

# Integrated bioinformatic and phenotypic analysis of RpoN-dependent traits in the plant growth-promoting bacterium *Pseudomonas fluorescens* SBW25

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## Summary

The alternative sigma factor RpoN is a key regulator in the acclimation of *Pseudomonas* to complex natural environments. In this study we show that RpoN is required for efficient colonization of sugar beet seedlings by the plant growth-promoting bacterium *Pseudomonas fluorescens* SBW25, and use phenotypic and bioinformatic approaches to profile the RpoN-dependent traits and genes of *P. fluorescens* SBW25. RpoN is required for flagellar biosynthesis and for assimilation of a wide variety of nutrient sources including inorganic nitrogen, amino acids, sugar alcohols and dicarboxylic acids. Chemosensitivity assays indicate that RpoN-regulated genes contribute to acid tolerance and resistance to some antibiotics, including tetracyclines and aminoglycosides. Gain of function changes associated with loss of RpoN included increased tolerance to hydroxyurea and Guanazole. Bioinformatic predictions of RpoN-regulated genes show a close correspondence with phenotypic analyses of RpoN-regulated traits and suggest novel functions for RpoN in *P. fluorescens*, including regulation of poly(A) polymerase. The reduced plant colonization ability observed for an *rpoN* mutant of *P. fluorescens* is therefore likely to be due to defects in multiple traits including nutrient assimilation, protein secretion and stress tolerance.

## Introduction

The alternative sigma factor RpoN regulates genes involved in nitrogen assimilation and environmental acclimation in a wide range of bacteria. *rpoN* is present in the genomes of many, but not all, *Proteobacteria* and is also present in bacteria from other taxonomical groups, including the *Firmicutes*, *Spirochaetes*, *Planctomycetes* and *Chlamydiae*. The mechanism and primary sequence of RpoN is unique among  $\gamma$ -proteobacterial sigma factors, and has features in common with eukaryotic transcription factors. RpoN recognizes a conserved *cis*-acting promoter motif, which is frequently adjacent to one or more binding sites for integration host factor (IHF). RpoN binds to RNA polymerase to form a closed holoenzyme complex that requires further activation by members of the NtrC class of enhancer-binding proteins (EBPs) (Studholme and Buck, 2000; Cases and de Lorenzo, 2001; Studholme and Dixon, 2003). Transcriptional activation by RpoN requires nucleotide hydrolysis by EBPs, which typically bind promoters at a distance from the transcription start site and contact RpoN by DNA looping (Carmona *et al.*, 1997; Studholme and Buck, 2000; Studholme and Dixon, 2003). DNA looping and supercoiling may be enhanced by the DNA-bending activity of IHF (Carmona *et al.*, 1997; Dworkin *et al.*, 1997; 1998; Palacios and Escalante-Semerena, 2000; Sze *et al.*, 2001). Environmental signal transduction occurs via activation or de-repression of EBPs, although general metabolic signals such as the stringent response signal (p)ppGpp and alterations in DNA supercoiling also affect a subset of RpoN-dependent promoters (Sze and Shingler, 1999; Carmona *et al.*, 2000; Sze *et al.*, 2001; Paul *et al.*, 2005; Bernardo *et al.*, 2006). As a regulatory mechanism RpoN confers tightly regulated, environment-specific gene expression over a wide range of expression levels, which may account for the recruitment of RpoN into the regulatory cascades of a diverse range of traits.

The well-characterized features of RpoN, EBPs and RpoN-binding promoters facilitate bioinformatic predictions of the components and functions of the RpoN-regulatory network. All RpoN-activating EBPs possess a conserved RpoN-activating ATPase domain (Pfam domain: PF00158), and most possess a conserved

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DNA-binding, helix–turn–helix (HTH) domain (typically PF02954 or PF00165). Bioinformatic predictions of RpoN binding sites give a consensus sequence of 5'-mrNrYTGGCACG-N4-TTG $\underline{C}$ WNNw-3', in which the underlined G and C nucleotides are situated at position –24 and –12 relative to the transcription start site (Barrios *et al.*, 1999; Dombrecht *et al.*, 2002; Cases *et al.*, 2003; Studholme and Dixon, 2004). Bacteria typically contain more RpoN-binding promoters than EBPs, as some EBPs, such as FleQ (AdnA), regulate transcription at multiple promoters (Jyot *et al.*, 2002; Robleto *et al.*, 2003). RpoN binding sites are also found upstream of regulatory proteins, such as the extracytoplasmic sigma factor HrpL of *Pseudomonas syringae* (Hendrickson *et al.*, 2000a), thereby placing additional genes under RpoN-dependent regulation. *Pseudomonas putida* KT2440 contains 22 EBPs and is estimated to contain around 55 RpoN-binding promoters (Cases *et al.*, 2003; Jurado *et al.*, 2003).

*Pseudomonas* strains lacking RpoN are typically compromised in host interactions, antagonistic interactions or survival in complex environments, but are generally able to grow *in vitro*, and are reported to tolerate environmental stresses such as extremes of heat, pH and osmolarity as well as wild-type bacteria (Hendrickson *et al.*, 2000b; Cases and de Lorenzo, 2001; Cases *et al.*, 2003). However, Pechy-Tarr and colleagues (2005) recently reported that an *rpoN* mutant of *Pseudomonas fluorescens* CHA0 was impaired in salt tolerance. Traits reported to be regulated by RpoN in *Pseudomonas* include nitrogen assimilation, dicarboxylic acid assimilation, motility, and virulence associated traits such as protein secretion, extracellular polysaccharide biosynthesis, quorum sensing and toxin biosynthesis (Hendrickson *et al.*, 2000a; Studholme and Buck, 2000; Cases and de Lorenzo, 2001; Cases *et al.*, 2003; Francisco *et al.*, 2003; Heurlier *et al.*, 2003; Penaloza-Vazquez *et al.*, 2004).

Empirical selection of assays for the functional characterization of RpoN mutants is inherently biased towards the known properties and predicted functions of RpoN-regulated genes, as outlined above. One way to challenge and confirm current hypotheses regarding the function of RpoN and RpoN-regulated proteins is to perform phenotypic analyses in a high-throughput format, comparing bacterial performance across a wide range of environmental conditions, a technique known as a phenoarray. In this study we use commercially available phenoarrays (Biolog Phenotype MicroArrays™) in conjunction with bioinformatics, mutagenesis and targeted phenotypic analyses to systematically investigate the role of RpoN in plant colonization by the model bacterium *P. fluorescens* SBW25 (SBW25). SBW25 was originally isolated from the sugar beet phyllosphere, and is a highly effective colonist

of plant roots and leaf surfaces (Bailey *et al.*, 1995). The genome of SBW25 has been sequenced by the Wellcome Trust Sanger Institute ([http://www.sanger.ac.uk/Projects/P\\_fluorescens](http://www.sanger.ac.uk/Projects/P_fluorescens)), and SBW25 has previously been used in experiments to identify plant-induced genes, to study the role of bacterial cellulose in plant colonization and biofilm formation, and to study bacterial adaptation to novel environments (Travisano and Rainey, 1998; Rainey, 1999; Preston *et al.*, 2001; Spiers *et al.*, 2002; Gal *et al.*, 2003; MacLean *et al.*, 2004; Jackson *et al.*, 2005; Zhang *et al.*, 2006).

