Genes expressed by the biological control bacterium *Pseudomonas protegens* Pf-5 on seed surfaces under the control of the global regulators GacA and RpoS

Teresa A. Kidarsa,1 Brenda T. Shaffer,1 Neal C. Goebel,2 Daniel P. Roberts,3 Jeffrey S. Buyer,3 Aaron Johnson,4 Donald Y. Kobayashi,5 T. Mark Zabriskie,2 Ian Paulsen6 and Joyce E. Loper1*

1 USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR, USA.
2 Department of Pharmaceutical Sciences, Oregon State University, Corvallis, OR, USA.
3 USDA-ARS Sustainable Agricultural Systems Laboratory, Beltsville, MD, USA.
4 J. Craig Venter Institute, Rockville, MD, USA.
5 Department of Plant Biology and Pathology, Rutgers University, New Brunswick, NJ, USA.
6 Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia.

**Summary**

Gene expression profiles of the biological control strain *Pseudomonas protegens* Pf-5 inhabiting pea seed surfaces were revealed using a whole-genome oligonucleotide microarray. We identified genes expressed by Pf-5 under the control of two global regulators (GacA and RpoS) known to influence biological control and secondary metabolism. Transcript levels of 897 genes, including many with unknown functions as well as those for biofilm formation, cyclic diguanylate (c-di-GMP) signalling, iron homeostasis and secondary metabolism, were influenced by one or both regulators, providing evidence for expression of these genes by Pf-5 on seed surfaces. Comparison of the GacA and RpoS transcriptomes defined for Pf-5 grown on seed versus in broth culture overlapped, but most genes were regulated by GacA or RpoS under only one condition, likely due to differing levels of expression in the two conditions. We quantified secondary metabolites produced by Pf-5 and gacA and rpoS mutants on seed and in culture, and found that production profiles corresponded generally with biosynthetic gene expression profiles. Future studies evaluating biological control mechanisms can now focus on genes expressed by Pf-5 on seed surfaces, the habitat where the bacterium interacts with seed-infecting pathogens to suppress seedling diseases.

**Introduction**

The soil bacterium *Pseudomonas protegens* Pf-5 (formerly *Pseudomonas fluorescens* Pf-5; Ramette et al., 2011) colonizes the surfaces of seeds and roots and can protect plants from infection by certain soil-borne fungal, oomycete and bacterial plant pathogens (Howell and Stipanovic, 1979; 1980; Xu and Gross, 1986; Kraus and Loper, 1992; Pfender et al., 1993; Rodriguez and Pfender, 1997; Sharifi-Tehrani et al., 1998). Secondary metabolite production is an important contributing factor for the biological control of many plant diseases by *Pseudomonas* spp. (Haas and Keel, 2003; Rezzonico et al., 2007; Weller et al., 2007), and Pf-5 produces a spectrum of secondary metabolites with antibiotic activity, including 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin, hydrogen cyanide, rhizoxin analogues and orfamide A (Howell and Stipanovic, 1979; 1980; Nowak-Thompson et al., 1994; Gross et al., 2007; Loper et al., 2008). A similar suite of antibiotics is produced by the biological control strain CHA0, which is closely related to Pf-5 (Haas and Keel, 2003). Antibiotic production by strains Pf-5 and CHA0 is regulated at multiple levels, including by the Gac/Rsm signal transduction pathway (Lapouge et al., 2008) and the stationary-phase sigma factor RpoS (αS; Sarniguet et al., 1995; Heeb et al., 2005).

In the Gac/Rsm signal transduction pathway, GacA acts as a positive transcriptional regulator of genes encoding the small regulatory RNAs, RsmX, RsmY and RsmZ (Heeb et al., 2002; Valverde et al., 2003; Kay et al., 2005). These small RNAs sequester the RNA-binding proteins RsmA and RsmE, relieving translational repression of genes having upstream binding sites for these repressor proteins (Valverde et al., 2003; Kay et al., 2005). GacA also has extensive effects on the transcriptome of *Pseudomonas* spp.: transcript levels of nearly 10% of the protein-encoding genes in the Pf-5 genome are influenced by GacA (Hassan et al., 2010), although this is
likely due largely to indirect regulation via other genes under translational regulation by the Gac/Rsm pathway, as in *Pseudomonas aeruginosa* (Brencic and Lory, 2009). Among the many genes regulated by GacA and its cognate sensor kinase GacS are those required for production of secondary metabolites and secreted exoenzymes, such as chitinase and the AprA protease (Lapouge et al., 2008; Hassan et al., 2010).

The RpoS sigma factor directs RNA polymerase to promoters of certain genes transcribed in response to stress or during the transition to stationary phase (Hengge-Aronis, 2002), directly or indirectly regulating the expression of a large set of genes having diverse functions (Schuster et al., 2004; Battesti et al., 2011). An rpoS mutant of Pf-5 shows reduced tolerance to oxidative and osmotic stress, as well as to freezing, starvation, UV irradiation and desiccation (Sarniguet et al., 1995; Stockwell and Loper, 2005). RpoS has differential effects on secondary metabolite production in Pf-5, with an rpoS mutant of Pf-5 overproducing pyoluteorin and DAPG, while failing to produce pyrrolnitrin (Pfender et al., 1993; Sarniguet et al., 1995).

Both the Gac/Rsm system and RpoS affect the ability of Pf-5 or CHA0 to act as biological control agents, gacS, gacA, rsmX, rsmY or rsmZ mutants of CHA0 are deficient in biological control of several plant diseases (Schmidli-Sacherer et al., 1997; Valverde et al., 2003; Zuber et al., 2003; Kay et al., 2005). An rpoS mutant of Pf-5 showed enhanced biological control of postemergence damping-off of cucumber caused by *Pythium ultimum* (Sarniguet et al., 1995), but a reduced ability to inhibit *Pyrenophora tritici-repentis* in wheat straw (Pfender et al., 1993). Because the Gac/Rsm system and RpoS have such broad effects on the physiology of the bacterium, it is unclear whether their influence on biological control is mediated solely through changes in secondary metabolite production versus other important traits, such as the ability to efficiently colonize seed or root surfaces.

To effect biological control of seed-infecting pathogens, Pf-5 must express genes needed to suppress target pathogens, as well as those required for growth and survival in the spermosphere, the region of the soil under the influence of seeds. The main energy source in the spermosphere is reduced carbon released from germinating seeds in the form of exudate (Nelson, 2004). Propagules of many fungal and oomycete plant pathogens can remain dormant in the soil for months or years but germinate quickly in response to seed exudates, producing mycelia that can infect seeds. Consequently, the spermosphere is often the initial point of interaction between pathogens infecting seeds and biological control microorganisms and represents a rich and dynamic habitat for microbial interactions (Nelson, 2004). Nutrients present in seed exudates are targets of competition between microorganisms and also may influence the expression of biocontrol traits by bacteria on the seed surface. Indeed, the availability and composition of carbon and other nutrients are major factors influencing antibiotic production by *P. protegens* Pf-5 and CHA0 (Nowak-Thompson et al., 1994; Kraus and Loper, 1995; Duffy and Défago, 1999; Haas and Keel, 2003; Haas and Défago, 2005; Valverde and Haas, 2008; Humair et al., 2009).

The primary focus of this work was to identify genes expressed under the control of GacA and RpoS by *P. protegens* Pf-5 inhabiting seed surfaces. Because both GacA and RpoS influence biological control by *Pseudomonas* spp., the GacA and RpoS transcriptomes of Pf-5 inhabiting the spermosphere are expected to include genes contributing to biological control. We defined and compared the GacA and RpoS transcriptomes of Pf-5 grown in culture and on seed surfaces. While many genes were regulated by GacA and RpoS both in culture and on seed surfaces, the majority of genes were regulated significantly under only one condition, thus greatly expanding the list of genes known to be under the control of these global regulators. This study provides evidence for the expression of hundreds of genes by Pf-5 inhabiting seed surfaces and highlights GacA- and RpoS-regulated genes that could contribute to biological control of seed-infecting pathogens.

