Short communication

A putative RNA-binding protein has a role in virulence in *Ralstonia solanacearum* GMI1000

ASHLEY FRANKS†, G. LOUISE MARK-BYRNE, J. MAXWELL DOW AND FERGAL O'GARA*

BIOMERIT Research Centre, Department of Microbiology, National University of Ireland, University College Cork, Cork, Ireland

SUMMARY

Molecular signals contained in root exudates are thought to induce specific transcriptional changes in bacteria within the rhizosphere, promoting the expression of genes associated with rhizosphere function. We have previously used *Pseudomonas aeruginosa* PAO1 as a model organism to identify novel genes with roles in rhizosphere competitiveness through analysis of those which showed altered transcription in response to root exudates. One such gene, *PA4753*, encodes a putative RNA-binding protein. Homologues of *PA4753* occur in other rhizosphere-associated bacteria, including the phytopathogen *Ralstonia solanacearum*. In this study, we have tested the role of this homologue, *Rsc1524*, in the virulence of *R. solanacearum* GMI1000. Disruption of *Rsc1524* resulted in a decrease in virulence in tomato and *Arabidopsis*. This was associated with an increase in the level of specific extra-cellular plant wall-degrading enzymes. Expression of *Rsc1524* was influenced by different plant root exudates and root exudate components, which suggests that it may play a role in molecular signalling between bacteria and plants underpinning wilt pathogenesis.

Molecular signalling between partners underpins the development of both the beneficial and pathogenic interactions that occur between plants and bacteria in nature. A greater understanding of these signalling processes may provide new strategies for the promotion of beneficial interactions and for the management of disease, with consequent benefits for agriculture (Morrissey *et al.*, 2004). Molecular signals secreted in root exudates are thought to induce specific transcriptional changes in bacteria within the rhizosphere, allowing the expression of genes associated with rhizosphere function. The effects of root exudates on the bacterial transcriptome have been investigated

recently in a model system using *Pseudomonas aeruginosa* PAO1 and exudates from sugar beet (*Beta vulgaris*) (Mark *et al.*, 2005). Functional analysis of the roles of a number of *P. aeruginosa* genes with altered expression has allowed the identification of several novel genes with roles in rhizosphere competitiveness (Mark *et al.*, 2005). Homologues of these genes occur in the genomes of both beneficial and pathogenic root-associated bacteria. This finding raises the question of the role of these homologues in other plant–microbe interactions occurring in the rhizosphere. In this study, we have examined the role of *Rsc1524*, a homologue of *PA4753* of *P. aeruginosa*, found in the root-infecting phytopathogen *Ralstonia solanacearum* GMI1000 (Boucher *et al.*, 1985).

Ralstonia solanacearum is capable of causing disease in a wide range of plant species, has a global distribution and is capable of surviving for prolonged periods in water or soil (Haywood, 2000). The process of pathogenesis starts with entry into the host through the roots. Inside the plant, *R. solanacearum* exhibits strong tissue-specific tropism, and extensive multiplication of bacterial cells in the xylem leads to a systemic infection of the plant, associated with wilting (Vasse *et al.*, 1995). The genome of GMI1000 has been fully sequenced and comprises two replicons: a 3.7-Mb chromosome and a 2.1-Mb mega plasmid (Salanoubat *et al.*, 2002).

PA4753 of *P. aeruginosa* encodes a conserved hypothetical protein of 104 amino acids that contributes to competitive ability in the rhizosphere (Mark *et al.*, 2005) and to swimming and swarming on agar plates (P. Kiely and F. O'Gara, unpublished work). *PA4753* contains a predicted RNA-binding domain, Pfam UPF0044 (<http://www.sanger.ac.uk/Software/Pfam/>), which is found in eubacteria, archaea and plants. On the basis of bioinformatic analysis, Till *et al.* (2001) have suggested that proteins with UPF0044 domains have a role in translation. A function in RNA binding has been suggested by structural and modelling studies (Ostheimer *et al.*, 2002). The crystal structure of the *Escherichia coli* protein YhbY, which contains a UPF0044 domain, has a fold that is similar to that of the C-terminal domain of translation initiation factor 3, which binds to 16S rRNA in the 30S ribosomal subunit (Ostheimer *et al.*, 2002). Furthermore, modelling studies

