Microbiology (2010), **156**, 2670–2681 **DOI** 10.1099/mic.0.039032-0

Agrobacterium tumefaciens ExoR represses succinoglycan biosynthesis and is required for biofilm formation and motility

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Received 12 February 2010
Revised 15 June 2010
Accepted 21 June 2010

The ubiquitous plant pathogen *Agrobacterium tumefaciens* attaches efficiently to plant tissues and abiotic surfaces and can form complex biofilms. A genetic screen for mutants unable to form biofilms on PVC identified disruptions in a homologue of the *exoR* gene. ExoR is a predicted periplasmic protein, originally identified in *Sinorhizobium meliloti*, but widely conserved among alphaproteobacteria. Disruptions in the *A. tumefaciens exoR* gene result in severely compromised attachment to abiotic surfaces under static and flow conditions, and to plant tissues. These mutants are hypermucoid due to elevated production of the exopolysaccharide succinoglycan, via derepression of the *exo* genes that direct succinoglycan synthesis. In addition, *exoR* mutants have lost flagellar motility, do not synthesize detectable flagellin and are diminished in flagellar gene expression. The attachment deficiency is, however, complex and not solely attributable to succinoglycan overproduction or motility disruption. *A. tumefaciens* ExoR can function independently of the ChvG–ChvI two component system, implicated in ExoR-dependent regulation in *S. meliloti*. Mutations that suppress the *exoR* motility defect suggest a branched regulatory pathway controlling succinoglycan synthesis, motility and biofilm formation.

INTRODUCTION

Agrobacterium tumefaciens is a well-studied plant pathogen best known for its ability to genetically transform host plants (Escobar & Dandekar, 2003). *A. tumefaciens* is a member of the family *Rhizobiaceae* and, although a pathogen, is closely related to nitrogen-fixing plant symbionts. The process of interkingdom gene transfer in *A. tumefaciens* is controlled in response to plant exudates, including certain phenolics and sugars, and requires a type IV secretion system (for reviews, see Christie *et al.*, 2005; Gelvin, 2003). Infection and gene transfer are dependent on attachment of the bacteria to the plant surface. Attachment and biofilm formation by *A. tumefaciens* C58 have been well established. Indeed, after initial attachment *A. tumefaciens* can assemble into complex biofilms on plant tissues and abiotic surfaces (Danhorn *et al.*, 2004; Merritt *et al.*, 2007; Ramey *et al.*, 2004).

Many bacteria efficiently adhere to surfaces, including a wide range of animal and plant pathogens. In these systems attachment is often mediated by cell-surface adhesins such as pili, flagella, curli and polysaccharides. Rhizobial attachment to plants is a critical early step in symbiosis, and probably involves several proteinacious adhesins, including the so-called Rap adhesins and rhicadhesin (Ausmees *et al.*, 2001; Russo *et al.*, 2006; Smit *et al.*, 1989), as well as cell-surface polysaccharides (Laus *et al.*, 2006). No analogous cell-surface adhesins have been definitively reported for *A. tumefaciens*, although it appears to produce a rhicadhesin-type molecule (Dardanelli *et al.*, 2003). Flagellar motility is required for efficient attachment of *A. tumefaciens* to a variety of surfaces, and non-motile mutants show deficiencies in pathogenesis (Chesnokova *et al.*, 1997; Merritt *et al.*, 2007). Several genes reported to be required for attachment and virulence, encoded within the Att cluster (Matthysse *et al.*, 2000), have been subsequently shown to be dispensable for these processes (Nair *et al.*, 2003). In general, little is known about the factors required for attachment or their coordination during the transition of *A. tumefaciens* to the sessile phase.

Biofilms can range from flat featureless layers to topographically complex assemblages, with cells adhering to

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Abbreviations: CV, crystal violet; qPCR, quantitative PCR; SDCM, spinning disk confocal microscopy.

A supplementary figure, showing alignment of ExoR sequences, and a supplementary table, listing oligonucleotide sequences used for molecular cloning and directed mutagenesis, are available with the online version of this paper.

surfaces and cohering to other cells (Hall-Stoodley *et al.*, 2004). The cells within biofilms are often enmeshed within a matrix largely composed of exopolysaccharide, but which may also contain proteins and nucleic acids (Branda *et al.*, 2005). Clonal growth of adherent cells, surface-based motility and matrix production drive the formation of mature biofilms. This maturation can be controlled at multiple points through a variety of regulatory pathways that respond to changing environmental conditions. In several different bacteria, regulators of exopolysaccharide biosynthesis have profound effects on the eventual structure and composition of the mature biofilm. For example, VpsR of *Vibrio cholerae* represses the *Vibrio* polysaccharide (*vps*) genes; mutants for this regulator overproduce Vps and form dense (rugose) biofilms, in comparison to the more diffuse wild-type biofilms (Lim *et al.*, 2007). In the plant vascular pathogen *Pantoea stewartii*, synthesis of the exopolysaccharide stewartan is controlled by the EsaR–EsaI quorum sensing system, which also regulates biofilm formation (Koutsoudis *et al.*, 2006). In *Sinorhizobium meliloti*, regulation of the exopolysaccharide succinoglycan influences the microbe's ability to form symbiotic nodules with host legumes. Several studies have described a potential role for succinoglycan in biofilm formation by *S. meliloti*, though a recent report has called this conclusion into question (Fujishige *et al.*, 2006; Rinaudi & Gonzalez, 2009; Wells *et al.*, 2007; Yao *et al.*, 2004). *A. tumefaciens*, like *S. meliloti*, also produces succinoglycan (Cangelosi *et al.*, 1987).

In *A. tumefaciens*, several regulators have been shown to influence attachment and biofilm formation (Fuqua, 2008). The FNR-type transcription factor SinR affects late stages of biofilm maturation, possibly in response to oxygen limitation (Ramey *et al.*, 2004). BigR, a repressor originally identified in *Xylella fastidiosa*, is conserved in *A. tumefaciens*; *bigR* mutants form abnormally dense biofilms on abiotic surfaces (Barbosa & Benedetti, 2007). Phosphorus limitation also results in enhanced biofilm formation through the PhoR–PhoB two-component regulatory system (Danhorn *et al.*, 2004).

In this study, we isolated an *A. tumefaciens* transposon mutant on the basis of its profound attachment deficiency. The transposon insertion disrupts the regulator *exoR*, previously shown to control succinoglycan production in *S. meliloti* (Reed *et al.*, 1991; Yao *et al.*, 2004). Null mutations in the *A. tumefaciens exoR* gene result in elevated succinoglycan levels, reduced flagellar motility, prevention of surface attachment and complete loss of biofilm formation.