**Results and discussion**

The major goal of our study was to determine the effects of GacA and RpoS on gene expression of Pf-5 grown on seed surfaces. We assessed the transcriptomes of Pf-5 and gacA and rpoS mutants on surface-sterilized pea seed, as well as the chemical composition of the pea seed exudate (Table S1). Initial experiments showed that the population size of Pf-5 increased rapidly in the first 24 h following seed inoculation (from ~10^7 cfu per seed to ~10^8 cfu per seed), whereas minimal additional growth occurred in the subsequent 24 h period. Our observation that the population size of Pf-5 on seed surfaces reaches carrying capacity at 24 h after the onset of seed imbibition is consistent with the temporal pattern of pea seed exudation, as the highest levels of exudation occur during the initial hours after the onset of imbibition, with exudation diminishing 24 h after sowing (Roberts et al., 1999; 2000). Preliminary reverse-transcriptase real-time PCR (RT-qPCR) analysis showed that wild-type and mutant bacteria grown on seed surfaces for 24 h displayed expression differences in genes previously found to be regulated by GacA or RpoS in culture, including genes involved in the production of secondary metabolites (data not shown). Furthermore, gene expression by biological control agents of seed-infecting pathogens is particularly important during the first 24 h after sowing when seed exudates...
trigger germination of pathogen propagules, which colonize seed during this period (Nelson, 2004). These considerations led us to choose the 24 h time point to assess the GacA and RpoS transcriptomes of PF-5 on seed surfaces. Genes regulated significantly by GacA or RpoS were identified from a microarray representing each of the 6147 annotated protein-coding genes in the PF-5 genome (Hassan et al., 2010) using the Significance Analysis of Microarrays (SAM) method (Tusher et al., 2001). In addition, we imposed a fold change criterion of approximately 1.4 (log2 ratio of 0.5 or greater), thereby eliminating genes having low fold changes while including genes that had moderate fold changes, many of which are in operons with other significantly regulated genes. Using these criteria, we identified 595 genes that were differentially expressed in the gacA mutant relative to PF-5 on seed and 476 genes that differed significantly between the rpoS mutant and PF-5 on seed (Fig. 1A and Table S2). A total of 897 genes were differentially expressed in either the rpoS or the gacA mutant or both relative to PF-5 on pea seed surfaces (Fig. 1A and Table S2). Because the detection of differential expression requires measurable transcript levels, these microarray experiments provide evidence for the transcription of these 897 genes by PF-5 on seed surfaces.

The GacA and RpoS transcriptomes of PF-5 inhabiting seed surfaces were compared with those of the bacterium grown in a rich culture medium, using previously published data for the GacA transcriptome of PF-5 in culture (Hassan et al., 2010) and a newly generated data set for the RpoS transcriptome in culture (Table S2). Cultures of PF-5 and the rpoS mutant were grown in the same medium and harvested at the same growth phase (early stationary phase) as was done in the previous study of the GacA transcriptome. This growth phase was appropriate for the RpoS microarray experiment because transcripts of rpoS are at high levels in early stationary-phase cultures of PF-5 (Hassan et al., 2010), and transcriptomes of an rpoS mutant and wild-type P. aeruginosa PAO1 differ markedly at early stationary phase (Schuster et al., 2004). After SAM analysis and imposition of the 1.4-fold change criterion to the RpoS microarray data, we identified a total of 262 genes that were differentially expressed in the rpoS mutant compared with PF-5, while 380 genes from the previously published GacA transcriptome (Hassan et al., 2010) met our fold change criterion and therefore were considered in this analysis (Fig. 1B; Table S2). RT-qPCR validation of a selected set of genes correlated well with the microarray data (Tables S3 and S4).

As an initial analysis, we looked at the degree of overlap in significantly expressed genes across all four experiments. The largest amount of overlap was between genes downregulated in both the gacA and the rpoS mutants on seed (142 genes; Fig. 1A). This is not surprising, given that GacA is known to positively influence both transcript levels of rpoS and accumulation of σS in PF-5 (Whistler et al., 1998; Hassan et al., 2010). In our study, transcript levels of rpoS (PFL_1207) did not differ significantly between the gacA mutant and wild-type PF-5 on seed surfaces. The large degree of overlap in the genes downregulated in both mutants may be due to posttranscriptional regulation of rpoS by GacA (Whistler et al., 1998; Heeb et al., 2005). Alternatively, GacA may have had larger effects on rpoS transcript levels at an earlier time, causing residual effects on transcript levels of downstream genes that were still apparent at the time point evaluated. Thus, genes regulated in the same direction in both mutants are likely regulated by GacA in a manner largely dependent on RpoS. Nevertheless, the majority of the significantly regulated genes were influenced by GacA or RpoS, but not both. These genes appeared to be controlled by GacA in an RpoS-independent manner or affected only when RpoS is completely lacking. For the 14 genes regulated in opposite directions by RpoS versus GacA on seed (Fig. 1A), RpoS-independent effects of
GacA may outweigh the influence that GacA has on RpoS expression. Similarly, comparison of the culture experiments show a total of 61 genes regulated in the same direction in gacA and rpoS mutants while only 12 genes were regulated in opposite directions in the two mutants (Fig. 1B).

For both GacA and RpoS, the vast majority of genes were significantly regulated either in culture or on seed surfaces but not under both conditions (Fig. 1C and D). This observation reinforces the importance of performing gene expression studies under conditions relevant to the process under study. Because a gene must be expressed to observe the effects of a mutation, we hypothesized that many of the genes that were significantly regulated under only one condition were not expressed well under the other condition. To estimate the relative expression of particular genes, we calculated the expression ratio of several target genes to the reference gene zwf (glucose-6-phosphate 1-dehydrogenase; PFL_4610) in wild-type Pf-5 grown under each condition (Fig. 2). We found that transcripts of genes that were significantly regulated under only one condition were at higher levels under that condition. These results do not explain all the observed differences between the two conditions, such as the 17 genes regulated in opposite directions by GacA or RpoS on seed versus in culture (Fig. 1C and D). Nevertheless, our results support our hypothesis that disparities between the lists of genes significantly regulated by GacA or RpoS on seed versus in culture are due in part to differing levels of expression in the two environments.

Role categories of genes in the GacA and RpoS regulons

Genes expressed by Pf-5 under the control of GacA and RpoS represent diverse aspects of bacterial physiology, falling into 18 functional role categories (http://cmr.jcvi.org/cgi-bin/CMR/shared/RoleList.cgi; Fig. S1). An overview of the GacA and RpoS regulons of Pf-5 was obtained through analysis of the role categories overrepresented in each set of regulated genes (Fig. S1 and Supplemental Text S1). Of particular note, the Hypothetical Proteins role category was overrepresented in the genes downregulated in both the gacA and the rpoS mutants on seed but not in culture. Of the 142 genes downregulated in both mutants on seed (Fig. 1A), 50 are hypothetical proteins, indicating that a large proportion of the genes in the GacA and RpoS regulons of Pf-5 grown on seed remain to be characterized. Also of note, the Transport and Binding Proteins role category is overrepresented in the list of genes upregulated in a gacA mutant on seed (Fig. S1). Thirty-six genes in this role category fall into the Amino Acids, Peptides and Amines subrole, which may be particularly important in nutrient acquisition by Pf-5 grown on seed or in culture (Fig. 1B).

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Pf-5 inhabiting pea seed surfaces, as analysis of the batch of seed used in the microarray study showed that amino acids are major components of the pea seed exudates (Table S1). The large number of GacA-regulated genes in this subrole could be due to higher expression of those genes by Pf-5 growing on seed surfaces versus in culture medium. For example, in Pf-5, transcript levels of PFL_0639, which encodes the urea ABC transporter permease UrtC, were 30-fold higher on seed surfaces than in culture (Fig. 2A).

Secondary metabolite and exoenzyme production

The gacA/gacS and rpoS mutants of Pf-5 were selected originally due to their altered production of the antifungal compounds pyoluteorin, DAPG and pyrrolnitrin (Corbell and Loper, 1995; Sarniguet et al., 1995; Whistler et al., 1998). gacA mutants also lack production of hydrogen cyanide (Whistler et al., 1998), rhizoxin and orfamide A (Gross et al., 2007; Hassan et al., 2010). In agreement with the known effects of these global regulators on antibiotic production by Pf-5, we observed a striking influence of GacA and RpoS on the transcript levels of secondary metabolite biosynthetic genes (Figs 3 and S2).

