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

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Received 6 September 1996/Accepted 29 August 1997

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 petunia and identified as the major vir gene inducer in that species (9). 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 gene-inducing activity (20).

The production of phenolic signals such as acetosyringone is controlled by a complex regulatory system in 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).

To verify if Bacto Agar contained a vir gene inducer, or an extract prepared from Bacto Agar (Difco) was added to liquid ANG medium (pH 5.6). For solid 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+) was inoculated in the
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, 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, 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 was detected, which 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 extract. When added to liquid ANG medium (pH 5.6) at a rate of 1 ml of methanolic fraction per ml of medium. A. tumefaciens NT1(pTiC58Tra, 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.

![Image](https://example.com/image.png)

**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, 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.

<table>
<thead>
<tr>
<th>TABLE 1. Analysis of agar for vir gene-inducing activity</th>
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<tbody>
<tr>
<td>Additions to ANG medium</td>
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<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Solid medium</td>
</tr>
<tr>
<td>Bacto Agar (15 g/liter)</td>
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<tr>
<td>Agarose (12 g/liter)</td>
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<tr>
<td>Liquid medium</td>
</tr>
<tr>
<td>Crude methanolic extract of Bacto Agar (0.2% [vol/vol])</td>
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<tr>
<td>Methanol (0.2% [vol/vol])</td>
</tr>
<tr>
<td>Acetosyringone (60 μM)</td>
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| * Solid and liquid cultures were grown for 48 and 24 h, respectively, and a sample was then taken for determination of eight determinations ± the standard deviations. ND, not determined.

**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 extract. When added to liquid ANG medium (pH 5.6) at a rate of 1 ml of methanolic fraction per ml of medium. A. tumefaciens NT1(pTiC58Tra, 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.

**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, 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.

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 C<sub>18</sub> column was used. The elution program was 5 mM unbuffered H<sub>3</sub>PO<sub>4</sub> (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 spectrometry 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.

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to P. Dion.

REFERENCES