## Results

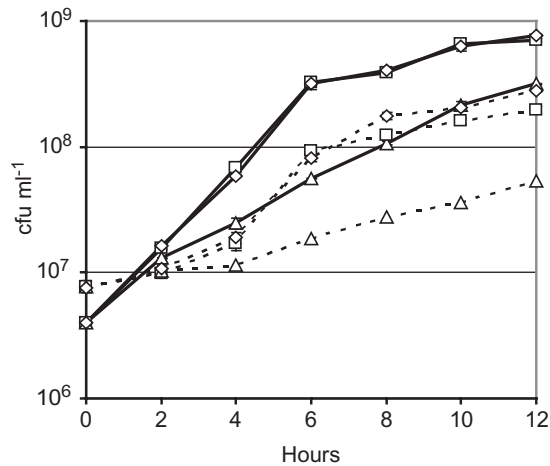
### *Construction of an RpoN mutant of P. fluorescens SBW25*

The genome of SBW25 encodes a single RpoN protein (PFLU0882), which contains all three conserved RpoN Pfam domains (PF00309, PF04963 and PF04552) and is similar to the RpoN proteins of *P. fluorescens* Pf-5, *P. fluorescens* PfO-1 and *P. syringae* (96% identity). The *rpoN* gene is located upstream of and in the same orientation as four genes: PFLU0881, a putative RpoN-modulating protein (PF02482); PFLU0880, a homologue of the PTS IIA-like nitrogen-regulatory protein PtsN (PF00359); PFLU0879, a hypothetical P-loop ATPase protein (PF03668); and PFLU0878, a putative phosphocarrier protein (PF00381). *rpoN* and flanking genes from PFLU0878 to PFLU0887 are conserved and syntenic in all completed *Pseudomonas* genomes (*P. aeruginosa* PAO1, *P. aeruginosa* PA14, *P. putida* KT2440, *P. entomophila* L48, *P. fluorescens* Pf-5, *P. fluorescens* PfO-1, *P. fluorescens* SBW25, *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *phaseolicola* 1448A).

Overexpression or inactivation of genes downstream of *rpoN* has previously been shown to alter expression of both RpoN-regulated and RpoN-independent genes (Merrick and Coppard, 1989; Begley and Jacobson, 1994; Powell *et al.*, 1995; Cases *et al.*, 1999; 2001; Boel *et al.*, 2003). We therefore constructed an unmarked, non-polar deletion mutant of the *rpoN* open reading frame (ORF) by gene replacement, as described under *Experimental procedures*. We refer to the deletion mutant as SBW25  $\Delta rpoN$  throughout this paper.

### *RpoN is important but not essential for growth in complex and minimal media*

SBW25  $\Delta rpoN$  was significantly impaired in growth in complex media [Luria–Bertani (LB)] and in M9-glucose when compared with SBW25 (Fig. 1). Growth was restored to near wild-type levels when the mutant was



**Fig. 1.** SBW25  $\Delta rpoN$  shows reduced growth compared with SBW25 in complex and minimal media. SBW25, SBW25  $\Delta rpoN$  and the complemented strain SBW25  $\Delta rpoN$  (pJJP6) were grown overnight in LB at 28°C, 200 r.p.m. and transferred to 7.5 ml of M9-glucose or LB. Cultures were incubated at 28°C, 200 r.p.m. and the OD<sub>600</sub> was recorded every 2 h. OD<sub>600</sub> was converted to cfu ml<sup>-1</sup> according to standard curves. Data shown are the average of three independent replicates. LB (solid line); M9 (dashed line); SBW25 (squares); SBW25  $\Delta rpoN$  (triangles); SBW25  $\Delta rpoN$  (pJJP6) (diamonds). Error bars show standard deviation.

complemented with plasmid pJJP6, which constitutively expresses *rpoN*. Complementation with the control plasmid pBroadgate-D had no effect on growth (data not shown). Supplementary glutamine has been reported to rescue growth defects observed with *rpoN* mutants of *P. aeruginosa* and *P. syringae* (Totten *et al.*, 1990; Hendrickson *et al.*, 2001; Francisco *et al.*, 2003). However, SBW25  $\Delta rpoN$  displayed only a slight increase in growth when cultured in LB supplemented with glutamine (data not shown). SBW25  $\Delta rpoN$  was unable to grow in M9 when glucose was replaced with dicarboxylic acids such as succinate (data not shown).

#### *RpoN* is required for rhizosphere competence and motility in SBW25

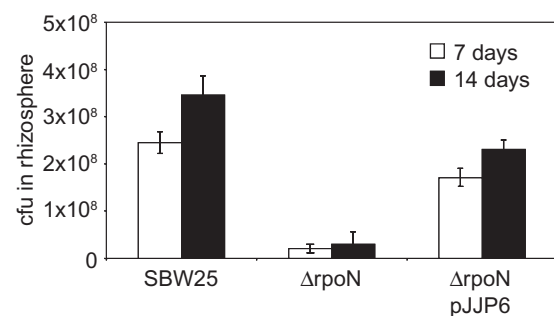
We assessed the ability of SBW25  $\Delta rpoN$  to colonize the sugar beet seedling rhizosphere using the colonization assay described by Rainey (1999) and Gal and colleagues (2003). SBW25  $\Delta rpoN$  was significantly compromised in its ability to colonize sugar beet roots (Fig. 2). Colonization could be rescued by complementation with pJJP6.

One trait known to contribute to plant colonization by SBW25, and predicted to be defective in SBW25  $\Delta rpoN$  is motility (Turnbull *et al.*, 2001). Electron microscopy and motility assays confirmed that SBW25  $\Delta rpoN$  is aflagellate and non-motile (Fig. 3). Flagellar biosynthesis could be restored by complementation with pJJP6 (Fig. 3), but not with pBroadgate-D (not shown). Intriguingly, prolonged (> 48 h) incubation of soft agar plates inoculated with

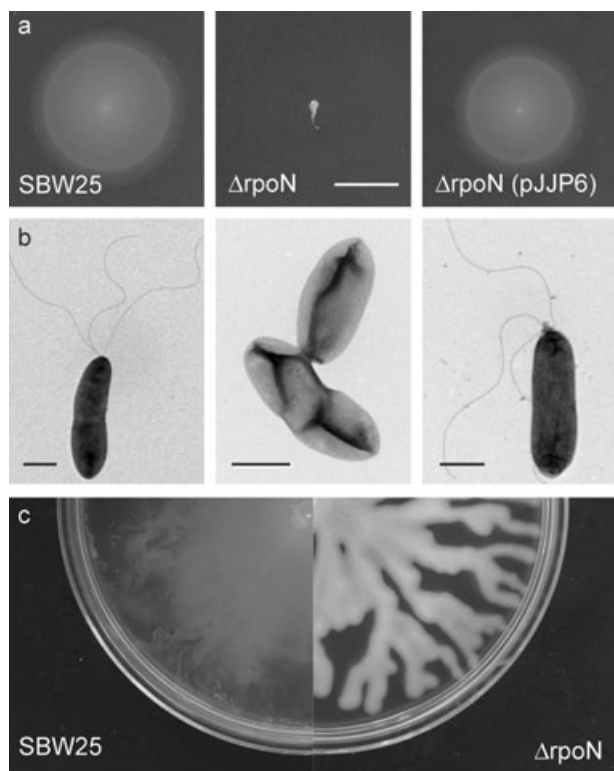
SBW25  $\Delta rpoN$  frequently resulted in the appearance of surface-colonizing bacteria (Fig. 3C), which appear to be spontaneous mutant derivatives of SBW25  $\Delta rpoN$ . SBW25  $\Delta rpoN$  showed no difference from SBW25 in adhesion assays (data not shown).