METHODS

Strains, plasmids, reagents and growth conditions. All strains and plasmids used in this study are described in Table 1. Buffers, antibiotics and media were obtained from Fisher Scientific and Sigma. DNA manipulations were performed in accordance with standard protocols (Sambrook *et al.*, 1989). DNA sequencing was performed with ABI Big Dye Terminator version 3.1 on an ABI 3730 sequencer operated by the Indiana Molecular Biology Institute. Oligonucleotides (see Supplementary Table S1, available with the online version of this paper) were obtained from Integrated DNA Technologies. Plasmids were electroporated into *A. tumefaciens* by a standard method (Mersereau *et al.*, 1990). *A. tumefaciens* derivatives were grown in AT minimal salts medium and 15 mM $(NH_4)SO_4$ and either 1 % (w/v) glucose (ATGN) or 0.4 % (w/v) succinic acid (ATSucN) as the carbon source (Tempé *et al.*, 1977). Antibiotics were used at the following concentrations (μg ml^{-1}): for *A. tumefaciens*, ampicillin (Ap) 50, gentamicin (Gm) 500; kanamycin (Km) 150; spectinomycin (Sp) 50; streptomycin (Sm) 2000; and for *Escherichia coli*, Ap 100, Gm 25, Km 25, Sp 50. Antibiotic selection was maintained throughout all experiments when using mutants generated by plasmid insertion. Calcofluor was added to media at 200 μg ml^{-1}. Swim agar contained 0.2, 0.25 or 0.3 % BactoAgar.

Identification of the *exoR* gene. The *A. tumefaciens sin2* and *sin3* mutants were isolated using a transposon mutagenesis screen described previously (Ramey *et al.*, 2004). A transposon mutant library was generated using the pTnMod-OKm′ plasposon system (Dennis & Zylstra, 1998). Two thousand mutants were evaluated using a microtitre plate adhesion assay (O'Toole *et al.*, 1999). Genomic DNA from these mutant isolates was purified and digested with *Bam*HI or *Sac*II, and fragments were ligated under dilute conditions to favour self-ligation. The resulting plasmids were transformed en masse into *E. coli* DH10B/λ*pir*. Plasmids isolated from Km^R colonies carried the plasposon insertion and flanking genomic DNA. The resulting plasmids were designated pSin2 and pSin3, and were sequenced using primers complementary to the transposon ends.

Molecular cloning and directed mutagenesis. In-frame deletions of the *exoR* and *exoA* genes were introduced via allelic replacement as described previously (Merritt *et al.*, 2007). Sequences flanking each side of the target gene were amplified by PCR, fused by standard ligation and cloned into pGEM-T (Promega) at which time the inserts were confirmed by sequencing. The resulting deletion fragments were excised by restriction digestion and ligated into suicide vector pKNG101 (Kaniga *et al.*, 1991). For *exoR*, the oligonucleotides *exoR*upfwd and *exoR*uprev were used to amplify the upstream fragment, and *exoR*downfwd and *exoR*downrev were used for the downstream fragment; for *exoA* oligonucleotides ExAUSS and ExAUSAS were used to amplify the upstream fragment, and ExADSS plus ExADSAS were used for the downstream fragment. The resulting plasmids were conjugated into *A. tumefaciens* C58 from the SM10/λ*pir* donor, and prototrophic Sm^R transconjugants were isolated on minimal media. Deletion mutants of the resident gene generated by excision of the plasmid were allowed to accumulate by culturing those derivatives that carried the integrated plasmid for several generations without antibiotic selection. Cultures were then plated on ATGN solid media, and colonies were selected for sucrose resistance. Candidates were purified and confirmed as deletion mutants by PCR amplification across the deletion and sequencing of the PCR product.

A plasmid-borne copy of the *exoR* gene was constructed by amplifying the *exoR* coding sequence by PCR and cloning into pBBR1-MCS5, generating pTWD100. The *exoR* coding sequence on pTWD100 was sequenced to ensure that it was free of mutations and properly fused to P_{lac}.

The *chvG* gene was mutagenized by plasmid integration. An internal 520 bp fragment of the *chvG* coding region (codons 154–329) was amplified using oligonucleotides *chvG* trunc fwd and *chvG* trunc rev, and cloned into pCR2.1-TOPO. The insert was excised as an *Eco*RI/*Xba*I fragment and ligated into pVIK112 (Kalogeraki & Winans, 1997) to generate pADT112. The resulting plasmid was conjugated from the *E. coli* Sm10/λ*pir* donor into C58, Δ*exoR*, Δ*exoA* and Δ*exoR*Δ*exoA* strains. Integrants were selected for Km^R on minimal

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
A. tumefaciens		
C58	Nopaline-type strain, pTiC58, pATC58	Sciaky *et al.* (1978)
Sin2	*exoR*::TnMod-OKm′, Km^R	This work
Sin3	*exoR*::TnMod-OKm′, Km^R	This work
BER9	*exoR*::*pRR100*	This work
PMM1	Δ*exoR* C58 derivative	This work
PMM2	Δ*exoRexoA* C58 derivative	This work
PMM4	Δ*flgE* C58 derivative	Merritt *et al.* (2007)
ADT1	*chvG*::*pADT112*	This work
ADT2	Δ*exoR*, *chvG*::*pADT112*	This work
ADT3	Δ*exoRexoA*, *chvG*::*pADT112*	This work
ADT10	Class I suppressor of PMM1	This work
ADT11	Class II suppressor of PMM1	This work
ADT12	Class I suppressor of PMM2	This work
E. coli		
S17-1/λ*pir*	λ*pir*, Tra^+, cloning host	Gift of V. de Lorenzo
SM10/λ*pir*	λ*pir*, cloning host	Miller *et al.* (1989)
DH10B/λ*pir*	λ*pir*, cloning host	Gift of David Bauer
DH5α F′	Cloning host	Woodcock *et al.* (1989)
TOP10	Cloning host	Invitrogen
Plasmids		
pTNMod-OKm′	Plasposon	Dennis & Zylstra (1998)
pGEM-T	Cloning vector	Promega
pCR2.1-TOPO	Cloning vector	Invitrogen
pKNG101	Cloning vector, Suc^S Sp^R	Kaniga *et al.* (1991)
pJZ383	P_{tac}::*gfp*, Sp^R, pVS replicon	J. Zhu; Cormack *et al.* (1996)
pRR100	*exoR* mutation vector, pVIK112 derivative	This work
pTD100	P_{lac}::*exoR*, pBBR1MCS-5 derivative	This work
pTWD114	*exoA* deletion vector, pKNG101 derivative	This work
pADT112	*chvG* mutation vector, pVIK112 derivative	This work

media, and disruption of the *chvG* coding region was confirmed by PCR amplification and sequencing. The site of integration results in a ChvG protein truncated at amino acid 329, prior to the histidine kinase and HATPase domains.

Sequences of all oligonucleotides used for molecular cloning and directed mutagenesis are available in Supplementary Table S1.

Static culture biofilm assays. Static culture biofilms were grown on sterile PVC coverslips suspended vertically in the wells of UV-sterilized 12-well polystyrene dishes. Cultures were inoculated at OD_{600} 0.05 in 3 ml ATGN broth and incubated at room temperature for 24–48 h. Adherence to PVC coverslips was visualized by staining with crystal violet (CV). Quantification of adherent biomass was achieved via solubilization of adsorbed CV in a standardized volume of 33 % acetic acid. Solubilized CV was measured as A_{600} to estimate relative amounts of adhered biomass. To visualize β-linked polysaccharides in adherent biomass, inoculated coverslips were washed in deionized water and stained in 1 % calcofluor (w/v double distilled water).

