INTRODUCTION

Bacterial biofilms are surface-associated multicell communities generally embedded in an extracellular polysaccharide matrix that acts to hold bacteria together, attached to an abiotic or biological surface. In contrast, the planktonic lifestyle is associated with growth of individual micro-organisms in liquid environments and frequently with the capacity for motility. These two different lifestyles confer different adaptive advantages depending on the local environmental conditions. The frequently changing environmental cues present in most ecological niches necessitate rapid adaptive responses, and thus bacteria have evolved finely tuned regulation systems to enable and control the transition between these two different ways of life. It has been proposed that the ability to carry out this transition efficiently and accurately plays a crucial role in the transition between planktonic and biofilm modes of growth, and for other morphological transitions, in many bacteria (Jonas et al., 2009). The ability to modulate the production of key adhesion factors in some bacteria is a typical example of the biological importance of c-di-GMP regulation. The adhesins in question include proteinaceous factors (Hinsa et al., 2003; Yousef & Espinosa-Urgel, 2007), exopolysaccharide (EPS; Borlee et al., 2010), adhesive curli fimbriae and pili (Pesavento et al., 2008), flagella (O’Toole & Kolter, 1998) and the adhesive holdfast of Caulobacter crescentus (Toh et al., 2008). Indeed, the matrix composition of biofilms typically consists of a complex mixture of EPS, proteins and nucleic acids (Branda et al., 2005).

Poly-β-1,6-N-acetyl-D-glucosamine (PGA) is a polysaccharide present in the extracellular matrix of biofilms in a wide range of bacteria (Bosse et al., 2010; Choi et al., 2009; Itoh et al., 2005; Izano et al., 2007, 2008; Mack et al., 1996; Wang et al., 2004). For instance, the Gram-negative periodontal pathogen Actinobacillus actinomycetemcomitans forms a PGA-dependent biofilm required to colonize the human oral cavity, where it has been implicated as the causative agent of localized juvenile periodontitis (Zambon, 1985). The dense biofilm formed by this orchestration of physiological changes required to switch between planktonic and biofilm modes of growth, and for other morphological transitions, in many bacteria (Jonas et al., 2009). The ability to modulate the production of key adhesion factors in some bacteria is a typical example of the biological importance of c-di-GMP regulation. The adhesins in question include proteinaceous factors (Hinsa et al., 2003; Yousef & Espinosa-Urgel, 2007), exopolysaccharide (EPS; Borlee et al., 2010), adhesive curli fimbriae and pili (Pesavento et al., 2008), flagella (O’Toole & Kolter, 1998) and the adhesive holdfast of Caulobacter crescentus (Toh et al., 2008). Indeed, the matrix composition of biofilms typically consists of a complex mixture of EPS, proteins and nucleic acids (Branda et al., 2005).

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N-Acetylglucosamine-dependent biofilm formation in Pectobacterium atrosepticum is cryptic and activated by elevated c-di-GMP levels

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The phytopathogenic bacterium Pectobacterium atrosepticum (Pba) strain SCRI1043 does not exhibit appreciable biofilm formation under standard laboratory conditions. Here we show that a biofilm-forming phenotype in this strain could be activated from a cryptic state by increasing intracellular levels of c-di-GMP, through overexpression of a constitutively active diguanylate cyclase (PleD*) from Caulobacter crescentus. Randomly obtained Pba transposon mutants defective in the pga operon, involved in synthesis and translocation of poly-β-1,6-N-acetyl-D-glucosamine (PGA), were all impaired in this biofilm formation. The presence of the PGA-degrading enzyme dispersin B in the growth media prevented biofilm formation by Pba overexpressing PleD*, further supporting the importance of PGA for biofilm formation by Pba. Importantly, a pga mutant exhibited a reduction in root binding to the host plant under conditions of high intracellular c-di-GMP levels. A modest but consistent increase in pga transcript levels was associated with high intracellular levels of c-di-GMP. Our results indicate tight control of PGA-dependent biofilm formation by c-di-GMP in Pba.