#### Phenotype MicroArray™ analysis of *RpoN*-dependent traits in SBW25

Previous analyses of *rpoN* mutants of *Pseudomonas* have identified numerous differences between wild-type and mutant strains, including differences in nutrient utilization, stress tolerance and host interactions (Köhler *et al.*, 1989; Totten *et al.*, 1990; Hendrickson *et al.*, 2000b; 2001; Alarcon-Chaidez *et al.*, 2003; Pechy-Tarr *et al.*, 2005). We used the Biolog Phenotype MicroArray™ (phenomicroarray) system to generate a detailed profile of phenotypic differences between SBW25 and SBW25  $\Delta rpoN$  that could account for the impaired rhizosphere competence of this strain. Assays were performed in duplicate by Biolog according to standard protocols (<http://www.biolog.com>; Bochner *et al.*, 2001). Eighteen 96-well phenomicroarrays {metabolic panels PM01 [Carbon (C) source], PM02A (C), PM03B [Nitrogen (N) source], PM06–PM08 (N source) and sensitivity panels PM09, PM10B, PM11, PM12B, PM13B, PM14A, PM15B, PM16A, PM17A, PM18C, PM19, PM20B} were used. *In vitro* growth experiments had shown that SBW25  $\Delta rpoN$  was unable to use succinic acid (standard Biolog C source) as sole C source, so succinic acid was replaced with glucose in nitrogen utilization panels PM03B and PM06–PM08. No modifications were made to panels PM09–PM20B, which contain Biolog complex media as a base medium.



**Fig. 2.** SBW25  $\Delta rpoN$  is impaired in the ability to colonize the sugar beet seedling rhizosphere. Sugar beet seeds were inoculated with SBW25, SBW25  $\Delta rpoN$  and the complemented strain SBW25  $\Delta rpoN$  (pJJP6) as described in *Experimental procedures*. Data shown are the average number of bacteria/rhizosphere from eight independently harvested seedlings. Error bars show standard error. The population density of SBW25  $\Delta rpoN$  is significantly lower than the population density of SBW25 and SBW25  $\Delta rpoN$  (pJJP6), and the population density of SBW25  $\Delta rpoN$  (pJJP6) is significantly lower than the population density of SBW25 at both 7 and 14 days after inoculation (determined by ANOVA,  $P < 0.001$ ).



**Fig. 3.** SBW25  $\Delta rpoN$  is aflagellate and non-motile. A. Plate assays show that SBW25  $\Delta rpoN$  (centre) is non-motile. Complementation of SBW25  $\Delta rpoN$  with the  $rpoN$ -expressing plasmid pJJP6 restores motility to near wild-type levels [SBW25 (left), SBW25  $\Delta rpoN$  pJJP6 (right)]. Plates contain 1:10 LB and 0.3% agar and were photographed 24 h after inoculation. Bar = 1 cm. B. Imaging of bacteria by transmission electron microscopy confirms that SBW25 (left) is flagellate and SBW25  $\Delta rpoN$  (middle) is aflagellate. Complementation with pJJP6 restores flagellar biosynthesis (right). The collapsed appearance of SBW25  $\Delta rpoN$  cells is representative of all cells observed in two independent experiments. Bar = 1  $\mu$ m. C. Prolonged incubation of swarm plates results in rapid surface colonization by SBW25 (left, photographed 48 h after inoculation), and mucoid, branched surface colonization by SBW25  $\Delta rpoN$  (right, photographed 72 h after inoculation). The surfaces of plates inoculated with wild-type SBW25 are completely covered with bacteria 72 h after inoculation (not shown).

SBW25 performed as well as, or better than, SBW25  $\Delta rpoN$  in almost all assays, with two notable exceptions. SBW25  $\Delta rpoN$  was able to tolerate higher concentrations of 3,5-Diamino-1,2,4-triazole (Guanazole) and hydroxyurea. Both of these chemicals inhibit ribonucleotide DP reductase (RNR), which catalyses the biosynthesis of dNTPs from corresponding ribonucleotides (Moore and Hurlbert, 1985; Nordlund and Reichard, 2006). Hydroxyurea is a specific inhibitor of class I RNR and has also been reported to inhibit folate metabolism and amidase activity, and to chelate iron (Gregoriou and Brown, 1979; Nigovic *et al.*, 2005; Nordlund and Reichard, 2006).

### *RpoN* is required for assimilation of a wide range of carbon and nitrogen sources

Interpretation of phenoarray results is complicated by the observation that SBW25  $\Delta rpoN$  shows reduced growth compared with wild-type SBW25 in both minimal (PM01–PM03B, PM06–PM08) and complex (PM09–PM20B) media (Fig. 1). Normalization of data, as described in *Experimental procedures*, allowed us to identify gains and losses of growth competences relative to overall differences in growth rates (Tables 1 and 2, Tables S1 and S2). SBW25  $\Delta rpoN$  was impaired in the ability to assimilate a wide range of organic and inorganic nutrients including: nitrate, urea, amino acids as carbon sources (e.g. valine, leucine, asparagine) or nitrogen sources (e.g. aspartate, asparagine); dicarboxylic acids (e.g. malate, succinate); sugar alcohols (e.g. myo-inositol, D-sorbitol); sugars (e.g. D-ribose, D-xylose); and nucleosides (e.g. uridine, cytosine).

The low signal strength or high level of variability observed between replicates for some assays meant that some results that were not identified as statistically significant ( $P > 0.05$ ) could be false negatives. This particularly affects assays in which the performance of SBW25 is low, but detectable, and the performance of SBW25  $\Delta rpoN$  is similar to negative control values (Tables 1C and 2C). Validation experiments confirmed that SBW25  $\Delta rpoN$  displays a growth defect in the presence of L-Histidine, D-Mannitol or D-Trehalose (Table 1C) as sole C source, and Inosine, L-Valine and L-Proline (Table 2C) as sole N source. Growth could be restored by complementation with pJJP6 (Figs S1 and S2). Normalization of phenoarray data also allowed us to identify assays in which SBW25  $\Delta rpoN$  performed relatively well compared with SBW25 (Tables 1B and 2B). Four of these C sources were tested in *in vitro* growth assays, which showed that SBW25  $\Delta rpoN$  grew better than SBW25 on adenosine as sole C source, and grew comparatively well on acetic acid, lactic acid and glycerol (Fig. S1).