Of all the genes of Pf-5 represented on the microarray, those in the pyoluteorin biosynthetic gene region were the most highly upregulated in an rpoS mutant both on seed surfaces and in culture, which is in agreement with the overproduction of pyoluteorin by the rpoS mutant observed previously (Pfender et al., 1993; Sarniguet et al., 1995).
et al., 1995; Whistler et al., 1998). The plt genes accounted for 16 of the 21 genes upregulated in an rpoS mutant under both conditions (Table S2 and Fig. 3A). Only two genes (PFL_2794 and 2795) were significantly downregulated (~2-fold) in a gacA mutant on seed, despite the strong downregulation of certain plt genes (e.g. pltL, PFL_2796, ~8.7-fold) by a gacA mutant in culture (Hassan et al., 2010). The stronger downregulation of plt genes in culture may be due to higher expression of these genes under this condition. Indeed, we found that pltL transcript levels were approximately 23-fold higher in culture than on seed surfaces (Fig. 2A).

The biosynthetic genes for DAPG, phlACBD, were highly regulated by GacA on seed surfaces, with phlD (PFL_5957) showing the greatest fold change (~25-fold) of any gene on seed surfaces (Table S2). Genes encoding the DAPG-specific hydrolyase PhlG and the putative permease PhIE were also significantly regulated by GacA on seed surfaces. phlF, which encodes a transcriptional repressor, was significantly upregulated in an rpoS mutant on seed. Although the phlACBD and phlE genes were upregulated by more than twofold on average in the rpoS mutant on seed, these differences were not significant due to variation between replicates. The DAPG biosynthetic genes showed no significant regulation by either mutant in culture, likely due to the medium used, which supports only very low levels of DAPG production (Hassan et al., 2010). This is supported by our finding that the phlD gene (PFL_5957) is expressed at approximately 12-fold greater levels on seed surfaces than in culture in wild-type Pf-5 (Fig. 2A). Similarly, genes of the pyrrolnitrin biosynthetic operon pmnABCD were downregulated (>3-fold) on seed surfaces in both the gacA and the rpoS mutants (Fig. 3A), but these differences were not significant in the rpoS mutant due to variability between replicates. In culture, only pmnA showed slight but significant downregulation in an rpoS mutant. The variability in level of regulation of the DAPG and pyrrolnitrin genes on seed may suggest that RpoS regulation of secondary metabolite gene expression is transient and/or highly dependent on the exact physiological state of the bacteria, resulting in more variation between replicates than was seen for GacA. Nevertheless, these results are consistent with overproduction of DAPG and lack of production of pyrrolnitrin by an rpoS mutant (Pfender et al., 1993; Sarniguet et al., 1995; Whistler et al., 1998).

Six genes in the cluster for production of the cyclic lipopeptide orfamide A, including the three biosynthetic genes PFL_2145–2147, showed significant downregulation in the gacA mutant on seed surfaces, whereas only the transcriptional regulator PFL_2150 showed significant regulation by GacA (Hassan et al., 2010) or RpoS in culture. Genes for biosynthesis of rhizoxin analogues were downregulated in all four experiments (Table S2 and Fig. 3A). However, the level and number of genes downregulated was greater on seed than in culture for both the rpoS and the gacA mutants.

To determine whether the observed changes in gene expression correlated with differences in secondary metabolite production, we evaluated the spectrum of secondary metabolites produced by Pf-5 and the rpoS mutant grown for 48 h in culture under the same conditions used for our microarray experiments. The rpoS mutant overproduced pyoluteorin and DAPG, moderately overproduced orfamide A, and lacked pyrrolnitrin and rhizoxin production (Table 1). Hassan and colleagues (2010) found that DAPG, pyoluteorin, pyrrolnitrin and rhizoxin were below detection and orfamide A was reduced 64-fold in a gacA mutant of Pf-5 grown under these culture conditions. We also quantified each of these compounds from extracts of seeds supporting populations of Pf-5 or the gacA or rpoS mutant. On seed, Pf-5 produced DAPG, pyrrolnitrin, orfamide A and rhizoxin, but did not produce detectable levels of pyoluteorin (Table 2). Comparisons between Pf-5 and the gacA and rpoS mutants on seed provided further evidence for the essential role of gacA and the differential influence of rpoS in the production of these compounds. Based on the strong regulation of pyrrolnitrin genes on seed, however, we expected greater production of this compound than was observed. Recovery of pyrrolnitrin may have been low because the majority of this compound is found in the cell pellet, which was not extracted separately in the seed experiments. In some cases, changes in secondary metabolite production were not accompanied by significant changes in gene expression, such as the increased production of orfamide A in an rpoS mutant. This could be due to post-transcriptional regulation or could indicate that the time point used for the

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**Table 1.** Concentrations of secondary metabolites in cultures of Pf-5 and an rpoS mutant.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pyoluteorin (ng ml⁻¹)</th>
<th>DAPG (ng ml⁻¹)</th>
<th>Pyrrolnitrin (ng ml⁻¹)</th>
<th>Rhizoxin (ng ml⁻¹)</th>
<th>Orfamide A (ng ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf-5</td>
<td>340 ± 100</td>
<td>9 ± 16</td>
<td>450 ± 110</td>
<td>220 ± 60</td>
<td>6 900 ± 2100</td>
</tr>
<tr>
<td>rpoS</td>
<td>35 800 ± 11 500</td>
<td>10 400 ± 4600</td>
<td>BD</td>
<td>BD</td>
<td>21 400 ± 4300</td>
</tr>
</tbody>
</table>

a. Values represent the sum of each compound (~one standard deviation) extracted from the supernatant and cell pellet of cultures grown in NBG/NG for 48 h at 20°C. BD indicates below detection.

b. Only the predominant rhizoxin analogue, rhizoxin WF-1360F, was quantified.

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microarray experiment did not coincide with observable differences in transcript levels. Overall, however, differences in secondary metabolite production were in agreement with observed changes in gene expression from the microarray experiments.

The most notable difference in secondary metabolite production between Pf-5 grown in culture versus on seed surfaces was that in culture, pyoluteorin was produced at a much higher level than DAPG, while on seed, DAPG, but not pyoluteorin, was detected. Biosynthesis of these two metabolites is coordinated by a complex regulatory network involving autoinduction and mutual repression (Schnider-Keel et al., 2000; Brodhagen et al., 2004; Baehler et al., 2005; Kidarsa et al., 2011) with carbon source composition being a major factor mediating their relative production (Nowak-Thompson et al., 1994; Kraus and Loper, 1995; Duffy and Défago, 1999). The carbon source for the culture experiments was glycerol, which is favourable for pyoluteorin production (Nowak-Thompson et al., 1994; Kraus and Loper, 1995; Duffy and Défago, 1999), while the main carbohydrates detected in pea seed exudates were sucrose, galactose, mannose and fructose (Table S1). While we cannot conclude that carbon source was responsible for the observed differences in secondary metabolite production or gene expression on seeds versus in culture, the data presented in this study indicate the value of assessing gene expression of a biological control agent grown on a plant surface.

Secreted enzymes, such as chitinases and proteases, function in nutrient acquisition and the interactions of bacteria with their microbial co-inhabitants and eukaryotic hosts. Genes encoding a chitinase and chitin-binding protein (PFL_2091 and 2090) were upregulated in the rpoS mutant and downregulated in the gacA mutant versus wild-type Pf-5 on seed. Accordingly, the rpoS mutant exhibited far more exo- and endochitinase activity than Pf-5 (Table 3), whereas the gacA mutant exhibited very low chitinase activity, as was reported by Loper and colleagues (2012). This is in agreement with the decrease in protein levels of chitinase and chitin-binding protein observed in a gacA mutant of *P. aeruginosa* PAO1 (Kay et al., 2006). GacA also is required for extracellular protease activity of Pf-5 (Whistler et al., 1998), and genes encoding the alkaline protease AprA (PFL_3210) and the adjacent protease inhibitor (PFL_3209) were significantly downregulated in a gacA mutant under both conditions. Another exoprotease gene (PFL_2483) was also downregulated significantly in the gacA mutant on seed (Table S2). These results provide evidence for the expression of genes encoding these exoenzymes by Pf-5 inhabiting seed surfaces.