Abbreviations: CR, Congo red; CV, crystal violet; DGC, diguanylate cyclase; EPS, exopolysaccharide; ESI, electrospray ionization; Pba, Pectobacterium atrosepticum; PGA, poly-β-1,6-N-acetyl-D-glucosamine.
bacterium is resistant to removal by agents such as detergents, proteases, heat, sonication and vortex agitation. For this reason, it is considered that A. actinomycetemcomitans harbours the dspB gene encoding a β-(1,6)-N-acetyl-D-glucosaminidase (E.C.3.2.1.52) to enable the release of single cells from the biofilm to allow dispersal and subsequent colonization of new surfaces (Kaplan et al., 2003). In Escherichia coli, the RNA-binding protein CsrA has been described as the main regulator of PGA production, by post-transcriptional repression of the pgaABCD operon (Wang et al., 2005). CsrA has also been associated with c-di-GMP metabolism in E. coli, in which this protein regulates the expression of several GGDEF proteins at the post-transcriptional level (Jonas et al., 2008). The regulation of PGA by c-di-GMP is not well defined, and recently c-di-GMP has been proposed to control expression of the E. coli pgaABCD operon at post-transcriptional and post-translational levels (Boehm et al., 2009; Tagliabue et al., 2010). A role for c-di-GMP in the regulation of PGA synthesis by the hmsHFRS operon of the animal pathogen Yersinia pestis has also been described (Bobrov et al., 2008, 2011; Kirillina et al., 2004). The hmsHFRS genes are essential for biofilm development in vitro, for colonization and eventual blockage of the proventriculus in fleas, and for colonization of the mouthparts of Caenorhabditis elegans (Darby et al., 2002; Hinnebusch & Erickson, 2008; Kirillina et al., 2004).

Despite extensive investigations into biofilm formation by human and animal pathogens, there is a comparative paucity of studies on biofilm formation in phytopathogens. Therefore, we decided to investigate the capacity for biofilm formation by a genomically sequenced strain (SCRI1043) of the blackleg-causing potato pathogen Pectobacterium atrosepticum. These investigations initially found that this strain had very poor biofilm-forming capacity under routine laboratory conditions. However, the results of a genetic approach revealed crypticity in biofilm formation capabilities and allowed us to identify N-acetylglucosamine as an EPS indispensable for robust biofilm assembly in this bacterium. The biofilm-forming phenotype in this strain could be activated from its cryptic biofilm assembly in this bacterium. The biofilm-forming phenotype in this strain could be activated from its cryptic biofilm assembly in this bacterium. The biofilm-forming phenotype in this strain could be activated from its cryptic biofilm assembly in this bacterium. The biofilm-forming phenotype in this strain could be activated from its cryptic biofilm assembly in this bacterium.

**METHODS**

**Bacterial strains, plasmids and culture conditions.**

Pectobacterium atrosepticum (Pba) SCRI1043 is a wild-type strain (Hinton & Salmond, 1987). E. coli β2163 (Demarre et al., 2005) was used as a donor strain for the conjugal transfer of plasmid pDS1028 into Pba. Plasmid pRP89 expresses PleD*, a mutant variant of the diguanylate cyclase (DGC) PleD from C. crescentus, from the vector pET11 (Paul et al., 2004). Plasmid pRP89 ΔNcol is a pRP89 derivative with an Ncol internal deletion of 1141 bp of the 1380 bp of pldD. Overnight and starting cultures of Pba were routinely grown in Luria-Bertani medium (LB) (per litre: 10 g tryptone, 5 g yeast extract, 5 g NaCl) at 25 °C. Minimal medium (MM) (Robertson et al., 1981) and Pel minimal medium (PMM) (Corbett et al., 2005) were used in 96-well microtitre plate biofilm assays. Where required, antibiotics and other compounds were added at the following final concentrations: ampicillin (Ap), 100 μg ml⁻¹; chloramphenicol (Cm), 25 μg ml⁻¹; kanamycin (Kn), 25 μg ml⁻¹; IPTG, 50 μM; Congo red (CR), 50 μg ml⁻¹; dispersin B (Kane Biotech), 50, 5 and 0.5 μg ml⁻¹; glucose, 0.2%; N-acetyl-D-glucosamine (NAG), 0.1%.

