The role of VirA and VirG phosphorylation in chemotaxis towards acetosyringone by *Agrobacterium tumefaciens*

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The Ti-plasmid-encoded two-component sensor-regulator system comprising VirA and VirG confers upon *Agrobacterium tumefaciens* the ability to respond chemotactically to nanomolar concentrations of *vir*-inducing phenolics such as acetosyringone. Non-phosphorylatable, mutant VirA and VirG proteins are incapable of replacing their wild-type counterparts in conferring this phenotype. This indicates that, like *vir*-gene induction in response to acetosyringone, chemotaxis to the same ligand involves phosphorylation of VirA and VirG. However, unlike *vir*-induction, deletion of the periplasmic domain of VirA severely curtails acetosyringone chemotaxis, suggesting that acetosyringone may mediate effects through more than one region of VirA. When introduced into strains expressing wild-type VirA and VirG, the non-phosphorylatable versions suppress chemotaxis towards acetosyringone, implying that mutant copies of VirA and VirG compete with their wild-type counterparts in interactions between VirA and VirG.

Introduction

Environmental sensing is of particular importance to *Agrobacterium tumefaciens* (Shaw, 1991). The bacterium's existence in the rhizosphere, coupled with a requirement to interact with wounded plants, has led to the evolution of a highly sensitive chemotaxis system (Ashby *et al.*, 1987, 1988; Loake *et al.*, 1988). Motility is important in rhizosphere competence (Shaw *et al.*, 1991) and chemotaxis towards plant exudates is a component of virulence (Hawes & Smith, 1989; Hawes *et al.*, 1988). The most intense chemotactic response for avirulent, Ti-plasmid-cured *A. tumefaciens* is towards micromolar levels of plant sugars (Loake *et al.*, 1988). The Ti-plasmid confers the additional ability to respond specifically to nanomolar amounts of wound-exuded phenolic compounds, such as acetosyringone (Ashby *et al.*, 1987, 1988). This property requires the Ti-plasmid virulence genes *virA* and *virG* (Shaw *et al.*, 1988). At higher acetosyringone concentrations, VirA and VirG mediate a second response, the induction of the other *vir*-operons, products of which act in concert to transfer T-DNA to the plant cell (Howard & Citovsky, 1990).

VirA is a 92 kDa transmembrane protein (Winans *et al.*, 1989), and forms the sensor part of a two-component system with the 30 kDa cytoplasmic transcriptional

regulator, VirG (Winans *et al.*, 1986). Phenolics are believed to interact with the second transmembrane (TM2) domain of VirA (Melchers *et al.*, 1989). The C-terminal cytoplasmic domain of VirA can be auto-phosphorylated, at histidine-474 (Huang *et al.*, 1990*b*; Jin *et al.*, 1990*b*), and can transfer this phosphate to an aspartate residue (D52) in VirG (Jin *et al.*, 1990*a, b*). Through its C-terminal domain (Powell & Kado, 1990), VirG binds specifically to the *vir*-box (Jin *et al.*, 1990*c*; Pazour & Das, 1990*b*; Powell *et al.*, 1989), a conserved regulatory element found upstream of most of the *vir*-genes. Non-phosphorylatable mutant VirA and VirG proteins fail to induce *vir*-gene expression, suggesting that this system behaves in a similar manner to other two-component regulators such as NtrB/C (Jin *et al.*, 1990*a, b, c*). Indeed, there is considerable scope for interactions between different two-component systems, such as between VirG and PhoB (Aoyama *et al.*, 1991). However, nothing is known of how VirA/G can mediate two responses to wound phenolics (Shaw *et al.*, 1988): chemotaxis at low concentrations (10^{-7} M); and *vir*-induction at higher ligand concentrations (10^{-5} M).

VirA is involved in additional interactions. Sugars, e.g. galactose and glucose, can also enhance *vir*-induction (Cangelosi *et al.*, 1990; Huang *et al.*, 1990*a*). This requires the products of *chvE*, a chromosomal gene encoding a homologue of the *Escherichia coli* periplasmic ribose/galactose binding protein, and *virA* and *virG*

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Table 1. *Bacterial strains and plasmids used*