GacA is thought to act largely at the post-transcriptional level to control secondary metabolite and exoenzyme production (Lapouge et al., 2008), and it is likely that many of the differences in transcript level are due to indirect effects of a gacA mutation. As discussed by Lapouge and colleagues (2008), the arrest of translation initiation due to unrestricted RsmA binding in a gacA mutant could lead to instability, and therefore lower levels, of transcripts with RsmA binding sites, such as *aprA*. Another possibility is that initial post-transcriptional repression led to downstream effects on transcription due to loss of autoinduction by a secondary metabolite. This could be the case for the pyoluteorin and DAPG biosynthetic regions, as both show increased transcription in the presence of the corresponding antibiotic (Schnider-Keel et al., 2000; Abbas et al., 2002; Brodhagen et al., 2004; Baehler et al., 2005). Another possible scenario is that the Gac/Rsm system affects the translation of transcriptional regulators, leading to indirect effects on the transcription of genes under control of the regulatory proteins.

The secondary metabolite and exoenzyme genes that were downregulated by RpoS may be transcribed directly by the RNA polymerase holoenzyme in which $\sigma^5$ is the sigma factor or be influenced indirectly by products of genes directly transcribed by the $\sigma^5$ holoenzyme. However, a number of secondary metabolite genes were upregulated in an rpoS mutant of Pf-5, such as those coding for pyoluteorin and DAPG production. The $\sigma^5$-RNA polymerase holoenzyme could transcribe genes encoding repressors of these compounds. Alternatively, $\sigma^5$ may serve as a negative regulator by competing for association with a limited pool of the RNA polymerase core with another sigma factor(s) required for production of DAPG.

### Table 2. Concentrations of secondary metabolites in extracts of pea seeds inoculated with Pf-5 or the gacA or rpoS mutant derivatives.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pyoluteorin (ng per seed)$^a$</th>
<th>DAPG (ng per seed)$^a$</th>
<th>Pyrrolnitrin (ng per seed)$^b$</th>
<th>Rhizoxin$^c$ (ng per seed)$^a$</th>
<th>Orfamide A (ng per seed)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf-5</td>
<td>BD</td>
<td>81 ± 38</td>
<td>Present</td>
<td>17 ± 4</td>
<td>320 ± 160</td>
</tr>
<tr>
<td>gacA</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>rpoS</td>
<td>190 ± 160</td>
<td>710 ± 290</td>
<td>BD</td>
<td>BD</td>
<td>580 ± 680</td>
</tr>
</tbody>
</table>

a. Seeds were inoculated with the indicated strain and extracted after 48 h incubation at 20°C. BD indicates below detection. Values are ± one standard deviation.
b. A peak corresponding to pyrrolnitrin was identified in analysis of the wild-type sample but was not sufficiently above background for quantification. This peak was not present in the gacA and rpoS mutant samples.
c. Only the predominant rhizoxin analogue, rhizoxin WF-1360F, was quantified.
and pyoluteorin, such as RpoD or RpoN (Schnider et al., 1995; Péchy-Tarr et al., 2005). Therefore, as suggested elsewhere (Sarniguet et al., 1995; Heeb et al., 2005), genes upregulated in an rpoS mutant may consist mainly of genes transcribed from promoters recognized by RNA polymerases containing sigma factors other than $\sigma^5$, resulting in increased transcription in the absence of RpoS.

**Novel biosynthetic genes expressed by Pf-5 on seed surfaces**

We performed k-means clustering analysis (Soukas et al., 2000) on all genes showing significant regulation in at least one experiment to identify genes showing similar expression patterns between experiments. Our cluster analysis highlighted a group of genes that were highly downregulated in a gacA mutant both on seed and in culture (Fig. S3). This group includes biosynthetic genes for two putative secondary metabolites also identified from the GacA transcriptome of Pf-5 grown in culture (Hassan et al., 2010). PFL_1028–1037 (novel compound A; Figs 3B and S2C) is similar to the toxoflavin gene cluster found in Burkholderia glumae (Kim et al., 2004; Suzuki et al., 2004). PFL_0258–0268 (novel compound B; Figs 3B and S2C) encodes several putative fatty acid desaturases and other biosynthetic genes that have orthologues in Burkholderia vietnamiensis G4, Burkholderia ambifaria IOP40-10 and MEX-5, and Streptomyces sp. Mg1 (http://img.jgi.doe.gov/). A third potential novel secondary metabolite region, PFL_5540–5551 (novel compound C; Figs 3B and S2), is also present in the genomes of the soil bacteria P. fluorescens WH6 (Kimbel et al., 2010) and Q2-87 (Loper et al., 2012) and Pseudomonas brassicacearum NFM421 (Ortet et al., 2011) and Q8r1-96 (Loper et al., 2012). A smaller region corresponding to PFL_5540–5547 and PFL_5551 is present in the genome of the insect pathogen Pseudomonas entomophila (Vodovar et al., 2006). Most of the genes in this region were significantly downregulated in both rpoS and gacA mutants on seed surfaces, while none of these genes were significantly regulated in culture (Table S2 and Fig. 3B). This is likely due to differing expression levels between the two conditions as PFL_5545 was expressed at approximately sevenfold higher levels on seed surfaces than in culture (Fig. 2A). A fourth region (PFL_4647–4657; novel compound D) encodes a non-ribosomal peptide synthetase. Genes in this region were moderately downregulated in a gacA mutant and upregulated in an rpoS mutant on seed surfaces (Table S2 and Fig. 3B). To our knowledge, the products of these four gene clusters have not yet been identified, but our results indicate that the genes are expressed by Pf-5 inhabiting seed surfaces and could therefore confer traits that contribute to the fitness of the bacterium or its interactions with other organisms in the spermosphere.

**Acetoin and 2,3-butanediol**

Acetoin and the related compound 2,3-butanediol are produced by some plant growth-promoting rhizobacteria, such as Bacillus subtilis GB03 and Bacillus amyloliquifaciens IN937a, and contribute to plant growth enhancement and systemic resistance induced by these rhizobacteria (Ryu et al., 2003). Pseudomonas chlororaphis O6 produces 2,3-butanediol (Han et al., 2006) and, based on similar gene content, Pf-5 also likely produces this compound (Loper et al., 2012). The Pf-5 genome also contains a gene cluster (PFL_2168-PFL_2173) predicted to encode proteins required for the catabolism of acetoin and 2,3-butanediol (Huang et al., 1994). These catabolism genes were upregulated in an rpoS mutant of Pf-5 on seed (Table S2), as previously reported for an rpoS mutant of P. aeruginosa PAO1 (Table 5; Schuster et al., 2004). The promoter for the aco operon contains the –24 and –12 consensus sequences for a $\sigma^4$-dependent promoter (Barrios et al., 1999), as was found in Pseudomonas putida PpG2 (Huang et al., 1994) and KT2440 (Cases et al., 2003) and several other bacteria (Xiao and Xu, 2007). Therefore, upregulation of the aco genes in the rpoS mutant may be due to loss of competition with $\sigma^2$ for binding of $\sigma^4$ to the core RNA polymerase. The upregulation of the aco operon in an rpoS mutant could influence the plant growth-promoting effects of rhizosphere bacteria, perhaps including Pf-5 itself, through increased catabolism of these compounds in soil.

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**Table 3. Chitinase activity of Pf-5 and the gacA and rpoS mutant derivatives.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>4-Methylumbelliferyl N,N′-diacetyl-β-d-chitobioside$^c$ (ng)</th>
<th>4-Methylumbelliferyl β-di-N,N′-triacety chloritriose$^c$ (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf-5</td>
<td>550 ± 10</td>
<td>120 ± 10</td>
</tr>
<tr>
<td>gacA</td>
<td>11 ± 0</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>rpoS</td>
<td>2290 ± 70</td>
<td>1020 ± 20</td>
</tr>
</tbody>
</table>

a. Values ± one standard deviation were determined from the supernatant of cultures grown for 4 days in KMB at 27°C.

b. Exochitinase (chitobiosidase) activity.

c. Endochitinase activity.
Biofilm formation, motility and c-di-GMP signalling

*Pseudomonas protegens* Pf-5 has three gene clusters coding for the production of exopolysaccharides that are known to be involved in biofilm formation by *P. aeruginosa*: alginate, Psl and Pel (Ryder *et al*., 2007; Ghafour *et al*., 2011). Genes of the *pslABDEFGHIJ* cluster (PFL\_4208–4216) were downregulated (~1.7- to ~3.7-fold) in both a *gacA* and an *rpoS* mutant on seed, while only PFL\_4208 and 4209 showed regulation in culture (Table S2 and Fig. S4). In *P. aeruginosa* PAO1, *psl* gene expression is positively regulated at the transcriptional level by RpoS (Table S5; Schuster *et al*., 2004) and negatively regulated at a post-transcriptional level by RsmA (Irie *et al*., 2010).