Flow cell culture biofilms: growth and analysis. Flow cell biofilms were cultured as described previously (Ramey *et al.*, 2004). Flow cells were purchased through the Technical University of Denmark and inoculated with diluted overnight cultures (100 µl per flow chamber, final OD_{600} 0.05). After inoculation, cells were permitted to attach for 60 min before the flow of ATGN media with appropriate antibiotics commenced at ~3 ml h^{-1}. Each strain was inoculated into triplicate chambers, and growing biofilms were examined by spinning disk confocal microscopy (SDCM) at 12 h intervals, beginning 24 h post-inoculation and continuing to 5 days post-inoculation. Microscopy was performed with a Nikon TE-200 spinning disk confocal microscope at 100× magnification fitted with a Uniblitz shutter driver and an Orca ER camera (Hamamatsu). SDCM images were acquired with the Perkin-Elmer ULTRAVIEW software package and analysed using an automated version of biofilm analysis software COMSTAT (Heydorn *et al.*, 2000) called *auto*COMSTAT, running in MATLAB 7.0 (Merritt *et al.*, 2007).

Plant attachment assays. Attachment assays with *Arabidopsis thaliana* ecotype WS used 7–10-day-old seedlings grown from ethanol-surface-sterilized seeds planted on half-strength Murashige–Skoog agar media. Roots were cut into 1 cm segments and transferred to sterile dishes containing 2 ml 1 mM $CaCl_2$ and 0.4 % sucrose, and inoculated to OD_{600} 0.01 with the appropriate derivative strain of *A. tumefaciens* C58 carrying a green fluorescent protein (GFP) reporter construct under constitutive expression (P_{tac}::*gfp*). Four root segments were inoculated per strain. After a given period of incubation in the dark at room temperature, the root segments were recovered, rinsed and resuspended in fresh calcium chloride/sucrose solution, and sealed under coverslips for hydration. Epifluorescence microscopy was performed on a Nikon E800 at 60× magnification with METAMORPH software.

Quantification of extracellular polysaccharide production. To quantitatively compare levels of secreted exopolysaccharide between strains, a standard anthrone assay was implemented (Morris, 1948).

This assay measures the total reducing sugar content in a given sample. Cultures were grown in AT media containing 0.4 % succinic acid as the carbon source to avoid addition of extraneous reducing sugars. At mid-exponential growth phase, samples were prepared by centrifugation to remove cells. This cell-free culture fluid was assayed for exopolysaccharide content via sulfuric acid hydrolysis in the presence of the colorimetric indicator anthrone. To assay, 250 µl anthrone solution (2 % w/v in ethyl acetate) was added to 1 ml cell-free culture fluid, then hydrolysed with 2.5 ml concentrated sulfuric acid. A_{620} was measured and normalized to the OD_{600} of each culture.

Quantitative PCR (qPCR). Primers were designed to amplify approximately 150 bp sequences from the upstream regions of the *exoY* (Atu3327) and *flgD* (Atu0579) reading frames. Primer sequences are included in Supplementary Table S1. Cultures were grown in ATGN media with the appropriate antibiotics to an OD_{600} of approximately 0.8, and RNA was isolated using Qiagen RNAprotect reagent and RNEasy Mini Prep kits. Samples were treated with DNase (Ambion) to remove contamination. cDNA was prepared using qScript cDNA Super Mix kit (Quanta Biosciences). qPCR was performed with PerfeCta SYBR Green FastMix Low Rox reagent (Quanta Biosciences), on a Stratagene MX3000P instrument. Samples values were normalized using a σ^{70} primer set and calibrated against wild-type results. Data are representative of two biological replicates, each of which consisted of three technical replicates.

RESULTS

To identify genes required for surface colonization, we screened a set of 2000 *A. tumefaciens* C58 transposon mutants for decreased adherence to 96-well PVC microtitre plates. Among the mutants isolated were two designated *sin2* and *sin3* (surface interaction deficient). These two mutants were severely impaired in biofilm formation on abiotic surfaces, and were strikingly hypermucoid when grown on solid medium (Fig. 1a–d). They are prototrophic, with growth rates identical to those of the wild-type in AT minimal medium.

The *sin2* and *sin3* mutants carry unique transposon insertions in Atu1715 (Goodner *et al.*, 2001; Wood *et al.*, 2001), with the *sin2* insertion at codon 253 and the *sin3* insertion at codon 215. This gene encodes a homologue of the ExoR protein of *Sinorhizobium meliloti*. ExoR is a negative regulator of exopolysaccharide synthesis in several rhizobia and is conserved among a wide range of alphaproteobacteria, including both plant and animal symbionts as well as free-living micro-organisms (see Supplementary Fig. S1, available with the online version of this paper). The ExoR sequence contains a series of tetratricopeptide repeat and SEL1 domains, sequence motifs implicated in protein–protein interactions (Blatch & Lassle, 1999; D'Andrea & Regan, 2003). Additionally, the ExoR sequence suggests the presence of an N-terminal signal sequence, and periplasmic localization has been demonstrated for ExoR of *S. meliloti* (Wells *et al.*, 2007). ExoR has no recognizable DNA-binding domains, but its expression is known to affect the transcript levels of target *exo* genes involved in synthesis of *S. meliloti* succinoglycan (Reed *et al.*, 1991; Yao *et al.*, 2004) as well as affecting transcription of flagellar genes (Yao *et al.*, 2004), primarily through interactions with the ExoS–ChvI two component system (Chen *et al.*, 2008; Wells *et al.*, 2007). The hypermucoid phenotype of the *S. meliloti exoR* mutant, caused by overproduction of succinoglycan, was the reason for its initial isolation (Reed *et al.*, 1991).

