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

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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.

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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 (σ^S; 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

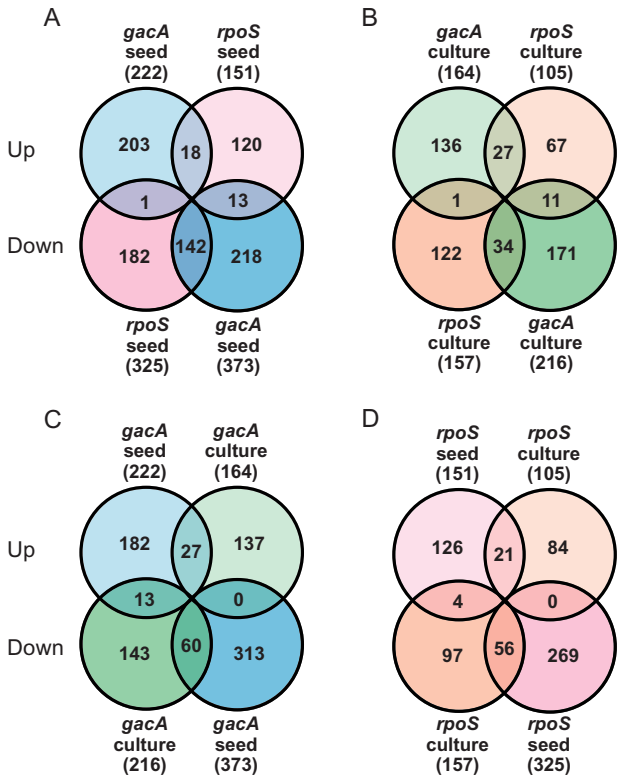

Fig. 1. Venn diagrams showing the number of significantly regulated genes unique to each experiment or shared between two experiments. Comparison of genes regulated by (A) GacA and RpoS on seed, (B) GacA and RpoS in culture, (C) GacA on seed versus in culture and (D) RpoS on seed versus in culture. The total number of significantly up- or downregulated genes for each experiment (out of the 6147 genes represented on the microarray) is shown in parentheses.

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 ($\log_2$ 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 σ^S 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 post-transcriptional 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