Therefore, the effect of GacA on transcript levels of the *psl* genes in Pf-5 could be due to the influence of GacA on RpoS expression or to mRNA instability caused by unrestricted RsmA binding in a *gacA* mutant, as discussed above. We also found that four genes of the *pefABCDEF* operon (PFL\_2972–2978) were modestly downregulated in a *gacA* mutant on seed. No genes in the alginate biosynthetic cluster (PFL\_1013–1024) showed significant regulation in any of the microarray studies. Pf-5 also contains homologues of *pgaABCD* (PFL\_0161–0164), which encode proteins involved in the synthesis and export of the biofilm polysaccharide poly-β-1,6-N-acetyl-D-glucosamine and are required for biofilm formation by *Escherichia coli* (Wang *et al*., 2004; Itoh *et al*., 2008), but are not present in *P. aeruginosa*. This operon was downregulated in both the *gacA* and the *rpoS* mutants of Pf-5 on seed, but not in culture. Proteins are also an important part of the biofilm matrix (Mann and Wozniak, 2012). The *lap* (large adhesion protein) genes encode a large cell-surface protein (LapA) and proteins involved in its secretion and regulation (Hinsa *et al*., 2003). LapA is required for biofilm formation in *P. fluorescens* WCS365 and *P. putida* OUS82 (Hinsa *et al*., 2003; Gjermansen *et al*., 2010). Genes encoding LapA (PFL\_0133) and a protein involved in LapA secretion (PFL\_0134) were upregulated in an *rpoS* mutant of Pf-5 on seed (Table S2). Overall, regulation of genes involved in biofilm formation by GacA and RpoS was more apparent on seed surfaces than in shaking cultures.

Biofilm formation and motility are considered to be opposing lifestyles. Plant growth-promoting rhizobacteria can form biofilms on plant roots and this may play a role in their rhizosphere competence (Rudrappa *et al*., 2008). In at least some environmental conditions, however, motility is more important than biofilm formation for competitive root colonization and biological control, as shown recently for *P. fluorescens* F113 (Barahona *et al*., 2010; 2011). In our microarray study, a limited number of motility-related genes were affected by RpoS or GacA, with the notable exception of five flagellar proteins (PFL\_1614–1616, 1632 and 1665) that were moderately downregulated in an *rpoS* mutant on seed.

The switch between biofilm formation and motility is regulated in many bacteria by levels of the second messenger cyclic diguanilate (c-di-GMP), with low c-di-GMP levels associated with motility and high levels leading to biofilm formation (Hengge, 2009). C-di-GMP is synthesized by diguanylate cyclases (DGCs) and broken down by phosphodiesterases (PDEs). The genome of Pf-5 includes 47 genes annotated as DGCs or PDEs or containing domains associated with DGC (GGDEF domain) or PDE (EAL domain) activity or both (Paulsen *et al*., 2005). Of these 47 genes, 12 were significantly regulated in one or more of the microarray experiments, with 11 regulated in at least one seed experiment and only two regulated in culture (Table S6). The GGDEF/EAL domain protein PFL\_5779, which had 2.8-fold lower transcript levels in a *gacA* mutant on seed, shares 70% amino acid identity with PP4959, a gene expressed by *P. putida* KT2440 living on maize roots (Matilla *et al*., 2011). Overexpression of PP4959 led to increased biofilm production, reduced motility and impairment of root tip colonization, while a mutation in PP4959 led to reduced biofilm formation (Matilla *et al*., 2011). A second GGDEF/EAL domain protein, encoded by PFL\_2061, was downregulated twofold in an *rpoS* mutant on seed. PFL\_2061 shows 87% amino acid identity with Pf01\_1887 of *P. fluorescens* Pf0-1. Mutation of Pf01\_1887 led to increased biofilm production in Pf0-1 (Newell *et al*., 2011).

In an attempt to correlate changes in gene expression with the phenotypes of *gacA* and *rpoS* mutants, we conducted motility and biofilm assays. We observed no significant difference in the swimming or swarming motility of the *rpoS* mutant (Table 4). The *gacA* mutant showed a complete inability to swarm, which was attributed to lack of orfamide A production (Hassan *et al*., 2010), but showed slightly enhanced swimming motility, similar to what was observed in a *gacS* mutant of *P. fluorescens* F113 (Barahona *et al*., 2011). The lack of a major effect on motility is consistent with the limited effects on the expression of motility-related genes seen on the arrays. Static biofilm assays showed that the *gacA* mutant is deficient in biofilm production (Fig. 4), which is consistent with

| Table 4. Swimming and swarming motility of Pf-5 and gacA and rpoS mutants. |
|---------------------------|----------------|----------------|
| Motility*               | Pf-5           | gacA           | rpoS           |
| Swimming (24 h)          | 19.6 ± 0.4     | 21.8 ± 0.5     | 19.8 ± 0.6     |
| Swimming (48 h)          | 36.9 ± 1.2     | 42.3 ± 0.6     | 37.7 ± 1.9     |
| Swarming (24 h)          | 33.8 ± 4.9     | 5.0 ± 0.0      | 34.9 ± 3.1     |

* a. Value represents the diameter of the colony (mm) ± one standard deviation. Five millimetre is the colony diameter at the time of inoculation.
previously published reports of reduced biofilm formation in gacA mutants of Pseudomonas sp. KL28 and P. aeruginosa PA14 (Parkins et al., 2001; Choi et al., 2007). The reduced biofilm formation of the gacA mutant of Pf-5 could be due to the reduced expression of exopolysaccharide genes described above. In contrast, the rpoS mutant exhibited enhanced biofilm production compared with wild-type Pf-5 (Fig. 4), as previously observed for an rpoS mutant of P. aeruginosa PAO1 (Heydorn et al., 2002). In Pf-5, enhanced biofilm formation by the rpoS mutant could be due to enhanced expression of LapA. Differences in c-di-GMP signalling could also play a role in the differing phenotypes of the gacA and rpoS mutants, possibly due to reduced expression of PFL_5779 in the gacA mutant and PFL_2061 in the rpoS mutant. As discussed above, these loci are homologous to genes having opposing effects on biofilm formation in other species of Pseudomonas. Further studies are needed to understand the complex regulation of the motility and biofilm phenotypes in Pseudomonas spp. under a range of conditions, including on seed and other plant surfaces. This regulation is likely to include post-translational control of target genes in addition to the transcriptional effects observed here.

GacA regulation of type VI secretion and RebB proteins

Genes encoding a type VI secretion system (T6SS; PFL_6074–6094) were downregulated in a gacA mutant relative to wild-type Pf-5 on seed surfaces (Table S2), as observed previously in culture (Hassan et al., 2010). The T6SS of Gram-negative bacteria serves as an apparatus for injection of effector proteins directly into target cells or secretion into the extracellular milieu (Mougous et al., 2006; Pukatzki et al., 2006; Records, 2011). T6SSs mediate both pathogenic and symbiotic relationships between bacteria and their hosts and also competitive and cooperative interbacterial interactions (Records, 2011). Pseudomonas aeruginosa contains three distinct T6SS loci and the T6SS of Pf-5 is related to one of these loci (HSI-I), which has been implicated in interbacterial interactions (Hood et al., 2010; Barret et al., 2011). The T6SSs of Pseudomonas syringae pv. syringae B728a and P. brassicacearum are also regulated by the Gac/Rsm system (Records and Gross, 2010; Lalucat et al., 2012). RebB proteins form refractile inclusion bodies (R-bodies) that have been associated with toxicity to parasitism when produced by obligate symbionts in the genus Caedibacter (Pond et al., 1989). R-bodies have been reported in some Pseudomonas spp., but their biological function in the genus is unknown (Lalucat et al., 1982; Wells and Home, 1983; Espuny et al., 1991). The rebB genes (PFL_0183 and PFL_0184) were strongly downregulated in a gacA mutant on seed (−23- and −7.7-fold) and moderately downregulated in culture (−3.0- and −2.3-fold).