**Molecular techniques.** Routine molecular biology techniques were performed according to standard protocols and the manufacturers’ instructions. RNA extractions for real-time RT-PCR were generally carried out from independent triplicate starter cultures of each strain, using the Qiagen RNeasy RNA purification kit (Qiagen) and following the manufacturer’s instructions. Total RNA (1 μg) treated with RNase-free Dnase I (Roche) was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) and random hexamers (Roche) as primers. Quantitative real-time PCR was performed on an iCycler iQ5 (Bio-Rad). Each 25 μl reaction contained 1 μl cDNA, 200 nM of each primer and iQ SyBrGreen Supermix (Bio-Rad). Control PCRs of the RNA samples not treated with reverse transcriptase were also performed to confirm the absence of contaminating genomic DNA. Samples were initially denatured by heating at 95 °C for 3 min, followed by a 35-cycle amplification and quantification programme (95 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s). Melting curve analysis was conducted to ensure amplification of a single product. The oligonucleotide sequences for quantitative PCR were: pgaA-F (5’-TTTGGCTCTGGCTTTCTCCG-3’), pgaA-R (5’-TCGCTATCGGATGGCAA-3’), recA-F (5’-GGTGAGCCTGTGATCTGCG-3’) and recA-R (5’-GACATCCGCTTAACCTCAGCC-3’). The efficiency of each primer pair (E) was determined by running 10-fold serial dilutions (four dilution series) of Pba genomic DNA as template and generating a standard curve by plotting the log of the dilution factor against the Ct value during amplification of each dilution. Amplification efficiency was calculated using the formula $E = (10^{1/ (Ct - C)} - 1) \times 100$, where α is the slope of the standard curve. The relative expression of the pgaA gene was normalized to that of recA, which was used as reference gene, as described by Takle et al. (2007).

**Mutagenesis strategy for Pba and mapping transposon insertion sites.** To generate random transposon mutants of the Pba wild-type strain, a Cm resistance (Cm r)-bearing transposon (pDS1028 plasmid; Smith, 2005) was mobilized from strain β2163 as described by Demarre et al. (2005). Approximately 10² Pba Cm transposon insertion mutants were obtained. These transposon mutants were pooled and electroporated en masse with 100 ng of pRP89 plasmid. Transformants were selected on LB plates supplemented with CR, Cm and Ap (the last to select for pRP89). Seven CR- colonies were isolated (PbaCm2 to PbaCm8) for further biofilm assays and their transposon insertion sites were determined as previously described (Pérez-Mendoza & de la Cruz, 2009). Briefly, 50 ng of genomic DNA from each PbaCm strain was digested with Csp61 endonuclease. Five nanograms of the digested genomic DNA was relaxed in a final volume of 20 μl and incubated overnight at 16 °C. Five micro litres of the ligation reaction was used as template for an inverse PCR using oligonucleotides pDS1028-F (5’-CATGTTGACAGCITATTACCG-3’) and pDS1028-R (5’-GGTTAAAGGCAACCAAATCTGC-3’). The amplified PCR products were purified from agarose gels and both ends were sequenced using primers pDS1028-F and pDS1028-R. DNA sequence homology searches were performed with the BLAST program from NCBI (Altschul et al., 1997) to determine precise positions of the transposon insertions.

**Biofilm assays.** The strains to be tested were grown overnight in 5 ml LB and diluted to OD₅₆₀ 0.1 in sterile media (LB, LB + glucose, LB + NAG, PMM or MM). Then, 200 μl samples of diluted culture
were aliquoted into the wells of sterile 96-well plates. Plates were left in a humid environment at 18, 25 or 30 °C for 4, 8, 18, 42, 66 or 162 h. For detachment assays, dispersin B dissolved in LB (to final concentrations of 50, 5 or 0.5 μg ml⁻¹) was added to a 2 day-old biofilm formed by Pba pRP89 and incubated for 6 h. The liquid from the wells was removed by aspiration and washed with 300 μl deionized water before 240 μl crystal violet (CV; 0.1% in water) was added to each well and left to stain for 1 h. The CV was removed by aspiration, and each well was washed carefully three times with 300 μl deionized water. Subsequently, 250 μl 50% ethanol was added to each well and the plate was gently agitated for at least 1 h. The purple colour was quantified by measurement of A₅₉₅ in a Sunrise microplate reader (Tecan).