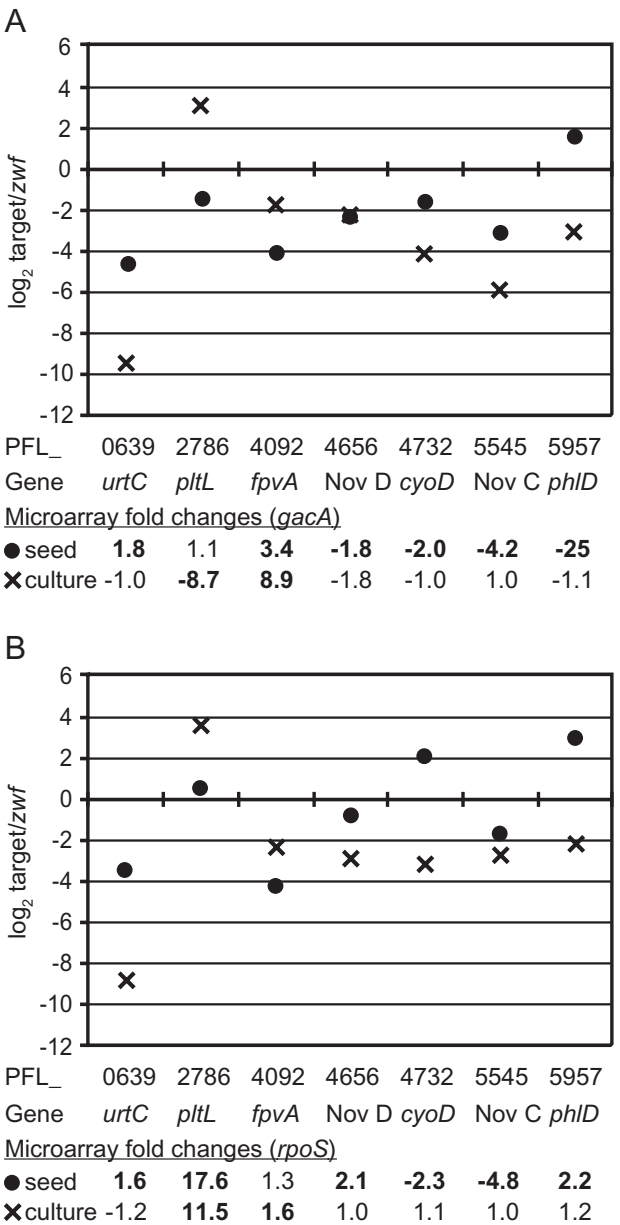

Fig. 2. Comparison of transcript levels of selected genes in wild-type Pf-5 grown on seed surfaces and in culture. Relative transcript levels of selected target genes in the wild-type Pf-5 RNA samples used for the seed and culture *gacA* (A) or *rpoS* (B) microarray experiments and the fold changes observed for those genes on the corresponding microarrays are shown. Each graph point represents the $\log_2$ expression ratio of the target gene in comparison with the reference gene *zwf* (PFL_4610) in wild-type Pf-5 grown on seed or in culture. Microarray fold changes in the indicated mutant relative to wild-type Pf-5 show that greater fold changes under one condition generally correlate with higher gene expression in that condition. Numbers in bold represent significant fold changes in the microarray experiments. PFL_4656 and PFL_5545 do not have gene names but are part of the biosynthetic gene clusters for potential novel compounds D and C respectively (see Fig. 3).