*Correspondence: E-mail: f.ogara@ucc.ie

†Present address: Department of Microbiology, Morrill Science Center IV North, University of Massachusetts Amherst, 639 North Pleasant Street, Amherst, MA 01003-9298, USA.

have indicated the presence of a highly basic conserved RNA-binding surface in all members of the family of UPF0044 domain proteins (Ostheimer *et al.*, 2002). Residues of this basic β -sheet surface in YhbY that are conserved amongst Gram-negative eubacteria include K6, K8, Q9, K12, H16, H44, K48, K50, R56, K59, Q76, I78, G79 and K80. PA4753 has identical amino acids at 11 of these 14 positions, with the conservative variation K59R and non-conservative variations K6E and Q9K. Rsc1524 has identical amino acids at nine of the 14 conserved positions, with conservative amino acid substitutions K8R, K12R and K59R and non-conservative variations K6A and Q9S.

Examination of the predicted proteome of *R. solanacearum* GMI1000 using BLASTP revealed the presence of a homologue of PA4753, designated Rsc1524 (BLASTP value $3e^{-14}$). Rsc1524 has 165 amino acids and, when compared with PA4753, shows 35% identical and 61% similar amino acids over 108 positions, which are located in the N-terminal part of the protein. The C-terminal part of Rsc1524 comprises an uncharacterized domain. The genomic context of Rsc1524 within the *R. solanacearum* GMI1000 genome is similar to that of PA4753 within the *P. aeruginosa* PAO1 genome (<http://bioinfo.genopole-toulouse.prd.fr/> and <http://v2.pseudomonas.com>). In preliminary experiments, Rsc1524 was cloned from *R. solanacearum* and used to complement a PA4753 mutant of *P. aeruginosa*. When introduced into a PA4753 mutant of *P. aeruginosa* PAO1, Rsc1524 restored swimming and swarming to *P. aeruginosa* wild-type levels. For these experiments, the Rsc1524 gene with a 500-bp upstream region was amplified by polymerase chain reaction (PCR) using the primers AFRsc1524F2 (5'-ACCGACTCTTCGCGGAAATCG-3') and AFRsc1524R2 (5'-CATTCATGGTGTGCTGCGTG-3'), and cloned into pCR2.1-Topo (Invitrogen), creating pT1524. The HindIII/XbaI fragment was excised from pT1524 and cloned into the HindIII/XbaI site of pBBR1MCS-2 (Kovach *et al.*, 1995) to create pBBR1524. This construct was electroporated into the PA4753 mutant (Mark *et al.*, 2005), and motility phenotypes were assessed on agar plates, as described by Rashid and Kornberg (2000). This functional complementation, together with the sequence relatedness of the encoded proteins, suggests that Rsc1524 and PA4753 are orthologues.

To investigate the role of Rsc1524 in the virulence of GMI1000, the Rsc1524 gene was inactivated by disruption with a gentamycin (Gm) resistance cassette. In brief, the HindIII/XbaI fragment was excised from pT1524 and inserted into HindIII/XbaI of pBlue-script (Fermentas), creating pB1524. A Gm resistance cassette, amplified from pBBR1MCS-5 (Kovach *et al.*, 1995), was inserted into the unique PstI site within Rsc1524 cloned in pB1524. Subsequently, the HindIII/XbaI fragment carrying the disrupted gene was excised and cloned into the allelic replacement vector pEX18Tc (Hoang *et al.*, 1998) to create pEXRsc15Gm24. This plasmid was electroporated into *R. solanacearum* GMI1000 as described previously (Allen *et al.*, 1991). Mutants arising from

double crossover events (Gm resistant and tetracycline sensitive) were selected as described previously (Liu *et al.*, 2001). One such mutant strain, GM1524, was confirmed by PCR to have a single disrupted copy of the Rsc1524 gene. For complementation of GM1524, the wild-type Rsc1524 gene was excised as a HindIII/XbaI fragment from pB1524 and ligated into pBBR1MCS-2 (Kovach *et al.*, 1995). This construct was transferred by electroporation into GM1524.