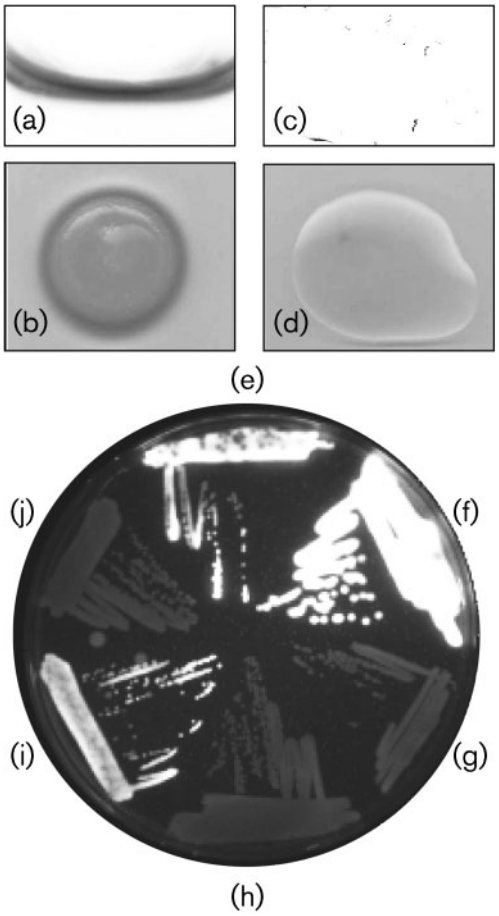

Fig. 1. Biofilm and mucoidy phenotypes of the *A. tumefaciens sin3* mutant and derivatives. (a)–(d) Wild-type *A. tumefaciens* C58 forms a biofilm on PVC coverslips (a), and a normal colony on minimal media (b), while the *sin3* mutant fails to form a biofilm on PVC coverslips (c) and is hypermucoid on solid media (d). (e)–(j) *A. tumefaciens* derivatives streak-isolated on ATGN medium with 1 % Calcofluor demonstrate differential production of β-linked polysaccharides. Wild-type fluorescence is visible in (e), while the hyperfluorescent *exoR* mutant can be seen in (f). The *exoA* mutant (g) is dim, as is the *exoRexoA* mutant (h). Addition of P_{lac}::*exoR* complements the hyperfluorescence of the *exoR* mutant (i), but not the dimness of the *exoRexoA* mutant (j). *exoR* mutant strains used in this assay were generated via Campbell integration.

Site-directed disruption of *exoR* using plasmid integration mutagenesis with an internal *exoR* fragment (C58-JR1), and independently by complete deletion of the gene (C58-PMM1), yielded prototrophic mutants with biofilm and hypermucoid phenotypes identical to the original transposon mutants. Complementation with an intact, plasmid-

borne copy of *exoR* expressed from the *lacZ* promoter (pTWD100, P_{lac}::*exoR*) effectively reversed these phenotypes. Except where noted, all results shown for the *exoR* mutant are from the complete deletion mutant C58-PMM1.

Overproduction of succinoglycan in the *A. tumefaciens exoR* mutant

Based on the findings from *S. meliloti*, it seemed likely that the hypermucoid phenotype observed in the *exoR* mutants was due to overproduction of succinoglycan. Accordingly, we examined the effect of abolishing succinoglycan synthesis in wild-type and *exoR* mutant backgrounds. ExoA has been characterized as a glycosyltransferase involved in addition of the first glucose in the octasaccharide repeat subunit during succinoglycan biosynthesis in *S. meliloti* (Reuber & Walker, 1993). The *exoA* gene was disrupted by an unmarked, non-polar deletion of the entire coding sequence to eliminate succinoglycan production in *A. tumefaciens*. *A. tumefaciens* derivatives were plated on standard minimal medium containing 1 % calcofluor, a fluorescent dye that binds to β-linked polysaccharides such as succinoglycan and fluoresces under UV light (Doherty *et al.*, 1988). Compared with wild-type cells (Fig. 1e), *exoR* mutant cells are hyperfluorescent, indicating the elevated production of β-linked polysaccharides (Fig. 1f), while *exoA* mutant cells are significantly less fluorescent than wild-type cells (Fig. 1g). The *exoRexoA* derivative has fluorescence equivalent to the *exoA* mutant and is non-mucoid, suggesting that the hypermucoidy and bright calcofluor fluorescence of the *exoR* mutant are dependent on the *exo* genes, and therefore primarily due to the overproduction of succinoglycan (Fig. 1h).

To determine relative levels of exopolysaccharide production, we evaluated each mutant using an anthrone-based assay. Soluble polysaccharides from cell-free supernatants were hydrolysed with sulfuric acid in the presence of the colorimetric indicator anthrone that detects reducing sugars (Morris, 1948). The *exoR* mutants produce approximately fourfold greater amounts of soluble exopolysaccharide than wild-type, whereas *exoA* and *exoRexoA* mutants produce roughly one-third of the wild-type amount during exponential-phase growth (Table 2).

Disruption of *exoR* elevates expression of the succinoglycan biosynthetic gene *exoY*

To determine whether ExoR regulates succinoglycan production via expression of *exo* genes, we performed qPCR on the *exoY* transcript in *A. tumefaciens* C58, Δ*exoR*, C58 (P_{lac}::*exoR*) and Δ*exoR* (P_{lac}::*exoR*) strains. This analysis revealed a striking 30-fold derepression of *exoY* expression in the *exoR* mutant that was fully reversed by provision of the plasmid-borne *exoR*. Expression of ExoR from this same plasmid in a wild-type background led to a slight reduction in *exoY* expression compared with the wild-type strain (Table 3). Analysis of an *exoT–lacZ* fusion in these same strains showed a similar, but slightly reduced, pattern of expression (data not shown). These findings demonstrate that *A. tumefaciens* ExoR exerts a significant negative regulation on the expression of *exo* genes, which probably explains the observed overproduction of succinoglycan in an *exoR* mutant.

The *exoR* adherence defect is not dependent on succinoglycan synthesis

The most striking visible phenotype of the *exoR* mutant is its hypermucoidy (Fig. 1). We hypothesized that the elevated production of exopolysaccharide reduced the efficiency or duration of surface interactions, yielding the observed biofilm deficiency. To test this directly, the *exoR* and *exoA* mutants were assayed for biofilm formation on the model abiotic surface PVC in a standard static culture biofilm assay. The *exoA* mutant revealed no attachment

Table 2. Biofilm and exopolysaccharide phenotypes of mutant derivatives

Strain	Biofilm A_{600}/OD_{600}* (SEM)	Exopolysaccharide A_{620}/OD_{600} (SEM)†
C58	1.81 (0.18)	3.02 (0.16)
Δ*exoR*	0.31 (0.04)	12.21 (0.40)
Δ*exoA*	1.17 (0.08)	0.94 (0.09)
Δ*exoRexoA*	0.23 (0.04)	1.03 (0.34)
chvG	2.85 (0.15)	2.25 (0.17)
chvG Δ*exoR*	0.21 (0.03)	5.90 (0.24)
chvG Δ*exoA*	1.38 (0.07)	1.75 (0.39)
chvG Δ*exoRexoA*	0.17 (0.03)	0.74 (0.63)
Δ*exoR* supp class I	1.47 (0.18)	2.87 (0.09)
Δ*exoR* supp class II	0.47 (0.08)	14.32 (0.43)
Δ*exoRexoA* supp	2.25 (0.30)	0.52 (0.12)

*CV absorbance of adherent biomass normalized to culture density.
†Anthrone absorbance normalized to culture density.

Table 3. Disruption of *exoR* affects transcription of *exoY* and *flgD* genes

Strain	$exoY_N$*	$flgD_N$*
C58	1.000 ± 0.35	1.000 ± 0.40
C58 (pTWD100, P_{lac}::*exoR*)	0.496 ± 0.54	1.225 ± 0.27
Δ*exoR*	30.172 ± 0.55	0.024 ± 0.26
Δ*exoR* (pTWD100, P_{lac}::*exoR*)	1.071 ± 0.23	1.106 ± 0.37
chvG	0.417 ± 0.12	0.585 ± 0.15

*The range given for expression, relative to C58, encompasses one SD.

deficiency, indicating that succinoglycan is not required for biofilm formation (Table 2, Fig. 2g). The *exoRexoA* double mutant, despite its inability to overproduce succinoglycan, exhibited an adherence defect similar to that of the *exoR* single mutant (Fig. 2j). Although succinoglycan overproduction may contribute to the overall biofilm deficiency of the *exoR* mutant, it is clearly not the sole cause of the defect.