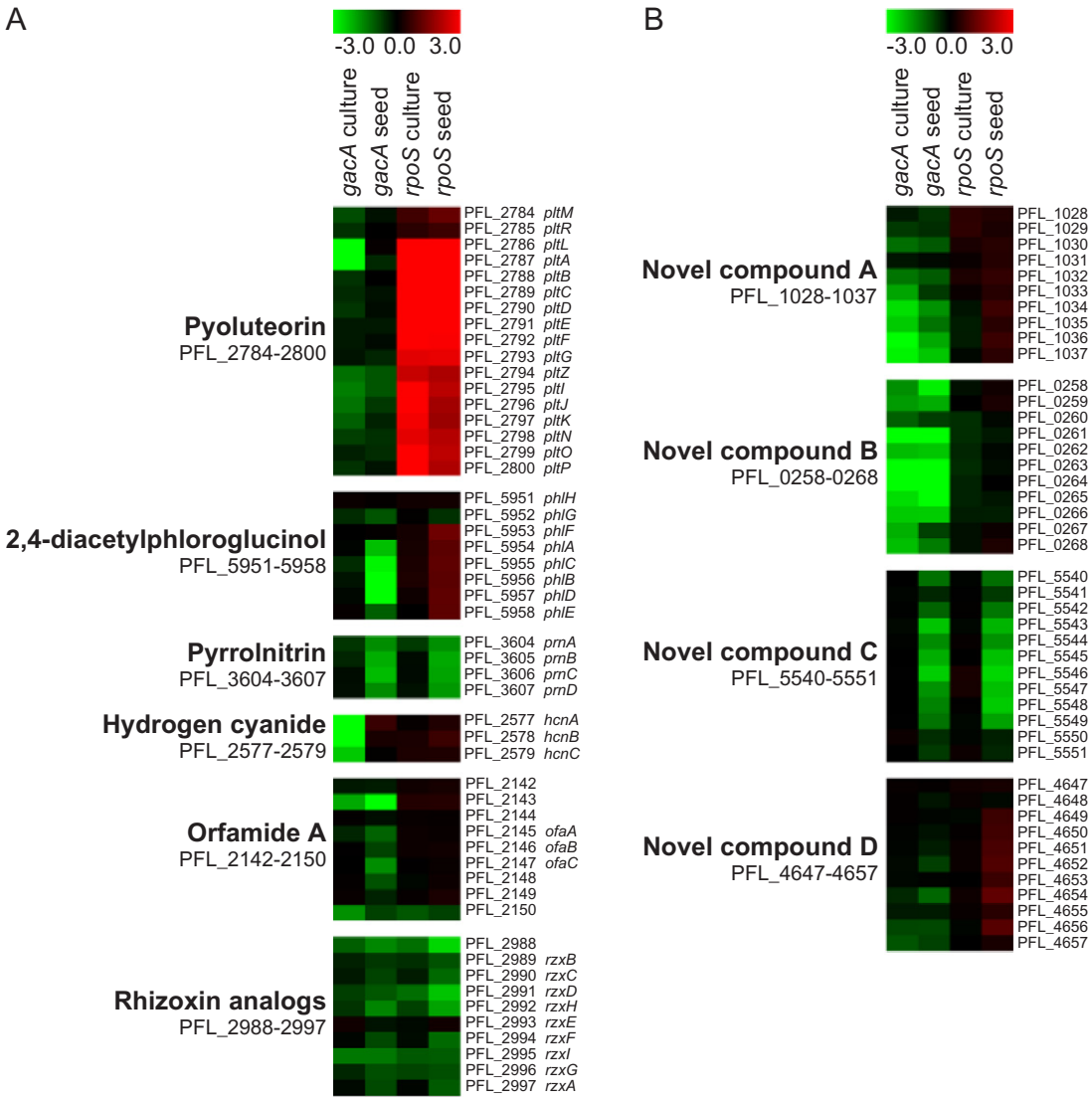

Fig. 3. Heat map showing $\log_2$ fold changes of genes in known (A) and putative (B) secondary metabolite biosynthetic gene clusters in a *gacA* or *rpoS* mutant grown on seed or in culture. The $\log_2$ fold change for each of the four microarray experiments is shown according to the colour scale.

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

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

Strain	Pyoluteorin (ng ml^{-1})[a]	DAPG (ng ml^{-1})[a]	Pyrrolnitrin (ng ml^{-1})[a]	Rhizoxin[b] (ng ml^{-1})[a]	Orfamide A (ng ml^{-1})[a]
Pf-5	340 ± 100	9 ± 16	450 ± 110	220 ± 60	6 900 ± 2100
rpoS	35 800 ± 11 500	10 400 ± 4600	BD	BD	21 400 ± 4300

a. Values represent the sum of each compound (± one standard deviation) extracted from the supernatant and cell pellet of cultures grown in NBGly for 48 h at 20°C. BD indicates below detection.
b. Only the predominant rhizoxin analogue, rhizoxin WF-1360F, was quantified.

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_2786, –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 hydrolase PhlG and the putative permease PhlE 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 *prnABCD* 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 *prnA* 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

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

Strain	Pyoluteorin (ng per seed)[a]	DAPG (ng per seed)[a]	Pyrrolnitrin (ng per seed)[a,b]	Rhizoxin[c] (ng per seed)[a]	Orfamide A (ng per seed)[a]
Pf-5	BD	81 ± 38	Present	17 ± 4	320 ± 160
gacA	BD	BD	BD	BD	BD
rpoS	190 ± 160	710 ± 290	BD	BD	580 ± 680

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.

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 σ^S is the sigma factor or be influenced indirectly by products of genes directly transcribed by the σ^S 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 σ^S-RNA polymerase holoenzyme could transcribe genes encoding repressors of these compounds. Alternatively, σ^S 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 3. Chitinase activity of Pf-5 and the *gacA* and *rpoS* mutant derivatives.

	4-Methylumbelliferone released from substrates (ng)[a]	
Strain	4-Methylumbelliferyl *N,N′*-diacetyl-β-D-chitobioside[b]	4-Methylumbelliferyl β-D-*N,N′,N″*-triacetylchitotriose[c]
Pf-5	550 ± 10	120 ± 10
gacA	11 ± 0	12 ± 2
rpoS	2290 ± 70	1020 ± 20

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.

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 σ^S, 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 (Kimbrel *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 amyloliquefaciens* 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 S5; Schuster *et al*., 2004). The promoter for the *aco* operon contains the –24 and –12 consensus sequences for a σ^{54}-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 σ^s for binding of σ^{54} 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.

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; Ghafoor *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 *pelABCDEFG* 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 diguanylate (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 Pfl01_1887 of *P. fluorescens* Pf0-1. Mutation of Pfl01_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[a]	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.