### *RpoN* contributes to acid tolerance in SBW25

Nutrient uptake is not the only trait required for successful rhizosphere colonization by *Pseudomonas*. Rhizosphere-colonizing bacteria also need to be able to tolerate biotic and abiotic stressors present in the plant rhizosphere, such as pH, osmolarity and antimicrobial chemicals produced by plant roots and other rhizosphere organisms. Biolog sensitivity panels assess bacterial performance in the presence of a wide range of antimicrobial chemicals (PM11–PM20B) and environmental stressors (PM9, PM10B). Sensitivity panels PM9–PM20B were analysed using the null hypothesis that the difference between strains in the presence of the stressor was equivalent to

**Table 1.** Carbon source utilization by SBW25 and SBW25  $\Delta rpoN$ .

Carbon source	Signal <sup>a</sup>		<i>P</i> <sup>b</sup>
	SBW25	$\Delta rpoN$	
<b>(A) SBW25 &gt; <math>\Delta rpoN</math></b>			
Itaconic acid	56.1	-2.1 <sup>c</sup>	0.012
L-Isoleucine	48.6	1.3 <sup>c</sup>	0.038
Quinic acid	52.6	6.3 <sup>c</sup>	0.027
D,L-Citramalic acid	45.1	2.6 <sup>c</sup>	0.042
L-Pyroglutamic acid	52.4	20.9	0.042
L-Valine <sup>d</sup>	35.4	6.9 <sup>c</sup>	0.012
L-Leucine	21.8	-4.0 <sup>c</sup>	0.044
L-Asparagine	26.0	0.92 <sup>c</sup>	0.034
D-Alanine	22.8	-0.4 <sup>c</sup>	0.043
L-Alanine	21.4	0.7 <sup>c</sup>	0.046
Myo-inositol <sup>d</sup>	19.4	-0.9 <sup>c</sup>	0.038
$\beta$ -Hydroxy-butyric acid	32.4	13.7	0.042
L-Proline	17.9	0.7 <sup>c</sup>	0.042
p-Hydroxy-phenylacetic acid	20.3	3.9	0.036
D-Galactonic acid- $\gamma$ -lactone	15.5	-0.8	0.042
D,L-Malic acid	24.1	8.1	0.042
D-Glucosamine	16.2	1.2 <sup>c</sup>	0.042
Tyramine	25.1	10.3	0.042
D-Glucosaminic acid	11.9	-0.9 <sup>c</sup>	0.038
D-Saccharic acid	12.3	-0.2 <sup>c</sup>	0.044
D-Glucuronic acid	11.0	-1.1 <sup>c</sup>	0.044
D-Sorbitol	11.5	-0.5 <sup>c</sup>	0.027
D-Galacturonic acid	11.0	0.7 <sup>c</sup>	0.044
Mucic acid	13.7	3.5	0.034
L-Malic acid	19.0	9.2	0.036
L-Alanyl-glycine	9.8	0.1 <sup>c</sup>	0.042
D-Ribose <sup>d</sup>	12.5	3.4 <sup>c</sup>	0.048
L-Ornithine	22.6	13.7	0.048
Adonitol	7.9	-0.8 <sup>c</sup>	0.033
Succinic acid <sup>d</sup>	7.8	0.2 <sup>c</sup>	0.042
D-Xylose	6.5	-0.8 <sup>c</sup>	0.036
Uridine <sup>d</sup>	8.4	1.9 <sup>c</sup>	0.034
L-Glutamic acid	17.5	12.4	0.044
L-Threonine	3.3	-0.8 <sup>c</sup>	0.042
Glycyl-L-proline	4.8	1.3 <sup>c</sup>	0.034
<b>(B) <math>\Delta rpoN</math> &gt; SBW25</b>			
L-Alaninamide	1.2 <sup>c</sup>	11.1	0.104
Sorbic acid	4.4 <sup>c</sup>	8.9	0.224
Adenosine <sup>d</sup>	6.7	11.2	0.069
Acetic acid <sup>d</sup>	4.0	7.8	0.056
L-Lactic acid <sup>d</sup>	5.5	8.3	0.076
Glycerol <sup>d</sup>	4.2	6.5	0.200
Malonic acid	7.0 <sup>c</sup>	8.8	0.250
Propionic acid	5.7	6.6	0.412
Bromo-Succinic acid	3.0	3.8	0.076
<b>(C) <math>\Delta rpoN \leq</math> negative control (<math>P &gt; 0.05</math>)</b>			
Fumaric acid	17.7	-0.6 <sup>c</sup>	0.096
L-Histidine <sup>d</sup>	37.2	-4.0 <sup>c</sup>	0.055
Succinamic acid	35.1	-3.9 <sup>c</sup>	0.084
D-Mannitol <sup>d</sup>	14.9	0.5 <sup>c</sup>	0.142
4-Hydroxy-benzoic acid	31.2	-4.1 <sup>c</sup>	0.106
D-Arabitol	40.0	4.7 <sup>c</sup>	0.144
m-Tartaric acid	8.2	-1.1 <sup>c</sup>	0.055
D-Trehalose <sup>d</sup>	10.8	1.8 <sup>c</sup>	0.062
L-Arabitol	15.3	-3.7 <sup>c</sup>	0.056
Capric acid	20.6	2.5 <sup>c</sup>	0.056
Putrescine	14.4	-0.8 <sup>c</sup>	0.056
Xylitol	7.9	-4.1 <sup>c</sup>	0.114

a. Given values are average signal levels corrected to the negative control and normalized to total signal on their plate; for each genotype the value is a mean of two replicates.

b. Probability, following FDR correction for multiple testing, of the null hypothesis that the true mean signal values are actually the same in the two genotypes.

c. No detectable signal based on average signal before correction or normalization relative to negative control, indicating a qualitative difference between SBW25 and SBW25  $\Delta rpoN$ .

d. Tested independently in *in vitro* C source utilization assays (Fig. S1). A complete set of results is listed in Table S1.

Substrates in each section are ordered from the largest to the smallest absolute differences between genotypes. (A) C sources on which SBW25 generates a higher signal compared with SBW25  $\Delta rpoN$  after normalization of signal values ( $P \leq 0.05$  after FDR correction). (B) C sources on which SBW25  $\Delta rpoN$  generates higher raw and normalized signal values compared with SBW25 ( $P > 0.05$ ). (C) C sources on which SBW25 generates a detectable signal and SBW25  $\Delta rpoN$  fails to give an average signal higher than the negative control value before normalization ( $P > 0.05$  after FDR correction).

**Table 2.** Nitrogen source utilization by SBW25 and SBW25  $\Delta rpoN$ .

Nitrogen source	Signal <sup>a</sup>		<i>P</i> <sup>b</sup>
	SBW25	$\Delta rpoN$	
<b>(A) SBW25 &gt; <math>\Delta rpoN</math></b>			
D-Glutamic acid	26.5	-2.8 <sup>c</sup>	0.042
Leu-Ala <sup>d</sup>	22.2	-4.3 <sup>c</sup>	0.037
$\gamma$ -Amino-N-butyric acid <sup>d</sup>	17.4	-5.2 <sup>c</sup>	0.045
Ala-His	25.3	3.6	0.042
L-Aspartic acid <sup>d</sup>	31.1	11.2	0.043
L-Asparagine <sup>d</sup>	25.8	10.1	0.042
Ile-Asn	19.2	7.7	0.038
Cytidine	12.2	-2.8 <sup>c</sup>	0.050
Urea <sup>d</sup>	6.8	-4.7 <sup>c</sup>	0.050
Nitrate <sup>d</sup>	6.9	-2.9 <sup>c</sup>	0.048
Val-Val	6.5	2.1	0.018
Glycine <sup>d</sup>	7.7	-0.9 <sup>c</sup>	0.042
D-Asparagine	3.7	-3.2 <sup>c</sup>	0.042
Ethanolamine	2.5	-3.9 <sup>c</sup>	0.003
<b>(B) <math>\Delta rpoN</math> &gt; SBW25</b>			
Lys-Met	2.2 <sup>c</sup>	6.6	0.045
Glu-Asp	-0.2 <sup>c</sup>	3.9	0.038
Ser-Asp	5.0	8.5	0.048
Phe-Met	0.8 <sup>c</sup>	4.2	0.048
Glu-Ser	1.1	4.3	0.038
Thr-Asp	5.2	8.3	0.038
Ser-Pro	0.1 <sup>c</sup>	2.9	0.049
Pro-Ser	2.6 <sup>c</sup>	5.3	0.043
Gln-Gly	5.0	7.6	0.042
Ser-Phe	3.2	5.4	0.049
Pro-Gly	0.5 <sup>c</sup>	2.6	0.038
Arg-Met	5.5	7.4	0.042
Thr-Met	0.3 <sup>c</sup>	2.2	0.038
Thr-Pro	0.8 <sup>c</sup>	2.6	0.042
Lys-Phe	3.0	4.7	0.049
Pro-Asp	1.2 <sup>c</sup>	2.3	0.049
<b>(C) <math>\Delta rpoN \leq</math> negative control</b>			
L-Proline <sup>d</sup>	28.7	-1.4 <sup>c</sup>	0.064
D,L-Lactamide	20.2	-1.3 <sup>c</sup>	0.061
Xanthosine	17.9	-0.1 <sup>c</sup>	0.057
Inosine <sup>d</sup>	17.0	-0.9 <sup>c</sup>	0.061
Guanosine	11.1	-2.6 <sup>c</sup>	0.054
Allantoin	8.1	-4.6 <sup>c</sup>	0.054
Nitrite	8.9	-3.8 <sup>c</sup>	0.055
L-Valine <sup>d</sup>	11.2	0.2 <sup>c</sup>	0.064
Cytosine	5.3	-5.2 <sup>c</sup>	0.055
N-Acetyl-D-glucosamine	6.7	-1.5 <sup>c</sup>	0.054
Uridine	5.7	-1.8 <sup>c</sup>	0.055

