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Chromosome and plasmid-encoded *N*-acyl homoserine lactones produced by *Agrobacterium vitis* wildtype and mutants that differ in their interactions with grape and tobacco

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Abstract

Agrobacterium vitis causes crown gall disease on grapevines. It also induces a specific necrosis on grape roots and a hypersensitive response (HR) on tobacco that are regulated by a complex quorum-sensing regulatory system. Strain F2/5 produces at least six *N*-acyl-homoserine lactones (AHLs) that function as signal molecules in quorum-sensing. The AHLs differ in acyl side chain length (8–16 carbons) as determined by gas chromatography/mass spectrometry and electrospray ionization tandem mass spectrometry. Mutant derivatives of F2/5 differ in ability to cause necrosis and the HR and show variable AHL profiles as determined by a thin-layer chromatography/biosensor assay. All wildtype *A. vitis* strains revealed the presence of long-chain AHLs regardless of tumorigenicity or ability to cause the HR. Whereas genes encoding long-chain AHLs are predicted to reside on the F2/5 chromosome, the determinants for short-chain AHLs were shown to be located on conjugal plasmids.

Keywords: Autoinducer; *Agrobacterium tumefaciens*; Rhizobiaceae; Quorum-sensing; Hypersensitive response

1. Introduction

N-acyl-homoserine lactone (AHL) mediated quorum-sensing (QS) is a means of cell–cell communication that is known to regulate different types of physiological functions such as light production, virulence, and biofilm formation in diverse species of bacteria [1]. Hallmarks of several well-studied QS systems in Gram-negative bacteria are the production of proteins encoded by the *luxI* and *luxR* gene families that function as synthases of AHLs and associated transcriptional regulators, respectively. LuxI type proteins generally synthesize AHLs from *S*-adenosyl-L-methionine and an appropriate acylated acyl carrier protein [2]. Structures of AHLs differ with regard to acyl chain length (those discovered thus far have four to eighteen carbons), and the presence of double bonds and/or hydroxyl or oxo substitutions at the third carbon. AHLs having greater than eight carbons in the acyl side chain are called long-chain whereas others are referred to as short-chain. AHLs may diffuse freely across bacterial membranes, or for long chain AHLs, they may exit bacteria via an efflux pumping system [3]. The structures of the LuxI-type protein, EsaI from the plant pathogen, *Pantoea stewartii* and LasI from the human pathogen *Pseudomonas aeruginosa* have been solved [3,4]. EsaI

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primarily synthesizes 3-oxohexanoyl-HL and was discovered to be monomeric and approximately spherical with a deep cleft that is believed to function in substrate binding. With regard to the LuxR family, TraR from *Agrobacterium tumefaciens* has also been solved, and highly conserved amino acid residues in domains that play essential roles in AHL and DNA binding have been identified [5].

A general paradigm for QS regulation is that at high bacterial cell densities AHLs reach a critical concentration above which they bind an amino-terminal domain of the LuxR transcriptional regulator. AHL binding often leads to a dimerization of the transcriptional regulator protein, leading to a conformation that allows the interaction of the complex with characteristic nucleotide sequences (lux boxes) found upstream of the regulated gene(s). Several variations of this scenario are known to occur in LuxR/I QS systems, and several bacteria possess more than a single pair of LuxI/R proteins and produce multiple AHLs [6]. Phylogenetic analyses suggests that LuxR/I proteins comprise two families with different functions and structure [7]. Family A including TraR from *A. tumefaciens*, requires binding of its cognate AHL, 3-O-C8-HL, for proper protein folding and dimerization into its active form and for protection against proteolysis [8,9]. A similar scenario was also identified for LuxR in *V. fischeri* and LasR in *P. aeruginosa* [10]. In contrast, binding of AHL for functionality is not necessary in Family B. For example, CarR and ExpR in *Erwinia carotovora* were shown to bind the promoter in the absence of autoinducer and EsaR functions as repressor in the absence of autoinducer [11,12]. In addition, a different CarR protein functions independently of AHL to control carbapenem antibiotic synthesis in *Serratia marcescens* [13].

