methanolic extraction of *vir* gene-inducing activity was repeated with four different batches of Bacto Agar and also with one batch of Noble agar (Difco) with similar results (data not shown).

A volume of 5 ml of the crude methanolic extract, in which *vir* gene-inducing activity had been detected, was subsequently fractionated by preparative thin-layer chromatography (TLC) on silica gel plates containing a fluorescent background indicator (TLC sheets precoated with silica gel 60 F-254; Merck), with hexane-acetone (2:1) as the solvent. A total of nine zones were delimited on the basis of fluorescence quenching under UV light and were numbered, zone 1 being that which was closest to origin of the chromatogram. Each zone was individually scraped from the plate and resuspended in acetone. The resulting mixture was shaken overnight at 27°C, filtered, and evaporated. The residue was dissolved in 200 μl of methanol to yield a particular TLC fraction. In total, nine fractions were

FIG. 1. Examination of *vir* gene induction with a methanolic extract of Bacto Agar fractionated by preparative TLC. Zones on the thin-layer chromatogram were delimited on the basis of UV fluorescence quenching and eluted, and the resulting extract fractions were used in induction experiments. Zones on the chromatogram and the corresponding extract fractions were numbered, the zone closest to the origin being identified as zone 1. Individual fractions were added to liquid ANG (pH 5.6) medium at a rate of 1 μl of methanolic fraction per ml of medium. *A. tumefaciens* NT1(pTiC58Tra^c, pSM243cd) was then transferred to the various media containing individual methanolic fractions. Bars on the histogram represent the level of *virB::lacZ* induction obtained after 24 h of incubation of the resulting cultures.

obtained from the original crude methanolic extract, each corresponding to a particular zone on the TLC plate. Three of these fractions exhibited *vir* gene-inducing activity (Fig. 1). Components in these active fractions migrated closer to the solvent front than an acetosyringone standard (data not shown).

The three TLC fractions with *vir* gene-inducing activity were analyzed by reversed-phase high-pressure liquid chromatography using UV detection at 260 and 280 nm. A 25-cm, reversed-phase, analytical Vydac C₁₈ column was used. The elution program was 5 mM unbuffered H₃PO₄ (pH ca. 2.5) with a gradient of 30 to 60% acetonitrile. The resulting chromatograms were complex, showing the presence of multiple components. Fraction 7 differed from the other two active fractions in showing a major high-pressure liquid chromatography peak that could not be resolved from an acetosyringone standard. For this reason, the low-resolution electron impact mass spectrum of fraction 7 was obtained with a Hewlett-Packard 5985-B mass spectrometer using electron impact ionization. This analysis showed that fraction 7 consisted mainly of palmitic and myristic acid and contained only very small amounts of phenolic compounds (data not shown). Commercial preparations of palmitic and myristic acid (Sigma), added alone or together at concentrations of up to 1 mM, had no *vir* gene-inducing activity. No signal at 196 atomic mass units, the molecular weight of acetosyringone, was detectable from fraction 7.

The demonstration of the presence of a *vir* gene-inducer in bacteriological agar should prove of interest to investigators involved in the study of agrobacterial *vir* gene induction, since solid induction media are used for certain applications such as screening of mutants altered in their *vir* gene induction properties. Background *vir* gene induction may be observed on such

media solidified with bacteriological agar, even in the absence of added acetosyringone or another *vir* gene inducer.

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