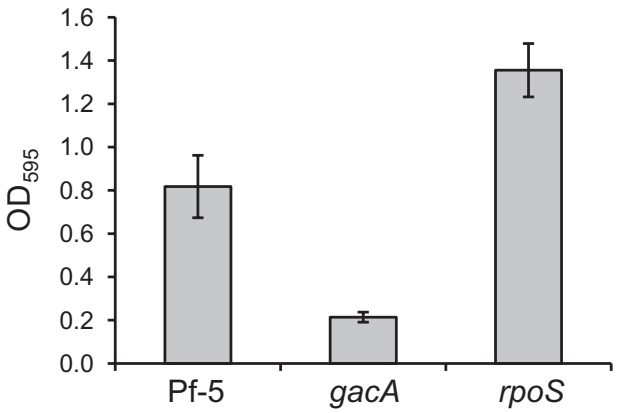

Fig. 4. Static biofilm assay. The formation of a static biofilm by wild-type Pf-5 and the *gacA* and *rpoS* mutants in a 96-well plate after 24 h growth in KMB was determined by absorbance at 595 nm of crystal violet absorbed by adhering biofilms after de-staining with ethanol. The experiment was performed twice with eight replicates per strain. Averages and standard deviations from one experiment are shown. A similar pattern was observed when NBGly or NBGlu media were used and at 48 h in all three media.

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; Lalaouna *et al*., 2012).

RebB proteins form refractile inclusion bodies (R-bodies) that have been associated with toxicity to paramecia 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 Horne, 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

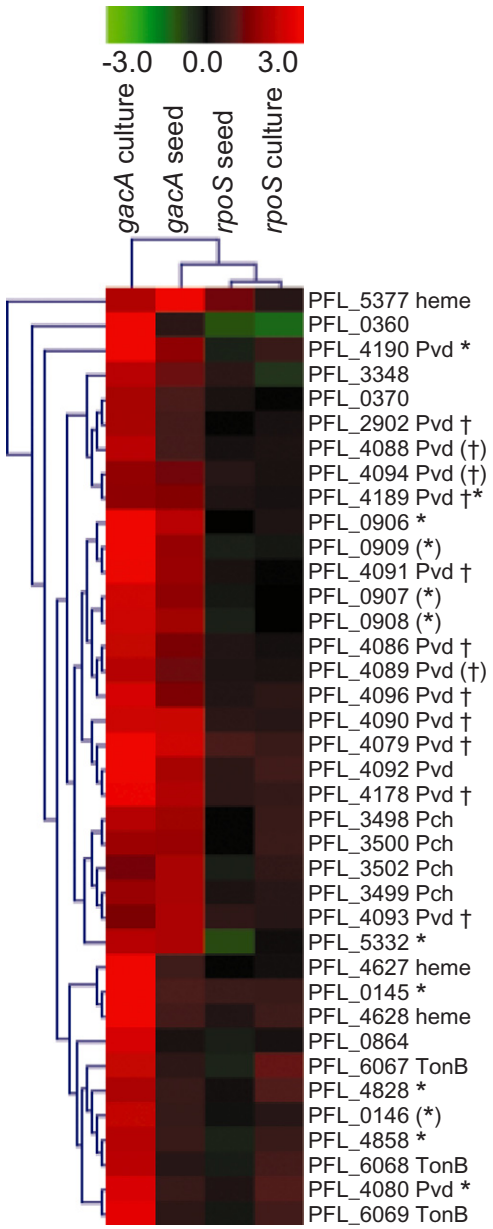

Fig. 5. Hierarchical tree showing a cluster of genes upregulated in a *gacA* mutant compared with Pf-5. Log_2 fold change ratios of the *gacA* or *rpoS* mutant compared with wild-type Pf-5 for each experiment are indicated by the colour scale. General functions of some of the genes are indicated. Pvd indicates genes present in one of the clusters involved in pyoverdine biosynthesis. Pch indicates genes present in the enantio-pyochelin 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.

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 enantio-pyochelin gene cluster, were also significantly upregulated in an *rpoS* mutant in culture, but not on seed (Table S7). Pf-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).

Pf-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 Pf-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 Pf-5, HasA, as well as ferrichrome (or ferrioxamine) and pyoverdines produced by *Pseudomonas* strains other than Pf-5 (Hartney *et al*., 2011). These results suggest that Pf-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 Pf-5 on seed and in culture.

Overall, a *gacA* mutant of Pf-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

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 downregulated in an *rpoS* mutant on seed, and orthologues of these genes are also downregulated in an *rpoS* mutant of *P. aeruginosa* PAO1 (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 cbb_3 oxidases. In *P. aeruginosa*, one cbb_3 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 Flp 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 *aphI*, which confers kanamycin resistance. The *rpoS* mutant of Pf-5 was described previously and contains a Tn*5* 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 OD_{600} = 2.0–2.4. Cultures were treated with RNAprotect Bacteria (Qiagen, Valencia, CA) for 10 min before cells were harvested by centrifugation. Cell pellets were stored at −80°C.

