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 virB3::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 acetylsyringone. 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, in addition to a variety of synthetic acetophenones, were identified as sinapyl and coniferyl alcohols (12). Petunia pollen and stigma tissue contain flavonoid glucosides with properties (7, 13). Production of phenolic compounds, such as chalcone derivatives, was recognized by systematically testing a large number of synthetic phenolic compounds (7, 13). Production of vir gene inducers by algae of the genera *Porphyra* and *Graecilus* has been mentioned (11).

We have previously reported that vir gene inducers inhibit 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) (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) 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 acetylsyringone 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 KH$_2$PO$_4$ and contained, in grams per liter, the following: KH$_2$PO$_4$, 15; MgSO$_4$ $\cdot$ 7 H$_2$O, 0.16; FeSO$_4$ $\cdot$ 7 H$_2$O, 0.005; CaCl$_2$ $\cdot$ 2 H$_2$O, 0.011; and MnCl$_2$ $\cdot$ 4 H$_2$O, 0.002. ANG medium also contained 2 g of glucose and 1 g of ammonium sulfate per liter. When desired, acetylsyringone 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), 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
TABLE 1. Analysis of agar for vir gene-inducing activity

<table>
<thead>
<tr>
<th>Addition to ANG medium</th>
<th>β-Galactosidase units obtained with strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NT1(pTiC58Tra&lt;sup&gt;a&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Solid medium</td>
<td>A136(pSM243cd)</td>
</tr>
<tr>
<td>Bacto Agar (15 g/liter)</td>
<td>2,427 ± 215</td>
</tr>
<tr>
<td>Agarose (12 g/liter)</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Liquid medium</td>
<td></td>
</tr>
<tr>
<td>Crude methanolic extract of Bacto Agar (0.2% [vol/vol])</td>
<td>317 ± 36</td>
</tr>
<tr>
<td>Methanol (0.2% [vol/vol])</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>Acetosyringone (60 μM)</td>
<td>1,135 ± 129</td>
</tr>
</tbody>
</table>

ND, not determined.

* 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.

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<sup>a</sup>, pSM243cd) solid culture, but not that prepared from the A136(pSM243cd) culture, strongly expressed β-galactosidase. To verify that the expression of β-galactosidase activity in strain NT1(pTiC58Tra<sup>a</sup>, 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 associated with Bacto Agar Agarose (12 g/liter) 26

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

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 NTI(pTiC58Tra<sup>a</sup>, pSM243cd) was then transferred to the various media containing individual methanolic fractions. Bars on the histogram represent the level of vir:B:lacZ induction obtained after 24 h of incubation of the resulting cultures.
media solidified with bacteriological agar, even in the absence of added acetosyringone or another vir gene inducer.

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REFERENCES