a. Given values are average signal levels corrected to the negative control and normalized to total signal on their plate; for each genotype the value is a mean of two replicates.

b. Probability, following FDR correction for multiple testing, of the null hypothesis that the true mean signal values are actually the same in the two genotypes.

c. No detectable signal based on average signal before correction or normalization relative to negative control, indicating a qualitative difference between SBW25 and SBW25  $\Delta rpoN$ .

d. Tested independently in *in vitro* N source utilization assays (Fig. S2). A complete set of results is listed in Table S2.

Substrates in each section are ordered from the largest to the smallest absolute differences between genotypes. (A) N sources on which SBW25 generates a higher signal compared with SBW25  $\Delta rpoN$  after normalization of signal values ( $P \leq 0.05$  after FDR correction). The complete data set includes a large number of peptide N sources (plates PM06-PM08) on which SBW25 generates a higher signal compared with SBW25  $\Delta rpoN$  ( $P \leq 0.05$ ). These are not listed in full here, but are described in Table S2. (B) N sources on which SBW25  $\Delta rpoN$  generates higher values compared with SBW25 after normalization of signal values ( $P \leq 0.05$ ). (C) N sources on which SBW25 generates a detectable signal and SBW25  $\Delta rpoN$  fails to give an average signal higher than the negative control value before normalization ( $P > 0.05$  after FDR correction).

the difference observed in the absence of the stressor (Table S3). For assays using more than one well to test a series of related stressors (e.g. testing different concentrations of an antimicrobial chemical) growth was assessed as a proportion of the maximum growth in wells testing that condition, with the null hypothesis that there was no difference between the strains (Table S4).

SBW25  $\Delta rpoN$  displayed lower tolerance to acidic pH ( $P = 0.03$ ), and similar tolerance to alkaline pH when compared with SBW25 (Table 3A and C and Table S4). However, SBW25  $\Delta rpoN$  showed increased tolerance to pH 9.5 relative to SBW25 in the presence of L-Arginine and L-Norvaline (Table 3B and Table S3). Osmosensitivity results indicated that SBW25  $\Delta rpoN$  shows increased sensitivity to some osmotic stressors, as previously reported for *P. fluorescens* CHA0 by Pechy-Tarr and colleagues (2005), but only KCl and sodium formate came close to satisfying the  $P < 0.05$  threshold (Table 3C and Table S4).

#### *Chemosensitivity assays reveal multiple roles for RpoN in antibiotic resistance*

SBW25  $\Delta rpoN$  displayed both increased and decreased tolerance to chemical stressors relative to SBW25 (Table 4 and Table S4). The term 'increased tolerance' is used here to refer to results where SBW25  $\Delta rpoN$  shows a distinct gain of function and outperforms SBW25 in terms of minimal inhibitory concentrations (as noted above for Guanazole and hydroxyurea), and to results where wild-type SBW25 shows pronounced inhibition as chemical concentrations increase, but mutant performance remains relatively high compared with its performance at the lowest inhibitor concentration used. It is conceivable that increased sensitivity in SBW25  $\Delta rpoN$  could lead to pronounced inhibition across all treatments, with no significant change in inhibition and signal strength as concentrations are increased beyond the initial condition. Therefore, as with nutrient source analyses, it is important that the results of these analyses are supported by inspection of raw values and validation of key results *in vitro*.

We observed significant differences between SBW25 and SBW25  $\Delta rpoN$  in 48 chemosensitivity assays, including seven assays in which SBW25  $\Delta rpoN$  outperformed SBW25 (Table 4B and Table S4). SBW25  $\Delta rpoN$  showed decreased tolerance to a wide range of chemicals, most notably to a wide range of inhibitors of protein synthesis such as aminoglycosides, tetracyclines, macrolides and fusidic acid. SBW25  $\Delta rpoN$  showed increased tolerance to RNR inhibitors, as noted above, and to toxic anions (Table 4A and B).

A subset of sensitivity results were validated using *in vitro* growth assays (Table 4, Fig. 4). Validation experiments confirmed that SBW25  $\Delta rpoN$  showed high toler-

**Table 3.** pH and osmotolerance of SBW25 and SBW25  $\Delta rpoN$ .

Assay	Mode of action	Signal <sup>a</sup>		<i>P</i> <sup>b</sup>
		WT	<i>rpoN</i>	
(A) SBW25 > $\Delta rpoN$				
Acid	pH	0.80	0.54	0.030
pH 4.5 + 5-Hydroxy-L-lysine	pH, decarboxylase	7.5	-2.3 <sup>c</sup>	0.025
pH 4.5 + Glycine	pH, decarboxylase	5.0	-0.4 <sup>c</sup>	0.016
(B) $\Delta rpoN$ > SBW25				
pH 9.5 + L-Arginine	pH, deaminase	15.8	18.5	0.039
pH 9.5 + L-Norvaline	pH, deaminase	14.6	16.2	0.045
(C) Additional results				
Sodium formate	Osmotic sensitivity	0.96	0.60	0.051
KCl	Osmotic sensitivity	0.76	0.63	0.061
Ethylene glycol	Osmotic sensitivity	1.23	1.16	0.158
NaCl	Osmotic sensitivity	0.47	0.43	0.125
Alkali	pH	1.23	1.29	0.427

**a.** Given values are those compared in the test for which the *P*-value is given, in assays with a single treatment, this is the corrected normalized signal as in earlier tables; for acid, alkali and osmotic sensitivity assays (where multiple tests are summarized in each line) the value shown describes bacterial tolerance to increasing concentrations of stressor, and is derived from average signal as a proportion of the maximum growth achieved in the assay, as described in *Experimental procedures*. A high test value indicates low sensitivity of growth to the stressor.

**b.** Probability, following FDR correction for multiple testing, of the null hypothesis that the true mean signal values are actually the same in the two genotypes.

**c.** No detectable signal based on average signal before correction or normalization relative to negative control, indicating a qualitative difference between SBW25 and  $\Delta rpoN$ . A complete set of results is listed in Table S3 (single wells) and Table S4 (multiple wells).