c-di-GMP extraction. c-di-GMP was extracted by heat, employing an adaption of the protocol described by Amikam et al. (1995). Three replicates of each Pba strain were grown in conical flasks with 5 ml LB in shaking waterbaths at 250 r.p.m. and incubated at 25 °C from a starting culture (diluted to an initial OD₆₅₀ of 0.1) for 24 h. Formaldehyde was added to a final concentration of 0.19% and the cells were then harvested by centrifugation (10 min at 3220 g). The pellet was washed in 1 ml ice-cold deionized water and centrifuged for 3 min at 15,700 g. The pellet was resuspended in 0.5 ml ice-cold deionized water and heated to 95 °C for 5 min. A volume of 925 μl ice-cold absolute ethanol was added to reach a final concentration of 65%. Nucleotides were extracted by vortexing for 30 s, followed by a centrifugation step (3 min at 15,700 g). Supernatants with extracted nucleotides were transferred to new tubes. A second extraction was carried out after adding 0.5 ml 65% ice-cold ethanol. The combined supernatants were then evaporated to dryness at 40 °C in a speed-vacuum. The pellet was resuspended by vigorous vortexing in a fixed volume of 10 mM ammonium acetate (pH 5.5) depending on the OD₆₅₀ of the original culture, to standardize all samples (300 μl per OD₆₅₀ unit). Samples were filtered through 0.45 μm pore-size GHP membranes (GHP Acrodisc; Pall) and 200 μl samples were analysed by reversed-phase-coupled HPLC-MS/MS. For quantification, a standard curve was established using synthetic c-di-GMP (Axxor) dissolved in ammonium acetate (10 mM, pH 5.5) at a range of concentrations (31.25 nM, 62.5 nM, 125 nM, 250 nM, 500 nM, 1 μM and 10 μM).

HPLC analysis and ESI-MS. All samples were analysed by HPLC, performed on an Agilent 1100 coupled to a 3 × 125 mm Waters Spherisorb 5 μm ODS2 (C-18) column. Running conditions were optimized using synthetic c-di-GMP as a reference. Electrospray ionization (ESI)-MS mass spectra were measured on an Esquire 6000 (Bruker Daltonics) and on a TSQ7000 (Finnigan) mass spectrometer. Matrix-assisted laser desorption ionization spectra were measured on a Reflex III spectrometer (Bruker Daltonics). To confirm the identity of the substance, relevant peaks were fragmented by ESI-MS using the positive ion mode. Three major ions were visible in the c-di-GMP fragmentation pattern: m/z 152 and 540 corresponded to products from single bond fragmentation, and 248 originated from double bond fragmentations. The area of the ion m/z 540 peak was used to estimate the amount of c-di-GMP in each sample (similar values were obtained with the replicates; data not shown).

Transduction of a pga mutation into a clean genetic background. The insertion PbaCam8 was moved into a clean genetic background by generalized transduction mediated by bacteriophage λM1, as previously described (Toth et al., 1997).

Potato root-binding assays. Strains of Pba to be tested were grown overnight to exponential phase in 10 ml LB broth at 25 °C. Root-binding assays were performed using 1 month old potato roots (cultivar Desiree) which had been removed from the plant, washed and surface-sterilized according to Coombs & Franco (2003), For each strain, 0.35 g roots were added to 40 ml of a 10⁷ c.f.u. ml⁻¹ culture in a 50 ml centrifuge tube, then gently agitated for 24 h. Following this, the roots were removed and washed vigorously five times for 5 min in 20 ml 10 mM MgSO₄. The roots were subsequently ground to a fine slurry, using an Ultra-Turrax T25 (IKA), with 10 ml 10 mM MgSO₄. From the resulting slurry, 100 μl was removed and serial dilutions were spread onto selective plates to enumerate bound Pba cells. Bacterial numbers in the inocula were verified by serial dilution and viable counts on selective plates. Results were expressed as the mean c.f.u. (g root)⁻¹ with three biological replicates per strain. Error bars represent ±1 SD.