Quantitative analysis of flow cell biofilms

Biofilms of the *exo* mutant strains were grown in a once-through continuous-flow cell system and analysed using SDCM, and the COMSTAT analysis program (Heydorn *et al.*, 2000). Microscopic examination of biofilm formation by *A. tumefaciens* C58 derivatives constitutively expressing GFP (pJZ383, P_{tac}::*gfp*) in flow cells was performed in real-time without disrupting the samples or ceasing flow. The *auto*COMSTAT program provides quantitative, statistically robust analysis of biofilm structure via processing of image stacks from multiple fields of view within the flow cell. SDCM images show that the *exoR* mutant demonstrates a severe biofilm formation deficiency and few attached cells (Fig. 2e), in comparison with the wild-type C58 (Fig. 2b), while the *exoA* mutant was fully competent for biofilm formation (Fig. 2h). As with the static coverslip biofilms, the flow cell biofilms of the *exoRexoA* double mutant were similar to *exoR* mutant biofilms (Fig. 2k). The biofilm deficiencies of both *exoR* and *exoRexoA* mutants were fully complemented by expression of plasmid-borne *exoR* (data not shown).

Quantitative COMSTAT analysis reveals statistically significant differences in such measurements as the amount of adherent biomass, average biofilm height and the maximum biofilm height for each strain (Table 4). These data support the qualitative assessment that the *exoA* mutant forms a wild-type biofilm, whereas the *exoR* and *exoRexoA* mutant strains are deficient in all measurements of biofilm formation that were quantified. These mutants occasionally bound as single cells or small cell clusters of five to six cells in height (Table 4), which rarely expanded further. Provision of plasmid-borne ExoR in either mutant background complemented the Δ*exoR* phenotype (Table 4). The overall flow cell phenotypes provide a more detailed view of attachment and biofilm structure

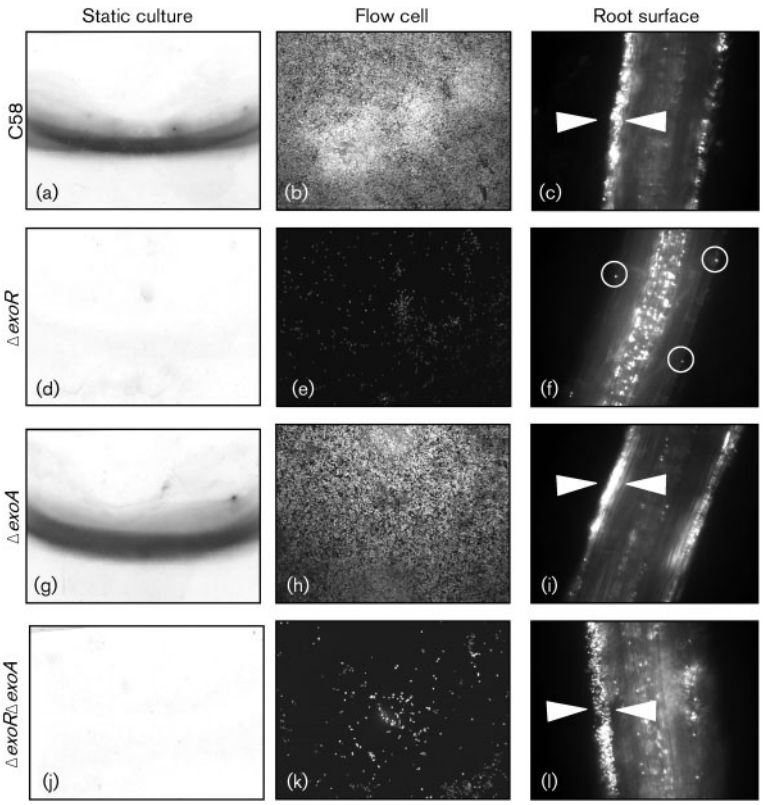

Fig. 2. Biofilm phenotypes of *exoR* and *exoA* mutants. Biofilms formed after 48 h static culture growth are shown in (a), (d), (g) and (j). (b), (e), (h) and (k) show biofilms grown under flow conditions for 72 h. Surface attachment to cuttings of *Arabidopsis thaliana* roots are shown in (c), (f), (i) and (l). Individually bound cells are indicated by circles in (f), while biofilms are indicated by arrowheads. Fluorescence of plant vasculature is due to autofluorescence, not microbial colonization of the vasculature. Wild-type cells (a, b and c) and Δ*exoA* cells (g, h and i) are competent for biofilm formation under all conditions, while Δ*exoR* (d, e and f) cultures do not form biofilms in any assay. Δ*exoRexoA* cells do not form biofilms on abiotic surfaces (j, k), but are competent for plant tissue attachment (l). Confocal microscopy was performed using a Nikon TE-200 spinning disk confocal microscope at ×100 magnification fitted with a Uniblitz shutter driver and an Orca ER camera (Hamamatsu) and Perkin-Elmer ULTRAVIEW software; epifluorescence microscopy was performed on a Nikon E800 at 60× magnification with METAMORPH software.

Table 4. COMSTAT analysis of flow-cell biofilms over 4 days

Values are means (±SEM) of 15 fields of view from three separate flow cells.

Strain*	Time (h)	Biovolume/area†	Mean thickness (μm)	Maximum thickness (μm)
Wild-type	48	0.17 (0.09)	0.26 (0.13)	4.58 (0.62)
	96	3.68 (0.67)	4.16 (0.72)	21.33 (2.23)
Δ*exoR*	48	0.00 (0.00)	0.00 (0.00)	3.00 (0.22)
	96	0.06 (0.01)	0.01 (0.00)	6.33 (0.42)
Δ*exoA*	48	0.05 (0.02)	0.07 (0.03)	5.08 (0.62)
	96	3.88 (0.81)	4.28 (0.88)	17.00 (5.00)
Δ*exoRexoA*	48	0.00 (0.00)	0.00 (0.00)	3.08 (0.45)
	96	0.04 (0.01)	0.88 (0.05)	6.50 (1.15)
Δ*exoR*, P_{lac}::*exoR*	48	0.23 (0.07)	0.35 (0.11)	4.67 (0.68)
	96	5.89 (0.34)	6.87 (0.35)	21.83 (2.71)
Δ*exoRexoA*, P_{lac}::*exoR*	48	0.43 (0.21)	0.26 (0.13)	4.83 (0.38)
	96	8.00 (1.85)	9.10 (1.97)	28.67 (4.44)

*All strains are derivatives of *A. tumefaciens* C58 carrying pJZ383 (P_{tac}::*gfp*).
†μm^3 fluorescent material per μm^2 surface attachment.

and are entirely consistent with the static culture phenotypes.