Iron acquisition and regulation

Hassan and colleagues (2010) observed that GacA negatively regulated many genes involved in iron acquisition by Pf-5 in culture, which is in agreement with previous studies showing an increase in siderophore production upon loss of GacA/GacS signalling in the closely related strain CHA0 (Schmidli-Sacherer et al., 1997; Duffy and Défago, 2000). Similarly, one of the most notable effects of a gacA mutation in Pf-5 grown on seed surfaces was the upregulation of genes involved in iron acquisition and homeostasis (Table S2 and Fig. S2C). Our cluster analysis identified a group of genes that were generally upregulated in the gacA mutant under both growth conditions, while showing no or moderate regulation by RpoS (Fig. 5). Nearly all the genes in this cluster have an iron-related function, including genes involved in the biosynthesis and uptake of pyoverdine and enantio-pyochelin, the two siderophore-mediated high-affinity iron uptake systems of Pf-5 (Table S7 and Fig. S2C; Youard et al., 2007; Hartney et al., 2011). The number of genes in the pyoverdine biosynthetic region that were significantly regulated by GacA was greater for the seed experiment than in culture. However, we attribute this to greater variability in the culture experiment because many of these genes showed greater fold changes in response to GacA in culture (Fig. 5 and Table S7; Hassan et al., 2010). This is in agreement with the greater influence of GacA in culture on transcript levels of pvdS (PFL_4190), which encodes the extracytoplasmic function (ECF) sigma factor required for transcription of the pyoverdine biosynthetic
genes (Fig. 5 and Table S2; Leoni et al., 2000; Wilson and Lamont, 2000; Swingle et al., 2008). A number of siderophore-related genes, particularly in the enantiopyochelin gene cluster, were also significantly upregulated in an rpoS mutant in culture, but not on seed (Table S7). PI-5 also produces a haemophore for high-affinity chelation and uptake of haem (Wandersman and Delepelaire, 2004), and genes encoding components of this system were significantly regulated by GacA on seed, as previously described in culture (Table S7). Indeed, PFL_5377, which encodes the HasA haemophore, was the most highly upregulated (17-fold) gene in the gacA mutant on seed surfaces (Table S2).

PI-5 has 45 TonB-dependent outer-membrane proteins (TBDPs), many of which are predicted to take up ferric complexes of siderophores produced by other microorganisms (Table S8; Hartney et al., 2011). TonB and the associated proteins ExbB and ExbD provide energy for uptake by TBDPs (Noinaj et al., 2010), and ECF sigma factors and sigma factor regulatory proteins function in TonB-dependent signalling for the subset of TBDPs that contain an N-terminal signalling domain (Ferguson et al., 2007). Many genes encoding components of the TonB uptake and signalling systems of PI-5 are highly regulated by GacA in culture (Hassan et al., 2010); far fewer were regulated by GacA on seed (Table S8). On seed, GacA influenced the transcript levels of genes encoding components of eight different TonB-signalling systems, which are predicted to take up the two siderophores and the haemophore produced by PI-5, HasA, as well as ferrichrome (or ferrioxamine) and pyoverdines produced by *Pseudomonas* strains other than PI-5 (Hartney et al., 2011). These results suggest that PI-5 expresses genes encoding for its three high-affinity iron-acquisition systems, as well as receptors for selected heterologous siderophores, on seed surfaces.

Other iron-related genes upregulated in a gacA mutant under both conditions are genes of the *fagA-fumC-orfX-sodA* operon (PFL_0906–0909; Fig. 5 and Table S2), which is expressed under iron-limited conditions and encodes non-iron-containing forms of fumarase and superoxide dismutase (Polack et al., 1996; Hassett et al., 1997a,b; Lim et al., 2012). A gene encoding a bacterioferritin-associated ferredoxin (PFL_4858), which is involved in the mobilization of iron from bacterioferritin under iron-starvation conditions (Andrews et al., 2003), was also upregulated in the gacA mutant of PI-5 on seed and in culture.

Overall, a gacA mutant of PI-5 showed a gene expression pattern mimicking bacteria grown under iron-limited conditions both in culture and on seed surfaces although differences in regulation, particularly of the TonB system, were observed between the two conditions. The mechanism by which GacA influences the expression of

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**Fig. 5.** Hierarchical tree showing a cluster of genes upregulated in a gacA mutant compared with PI-5. Log$_2$ fold change ratios of the gacA or rpoS mutant compared with wild-type PI-5 for each experiment are indicated by the colour scale. General functions of some of the genes are indicated. Pvd indicates genes present in one of the clusters involved in pyoverdine biosynthesis. Pch indicates genes present in the enantiopyochelin biosynthetic gene cluster. Heme indicates genes in one of the clusters involved in haem uptake. TonB indicates genes encoding the TonB-ExbB-ExbD complex. Genes marked with a cross contain a putative PvdS binding site in their promoter (Hassan et al., 2010). Genes marked with an asterisk contain a putative Fur-binding motif in their promoter (Hassan et al., 2010). A cross or asterisk in parentheses indicates that the gene is predicted to be part of an operon with either a PvdS- or a Fur-binding motif in the promoter upstream of the first gene of the operon.
iron-regulated genes is not known, but a motif similar to the binding site for Fur (ferric uptake regulator) is present upstream of many genes upregulated by GacA (Fig. 5; Hassan et al., 2010). Therefore, we speculate that the upregulation of some iron-regulated genes by GacA is mediated by Fur, which, in the presence of iron, functions as a repressor of iron-related genes in Pseudomonas spp. (Cornelis et al., 2009).

The respiratory chain and regulation by Anr

Pseudomonas spp. have extensively branched respiratory chains with multiple terminal oxidases, which help the bacteria adapt to diverse environments (Arai, 2011). Five of the seven terminal oxidase gene clusters in Pf-5 showed significant regulation in at least one of the four microarray experiments, and most exhibited quite different regulation on seed surfaces versus in culture (Table S9). Genes encoding one terminal oxidase (PFL_0061–0064) are among those most highly down-regulated in an rpoS mutant on seed, and orthologues of these genes are also downregulated in an rpoS mutant of P. aeruginosa PA01 (Table S5; Schuster et al., 2004). Our k-means analysis identified a cluster that encompasses 12 of the 13 genes that were upregulated in a gacA mutant on seed surfaces and downregulated in a gacA mutant in culture (Table S2 and Fig. S5). This cluster includes PFL_1922–1925, which encode one of two adjacent cytochrome cbbα oxidases. In P. aeruginosa, one cbbα cluster is expressed more highly under oxygen-limited conditions, and requires the transcriptional regulator Anr (anaerobic regulator of arginine deiminase and nitrate reductase) for expression (Comolli and Donohue, 2004). We identified a putative Anr binding site upstream of the PFL_1922–1925 operon and several other genes that were strongly downregulated in the gacA mutant in culture, but either upregulated or not regulated on seed (Fig. S5). Therefore, the difference in regulation of these genes may be due at least partially to reduced expression of anr (~2.2-fold) in a gacA mutant in culture but not on seed surfaces. A difference in anr expression would be expected if oxygen is more available to bacteria growing on seed surfaces than in culture, as was likely under our experimental conditions.

Other genes regulated by GacA and RpoS in Pf-5 growing on seed surfaces

Our k-means cluster analysis identified two clusters containing genes that were downregulated in both the gacA and the rpoS mutants on seed, but showed little or no regulation by either GacA or RpoS in culture (Fig. S4). Genes encoding putative type I pilus proteins (PFL_3951–3955) and Fip pilus proteins (PFL_0691–0698; Table S5; Bernard et al., 2009) fall in these two clusters, in addition to many genes having unknown functions. Examples of the latter include PFL_2151–2155, which is adjacent to the orfamide biosynthetic gene region, and PFL_0699–0702, adjacent to the Flp pilus operon, which are among the genes most highly regulated by GacA or RpoS on seed surfaces (Fig. S4 and Table S2). Of the 112 genes in the two clusters, 36 (32%) are annotated as hypothetical proteins compared with approximately 19% for the genome as a whole, again indicating that a relatively large number of the genes regulated by RpoS and GacA on seed surfaces are uncharacterized.