(A) Assays where SBW25 gives a higher signal compared with SBW25  $\Delta rpoN$  after normalization of signal values ( $P < 0.05$  after FDR correction).

(B) Assays where SBW25  $\Delta rpoN$  shows higher tolerance compared with SBW25 after normalization of signal values ( $P < 0.05$  after FDR correction). (C) Additional pH and osmotolerance results discussed in main text ( $P > 0.05$  after FDR correction).

ance to hydroxyurea and methyl viologen and relatively low tolerance to kanamycin, neomycin, tetracycline and nalidixic acid. Interestingly, sublethal concentrations of hydroxyurea induced visible flocculation in SBW25, but not in SBW25  $\Delta rpoN$ . We also tested two chemicals that gave inconsistent results in chemosensitivity assays in terms of the relative performance of the two strains in individual wells, cupric chloride and boric acid (adjusted to a neutral pH). SBW25  $\Delta rpoN$  grew better than SBW25 in the presence of high concentrations of boric acid, and grew well compared with SBW25 at high concentrations of cupric chloride, despite performing worse than SBW25 at lower concentrations, confirming that this interaction cannot easily be assigned to a single class (Fig. 4).

#### *Hidden Markov model-based prediction of RpoN-dependent promoters in Pseudomonas fluorescens SBW25*

In the final phase of this study we performed bioinformatic analyses to predict the identity and function of RpoN-regulated genes in the unpublished genome of SBW25, with the dual goals of identifying genes linked to the RpoN-dependent traits observed in experimental analyses, and identifying RpoN-regulated genes and traits that had not been tested in the phenotypic analyses described above, but which could be involved in plant colonization by SBW25.

We searched the unpublished SBW25 genome ([http://www.sanger.ac.uk/Projects/P\\_fluorescens](http://www.sanger.ac.uk/Projects/P_fluorescens)) for putative RpoN binding sites using a profile hidden Markov model (HMM) *rpoN* promoter matrix based on 186 sequences from a wide range of *Proteobacteria* (Barrios *et al.*, 1999). We used the frequency of intergenic hits (Studholme and Dixon, 2004) and the similarity of downstream ORFs to previously described RpoN-regulated genes as criteria to restrict the output from this analysis to intergenic sequences scoring at least 14. Table 5 lists RpoN-binding motifs that score > 14 and are predicted to be: (i) intergenic and (ii) in the correct orientation and location to act as a promoter for an adjacent ORF. Further information on the genomic context of putative RpoN binding sites is available in Table S5, which lists operon predictions, BLASTP results (Genbank; <http://www.ncbi.nlm.nih.gov/BLAST>; Altschul *et al.*, 1990), Pfam domain predictions (Pfam, Finn *et al.*, 2006), IHF binding site predictions (RegulonDB, Salgado *et al.*, 2006), and EBP binding site predictions for FleQ, NorR and NtrC, based on previously described HMM [FleQ (*P. aeruginosa*): Jyot *et al.*, 2002]; NorR (*Escherichia coli*): Tucker *et al.*, 2005]; and NtrC (*E. coli*): Salgado *et al.*, 2006].

FleQ and NtrC predictions successfully identified sites that are likely to act as EBP binding sites, based on the similarity of downstream genes to genes shown to be regulated by FleQ or NtrC in previous studies, as discussed below. However, the NorR HMM only identified

**Table 4.** Chemosensitivity of SBW25 and SBW25  $\Delta rpoN$ .

Assay	Mode of action	Signal <sup>a</sup>		<i>P</i> <sup>b</sup>
		WT	<i>rpoN</i>	
<b>(A) SBW25 &gt; <math>\Delta rpoN</math></b>				
Demeclocycline	Protein synthesis, 30S subunit, tetracycline	0.70	0.39	< 0.001
Doxycycline	Protein synthesis, 30S subunit, tetracycline	0.92	0.39 <sup>c</sup>	0.007
Cinnamic acid	Respiration, ionophore, H <sup>+</sup>	1.36	0.88	0.007
Neomycin <sup>d</sup>	Protein synthesis, 30S subunit, aminoglycoside	0.74	0.39 <sup>c</sup>	0.007
Amikacin	Protein synthesis, 30S subunit, aminoglycoside	0.71	0.39	0.007
Lauryl sulfobetaine	Membrane, detergent, zwitterionic	0.80	0.52 <sup>c</sup>	0.007
Chelerythrine	Protein kinase C inhibitor	1.42	1.20	0.012
Oleandomycin	Protein synthesis, 50S subunit, macrolide	0.99	0.83	0.012
Procaine	Ion channel inhibitor, Na <sup>+</sup>	1.06	0.86	0.012
Harmaline	Imidazoline binding sites, agonist	1.48	1.23	0.012
Geneticin (G418)	Protein synthesis, 30S subunit, aminoglycoside	1.41	0.69	0.013
Fusidic acid	Protein synthesis, elongation factor	1.13	0.91	0.025
Nalidixic acid <sup>d</sup>	DNA unwinding, gyrase, quinolone	1.40	1.02	0.025
Minocycline	Protein synthesis, 30S subunit, tetracycline	0.90	0.54 <sup>c</sup>	0.027
Rolitetracycline	Protein synthesis, 30S subunit, tetracycline	1.26	0.65	0.027
Cobalt chloride	Toxic cation	0.73	0.59	0.027
Cytosine arabinoside	Nucleic acid analogue, pyrimidine	1.35	1.17	0.027
Orphenadrine	Cholinergic antagonist	1.08	0.82	0.030
Kanamycin <sup>d</sup>	Protein synthesis, 30S subunit, aminoglycoside	0.74	0.47	0.030
<b>(B) SBW25 <math>\Delta rpoN</math> &gt; SBW25</b>				
D,L-Serine hydroxamate	tRNA synthetase	0.60	0.77	0.009
Cupric chloride <sup>d</sup>	Toxic cation	0.88	1.00	0.012
Hydroxyurea <sup>d</sup>	Ribonucleotide DP reductase, antifolate	0.41	0.81	0.025
Sodium cyanate	Toxic anion	0.96	1.05	0.027
Sodium arsenate	Toxic anion, PO <sub>4</sub> analogue	0.59	0.70	0.030
Sodium bromate	Toxic anion	0.94	1.26	0.049
Potassium tellurite	Toxic anion	0.95	1.19	0.049
<b>(C) Additional results</b>				
Guanazole	Ribonucleotide DP reductase	0.99	1.28	0.125
Boric acid <sup>d</sup>	Toxic anion	0.50	0.54	0.119
Methyl viologen <sup>d</sup>	Oxidizing agent	0.76	1.06	0.056
Tetracycline <sup>d</sup>	Protein synthesis, 30S ribosomal subunit	1.31	0.70	0.056

**a.** The value shown describes bacterial tolerance to increasing concentrations of stressor, and is derived from average signal as a proportion of the maximum growth achieved in the assay as described in *Experimental procedures*. A high test value indicates low sensitivity of growth to the stressor.

**b.** Probability, following FDR correction for multiple testing, of the null hypothesis that the true mean signal values are actually the same in the two genotypes.

**c.** No detectable signal based on average signal before correction or normalization relative to negative control, indicating a qualitative difference in performance.

**d.** Tested independently in *in vitro* chemosensitivity assays (Fig. 4). A complete set of results is listed in Table S4.

(A) Assays where SBW25 gives a higher signal compared with SBW25  $\Delta rpoN$  after normalization of signal values ( $P < 0.03$  after FDR correction).