ExoR is required for binding to plant root surfaces

Plant-root-binding assays were performed to evaluate the influence of the *exoR* mutation and succinoglycan production on associations with plant tissues. Sterile segments of *Arabidopsis thaliana* roots were incubated with the *A. tumefaciens* derivatives harbouring the pJZ383 plasmid expressing GFP. Root segments were washed to remove loosely associated bacteria and examined under epifluorescence microscopy. Both C58 wild-type and *exoA* mutants were able to bind *Arabidopsis thaliana* root cuttings (Fig. 2c and i), but *exoR* mutants were dramatically deficient (Fig. 2f). Surprisingly, however, the *exoRexoA* double mutant bound efficiently to root tissue (Fig. 2l). This finding illustrates that in contrast with results from abiotic surfaces, overproduction of succinoglycan is a dominant factor in the plant-binding deficiency of *exoR* mutants.

The *exoR* mutant's reduced propensity for root binding in suspension is not reflected in simple virulence assays in which *A. tumefaciens* is manually inoculated into plant wounds. When introduced onto sterile potato slices, the *exoR* mutant formed tumours indistinguishable in size and number from the wild-type (data not shown).

ExoR can function independently of the ChvG–ChvI two component system

The *S. meliloti* ExoR protein is secreted into the periplasm. Due to this unconventional location for a regulator, we began to consider possible intermediates through which *A. tumefaciens* ExoR might act. The first gene we targeted was *chvG*, a two-component-type histidine kinase homologous to the *S. meliloti* gene *exoS*. In *S. meliloti*, ExoR is reported to function through interactions with ExoS and its cognate response regulator ChvI, that together control production of multiple exopolysaccharides, including succinoglycan (Belanger *et al.*, 2009). A point mutation that decreases ChvI activity (K214T) has been described that suppresses *exoR* mutant phenotypes in that system (Wells *et al.*, 2007; Yao *et al.*, 2004). Therefore, we created a *chvG* mutation via Campbell integration in wild-type and the *exoR*, *exoA* and *exoRexoA* mutant backgrounds to examine potential interactions in *A. tumefaciens*. All *chvG* mutants exhibited sensitivity to growth on LB medium, consistent with previous reports on *A. tumefaciens* (Charles & Nester, 1993; Mantis & Winans, 1993) and *S. meliloti* (Belanger *et al.*, 2009). If *exoR* influences exopolysaccharide production exclusively through the ChvG–ChvI two component system, we would anticipate that a *chvG* null mutation would eliminate exopolysaccharide overproduction in an *exoRchvG* double mutant, consistent with observations in *S. meliloti* (Wells *et al.*, 2007). In contrast with that hypothesis, the *exoRchvG* mutant retained elevated levels of exopolysaccharide production, although these were somewhat reduced relative to the *exoR* mutant (Table 2), indicating that ExoR can affect succinoglycan production independently of ChvG. In contrast with the hypermucoid *exoR* phenotype, we found that the *chvG* mutant was slightly reduced in exopolysaccharide production as measured by the anthrone assay. Furthermore, expression of the *exoY* gene is 60 % reduced in the *chvG* mutant background, compared with a 30-fold derepression in the *exoR* background, indicating a modest role for ChvG in positively regulating *exo* gene expression (Table 3).

Additionally, we found that biofilm formation in the *chvG* mutant not only occurs but is also, in fact, slightly increased compared with the wild-type (Table 2). The *exoR* mutation is also dominant over the *chvG* mutation in both

the succinoglycan-positive and -negative backgrounds. This indicates that the *exoR* regulation of biofilm formation occurs independently of the *chvG* signal transduction pathway. Interestingly, the *exoAchvG* double mutant is somewhat reduced in biofilm formation, demonstrating that the modestly elevated *chvG* biofilm phenotype may be dependent on succinoglycan production. Thus, it appears that *chvG* has a mild negative influence on succinoglycan production – the mutant yields a slight increase in biofilm formation – but that this phenotype is independent of regulation by ExoR.

ExoR mutants exhibit reduced motility

Examination of *exoR* mutants revealed that they were severely deficient in swimming motility, forming a small ring relative to the wild-type after 4–7 days incubation on swim agar (0.25 % agar) (Fig. 3a and b). The *exoA* mutant was proficient for swimming motility with no visible mucoidy (Fig. 3c). Close examination of the *exoR* mutant swim plate revealed an accumulation of mucoid material on the surface at the site of inoculation, and it was plausible that this mucoid material inhibited motility. The *exoRexoA* double mutant, however, exhibited similarly attenuated motility, indicating that the motility deficiency of the *exoR* mutant is independent of succinoglycan overproduction (Fig. 3d). The *exoR* and *exoRexoA* mutant motility phenotypes were fully complemented by introduction of the P_{lac}::*exoR* plasmid (Fig. 3e and f). The swim plate phenotypes were consistent with microscopic examination of cells that were stained to visualize flagella, in which *exoR* and *exoRexoA* mutant cultures had predominantly aflagellate cells, while the *exoA* mutant was flagellated equivalent to wild-type (data not shown). These findings suggest that ExoR independently regulates both motility and succinoglycan production.

The absence of flagella suggested a defect in synthesis or assembly of flagellin. Flagellin levels were examined by immunoblotting using cross-reactive antibody raised against *Caulobacter crescentus* flagella. While the wild-type strain produced easily observable levels of flagellin, the protein was undetectable in the Δ*exoR* mutant, similar to aflagellate Δ*flgE* (flagellar hook) and Δ*flaABC* (flagellin) mutants (Fig. 3j).

qPCR was used to examine expression levels of *flgD*, the scaffold for flagellar hook assembly. Consistent with swim plate phenotypes and flagellin levels, *flgD* expression is nearly abolished in the *exoR* mutant relative to the wild-type. The plasmid-borne ExoR restores wild-type expression levels of *flgD*. A modest decrease in *flgD* expression is observed in a *chvG* mutant as well (Table 3).

Although the *exoR* and *exoRexoA* deletion mutants were clearly reduced in flagellation, a small number of flagella were always observed, consistent with the reduced but significant swim rings observed on motility agar. The swim plate environment appears to efficiently select for sup-