Strain	Characteristics*	Reference
<i>A. tumefaciens</i> C58C1	Ery ^r , Cm ^r Ti-plasmid-cured	Van Larebeke <i>et al.</i> (1974)
Ery Cm		
<i>E. coli</i> C2110	<i>polA</i> , <i>rha</i> , <i>his</i> , Nal ^r	Prince & Borlam (1985)
Plasmid	Characteristics	Reference
pVK219	Km ^r , <i>virAB</i> , IncP	Knauf & Nester (1982)
pVK225	Km ^r , <i>virGCDE</i> , IncP	
pVK257	Km ^r , <i>virABGC</i> , IncP	
pSG687	<i>virGD52N</i> , Km ^r , Sp ^r , IncW	Jin <i>et al.</i> (1990a)
pSG689	<i>virG</i> , Km ^r , Sp ^r , IncW	
pTB108	<i>virA</i> , Cb ^r , Tc ^r , IncW	Jin <i>et al.</i> (1990b)
pRS0824	<i>virAH474Q</i> , Cb ^r , Tc ^r , IncW	
pIB100	<i>virAG</i> , Cb ^r , IncW	Ankenbauer <i>et al.</i> (1991)
pIB415	<i>virAD63/240</i> , <i>virB::lacZ</i> , Cb ^r , Tc ^r , IncP	

* Nal, nalidixic acid; Cb, carbenicillin; Ery, erythromycin; Km, kanamycin; Sp, spectinomycin; ^r, resistant.

(Cangelosi *et al.*, 1990; Huang *et al.*, 1990a). Deletion of the periplasmic domain of VirA abolishes this effect, suggesting that ChvE interacts with this region (Cangelosi *et al.*, 1990). This domain contains a sequence conserved with the methyl-accepting chemotaxis protein Trg (Cangelosi *et al.*, 1990) which in *E. coli* interacts with the ribose- and galactose-binding proteins (Hazelbauer *et al.*, 1990). Interestingly, *chvE* mutants are defective in sugar chemotaxis (Cangelosi *et al.*, 1990). The periplasmic domain of VirA also appears to be involved in pH sensing (Melchers *et al.*, 1989).

VirA is clearly a multifunctional protein, but exactly how it interacts with VirG and the cytoplasmic signalling pathway to mediate chemotaxis towards phenolics is unclear. In this paper, we report data from experiments designed to investigate the role of VirA/G phosphorylation in chemotaxis towards acetosyringone.

Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1.

Media and chemicals. *E. coli* and *A. tumefaciens* were grown in LAB M nutrient broth number 2 (Amersham). *A. tumefaciens* was also cultured in Min A + glucose (Miller, 1972). Chemotaxis medium consisted of 0.1 mM-EDTA, pH 7.0 and 10 mM-KH₂PO₄, pH 7.0 (Adler, 1973). Swarm plates were prepared from nutrient broth supplemented with 0.17% Bacteriological agar Number 1 (Oxoid). Antibiotics (Sigma) were used as previously described (Leemans *et al.*, 1981). All other reagents were from BDH, unless otherwise stated.

Preparation of plasmids. Plasmids pSG687, pSG689, pIB100, pIB415, pTB108 and pRS0824 were purified from 100 ml overnight cultures of *E. coli* C2110 using Qiagen plasmid midi columns (Diagen).

Preparation of strains. Plasmids carrying copies of *virA* and *virG* were electroporated into *A. tumefaciens* backgrounds carrying cosmid clones. Cultures (5 ml) of the recipient strain were grown overnight with

antibiotic selection. Two successive 1.5 ml cell samples were pelleted in the same Eppendorf tube, washed three times in 1 ml ice-cold 1 mM-HEPES buffer, pH 7.0, and once in 1 ml chilled sterile 10% (v/v) glycerol. The cells were resuspended in 40 µl 10% (v/v) glycerol (4 °C) and 3 µl plasmid DNA, prepared by the alkaline lysis 'miniprep' method (Sambrook *et al.*, 1989) was mixed in. The mixture was transferred to an ice-cold 0.2 cm electroporation cuvette (Bio-Rad) and subjected to a 2.5 kV pulse dissipated at 200 Ω, 25 µF using a Gene Pulser (Bio-Rad). Successful transformations were associated with a time constant in the order of 4.7 ms. Immediately after transformation, 1 ml nutrient broth was added to the cuvette and the cells incubated with shaking at 27 °C for 2 h. Following pelleting and resuspension in 100 µl nutrient broth the cells were plated out and grown with appropriate antibiotic selection. Plasmid presence was verified by restriction pattern analysis of DNA prepared by the alkaline lysis 'miniprep' method. Motile recombinant populations were selected by three sequential rounds of swarming on selective plates (Loake *et al.*, 1988). Culture purity was confirmed microscopically using a Nikon Optiphot microscope equipped with phase-contrast optics and by a plate test for the production of 3-keto-lactose (Bernaerts & De Ley, 1963).