RESULTS

High levels of c-di-GMP promote biofilm formation in Pba

Pba strain SCRI1043 did not exhibit appreciable biofilm formation when grown in LB under routine laboratory conditions (Fig. 1a). Different rich media (LB supplemented with 0.2% glucose or 0.1% NAG) and minimal media (MM [Robertsen et al., 1981] and PMM [Corbett et al., 2005]) were tested under various growth conditions (at 18, 25 or 30 °C for 4, 8, 18, 42, 66 or 162 h) without any appreciable biofilm formation being observed, using a standard 96-well microtitre plate biofilm assay (data not shown). Preliminary studies suggested that c-di-GMP could positively regulate biofilm formation in this bacterium (data not shown). To confirm this result, the impact of expressing a heterologous DGC (PleD⁺) in Pba was tested, with the aim of generating an increase in intracellular levels of this second messenger and determining its impact on biofilm formation. PleD⁺ is a mutant variant of the well-characterized DGC PleD from C. crescentus. PleD⁺ contains four point mutations which generate a constitutively active DGC, independent of its phosphorylation status (Aldridge et al., 2003). Overexpression of PleD⁺ in Pba (Pba pRP89) produced a strong and visually obvious biofilm that was easily quantifiable after CV staining in microtitre plates (Fig. 1). Plasmid pRP89 promoted biofilm formation in all media (LB, LB + glucose 0.2%, LB + NAG 0.1%, PMM and MM; data not shown) and all conditions tested (at 18, 25 and 30 °C; data not shown), showing appreciable biofilm formation from 4 h up to more than 6 days (Fig. 1c). A derivative of pRP89 containing an internal NcoI deletion in pleD⁺ was used as a negative control (pRP89ΔNcoI, see Methods). This NcoI deletion abolished biofilm formation in Pba, confirming the activating effect of PleD⁺ on biofilm biogenesis (Fig. 1). MS was used to confirm that PleD⁺ overexpression did indeed generate an increase in intracellular levels of c-di-GMP in Pba (see Methods). A cell extract sample from an overnight culture of Pba harbouring pRP89 contained 0.726 μM c-di-GMP. In contrast, a parallel cell extract from Pba harbouring pRP89ΔNcoI contained undetectable levels of c-di-GMP. These results strongly suggested that the promotion of biofilm formation generated by overexpression of PleD⁺
was caused by an increase in intracellular levels of the second messenger c-di-GMP.

**Identification of c-di-GMP-modulated genes involved in Pba biofilm formation**

A strategy was designed to identify genes involved in the synthesis or secretion of the unknown biofilm-promoting factor. A positive CR phenotype (CR\(^{+}\)) was observed in wild-type Pba under conditions of high c-di-GMP levels (Pba with pRP89; Fig. 2a). CR binds to \(\alpha\)-D-glucopyranosyl units, basic or neutral polysaccharides, and to some proteins (Spiers et al., 2002). These types of molecules have been described in a wide range of bacteria as c-di-GMP-regulated molecules involved in cell aggregation and biofilm formation (Borlee et al., 2010; Cotter & Stibitz, 2007; Lee et al., 2007; Tamayo et al., 2007). Therefore, we hypothesized that it was likely that the unknown c-di-GMP-regulated substance might be required for both biofilm formation and the easily screened CR\(^{+}\) phenotype in Pba.

Random transposon mutagenesis of the Pba wild-type strain was performed using a Cm\(^{+}\)-bearing transposon from pDS1028 (Smith, 2005). Approximately \(10^5\) random Pba Cm\(^{+}\) transposon insertion mutants were obtained, and these were pooled and transformed en masse with the pRP89 plasmid. Transformants were selected on LB plates supplemented with CR, Cm and Ap (the last to select for pRP89). A parallel transformation of the Pba wild-type strain with the pRP89 plasmid was performed as a control, with the transformants selected on plates containing CR and Ap. All the Pba wild-type Ap-resistant colonies showed a CR\(^{-}\) phenotype. However, among the mutagenized Pba Cm\(^{+}\) Ap\(^{+}\) red colonies (CR\(^{+}\)), a few white colonies were found (Fig. 2b), and seven of these white colonies, exhibiting a CR\(^{-}\) phenotype, were investigated further. pRP89 plasmid DNA from the seven selected colonies was extracted and used to independently retransform the Pba.