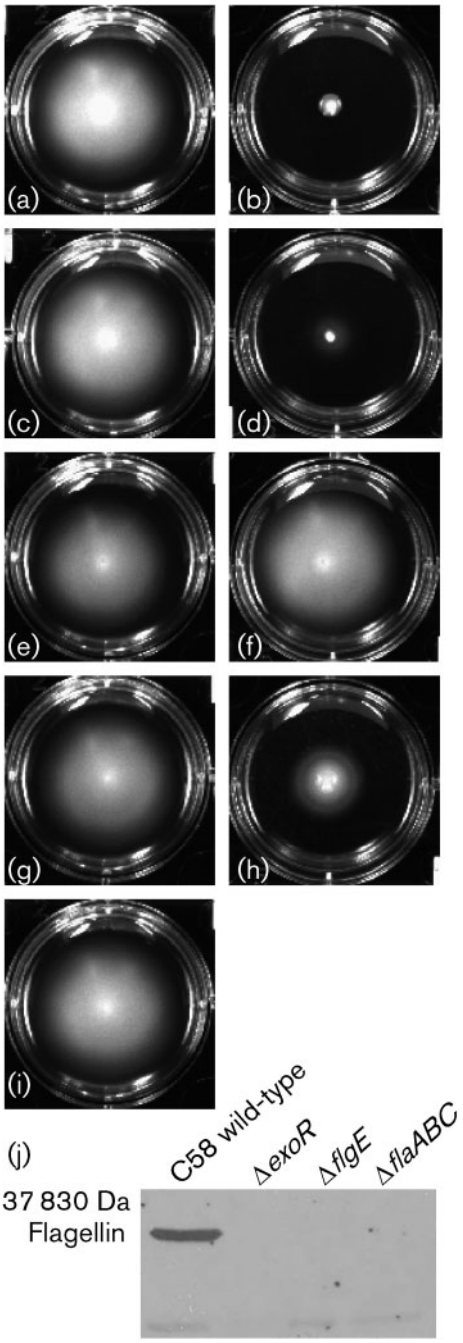

Fig. 3. ExoR null mutants are reduced in swimming motility and production of flagellin. Wild-type cells (a) are fully motile in 0.25 % swim agar, while Δ*exoR* mutants are non-motile (b). Succinoglycan−Δ*exoA* mutants have wild-type motility (c), but Δ*exoRexoA* mutants are deficient in motility (d), demonstrating that succinoglycan is not required for motility and that ExoR acts independently to regulate succinoglycan production and motility. Both the Δ*exoR* (e) and Δ*exoRexoA* (f) non-motile phenotypes are complemented by addition of P_{lac}::*exoR*. Class I suppressors of Δ*exoR* show wild-type motility (g), while class II suppressors show a partial restoration of motility (h). Suppressors of Δ*exoRexoA* are fully motile (i). Western blotting shows a reduction of flagellin in Δ*exoR* mutants compared with wild-type C58 (j).

pressor mutants. In each repetition of the swim assay, suppressors of the motility-deficient phenotype of the *exoR* and *exoRexoA* strains were observed at the edges of the swim ring. The suppressors of the *exoR* mutation fell into two classes: class I suppressors form dry, non-mucoid colonies, are apparently wild-type for motility and exopolysaccharide production, and form wild-type biofilms in the static culture coverslip assay (Fig. 3g, Table 2). Class II suppressors form mucoid colonies and accordingly produce exopolysaccharide at levels as high as *exoR* mutants. Class II mutants exhibit motility levels intermediate between the non-motile *exoR* mutants and the wild-type cells, but are still unable to form biofilms on coverslips (Fig. 3h, Table 2). All observed suppressors of the *exoRexoA* mutant exhibited dry colony morphologies, wild-type motility, and improved biofilm formation (Fig. 3i, Table 2). Introduction of a plasmid-borne copy of *exoR* into the non-mucoid, motile and biofilm-proficient class I mutants had no significant effect on these phenotypes (data not shown). In contrast, introduction of the *exoR* plasmid into the class II suppressors (mucoid, partially motile and biofilm-deficient) complemented all of these phenotypes to wild-type levels. These observations are consistent with the class I suppressors already functioning as nearly wild-type, and therefore not being affected by the presence of *exoR*. The class II suppressor mutations partially correct the motility deficiency, and thus provision of *exoR* restores full motility as well as correcting the remaining mutant phenotypes.

Together, these data suggest that we have identified suppressor mutations at two discrete points in the ExoR-dependent regulatory pathway. A previously reported suppressor of the *S. meliloti exoR* mutant mapped to *chvI* (Wells *et al.*, 2007). As such, we conducted sequence analysis of the *chvGI* region, including its upstream sequences, in the original *A. tumefaciens exoR* mutant and its suppressors. This analysis revealed all of these sequences to be identical to the corresponding sequences in wild-type C58. Thus, the isolated suppressor mutations must have occurred outside the *chvGI* region, and ChvG and ChvI are not required for ExoR regulation of these functions.

DISCUSSION

A. tumefaciens is a widely prevalent soil microbe, but is best known as a plant pathogen. Surface attachment is a required early step in the initiation of the infection process, and likewise is required for biofilm formation. In this study, we performed a screen for biofilm-deficient mutants of *A. tumefaciens* C58 and isolated two independent mutations in the *exoR* gene. ExoR is a regulator known to be required for nodulation through control of succinoglycan in the nitrogen-fixing alfalfa symbiont *S. meliloti* (Doherty *et al.*, 1988; Reed *et al.*, 1991). We found that *A. tumefaciens* C58 *exoR* null mutants are deficient in surface attachment, and therefore do not form biofilms on either plant tissues or abiotic surfaces. ExoR is a negative regulator of succinoglycan synthesis, and an *exoR* null mutant overproduces this exopolysaccharide. Succinoglycan overproduction requires the *exo* genes, and the expression of at least one of these genes is significantly derepressed in the *exoR* mutant. The attachment deficiency on abiotic model surfaces is not simply due to succinoglycan overproduction, but apparently involves other functions influenced by ExoR. At least one other function clearly under ExoR regulatory control is motility; *exoR* mutants exhibit reduced motility that corresponds to a loss of flagellation, and markedly reduced flagellar gene expression.

The motility deficiency of the *exoR* mutant is unlikely to be entirely responsible for its biofilm deficiency. In a separate study, we investigated the role of motility during *A. tumefaciens* biofilm formation (Merritt *et al.*, 2007). Although markedly reduced for biofilm formation in non-flowing systems, an aflagellate *A. tumefaciens flgE* mutant is highly competent for biofilm formation in flow cells. To exclude the possibility of a synthetic effect between motility and succinoglycan production on biofilm formation, we examined static culture biofilms of a *flgE exoA* double mutant. The double mutant exhibited the non-motile phenotype of the *flgE* parent strain, the succinoglycan production deficiency of the *exoA* parent strain, and was identical in biofilm formation to the *flgE* parent strain (data not shown). In contrast with the non-motile *flgE* mutant, the *exoR* mutant shows similarly compromised attachment under both non-flowing and flowing conditions. Also, although the *exoRexoA* mutant is reduced in swimming motility and abiotic surface attachment, it does form biofilms on plant tissue, indicating that its motility deficiency is not the underlying cause of its biofilm phenotype.

ExoR is broadly conserved across the alphaproteobacteria, including the plant symbionts *Rhizobium etli*, *Rhizobium leguminosarum* and *Mesorhizobium loti*, in animal pathogens such as *Brucella suis* and *Brucella abortus*, and even in free-living bacteria such as *Rhodopseudomonas palustris*. The amino acid sequence is highly conserved, with 40 invariant residues spread throughout the length of the sequence (Supplementary Fig. S1). In all homologues, a conserved secretion signal motif is located at the N terminus of the translation product. Tetratricopeptide repeat domains, and the closely related SEL-1 domains that extend through the ExoR sequence are implicated in protein–protein interactions (Mittl & Schneider-Brachert, 2007). This suggests that ExoR exerts its regulatory effects via interaction with other regulatory or signal transduction system(s). The sequence similarities imply an analogous biochemical function(s) for ExoR in each microbial system.