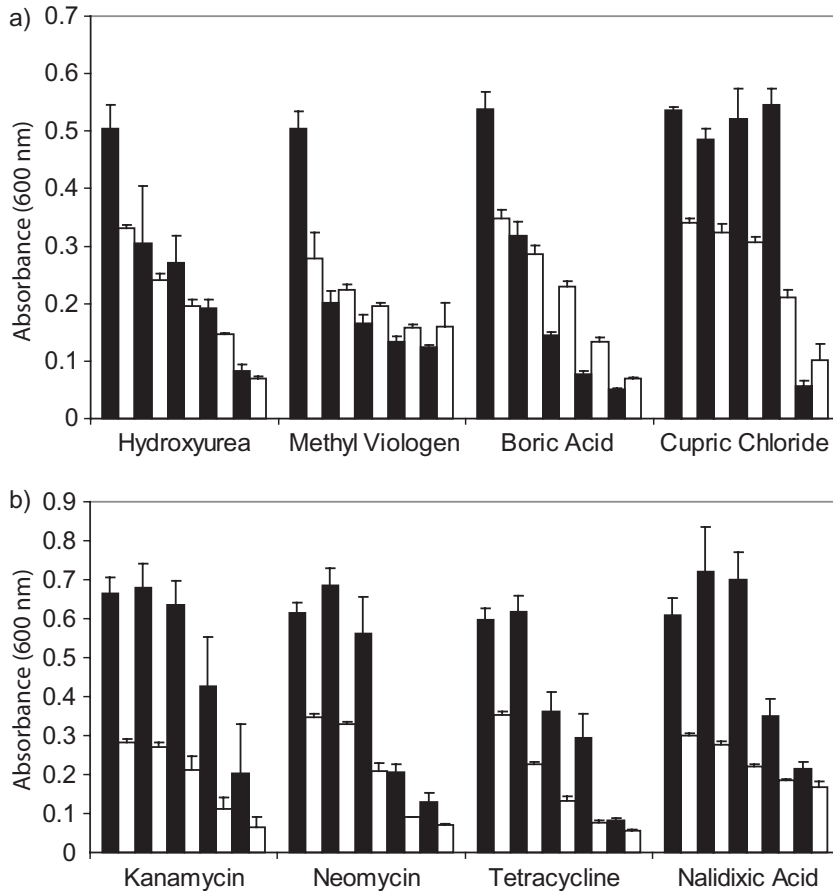
(B) Assays where SBW25  $\Delta rpoN$  shows higher tolerance compared with SBW25 after normalization of signal values ( $P < 0.05$  after FDR correction). (C) Additional results discussed in main text ( $P > 0.05$  after FDR correction).

sequences that were intragenic, or overlapped the putative RpoN binding site, indicating that although SBW25 contains a *norR*-like EBP gene (PFLU5143), the *E. coli* NorR recognition sequence is not well conserved in SBW25. Errors in start codon and ORF prediction in the draft genome of SBW25 could lead to a small number of promoters being overlooked, so we manually curated high-scoring binding sites located in the N-terminal regions of predicted ORFs to identify additional 'intragenic' binding sites that are strong candidate promoters (Table 5, italics).

The predicted functions of many of the genes in Table 5 and Table S5 show close correlations with traits associated with RpoN in phenotypic analyses. For

example, Table S5 lists six operons containing 24 genes known to be linked to flagellar biosynthesis, which collectively account for the loss of flagellar biosynthesis and motility in SBW25  $\Delta rpoN$  (Fig. 3). Five of these flagellar operons are associated with putative binding sites for the flagellar EBP FleQ. Table 5 and Table S5 also list genes implicated in myo-inositol (PFLU2385), ribose (PFLU0469) and dicarboxylic acid (PFLU4955) assimilation, all of which correspond to loss of function traits for SBW25  $\Delta rpoN$ .

We identified RpoN and NtrC binding sites upstream of several genes predicted to be involved in nitrogen assimilation, including *glnA* (glutamine synthetase, PFLU0348), *ureE* (urease, PFLU0563), a putative amino acid trans-



**Fig. 4.** Validation of chemosensitivity data. Growth assays were performed as described under *Experimental procedures*. Data shown are the average of four replicates  $\pm$  standard deviation. SBW25 (black), SBW25  $\Delta rpoN$  (white). Bacterial performance in a control treatment (no inhibitor, 0) and in four different inhibitor concentrations is shown from left to right in order of increasing concentration for each treatment. All assays were performed in LB and the pH of inhibitor treatments was adjusted to pH 7.0.

A. Toxicity assays. Concentrations used: Hydroxyurea: 25, 50, 100, 200 mM; Methyl Viologen: 0.8, 1.6, 3.2, 6.4 mM; Boric Acid: 0.140, 0.28, 0.56, 1.125  $\mu\text{g ml}^{-1}$ ; Cupric Chloride: 0.2, 0.4, 0.8 and 1.6  $\mu\text{g ml}^{-1}$ . Absorbance was measured 4 h after inoculation into test media.

B. Antibiotic resistance assays. Concentrations used: Kanamycin: 2.0, 4.0, 8.0 and 16.0  $\mu\text{g ml}^{-1}$ ; Neomycin: 3.0, 6.0, 12 and 24  $\mu\text{g ml}^{-1}$ ; Tetracycline 0.32, 0.64, 1.28 and 2.56  $\mu\text{g ml}^{-1}$ ; Nalidixic Acid: 5, 10, 20, 40  $\mu\text{g/ml}$ . Absorbance was measured six hours after inoculation into test media.

porter (PFLU1000) and the nitrite/nitrate sensor *narX* (PFLU2337). The association of RpoN, and in some cases NtrC, binding sites with at least 11 operons containing nitrate and amino acid transport genes, and numerous operons encoding enzymes and regulatory proteins associated with nitrate, urea and amino acid assimilation is consistent with the poor performance of SBW25  $\Delta rpoN$  on nitrate, urea and many amino acids. However, it is interesting to note that although *glnA* and two ammonium transport genes (PFLU5952 and PFLU1747) are in operons with putative RpoN binding sites, SBW25  $\Delta rpoN$  was able to grow on ammonium as sole nitrogen source. There are at least two possible explanations for this. First, *glnA* (glutamine synthetase, PFLU0348) may be regulated by multiple sigma factors. Some *glnA* promoter regions have been shown to contain both Sigma-70 (*glnAp1*)- and RpoN (*glnAp2*)-dependent promoters along with one or more minor promoters that ensure *glnA* expression (Reitzer and Schneider, 2001; Schwab *et al.*, 2007). This hypothesis is supported by BPROM analysis (SoftBerry, <http://www.softberry.com>) of the upstream region of *glnA*, which identified a putative Sigma-70 binding site 56 bp upstream of the start codon of *glnA*. Second, it is possible that glutamine synthesis in

*P. fluorescens* SBW25 can be catalysed by multiple glutamine synthetase enzymes, only some of which are regulated by RpoN. The genome of *P. fluorescens* SBW25 contains seven proteins with the glutamine synthetase-related Pfam domain Gln-synt\_C (PF00120), of which only two, PFLU0340 and PFLU2323, have putative RpoN-dependent promoters. However, only PFLU0348 contains the Gln-synt\_N domain that has been proposed to function in ammonia binding (Gill and Eisenberg, 2001).