Conclusions

This study constitutes, to our knowledge, the first report on the transcriptome of bacteria on seed surfaces and provides evidence for the expression of hundreds of genes by P. protegens Pf-5 in this environment. Here, we show that GacA and RpoS, two global regulators known to affect biological control activity by Pseudomonas spp. (Haas and Keel, 2003; Haas and Défago, 2005), influence the transcription of a total of 897 genes in Pf-5 on pea seed surfaces. These genes code for diverse functions, including the production of antibiotics, exoenzymes, biofilm formation, motility, type VI secretion and iron homeostasis, many of which are known to contribute to biological control or colonization of seed or root surfaces by Pseudomonas spp. GacA and RpoS influenced the spectrum of antibiotics produced by Pf-5 on seed surfaces, and we identified four gene clusters for the biosynthesis of possible novel secondary metabolites that were expressed under the control of GacA or RpoS by Pf-5 on seed surfaces. We found that only a fraction of the GacA- and RpoS-regulated genes on seed surfaces overlapped with those identified in culture. Thus, we greatly expanded the list of genes in the GacA and RpoS regulons of Pf-5 by performing experiments in both conditions. Perhaps not surprisingly, a greater proportion of the genes identified in the seed experiments are uncharacterized as compared with those regulated in culture, opening future avenues of study into the functions of these genes and their potential roles in biological control.

Two major differences between growth on a seed surface versus growth in culture that may affect gene expression are the ability of the bacteria to attach to a solid surface and, perhaps more importantly, the nutrients provided by seed exudates versus a rich culture medium. Growth on a solid surface may explain the greater number of genes involved in biofilm formation regulated on seed surfaces, while nutrient composition likely influenced the suite of secondary metabolites produced and the expression of transport genes. Because the composition of seed
exudates varies among plant species (Roberts et al., 1999), we expect the set of genes expressed by a biological control agent to differ in the spermospheres of different plants. While not a perfect model for the conditions encountered by biological control organisms in the field, the experimental system used for our seed microarray is a much closer approximation to the field environment than growth in culture.

To date, most biological control traits have been identified first due to their importance in antagonism of a target pathogen in culture; typically, those traits then are tested for their roles in plant disease suppression in a greenhouse or field environment (Thomashow and Weller, 1988; Haas and Défago, 2005). In many cases, however, the traits of a biological control agent that are responsible for antagonism of the pathogen in culture contribute little to suppression of the disease caused by that pathogen (Lindow, 1988; Paulitz and Loper, 1991; Kraus and Loper, 1992). The limited overlap between the regulons defined in culture versus on seed surfaces highlights a pitfall in formulating hypotheses regarding the activity of a biological control bacterium on plant surfaces based on the behaviour of the bacterium in culture. This study will help focus future efforts to identify mechanisms of biological control specifically on genes expressed by a bacterial antagonist on plant surfaces, the environment where it must interact with a target pathogen to provide effective biological control.

Experimental procedures

Cell culture and seed treatment

A gacA mutant of Pf-5 was constructed previously (Corbell, 1999) by replacing 626 bp internal to gacA (nt 1–626) with aphl, which confers kanamycin resistance. The rpoS mutant of Pf-5 was described previously and contains a Tn5 insertion in the rpoS gene (Sarniguet et al., 1995). For the rpoS culture microarrays, three replicate cultures of Pf-5 and the rpoS mutant were grown in 20 ml of Difco™ Nutrient Broth (Becton, Dickinson and Company, Sparks, MD) amended with 1% glycerol (NBGly) in a 125 ml Erlenmeyer flask with shaking (200 r.p.m.) at 20°C until they reached OD600 = 0.5. Cultures were treated with RNAprotect Bacteria (Qiagen). Pellets were stored at −80°C.

RNA extraction

DNA was subjected to an on-column DNase treatment (RNaseasy Mini Kit with DNase I;Qiagen) and used to prepare cDNA. A volume of 1.2 ml of Buffer QRL1 with 10 µl of 2-mercaptoethanol per ml was added, and the mixture vortexed another 2–3 min. After settling, the liquid was transferred to a microfuge tube and spun 1 min at maximum speed to remove residual beads. A volume of 1.25 ml of supernatant was used to complete the RNA isolation according to the provided protocol. All RNA was subjected to an on-column DNase treatment (RNaseasy Mini Kit with DNase I; Qiagen) and used to provide PCR to confirm removal of detectable DNA. RNA quality was analysed on a BioAnalyser 2100 (Agilent, Palo Alto, CA) by the Center for Genome Research and Biocomputing (CGRB) Core Laboratories, Oregon State University, Corvallis, OR.

Microarray hybridizations and analysis

A P. fluorescens Pf-5 genomic microarray (Hassan et al., 2010), composed of 70mer DNA oligonucleotides representing each of the 6147 annotated protein-coding genes spotted in triplicate was used for the rpoS culture array experiments. An array prepared in the same manner but with four replicates of each oligonucleotide was used for the seed array experiments. cDNA-labelling was conducted using an indirect labelling method as previously described (Peterson et al., 2004) using 6 µg of total RNA per reaction. Each microarray experiment included three biological replicates, with two technical replicates comprising a dye-flip for each biological replicate. Hybridizations were conducted as previously described (Peterson et al., 2004). Slides were scanned at 10 µm resolution using an Axon GenePix4200A (seed experiments) or 4000B (culture experiments) scanner. Tiff images of hybridized arrays were processed using TIGR-Spotfinder, and the data sets normalized by applying the LOWESS algorithm, using block mode and a smooth parameter of 0.33 in the TIGR-MIDAS package. Statistical analysis was performed on log2-transformed signal ratios of the replicate spots using the Significance Analysis of Microarrays (SAM) algorithms (Tusher et al., 2001). All results described for the seed array experiments were found to be significant using a false

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discovery rate (FDR) of less than 0.5%. For the rpoS culture experiment, all results were significant at a FDR of less than 5%. A higher FDR was allowed for this experiment because the slides contained only three replicate spots (versus four for the seed experiments), which led to lower statistical significance. In addition, a minimum log, ratio of 0.5 (corresponds to a fold change of ~ 1.4) was imposed on all data as an additional criterion for significance. To identify sets of genes that displayed similar patterns of expression between experiments, k-means clustering analysis (Soukas et al., 2000) with support (KMS) was performed on all genes showing significant regulation in at least one set of experiments using the TIGR Multi-Experiment Viewer software (Saeed et al., 2003).

Ten k-means runs with a maximum of 50 iterations per run generated a total of 19 consensus clusters with a threshold co-occurrence of 80% and 31% of genes unassigned to a cluster. Gene trees were subsequently generated by average linkage hierarchical clustering with Euclidean distance as the distance metric.

All microarray data presented are in accordance with the Microarray Gene Expression Data Society’s minimum information about microarray experiment recommendations (Brazma et al., 2001). Descriptions of the microarray experiments, data analysis and array design have been deposited into GEO (http://www.ncbi.nlm.nih.gov/geo/) and can be accessed using the Accession No. GSE38633.

Array validation and RT-qPCR

The rpoS culture microarray data were validated by RT-qPCR of selected genes as previously described (Hassan et al., 2010). For validation of the seed array data, RT-qPCR was conducted on selected genes as follows. cDNA was derived from total RNA through reverse transcription using Superscript II and random hexamers (Invitrogen, Life Technologies, Grand Island, NY). Real-time PCR was performed on the cDNA using SYBR Green on a Roche LightCycler (Roche, Indianapolis, IN, USA). Primers were designed using the LightCycler Probe Design Software and are listed in Table S10. Melting curve analysis of products was used to verify amplification of a specific product. The zwf gene (PFL_4610), which encodes glucose-6-phosphate 1-dehydrogenase, was used as the reference gene. The fluorescence per cycle data for each reaction was imported into the LinRegPCR program (Ramakers et al., 2003; Ruijter et al., 2009). LinRegPCR was used to determine the cycle threshold (Ct) value for each reaction and to determine the average amplification efficiency of each primer set. These values were used for relative quantification of each target gene in comparison with the reference gene by the Pfaffl method (Pfaffl, 2001).

For comparison of relative gene expression on seed surfaces versus in culture, cDNA was synthesized as described above from equal amounts of RNA from all wild-type Pf-5 samples used for the microarray experiments. RT-qPCR was conducted as described above on the target genes and the control gene zwf. LinRegPCR was used to calculate the relative number of starting molecules (N0) for each reaction using the Cq value for each sample and the average amplification efficiency of each primer set. The target/zwf N0 ratio was then calculated for each gene on seed and in culture.