![Fig. 1](http://mic.sgmjournals.org/3343)
wild-type. All seven transformations produced only CR+ colonies, indicating that the CR− phenotype observed in the original transposon mutants was a consequence of chromosomal transposon insertions and not due to a mutation in pRP89. Furthermore, a selected insertion (PbaCm8) was reintroduced into the wild-type Pba background by generalized transduction using bacteriophage wM1 (Toth et al., 1997). Two of these transductants were transformed with pRP89, generating CR+ colonies. This confirmed that the CR+ phenotype observed in the original transposon mutant was indeed a direct consequence of the transposon insertion.

The transposon insertion points of each of the seven mutants were determined (Bell et al., 2004; see Methods). Sequence analysis showed that, in all the mutants, the transposon had inserted into the same region of the Pba genome (interrupting ECA4452, ECA4453 or ECA4454; Fig. 3) and that this locus constituted a putative operon. Furthermore, six of the seven insertion points were different, indicating that these mutants were not siblings and arose from independent transposition events (Fig. 3).

### PGA is required for Pba biofilm formation

Biofilm assays were carried out with the seven selected CR− transposon insertion mutants (PbaCm2–PbaCm8, each bearing pRP89), together with positive (Pba pRP89) and negative controls (blank and Pba pRP89 ΔNcol). Fig. 4 clearly shows that all of these CR− transposon insertion mutants were also impaired in biofilm formation.

The ECA4451–54 genes display sequence homology and synteny with pga/ica operons of other bacteria (e.g. the proteins encoded by ECA4451–54 share 59, 70, 83 and 41 % similarity with the PgaABC and D proteins of E. coli, respectively). The pga operon has been described as a locus involved in synthesis, translocation and possibly surface docking of PGA in E. coli (Wang et al., 2004). A commercially available version of a PGA-degrading enzyme (dispersin B) has been used to confirm the role of PGA in biofilm formation by various bacteria (Izano et al., 2007, 2008; Kaplan et al., 2004; Parise et al., 2007). The presence of dispersin B at 50 μg ml−1 (or at 5 and 0.5 μg ml−1; data not shown) in LB media in a standard biofilm assay completely prevented biofilm formation by Pba, despite high levels of c-di-GMP in the assayed strain (Pba pRP89; Fig. 4). Furthermore, subsequent addition of dispersin B completely disrupted a previously formed robust (48 h) Pba biofilm, within 6 h of treatment (Fig. 4). These findings strongly suggest that PGA functions as an essential biofilm matrix polysaccharide in Pba.

### Influence of PGA on Pba interaction with the host plant

As PGA was shown to play an essential role in the attachment of Pba to an abiotic surface (Fig. 4), we then investigated whether PGA also plays a role in binding of Pba to biotic surfaces. The root-binding ability of a selected PGA biosynthesis mutant (PbaCm8) was evaluated and compared with the wild-type, under physiological conditions or high intracellular levels of c-di-GMP (presence of pRP89). The ability of wild-type Pba to bind/colonize potato roots was more than 20-fold higher than that of PbaCm8 in the presence of high levels of c-di-GMP.
(Fig. 5). However, no statistically significant difference was observed between wild-type *Pba* and *PbaCm8* in the absence of pRP89 under the conditions tested (Fig. 5). It is also noteworthy that there was a reduction in the root-binding ability of *PbaCm8 pRP89* \(\Delta\text{NcoI}\) compared with *PbaCm8 pRP89*, implying the presence of (an)other, unknown c-di-GMP-regulated factor(s) in *Pba*, apart from PGA, involved in binding to the host plant. No significant differences between wild-type *Pba* and *PbaCm8* in the tuber rotting virulence assay, performed as described previously (Coulthurst et al., 2006), were observed (data not shown).