Inocula for pea seed microarrays were overnight cultures of Pf-5 and the *gacA* or *rpoS* mutant grown in 50 ml of NBGly in a 250 ml Erlenmeyer flask with shaking (200 r.p.m.) at 27°C. Cultures were centrifuged 5 min at 5000 *g* and the pellet washed with sterile distilled water. Washed cells were diluted to OD_{600} = 0.5 (~ 5×10^8 cfu ml^{-1}) in sterile distilled water. Pea seeds (*Pisum sativum* cv. Sugar Snap) were surface sterilized in 95% ethanol for 2 min, followed by treatment with 20% bleach for 15 min. Seeds were washed three to four times in sterile distilled water (10 min each). Seeds that swelled during sterilization (indicating broken seed coats) were discarded. Intact seed (180 per sample) were placed in the OD_{600} = 0.5 bacterial solutions for 5 min and then transferred onto sterile moistened filter paper (Whatmann #3) in Petri dishes (20 seed per dish). Petri dishes were placed in a sealed box with wet paper towels to maintain humidity and incubated at 20°C for 24 h. After 24 h, the 180 seed were combined and placed in a 66% (v/v) solution of RNAprotect Bacteria (Qiagen). Seed were vortexed at maximum speed for 30 s to remove bacteria from the seed surfaces. The liquid was decanted into a centrifuge tube, incubated for 5 min, and centrifuged 10 min at 5000 *g*. Pellets were stored at −80°C.

RNA isolation

RNA extractions were done using the RNA/DNA Midi Kit (Qiagen) according to the provided protocol. For seed experiments, 300 μl of TE buffer with lysozyme (1 mg ml^{-1}) and ~ 250 μl volume of acid washed glass beads (212–300 μm; Sigma-Aldrich, St. Louis, MO) were added to the thawed bacterial pellet and the pellet vortexed for 5 min at the maximum setting. A volume of 1.2 ml of Buffer QRL1 with 10 μl of 2-mercaptoethanol (Sigma-Aldrich) 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 (RNeasy Mini Kit with DNase I; Qiagen) and used to perform 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 log_2-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

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 $\log_2$ 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 (C_T) 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 (N_0) for each reaction using the C_T value for each sample and the average amplification efficiency of each primer set. The target/*zwf* N_0 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 (OD_{600} = 0.5) of Pf-5, the *gacA* mutant or the *rpoS* mutant, as described above. Seeds 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 triacontane was added as an internal standard. Samples for amino acid analysis were derivatized with 50 µl of *N*-methyl-*N*-(t-butyldimethylsilyl)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

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 *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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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Role categories for genes significantly regulated by GacA or RpoS on seed surfaces or in culture. Values are expressed as a ratio of the observed number of regulated genes in each role category divided by the expected number of genes from that role category. The number of expected genes was calculated by assuming proportional representation of each role category in the set of significantly up- or downregulated genes for each experiment. Role categories marked with an asterisk were significantly overrepresented as determined by a Fisher's exact test with Bonferroni correction ($P < 0.05$). Role categories are from JCVI (http://cmr.jcvi.org/cgi-bin/CMR/shared/RoleList.cgi). The total number of genes in each role category is indicated in parentheses. Certain genes fit in more than one role category and therefore are counted more than once.

Fig. S2. Differential gene transcript levels observed in microarray experiments. Transcript levels in (A) an *rpoS* mutant versus wild-type Pf-5 in culture, (B) an *rpoS* mutant versus wild-type Pf-5 on seed or (C) a *gacA* mutant versus wild-type Pf-5 on seed are shown. Each point is one of the 6147 annotated Pf-5 genes included on the microarray with the *x*-axis showing gene order and the *y*-axis showing the $\log_2$ ratio of transcript abundance in the mutant relative to wild-type Pf-5. The identities of highly regulated, well-characterized gene clusters are shown. A, B, C and D refer to the potential secondary metabolite biosynthetic gene clusters PFL_1028–1037, PFL_0258–0268, PFL_5540–5551 and

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. Log_2 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. Log_2 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 assembly 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. Log_2 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.