Preliminary experiments revealed that there was no difference in growth rates between GMI1000, GM1524 and the complemented mutant when grown in complex (BG), minimal (MM) or low nutrient (YMM) (MM supplemented with 0.1% yeast extract and 0.5% glycerol) medium. The compositions of these media are given in Boucher *et al.* (1985). To assess the effect of mutation of Rsc1524 on virulence, assays were conducted using *Arabidopsis thaliana* Col-0 and tomato cv. Super Marmande. GMI1000, GM1524 and the complemented GM1524 strain were soil inoculated onto either the unwounded roots of tomato plants 15 days after seed germination (26 plants per condition per trial) at a concentration of 5×10^6 colony-forming units (cfu)/g soil (Tans-Kersten *et al.*, 2001), or the unwounded roots of *Arabidopsis* plants 3 weeks after seed germination (24 plants per condition per trial) at a concentration of 10^8 cfu/plant. Equivalent volumes of sterile water acted as a control. Plants were grown at 25 °C, 70% humidity, with a 16-h light/8-h dark lighting cycle. Wilting symptoms were scored daily postinoculation on a scale from 0 (for no wilting) to 4 (for 75%–100% of the plant wilted). The disruption of Rsc1524 decreased virulence in both tomato and *Arabidopsis*, as revealed by a decrease in the rate of appearance of symptoms and a reduced number of plants wilted by the end of the experiment, at 14 days after inoculation (Fig. 1).

To explore the possible mechanisms by which Rsc1524 affected virulence, the effects of mutation on the production of a number of factors associated with virulence, such as extracellular enzymes and extracellular polysaccharide (EPS), and the virulence-associated function of motility were assayed as described previously (Liu *et al.*, 2001, 2005). The disruption of Rsc1524 had no effect on motility examined using agar plate assays (Liu *et al.*, 2001; Tans-Kersten *et al.*, 2004). Rsc1524 mutation also had no effect on the production of EPS in liquid medium (Huang *et al.*, 1993), or on the production of endoglucanase (Egl) or pectin methyltransferase (Pme) as assayed on plates (Fig. 2). However mutation of Rsc1524 led to an increase in polygalacturonase (Peh) production that could be restored to wild-type levels through complementation with the cloned gene Rsc1524 (Fig. 2).

We were interested to determine whether plant root exudates and their components could influence the expression of Rsc1524, as was seen with the orthologue PA4753 in *P. aeruginosa*. The major components of root exudates are thought to be monosaccharides, amino acids and organic acids (Lugtenberg *et al.*, 2001; Lugtenberg and Dekkers, 1999; Simons *et al.*, 1996). To monitor

Fig. 1 Virulence of *Ralstonia solanacearum* strains inoculated onto 15-day-old tomato plants (cv. Super Marmande) (a) and 3-week-old *Arabidopsis thaliana* Col-0 plants (b). GMI1000 (■), GMI1524 (▲) and the complemented strain (×) were soil inoculated at a concentration of 10^6 colony-forming units (cfu) per gram of soil for tomato plants and 10^8 cfu/plant for *A. thaliana* plants. Water (♦) acted as a control. Twenty-six tomato and 24 *A. thaliana* plants were used for each treatment. Plants were rated daily postinoculation on a disease index scale from 0 (no wilting) to 4 (75%–100% wilted). The data given represent one experiment that was repeated two further times with essentially the same outcome.