ExoR has been extensively investigated in *S. meliloti*, where it was originally identified as a repressor of succinoglycan production (Reed *et al.*, 1991). Our results reveal that ExoR is also a negative regulator of succinoglycan production via

exo gene repression in *A. tumefaciens*. In both bacteria, mutation of *exoR* causes hypermucoidy and an increase in *exo* gene expression (Reed *et al.*, 1991; Chen *et al.*, 2008) (Table 3). Additionally, ExoR appears to be required for flagellar motility in both organisms (Yao *et al.*, 2004). However, other phenotypes resulting from disruption of *exoR* in the two bacteria are strikingly different. Succinoglycan is required for *S. meliloti* to form functional symbiotic relationships with host legumes (Leigh *et al.*, 1987), and the *exoR* mutant is also symbiotically deficient. In contrast, succinoglycan is dispensable for crown gall disease in *A. tumefaciens* (Cangelosi *et al.*, 1987) and we find that *exoR* mutants form tumours efficiently upon manual inoculation of model plants such as potato. Wells *et al.* (2007) reported that an *exoR*::Tn*5* mutation, which results in high-level succinoglycan synthesis, increases *S. meliloti* biofilm formation on abiotic surfaces. Disruption of succinoglycan synthesis (*exoY*::Tn*5*) correspondingly decreased biofilm formation in a similar assay (Fujishige *et al.*, 2006). Our isolation of two separate *A. tumefaciens exoR* transposon mutants based on their inability to form biofilms, which were found to overproduce succinoglycan, directly contrasts with these phenotypes. Consistent with this, an *A. tumefaciens* mutant unable to synthesize succinoglycan (*exoA* disruption) is unaffected with respect to biofilm formation. However, a more recent report for *S. meliloti* is in agreement with our findings, demonstrating that succinoglycan-deficient mutants form normal biofilms, but that exopolysccharide II (galactoglucan) is required (Rinaudi & Gonzalez, 2009). Another difference is the observation that the *S. meliloti exoR*::Tn*5* mutant is auxotrophic, while the *A. tumefaciens exoR* mutant strains are uniformly prototrophic.

S. meliloti ExoR associates with and inhibits the ExoS sensor kinase, and through it, the response regulator ChvI (Chen *et al.*, 2008; Wells *et al.*, 2007). ExoS and ChvI control exopolysaccharide production and apparently inhibit motility in *S. meliloti* (Belanger *et al.*, 2009; Wells *et al.*, 2007). We examined an *A. tumefaciens chvG* mutant and observed decreased expression of *exoY* and a slight decrease in exopolysaccharide production, indicating that ChvG exerts a mild positive regulation of exopolysaccharide levels. A *chvG* mutation also slightly increases *A. tumefaciens* biofilm formation. We have observed that a *chvG* mutation modestly decreases flagellar gene expression (i.e., the *flgD* transcript), although this does not affect motility (data not shown).

S. meliloti exoR mutants are reported to be non-motile and to exhibit an elevated biofilm phenotype. Mutations that decrease ChvI activity can suppress the motility defect of an *exoR* mutant and restore wild-type biofilm levels (Wells *et al.*, 2007). In contrast, in *A. tumefaciens*, ExoR regulation of biofilm formation, motility and exopolysaccharide production appears not to require the ChvG (ExoS)/ChvI two-component system. Loss of a functional ChvG/I does not restore wild-type biofilm, or exopolysaccharide phenotypes to an *exoR* mutant. In further contrast with *S. meliloti* reports, mutation of ChvG/I in an *A. tumefaciens exoR* mutant does not restore motility (data not shown). Therefore, we conclude that *A. tumefaciens* ExoR must be able to function through a signalling pathway distinct from ChvG/I.

One way to define such a pathway is by isolating mutants in which *exoR* mutant phenotypes are suppressed. The two classes of *exoR* mutant suppressors we have isolated thus far indicate a branched regulatory pathway. Class I suppressors cause reversion to wild-type levels of both motility and exopolysaccharide production, and these suppressors regain competence for biofilm formation. Class II suppressors still overproduce exopolysaccharide and remain mucoid, but have regained intermediate motility compared to the wild-type. The simplest explanation is that the intermediate motility of the class II suppressors is due to excess exopolysaccharide, blocking motility by increasing local viscosity. Class II suppressors remain incompetent for biofilm formation, another indication that the biofilm deficiency of the *exoR* mutant is not solely due to the loss of motility. The single class of suppressors (Class I-like) isolated for the *exoRexoA* mutant have regained wild-type levels of motility and biofilm formation, and remain succinoglycan-deficient (due to the *exoA* mutation). Provision of wild-type *exoR* in both class I and class II suppressor mutants leads to phenotypes consistent with the current model. Similar suppressors of *exoR* mutations in *S. meliloti* consistently map to the *exoS* and *chvI* genes (Wells *et al.*, 2007), reflecting the ExoR–ExoS interaction. In the *A. tumefaciens* suppressors described here, the *chvG* and *chvI* genes are not mutated.

A mechanism by which ExoR functions via its inhibition of ExoS/ChvI, while strongly supported in *S. meliloti*, does not seem to apply to the phenotypes we have examined in *A. tumefaciens*. It is possible that ExoR influences a single regulatory pathway to exert the wide variety of effects we observe. A cytoplasmic regulatory circuit implicated in control of exopolysaccharide production and motility in *S. meliloti* includes the zinc finger protein MucR, the LuxR-type regulators VisN/R and their interacting partner Rem (Bahlawane *et al.*, 2008). Homologues of each of these regulators are present in *A. tumefaciens* C58, and these may be indirect targets for ExoR regulation. Indeed, expression of the MucR homologue Ros is elevated in the Δ*exoR* mutant, which may account for the hypermucoidy of this mutant (A. D. Tomlinson and C. Fuqua, unpublished data). Alternatively, ExoR may be influencing multiple, discrete pathways, including ChvG/I, some of which impinge upon the process of surface attachment. Finally, a more indirect role is possible. For example, given its location in cells, ExoR may serve to sense the state of the periplasm and thereby influence multiple aspects of cellular physiology. Future experiments will discriminate between these models by identifying the partners with which ExoR interacts, as well as the downstream targets of ExoR regulation. These studies will also elucidate whether ExoR functions in response to specific environmental cues or is a

required but non-modulated component of these regulatory pathways.

ACKNOWLEDGEMENTS

We wish to acknowledge José Rodriguez, Rania Rizk and Melanie Lawler for valuable input on this project. Yves Brun generously provided the anti-flagellin antibody. Thanks also to Juan González for the anthrone assay, and both Juan González and Lois Banta for helpful discussions. The Indiana University Light Microscopy Imaging Center provided valuable assistance and microscopy for this study. Daniel Kearns provided useful suggestions for the manuscript. A. D. T. and B. R.-H. were appointees on the Indiana University Genetics, Molecular and Cellular Sciences Training Grant T32-GM007757. This project was supported by National Institutes of Health grant RO1-GM080546 (C. F.) and through a grant from the Indiana University META-Cyt program funded in part by a major endowment from the Lilly Foundation (C. F.).

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Edited by: M. F. Hynes