### The secondary messenger c-di-GMP regulates expression of the PGA operon at the transcriptional level in *Pba*

The results described above revealed that high levels of c-di-GMP promote the production of PGA, a polysaccharide matrix that, as in some other bacteria, is required for biofilm formation in *Pba*. To analyse the expression of the *Pba* PGA operon in the presence of high levels of c-di-GMP, transcript levels of *pgaA* were assessed by real-time RT-PCR in the *Pba* wild-type strain in the presence (*Pba pRP89*) or absence (*Pba pRP89* \(\Delta\text{NcoI}\)) of a functional

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**Fig. 4.** Biofilm formation by strains of *Pba* SCRI1043 with insertions in the PGA biosynthetic operon and expressing *PleD*\*.

All strains were grown statically for 48 h in LB in a 96-well microtitre plate, with or without dispersin B (DispB\_1; 50 \(\mu\)M) at 25 °C. For the detachment assay (DispB\_2), a 48 h pre-grown biofilm of *Pba* SCRI1043 expressing *PleD*\* was supplemented with dispersin B at 50 \(\mu\)M (final concentration) and incubated for a further 6 h. Similar growth of all strains was confirmed by measuring their OD\_600 before analysis of biofilm formation by CV staining (\(A_{595}\)). CV staining was quantified from eight different wells for each strain; error bars, SD.

**Fig. 5.** Effect of PGA on the *Pba* SCRI1043 interaction with the host plant, potato. The figure shows a root-binding assay comparing the ability of different strains of *Pba* to interact with the potato plant. The graph shows the number of c.f.u. of wild-type *Pba* and a selected *pga* mutant (*PbaCm8*), carrying pRP89 or pRP89\(\Delta\text{NcoI}\), recovered from being bound to potato roots after 24 h incubation (see Methods). Bars represent the mean of three biological replicates; error bars, SD; a, b and c denote analysis of variance categories for significant differences at \(P<0.05\).
PleD*. High levels of c-di-GMP (Pba pRP89) produced a twofold increase in transcription of pgaA in Pba (2.03 ± 0.25). This modest upregulation of transcript levels of pgaA in the presence of high c-di-GMP levels may suggest that this is not the only route through which c-di-GMP affects Pba biofilm formation.

**DISCUSSION**

The second messenger c-di-GMP has emerged as a crucial molecule in the transition between motile and sessile states, in what has been termed ‘stick-or-swim’. Consequently, various studies correlating c-di-GMP and biofilm formation in diverse bacteria are under way. More investigation will be required to fully understand how bacteria ‘decide’ how and when to modulate production of this signal during physiological transitions under different environmental conditions.

To the best of our knowledge, this is the first report describing the ability of Pba to form significant biofilms. Biofilm formation was induced under laboratory conditions, but only in the presence of artificially high levels of c-di-GMP. This crypticity of effective biofilm-forming capacity suggests that the pathogen could produce biofilms in response to other environmental or physiological cues that exist outside a laboratory environment, for example in response to plant signals that may operate via modulation of c-di-GMP levels in the pathogen. In other plant-interacting bacteria (Rhizobium leguminosarum), induction of c-di-GMP-dependent cellulose biosynthesis by plant products has also been suggested (Ausmees et al., 1999). Indeed, in addition to the c-di-GMP-related domains, GGDEF and EAL proteins often contain other sensory or signal transduction domains. This modularity suggests a great flexibility and range of functions for c-di-GMP signalling (Römling et al., 2005).

The development of a genetic approach to identify genes involved in Pba biofilm formation in the presence of high levels of c-di-GMP allowed us to identify PGA as a key EPS. The random coverage of the Pba genome in the mutagenesis, together with the observation that all of the mutants impaired in biofilm formation carried insertions in the same operon (ECA4451–54), emphasizes the key role for this cluster in Pba biofilm formation. The role of PGA in Pba biofilm formation was further confirmed by the observation that addition of β-(1,6) N-acetyl-d-glucosaminidase, or dispersin B, to the media completely prevented and disrupted Pba biofilms.