Notable examples of QS systems in Rhizobiaceae include the TraR/I system in *A. tumefaciens* C58 that regulates conjugal transfer of the Ti plasmid and the SinR/I system in *S. meliloti* Rm1021 that regulates production of EPSII and nodulation. For C58, there is a single synthase gene, *traI*, that resides on the Ti plasmid and is responsible for production of the AHL, 3-O-C8-HL [14]. The AHL synthase, SinI of *S. meliloti* is responsible for production of multiple long-chain AHLs including C12-HL, 3-O-C14-HL, 3-O-C16:1-HL, C16:1-HL, and C18-HL [15]. Short-chain AHLs are also produced by Rm1021 and have been shown to be encoded by a second functional QS system [15,16].

Agrobacterium vitis causes crown gall, a serious disease of grape that can lead to poor growth and death of vines [17]. In addition to causing tumors, *A. vitis* induces a tissue-specific necrosis on its grape host and a hypersensitive-like response (HR) on non-host plants, such as tobacco [18]. Necrosis is induced by tumorigenic and non-tumorigenic *A. vitis* strains and is induced on diverse grape genotypes [19]. The significance of necrosis on vine root mass and vine growth have not been determined however it is hypothesized as a possible means whereby the bacterium invades the vine from the soil. We have previously generated mutants of *A. vitis* strain F2/5 that are altered in their ability to cause grape necrosis and the HR. One mutant, M1154, that is completely necrosis and HR negative, was found to have the Tn5 insertion in a *luxR* homolog named *aviR* [20]. M1154 produces fewer AHLs than wildtype F2/5. Recently a second *luxR* type gene, *avhR* was reported in strain F2/5. It is also involved in expression of grape necrosis and HR-negative phenotypes [21]. The *avhR* mutant produces an AHL profile that has the same number of signals as wildtype F2/5, however signal strength is noticeably stronger than the wildtype strain.

A major aim of this paper was to identity the AHLs that are produced by strain F2/5. In addition, AHL profiles from F2/5 were compared to those of mutant derivatives that express altered necrosis and HR phenotypes. Profiles are also compared for other *A. vitis* strains that differ with regard to the type of Ti plasmid they carry and their ability to induce an HR on tobacco. Previously, two large conjugal plasmids carried by F2/5 were partially characterized [22]. In this paper we demonstrate that both of the plasmids carry genes for synthesis of short-chain AHLs and do not carry determinants that encode HR or necrosis induction.

2. Materials and methods

2.1. Bacterial strains and media

Bacterial strains are listed in Table 1. All strains of *A. vitis* were cultured on Potato Dextrose Agar (PDA) (Difco Laboratories, Detroit, MI) or PDA amended with kanamycin (50 μg/mL). Strains Tm4, AB3 and S4 carry well-studied octopine or vitopine types of Ti plasmids [26,27,29]. Strains F2/5 [23] and CG506 [28] are non-tumorigenic, induce an HR and grape necrosis. F2/5 also prevents crown gall from forming at wounds on grape i.e. functions as a biological control of the disease [30]. The F2/5 mutants [18] M1154 and M1320 carry a single Tn5 insertion in *aviR* and *avhR*, respectively. Mutant M6 carries a single insertion in a putative gene associated with fatty acid biosynthesis and M852 carries a single insertion in an unknown ORF. *A. tumefaciens* strain UBAPF2 (C58 derivative cured of its Ti plasmid) [24] and UBAPF2 transformed with F2/5 plasmids that are associated with octopine and tartrate utilization (pOc and pTr respectively) [22] were grown on PDA or on Luria-Bertani (LB) medium. The *A. tumefaciens* biosensor strain NTL4 (pZLR4) was grown on LB containing 40 μg gentamicin and 100 μg carbenicillin/mL [25].

2.2. HR and necrosis phenotypes

Assays for grape necrosis and tobacco HR were done as previously reported [18]. Cultures were grown on PDA for 20–24 h and then cells were suspended in sterile distilled water (SDW) to $OD_{600} = 1.5$ (about 1.0×10^9 colony-forming units (CFU)/mL). Suspensions were dilution