Fig. 2 Plate assays for the production of extracellular proteins by colonies of GMI1000 (a), GMI1524 (b) and the complemented strain (c). Endopolygalacturonase (Peh) was detected as a clear zone on a cloudy background, endoglucanase (Egl) as a yellow zone on a red background after Congo red staining, and pectin methyl esterase (Pme) as a purple zone on a pink background after ruthenium red staining. The clear zones in the Pme assay are a result of the further breakdown of pectic products by Peh. An increase in Peh activity was observed in GMI1524 in comparison with GMI1000. The complemented mutant produced a zone of clearing similar to that of GMI1000.

the expression of *Rsc1524* in response to root exudates or root exudate components, the 5'-end of the *Rsc1524* gene was ligated to a promoterless *uidA* gene, encoding β -glucuronidase (GUS), and introduced into the chromosome. For this, a 600-bp fragment that included the 5'-end of the *Rsc1524* gene and upstream sequences was amplified using the primers AFP30 (5'-AACTC-GAGTGCTGGAACCGACTCTTC-3') and AFP31 (5'-AATCTA-GATGGTGTCTAAAGCTCGATG-3'). The DNA fragment was cloned into the *XhoI/XbaI* restriction site of pVO155 (Oke and Long, 1999), and this construct was introduced into GMI1000 by electroporation. The integration of this construct into the chromosome created a *cis* merodiploid of *Rsc1524*, leaving an intact functional copy of the gene; this was confirmed by PCR. GUS activity was assayed as described previously (Brown *et al.*, 2007; Jefferson *et al.*, 1987) with 100 mM *p*-nitrophenyl glucuronide (PNPG) as a substrate.

The effect of different carbon sources within root exudates on the expression of *Rsc1524* was assayed in bacteria grown in

MM substituted with 0.5% of the carbon source to be tested, and compared with the expression of bacteria grown in YMM. *Rsc1524* showed complex regulation in response to various carbon sources (Fig. 3). Expression was maximal in the presence of fructose and sucrose, whereas glucose gave considerably less expression.

The effect of root exudates on the expression of *Rsc1524* was assessed using exudates from a range of plants (tomato cv. Super Marmande, *Arabidopsis thaliana* Col-0, *Beta vulgaris* varieties Celt and Roberta and winter wheat) that included both hosts and non-hosts for *R. solanacearum* (Haywood, 2000). Exudates were prepared, as described previously, from plants grown in vermiculite containing 1 : 10 sterile Hoagland's plant nutrient solution (Mark *et al.*, 2005). Exudates were added (10% v/v) to 1 : 10 BG medium, which contains a mix of peptone, amino acids, yeast extract and glucose, and *Rsc1524* expression was determined as described previously. As a control treatment, 1 : 10 BG medium was supplemented with 1 : 10 sterile Hoagland's plant nutrient solution. The addition of exudates did not affect the expression of *Rsc1524* in the early growth phases. However, in the early stationary phase [optical density at 600 nm (OD_{600}) of approximately 0.5], expression was lower in the presence of exudates than in the control (Fig. 4). All exudates showed this effect. These findings, together with the observations on the effects of different carbon sources (Fig. 3), suggest that the expression of *Rsc1524* is under complex control, and may respond to multiple environmental cues. As *Rsc1524* regulates Peh production, it would be of interest to examine the effects of polygalacturonate, and perhaps other plant cell wall polymers, on the expression of *Rsc1524*.

The regulation of virulence factors and their contribution to virulence in GMI1000 are complex (Brown and Allen, 2004; Genin and Boucher, 2002; Liu *et al.*, 2005; Salanoubat *et al.*, 2002), and require coordinated expression during adaptation from the soil to

Fig. 3 Expression of *Rsc1524* in response to various carbon sources. Expression was determined using a strain with an *Rsc1524::uidA* chromosomal fusion grown in MM supplemented with glucose, trehalose, glutamate, fructose or sucrose (0.2% w/v) as the sole carbon source. The data represent the average of a minimum of three replicates. The standard deviation is shown as bars. There was no difference in growth in the different conditions.

Fig. 4 Expression of *Rsc1524* in response to root exudates from different plants. Expression was determined using a strain with an *Rsc1524::uidA* chromosomal fusion grown in 1 : 10 BG medium supplemented with root exudate from sugar beet (*Beta vulgaris*) variety Celt (BVC), *Beta vulgaris* variety Roberta (BVR), tomato cv. Super Marmande (Tomato), *A. thaliana* Col-0 (Arabidopsis), winter wheat (Wheat) and 1 : 10 Hoagland's medium control (Hoags). 1 : 10 BG medium (No supplement) was included as a comparative control. Cultures were sampled at an optical density at 600 nm (OD_{600}) of approximately 0.5. No differences were observed at earlier growth stages. The data represent the average of a minimum of three replicates. Standard deviations are shown as bars.