Preparation of attractants. Stock solutions of acetosyringone (Janssen Chimica) were prepared in decimal concentration decrements from 10⁻² to 10⁻⁵ M using ethyl acetate:methanol (1:1) as a solvent. Solutions (10⁻⁵ to 10⁻⁸ M) were prepared for assays by dilution of these stocks with 1000 vols of chemotaxis medium. This ensured a constant and low concentration of solvent in all assays. Control solutions consisted of chemotaxis medium with an equal concentration of solvent to that in assay solutions, but no acetosyringone.

Chemotaxis assays. Cultures (50 ml) of the strains to be assayed were grown for 16 h to exponential phase in Min A + glucose. Samples (40 ml) were pelleted at 7000 g for 10 min in sterile tubes. The pellets were resuspended and washed in 40 ml chemotaxis medium. This was repeated, concentrating the pellets in 30, 20, 15 and finally 10 ml of chemotaxis medium. Blindwell assays (Shaw *et al.*, 1988) were performed using 200 µl of cell suspension in Boyden chambers (Armitage *et al.*, 1977). Nuclepore 13 mm diameter polycarbonate filters with 8 µm pores (Sterilin) were used in assay chambers. After 2 h inversion the number of cells passing through the membranes was counted using a Coulter Electronics Multisizer II, fitted with a 30 µm aperture (Shaw *et al.*, 1988). The density of the culture initially

introduced into the chambers was also ascertained. Control values were obtained from parallel assays using chemotaxis medium as attractant. All assays were repeated in triplicate for each strain and attractant concentration. Three separate Multisizer readings were taken for each assay.

Presentation of results. Results were presented in the form of the Chemotactic Index (CI) which is a measure of the proportion of cells in the bacterial population attracted towards the attractant. The CI corrects for differing initial cell density, and arises from the observation that for a given strain, the proportion of cells attracted to a particular attractant concentration is a constant, over the range of cell densities used in the experiments described in this paper (data not shown). To calculate the CI, Multisizer readings were converted to numbers of cells per ml. Control values were subtracted from each point to give a figure for number of cells attracted solely by the acetosyringone. To correct for differences in original culture density, the data were then divided by the cell density of the suspension introduced into the chambers. Averaged figures were then converted to percentages of cells attracted by the compound (the CI) and graphically presented. The CI is thus a reproducible value, and permits comparison from one experiment to the next. In an average experiment, a CI of 2 represents approximately 10^7 bacteria per ml in the attractant chamber.

$$CI = \frac{[\text{Cells in upper chamber}] - [\text{Cells in control assay upper chamber}]}{[\text{Cells initially introduced to lower chamber}]} \times 100\%$$

Results

Recently, a number of site-specific mutations in *virA* and *virG* (see Table 1) have been constructed and made available by the group of Prof. E. W. Nester (Seattle) (Cangelosi *et al.*, 1990; Jin *et al.*, 1990a, b). pSG687 and pRS0824 encode mutant *VirG* and *VirA* proteins, respectively, in which the phosphorylated residue has been altered. Neither protein can mediate *vir*-induction (Jin *et al.*, 1990a, b). pIB415 expresses a mutant *VirA* protein, in which residues 63–240 (essentially the whole periplasmic domain) have been deleted. This mutant is still capable of mediating *vir*-induction (Cangelosi *et al.*, 1990). pTB108 (Jin *et al.*, 1990b) and pSG689 (Jin *et al.*, 1990a) express wild-type *VirA* and *VirG*, respectively.

To investigate the pathway effecting acetosyringone chemotaxis, the ability of these mutant proteins to confer this phenotype upon *A. tumefaciens* was investigated. Due to plasmid incompatibility, it was necessary to analyse the wild-type and mutated *virA* and *virG* (on IncW plasmids) in *A. tumefaciens* backgrounds carrying *vir*-gene combinations on the IncP cosmids pVK219 (*virAB*) pVK225 (*virGCDE*) and pVK257 (*virABGC*) (Knauf & Nester, 1982). Plasmids were introduced into these backgrounds by electroporation (see Methods). Presence of both plasmids in each strain was confirmed by restriction pattern analysis of DNA purified by mini-prep (see Methods). Motile populations of bacteria were then selected by swarm plate, and assayed for chemotaxis by Blindwell assay against several acetosyringone concentrations (see Methods).