Quantification of secondary metabolite production

Extraction and HPLC analysis of secondary metabolites from cells and culture supernatants were performed as described previously (Whistler et al., 2000; Hassan et al., 2010) on cultures of Pf-5 and the rpoS mutant grown in NBGly at 20°C for 48 h with shaking (200 r.p.m.). For quantification of secondary metabolites produced by bacteria grown on pea seed surfaces, pea seeds were surface sterilized and treated with bacterial suspensions (OD600 = 0.5) of Pf-5, the gacA mutant or the rpoS mutant, as described above. Seeded were incubated in Petri dishes with sterile moist filter paper at 20°C for 48 h. Forty seed per sample were vortexed in 20 ml of sterile distilled water for 30 s and sonicated for 30 s. Then 10 ml ethyl acetate was added and the samples vortexed another 30 s. The liquid was transferred to another tube and centrifuged 5 min at 5000 g. The ethyl acetate phase was collected and the aqueous phase extracted with another 10 ml of ethyl acetate. The ethyl acetate phases were combined and dried under vacuum. HPLC analysis was as previously described (Hassan et al., 2010). Values presented are means of three replicate cultures or three replicate seed samples.

Seed exudate analysis

Pea seeds (3.75 g) in a 250 ml Erlenmeyer flask were surface disinfested by incubation in 25 ml of 20% bleach for 20 min followed by three 5 min rinses in sterile distilled water. Sterile distilled water (10 ml) was added to each replicate flask and the flasks shaken at 125 r.p.m. at room temperature (approximately 22°C) in the dark for 24 h. At sampling (24 h), the aqueous contents of the flasks were decanted and checked for microbial contamination by spotting 10 µl aliquots onto nutrient agar. Non-contaminated replicate samples were frozen until used in analysis. All analyses had six replicate samples. Total carbohydrates in the samples were estimated by the anthrone assay (Morris, 1948) with glucose as the standard. Total amino acids in samples were estimated by the ninhydrin assay (Spies, 1957) with L-leucine as the standard.

All chemicals were obtained from Sigma-Aldrich. Exudate samples were evaporated to dryness in vacuo and triaconone was added as an internal standard. Samples for amino acid analysis were derivatized with 50 µl of N-methyl-N-(tbutyldimethylsilyl)trifluoroacetamide (MTBSTFA) containing 1% t-butyldimethylchlorosilane and 50 µl of anhydrous dimethylformamide at 70°C for 30 min (Goh et al., 1987). Samples for carbohydrate and organic acid analyses were first reacted with 50 µl of methoxyamine hydrochloride in anhydrous pyridine (20 mg ml⁻¹) at 40°C for 90 min, followed by derivatization with 50 µl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) at 40°C for 120 min (Fiehn et al., 2000). Derivatized samples were analysed on a Perkin-Elmer Clarus 500 GC-MS using a DB-5MS column, 25 m long × 0.2 mm i.D. × 0.33 µm film thickness, and helium as the carrier gas at 1 ml min⁻¹. The initial oven temperature was 80°C with a splitless injection of 0.5 µl followed by a 1 min hold time. The oven was then ramped to 325°C at 5°C min⁻¹ with a final hold time of 15 min. Electron impact spectra were collected from 100 to 700 amu. Chromatograms were processed with AMDIS software (NIST) and compounds were identified by relative retention time and mass spectral.

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comparison, and quantified with compound-specific response factors, using authentic standards run separately. The detection limit was 0.1 μg per seed for all compounds.

Chitinase assay

Chitinase activity was determined from cultures grown in 5 ml of King’s Medium B (King et al., 1954) broth for 4 days with shaking (200 r.p.m.) at 27°C. Ten microlitres of culture supernatant was added to 90 μl of substrate from a fluorometric chitinase assay kit (Sigma-Aldrich) and processed according to the manufacturer’s specifications. Two replicate cultures were evaluated in each of two independent experiments.

Biofilm and motility assays

Biofilm assays were performed in polystyrene 96-well plates as described previously (Gross et al., 2007). Assays were performed in KMB, NBGly and NB supplemented with 2% glucose (NBGlu) and cell adhesion measured at 24 h and 48 h. The experiment was repeated twice with eight replicates per treatment. Swimming and swarming assays were conducted as previously described (Hassan et al., 2010). The experiment was performed twice with seven or eight replicates per treatment.

Identification of Anr binding sites

Known Anr binding sites from \textit{P. aeruginosa} PAO1 listed in the PRODORIC Database Release 8.9 (http://prodoric.tu-bs.de/) were used to build a Hidden Markov Model (HMM) profile using the HMMER2 (Eddy, 2011; Finn et al., 2011) tool in Unipro UGENE v1.10.4 (Okonechnikov et al., 2012). This HMM was used to search the Pf-5 genome. The top-scoring putative Pf-5 binding sites were combined with the PAO1 sites to build a new HMM profile. This profile was used to search the Pf-5 genome again. Sites in promoter regions with an e-value less than 0.1 were considered to be possible Anr binding sites.

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References


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PFL_4647–4657 respectively. T6SS refers to the type VI secretion system gene cluster.

**Fig. S3.** Hierarchical tree showing a cluster of genes down-regulated in the gacA mutant compared with Pf-5. Log₂ fold change ratios of the gacA or rpoS mutant compared with wild-type Pf-5 for each experiment are indicated by the colour scale. General functions or gene products of some of the genes are indicated. DAPG, Ofa, Novel A and Novel B indicate genes in the biosynthetic gene clusters for 2,4-diacetylphloroglucinol, orfamide A, and novel compounds A and B. T6SS indicates genes in the type VI secretion system gene cluster.

**Fig. S4.** Hierarchical trees showing clusters of genes down-regulated in a gacA or rpoS mutant on seed surfaces. Log₂ fold change ratios of the gacA or rpoS mutant compared with wild-type Pf-5 for each experiment are indicated by the colour scale. General functions of some of the genes are indicated. Prn, Rzx and Novel C indicate genes in the biosynthetic gene clusters for pyrrolnitrin, rhizoxin and novel compound C. Psl and PGA indicate biosynthetic genes for the exopolysaccharides Psl and poly-β-1,6-N-acetyl-D-glucosamine respectively. Oxidase indicates terminal oxidase subunit or oxidase accessory protein genes. Flp pilus indicates genes in an operon encoding Flp pilus proteins and type I pilus indicates genes in an operon encoding type I pilus proteins.

**Fig. S5.** Hierarchical tree showing a cluster of genes down-regulated in the gacA mutant compared with Pf-5 in culture but not regulated or upregulated in the gacA mutant grown on seed surfaces. Log₂ fold change ratios of the gacA or rpoS mutant compared with wild-type Pf-5 for each experiment are indicated by the colour scale. General functions of some of the genes are indicated. HCN indicates genes in the hydrogen cyanide biosynthetic gene cluster. Oxidase indicates terminal oxidase subunit or oxidase accessory protein genes. Anr indicates genes with a predicted Anr binding site in their promoter regions. Anr in parentheses indicates genes in an operon with a predicted Anr binding site upstream of the first gene of the operon.

**Table S1.** Analysis of pea seed exudates.

**Table S2.** Fold change of significantly regulated genes in mutant compared with wild-type Pf-5.

**Table S3.** RT-qPCR microarray validation results for the rpoS mutant versus Pf-5 in culture.

**Table S4.** RT-qPCR microarray validation results for the gacA and rpoS mutants versus Pf-5 on seed surfaces.

**Table S5.** Genes regulated by RpoS in *P. protegens* Pf-5 and *P. aeruginosa* PAO1.

**Table S6.** Fold change of significantly regulated GGDEF and EAL domain protein genes.

**Table S7.** Influence of GacA and RpoS on expression of genes for high-affinity iron-acquisition systems of Pf-5 on seeds and in culture.

**Table S8.** Genes encoding components of TonB-dependent cell surface signalling systems regulated by GacA or RpoS (this study) or iron starvation (Lim et al., 2012).

**Table S9.** Fold change of significantly regulated terminal oxidase genes.

**Table S10.** Primers used for RT-qPCR validation.

**Supplemental Text S1.** Role categories of genes in the GacA and RpoS regulons.