the plant environment. After long periods in the soil, the bacterium can gain nutrients from exudates in the rhizosphere before migration through intercellular spaces, penetration of the xylem, initial multiplication in the nutrient-poor xylem fluid (Bialczyk and Lechowski, 1995; Schell, 2000) and multiplication to large numbers that may involve mobilization of plant cell wall polymers as carbon and nitrogen sources. The ability to sense such environmental changes and to respond by appropriate changes in the expression of virulence genes is undoubtedly a key to the successful establishment of disease. For example, the *hrp* genes of *R. solanacearum* and other phytopathogenic bacteria are induced during growth in synthetic minimal medium, but the level of *hrp* gene induction depends on the nature of the supplemented carbon source and other environmental signals (Arlat *et al.*, 1992; Brito *et al.*, 1999; Rahme *et al.*, 1992; Wei *et al.*, 1992). *Rsc1524* may act in a regulatory pathway that links the

perception of environmental signals to the appropriate level of expression of virulence factors.

Is there a link between the overproduction of Peh and the loss of virulence of the *Rsc1524* mutant? Previous studies have shown that, although Peh, Egl and Pme all contribute to the ability to cause disease, none of them is essential; Peh appears to make the least contribution (Denny *et al.*, 1990; Huang and Allen, 2000). GMI1000 encodes three Pehs: PehA (an endopolygalacturonase), PehB and PehC (both exopolygalacturonases). The loss of PehC has no effect on the virulence of GMI1000, whereas the loss of PehA or PehB, or both PehA and PehB, results in a slight decrease in the virulence of GMI1000 (Liu *et al.*, 2005). This is in contrast with previous studies of other *R. solanacearum* strains, where the effects of these mutations are additive (Denny *et al.*, 1990; Huang and Allen, 1997, 2000; Schell *et al.*, 1994). Surprisingly, the disruption of genes encoding all three Peh

enzymes in both GMI1000 and K60 results in a strain that is slightly more virulent than the wild-type (Gonzalez and Allen, 2003, Liu *et al.*, 2005). This has prompted suggestions that the increased virulence of the triple deletion mutant may be a result of the absence of activation of plant defences by pectic breakdown products during disease. We speculate that, in a converse fashion, enhanced production of Peh in the *Rsc1524* mutant may increase plant resistance to the pathogen through the increased production of pectic fragments. Similar considerations could apply to the deregulated production of other virulence determinants, which may lead to the triggering of plant defences.

It is of note that *Rsc1524* and *PA4753* regulate different targets in *Ralstonia* and *Pseudomonas*, yet *Rsc1524* is able to complement the motility defect in a *PA4753* mutant, demonstrating an apparently orthologous function for the two proteins. The molecular basis for this pattern of regulation is unknown, but it would be of interest to see whether Pfam UPF0044 domains from a wider range of bacteria, including those that are not associated with plants, have the same effect in the *PA4753* mutant. In addition to the Pfam UPF0044 domain, *Rsc1524* has an uncharacterized C-terminal extension. The presence or absence of this extension seems to define two subfamilies of proteins with Pfam UPF0044; those with an extension are found in a range of bacteria, including *Burkholderia* spp. and other *Ralstonia* spp., whereas proteins with a 'stand alone' Pfam UPF0044 domain are more widely distributed and occur in many other organisms, including plant-associated bacteria such as *Pseudomonas fluorescens*, different pathovars of *Pseudomonas syringae*, *Xylella fastidiosa* and *Xanthomonas* spp.

In summary, this work shows that *Rsc1524* is a gene with a role in virulence in *R. solanacearum*, whose expression is regulated by the bacterial environment. The mechanism of action of *Rsc1524* in the regulation of virulence is obscure. *Rsc1524* has a putative RNA-binding domain that is widely distributed in bacteria, including those associated with plants. It remains to be seen whether these proteins represent a new family of regulators that control functions that influence the outcome of plant-microbe interactions.

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