Fig. 1. Response curves showing chemotaxis towards varying concentrations of acetosyringone by *A. tumefaciens* derivatives harbouring either ■, pVK257 (*virABGC*); ○, pVK219 (*virAB*); ●, pVK219 and pSG687 (*virGD52N*); or □, pVK219 and pSG689 (*virG*). Blindwell assays and data processing as in Methods. Error bars indicate one standard deviation from the mean.

The requirement for *VirG* phosphorylation in mediating chemotaxis

Fig. 1 depicts the results of acetosyringone chemotaxis experiments, using *A. tumefaciens* harbouring either pVK219 (*virAB*) or pVK257 (*virABGC*) alone, and strains harbouring both pVK219 and pSG687 (*virGD52N*) or pVK219 and pSG689 (*virG*). In agreement with our previous results (Shaw *et al.*, 1988), pVK257 can confer chemotaxis to acetosyringone, with a peak of attraction at 10^{-7} M. Similar results were obtained from strains harbouring pIB100, which carries wild-type *virA* and *virG* only (data not shown). The fact that pVK219-harboring cells lack this response, shows that *virA* alone is insufficient to mediate chemotaxis to acetosyringone. However, the introduction into pVK219-harboring cells of pSG687 demonstrates that the non-phosphorylatable mutant of *VirG*, is not able to complement this deficit, when compared to the wild-type *VirG* encoded by pSG689. This indicates that phosphorylation of *VirG* is necessary for acetosyringone chemotaxis.

The requirement for *VirA* phosphorylation in mediating chemotaxis

Fig. 2 illustrates chemotaxis assays in which the test strains all harboured pVK225 (*virGCDE*) either alone, or in combination with pRS0824 (*virAH474Q*), or pTB108 (*virA*). pVK257 data from Fig. 1 is shown by way of comparison. In accordance with our previous results (Shaw *et al.*, 1988), pVK225 is incapable of conferring chemotaxis towards acetosyringone, demonstrating that *virG* alone is insufficient for this response. Addition of the non-phosphorylatable *VirA* (encoded by pRS0824)

Fig. 2. Response curves showing chemotaxis towards varying concentrations of acetosyringone by *A. tumefaciens* derivatives harbouring either ■, pVK257 (*virABGC*); ○, pVK225 (*virGCDE*); ●, pVK225 and pRS0824 (*virAH474Q*); or □, pVK225 and pTB108 (*virA*). Blindwell assays and data processing as in Methods. Error bars indicate one standard deviation from the mean.

provides very marginal chemotactic competency compared to the wild-type situation conferred by pVK257 or pVK225 and pTB108 in combination. The non-phosphorylatable derivative of *virA* is thus incapable of complementing for the wild-type protein, suggesting that phosphorylation of VirA is required for acetosyringone chemotaxis.

The role of the periplasmic region of VirA

In Fig. 3, the effect of the periplasmic deletion of *virA* upon chemotaxis is shown in comparison with the data for pVK257 from Fig. 1. pIB415 (*virA*Δ63/240) which encodes a truncated version of VirA, missing the periplasmic portion of the protein, is able only partially to restore chemotactic ability in concert with wild-type *virG* carried by pSG689. The peak is substantially smaller and shifted to a higher concentration, relative to the wild-type situation. This suggests that while the periplasmic domain of VirA is non-essential for *vir*-induction by acetosyringone, it is important in acetosyringone chemotaxis.

The interaction between wild-type and mutant two-component regulatory system molecules

From Fig. 4 it is apparent that the addition of mutant copies of VirA and VirG (encoded by pRS0824 and pSG687, respectively) to a background harbouring their wild-type counterparts (encoded by pVK257) decreases chemotactic competency. Using half-logarithmic increments, it appears that mutant copies of VirA and VirG in a wild-type background cause a shift in the

Fig. 3. Response curves showing chemotaxis towards varying concentrations of acetosyringone by *A. tumefaciens* derivatives harbouring either ●, pVK257 (*virABGC*); or ○, pSG689 (*virG*) and pIB415 (*virA*Δ63/240). Blindwell assays and data processing as in Methods. Error bars indicate one standard deviation from the mean.