The importance of PGA in the ecology of Pba is highlighted by the significant reduction in plant-binding capacity observed in a PGA mutant compared with the wild-type (Fig. 5). However, this reduction was only observed under ‘high PGA-production conditions’ (high levels of c-di-GMP, directed by pRP89). This result suggests that the hypothetical environmental cue(s) responsible for elevating c-di-GMP levels in order to switch on secreted PGA production do(es) not seem to operate in our laboratory potato root-binding assay. Future experiments will focus on identifying the environmental signals involved in triggering PGA-dependent biofilm formation in Pba. On the other hand, the small increase in root-binding ability of a PGA mutant strain (PbaCm8) still induced by the presence of pRP89 (Fig. 5) suggests the presence of (an)other c-di-GMP-regulated adhesion factor(s) with an important role in binding to plant roots. Recent work in our laboratory is consistent with this idea; specifically, the identification of a proteinaceous adhesion factor (MRP) with a key role in root binding and whose production/secretion is regulated by c-di-GMP in Pba (Pérez-Mendoza et al., 2011). The absence of an observable difference in the tuber rotting assay between a PGA mutant and the wild-type is perhaps unsurprising because, in this assay (Coulthurst et al., 2006), Pba is inoculated directly into the tuber, minimizing the impact of any bacterial attachment/colonization defect.

One of the most intriguing properties of c-di-GMP is the wide-ranging ability of this molecule to regulate functions at different levels [e.g. transcriptional regulation (Cotter & Stibitz, 2007), allosteric regulation (Paul et al., 2007; Ross et al., 1990), interaction with transcription factors (Hickman & Harwood, 2008) or directly with RNA molecules (Jonas et al., 2008; Sudarsan et al., 2008)]. In the particular case of EPS, its biosynthesis may be regulated at both transcriptional and post-transcriptional levels in different organisms. In Pseudomonas aeruginosa, the synthesis of the three known EPSs involved in biofilm formation, alginate, Pel and Psl, is regulated by c-di-GMP (Ryder et al., 2007). In one classic example, the cellulose synthase activity of Glucanacetobacter xylinus is allosterically controlled by c-di-GMP (Ross et al., 1987). Similarly, an allosteric activation for the glycosyltransferase HsmR, involved in PGA synthesis of Y. pestis, has also been suggested (Bobrov et al., 2008). Notwithstanding the growing evidence for involvement of this second messenger in NAG-dependent biofilm formation in diverse bacteria, there appear to be multiple ways in which c-di-GMP exerts its action in biofilm regulation. In E. coli, the second messengers c-di-GMP and ppGpp regulate biofilm induction upon ribosomal stress. Biofilm induction involves the upregulation of at least two components of the E. coli PGA biosynthesis machinery (PgaA and PgaD; Boehm et al., 2009). The molecular mechanism through which these two secondary messengers regulate PGA production remains to be elucidated. However, the unaltered levels of β-galactosidase activity observed (with a transcriptional lacZ fusion to the pga promoter following perturbations of ppGpp or c-di-GMP levels) argue in favour of post-transcriptional regulation (Boehm et al., 2009). By contrast, Tagliabue et al. (2010) have shown that c-di-GMP positively upregulates transcript levels of the E. coli pgaABCD operon. Furthermore, those authors demonstrate that PGA production does not simply respond to intracellular c-di-GMP concentration, but specifically...
requires the DGC activity of the YddV protein and is not affected by the overexpression of two other active DGCs, AdrA and YcdT (Tagliabue et al., 2010).

Pba encodes a GGDEF protein (EC1681) displaying 38% identity with YddV. However, we demonstrated that an increase in the intracellular level of c-di-GMP, generated by overexpression of a heterologous DGC from C. crescentus, is enough to promote formation of a robust biofilm in Pba. At least in part, this c-di-GMP effect seems to be mediated by an increase in the transcript levels of the pgaABCD operon. Nevertheless, the modest extent of the upregulation observed may suggest that this is not the only route through which c-di-GMP affects Pba biofilm formation.

ACKNOWLEDGEMENTS

Work in the Cambridge lab was funded by the BBSRC, UK. D. P. M. was initially supported by a postdoctoral EMBO long-term fellowship and later by a JAE postdoctoral contract from Consejo Superior de Investigaciones Científicas (CSIC), Spain. D. Rodríguez Carbonell and S. Muñoz are acknowledged for technical assistance. We thank members of the Salmond group for helpful suggestions and Rafael Leon for his help in providing potato plants.

REFERENCES


Edited by: H.-M. Fischer