Fig. 4. Response curves showing chemotaxis towards varying concentrations of acetosyringone by *A. tumefaciens* derivatives harbouring either ○, pVK257 (*virABGC*); ●, pVK257 and pSG687 (*virGD52N*); or □, pVK257 and pRS0824 (*virAH474Q*). Blindwell assays and data processing as in Methods. Error bars indicate one standard deviation from the mean.

chemotaxis optimum towards higher attractant concentrations (data not shown). As the *vir*-genes on these plasmids are expressed from their normal promoters, it is to be expected that wild-type and mutant proteins would be transcribed at equivalent levels. This suggests that the mutant copies of VirA and VirG interfere with normal signalling in a wild-type background.

Discussion

The non-phosphorylatable versions of VirA and VirG proteins are not competent to mediate chemotaxis towards acetosyringone. This deficiency when compared to their wild-type counterparts implies a requirement for phosphorylation if chemotaxis is to occur efficiently. The

implicit suggestion that phospho-VirA and phospho-VirG are required for chemotaxis is consistent with the previously reported phosphorylation of VirG by phospho-VirA during the induction of *vir*-gene expression (Jin *et al.*, 1990*a, b*). It would appear that VirA, the sensor, is capable of sensing a broad band of acetosyringone concentrations, and effecting two responses, depending upon the sensed concentration. Phospho-VirG is the active, stable, transcriptional activator, capable of sequence-specific, high-affinity DNA binding (Jin *et al.*, 1990*a, c*; Pazour & Das, 1990*a, b*; Powell *et al.*, 1989; Powell & Kado, 1990). How it can fulfil this role at high concentrations of acetosyringone and mediate chemotaxis at lower concentrations is not yet clear.

A possible explanation for the ability of VirA and VirG to mediate two different effects, in response to differing concentrations of the ligand would be that phospho-VirG has a high affinity for a component of the cytoplasmic chemotaxis signalling pathway (possibly a CheA homologue) and a somewhat lower affinity for *vir*-box DNA. Thus at low concentrations of the ligand ($\ll 1$ molecule per receptor) the small amounts of phospho-VirG produced would preferentially interact with the chemotaxis pathway. At higher concentrations (> 10 molecules per receptor) sufficient phospho-VirG is produced to participate in significant *vir*-box binding, and hence *vir*-induction.

The periplasmic region of VirA has been shown to be essential for *vir*-induction by sugars through the ChvE pathway (Cangelosi *et al.*, 1990). It has also been shown not to be involved in chemotaxis towards sugars, where a mechanism involving ChvE and a putative Trg analogue are thought to effect the response. The role of this region of the molecule in the cell's response to acetosyringone is unclear. The putative acetosyringone binding site of VirA is thought to be associated with the transmembrane regions of the molecule (Cangelosi *et al.*, 1990; Melchers *et al.*, 1989). Deletion of the periplasmic region is reported to have a variety of effects on *vir*-induction, with one report of no change (Melchers *et al.*, 1989) and another describing increasing sensitivity of response (Cangelosi *et al.*, 1990). The reported increased sensitivity to acetosyringone is thought to result from the molecule adopting a non-wild-type conformation in the membrane. The periplasmic deletion appears to severely curtail chemotaxis towards acetosyringone, which may be simply an effect of the altered conformation. However, the data does indicate that the periplasmic domain, while inessential for *vir*-induction, is required for chemotaxis. This suggests that VirA may possess more than one site with differing affinities for acetosyringone. It is also possible that acetosyringone does not interact directly with VirA, but rather with one or more

binding proteins in the periplasm, with different ligand affinities. Differential concentration effects would then be achievable by these proteins interacting with different domains of VirA.

The addition of mutant copies of VirA and VirG to the wild-type chemotaxis system encoded by pVK257 decrease its effectiveness. The ability to respond to the stimulus is reduced, with a shift in the chemotaxis peak towards higher ligand concentrations. In the case of *virA* mutants, it is hypothesized that of the relatively small number of acetosyringone molecules interacting with cell surfaces, a proportion interact with mutant molecules to no effect and a proportion elicit a chemotaxis-inducing signal through the wild type. This decreases the number of cells attracted. The explanation of the results following the addition of mutant *virG* is thought to be that the cell's ability to respond to acetosyringone is depleted because its proportion of competent signal intermediates is lessened. Mutant non-phosphorylatable VirG molecules compete with wild-type intermediates for association with VirA, meaning that although acetosyringone presence is detected by VirA, the chemotactic signal is transmitted at lower levels.

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