*Pseudomonas* proteins are the closest BLASTP hits for most of the ORFs that have candidate RpoN binding sites, with the majority showing the highest similarity to genes in the genomes of *P. fluorescens* Pf-5 and PfO-1. One exception is PFLU3582, which is most similar to a protein from *Ralstonia solanacearum*, and lies in a cluster of genes, from PFLU3581 to PFLU3586, which are syntenic with genes in two *R. solanacearum* genomes (*R. solanacearum* UW551 RR04192-RR01488 and GMI1000 RSc3411-RSc3407). The PFLU3582 gene cluster may have been transferred between bacteria with an operational RpoN-binding promoter, as RSc3410 has a high scoring RpoN promoter (5'-TGGCATGC CCATTGCA-3', score = 17.40).

**Table 5.** Candidate RpoN binding sites in *Pseudomonas fluorescens* SBW25.

Score	RpoN binding site Sequence	Identifier	Gene adjacent to and in the same orientation as promoter motif Blast Hit (UniProt, GenBank)
19.09	<b>TGGCACGGCCTTTGCT</b>	PFLU0551	YP_610348 (8e-177), <i>P. entomophila</i> L48, Leu/Ile/Val (LivK) ABC transporter
18.72	<b>TGGCACAGGCCTTGCT</b>	PFLU2385	YP_274540 (7e-151), <i>P. s. pv. phaseolicola</i> 1448A, Myo-inositol 2 dehydrogenase
18.72	<b>TGGCACAGCCCTTGCT</b>	PFLU2216 <sup>a</sup>	ZP_01712546 (7e-179), <i>P. putida</i> GB-1, TonB-dependent siderophore receptor
18.27	<b>TGGCACAGCACTTGCT</b>	PFLU2218 <sup>a</sup>	NP_420990 (1e-98), <i>Caulobacter crescentus</i> CB15, Peptidase S58, DmpA
17.82	<b>TGGCACACCGCTTGCA</b>	PFLU2220	ZP_00977154 (3e-152), <i>Burkholderia cenocepacia</i> PC184, Dipeptide transporter
17.70	<b>TGGCACCGTTTTGCT</b>	PFLU2802	ZP_00900376 (5e-57), <i>P. putida</i> F1, Isoquinoline 1-oxidoreductase alpha subunit
17.68	<b>TGGCATGCAATTTGCT</b>	PFLU0348	YP_257530 (0.0), <i>P. fluorescens</i> Pf-5, Glutamine synthetase, type I
17.61	<b>TGGCATGCACCTTGCT</b>	PFLU4731	YP_261568 (5e-62), <i>P. fluorescens</i> Pf-5, Flagellar basal body rod protein FigB
17.30	<b>TGGCACGGCCTTGCG</b>	PFLU4666	YP_347467 (0.0), <i>P. fluorescens</i> PfO-1, Hypothetical DUF404-DUF407 protein
17.18	<b>TGGCACCCCTTTGCT</b>	PFLU4440	YP_347267 (1e-32), <i>P. fluorescens</i> PfO-1, Flagellar hook-basal body protein FliE
17.14	<b>TGGCACCGTTGCTGCT</b>	PFLU2730	YP_260368 (0.0), <i>P. fluorescens</i> Pf-5, Alkanesulfonate monooxygenase
17.05	<b>TGGCACGGCCTTGCG</b>	PFLU4717	YP_261555 (0.0), <i>P. fluorescens</i> Pf-5, C4-dicarboxylate transport protein, GltP
16.89	<b>TGGCACGGCACCTTGCA</b>	PFLU4741	NP_791673 (2.3), <i>P. s. pv. tomato</i> DC3000, Hypothetical protein
16.87	<b>TGGCATAACACTTGCT</b>	PFLU4429	YP_347277 (5e-63), <i>P. fluorescens</i> PfO-1, Nitrate transporter protein
16.83	<b>TGGTACGGCCTTGCT</b>	PFLU1000	YP_346705 (7e-176), <i>P. fluorescens</i> PfO-1, Amino acid ABC transporter
16.83	<b>TGGCACGGCCCTTGCA</b>	PFLU4833	YP_261689 (1e-63), <i>P. fluorescens</i> Pf-5, Hypothetical protein
16.83	<b>TGGCACGGCTCTTGCA</b>	PFLU0469	NP_792793 (5e-144), <i>P. s. pv. tomato</i> DC3000, D-Ribose-binding periplasmic protein
16.76	<b>TGGCACAGCCTTGCT</b>	PFLU4955	YP_350187 (0.0), <i>P. fluorescens</i> PfO-1, Dicarboxylate MFS transporter
16.66	<b>TGGCAAGGTTATTGCA</b>	PFLU2323	YP_275098 (0.0), <i>P. syringae</i> pv. <i>phaseolicola</i> 1448A, Glutamine synthetase, type III
16.58	<b>TGGTACAGCTTTGCT</b>	PFLU1747	YP_261322 (3e-161), <i>P. fluorescens</i> Pf-5, Ammonium transporter
16.58	<b>TGGTACAGCTTGCT</b>	PFLU2058	YP_261247 (7e-70), <i>P. fluorescens</i> Pf-5, NADH-dependent FMN reductase MsuE
16.52	<b>TGGCACGCACCTTGCT</b>	PFLU5239 <sup>b</sup>	YP_262347 (0.0), <i>P. fluorescens</i> Pf-5, Poly(A) polymerase PcnB
16.51	<b>TGGCACGACTCATGCT</b>	PFLU1139	YP_276039 (2e-129), <i>P. s. pv. phaseolicola</i> 1448A, Amino acid ABC transporter
16.45	<b>TGGCGCAGCCTTGCT</b>	PFLU4609	YP_347515 (3e-180), <i>P. fluorescens</i> PfO-1, Nitrate transporter, NarK
16.36	<b>TGGCCCACTCTTGCT</b>	PFLU4430	YP_347276 (1e-74), <i>P. fluorescens</i> PfO-1, Flagellar hook-length control protein, FliK
16.32	<b>TGGCACGGTCGATGCT</b>	PFLU1992	YP_237006 (6e-120), <i>P. s. pv. syringae</i> B728a, Amidase, tRNA amidotransferase
16.25	<b>TGGCATGTGCCTTGCT</b>	PFLU4607	YP_347517 (0.0), <i>P. fluorescens</i> PfO-1, Nitrate/sulfonate transport protein, NasS
16.24	<b>TGGCACACTTTGCT</b>	PFLU2617	YP_348821 (2e-143), <i>P. fluorescens</i> PfO-1, Amino acid ABC transporter
16.22	<b>TGGCACGAACTTGCT</b>	PFLU3746	YP_349364 (0.0), <i>P. fluorescens</i> PfO-1, Sugar ABC transporter
16.16	<b>TGGCACAGAGCTTGCG</b>	PFLU3582	ZP_00443209 (2e-84), <i>Ralstonia solanacearum</i> UW551, Amino acid transporter
16.05	<b>TGGCACGCAGACTGCA</b>	PFLU0446	YP_346179 (0.0), <i>P. fluorescens</i> PfO-1, NhaP-type Na <sup>+</sup> /H <sup>+</sup> antiporter
15.99	<b>TGGCCTGGGTTTTGCA</b>	PFLU4026	NP_792721 (4e-121), <i>P. s. pv. tomato</i> DC3000, Nitrogen assimilation regulator, Nac
15.93	<b>TGGCACAGACCTTGCA</b>	PFLU3425	YP_236169 (0.0), <i>P. s. pv. syringae</i> B728a, Nitrite/sulfite reductase
15.85	<b>TGGCACGATGCTGCT</b>	PFLU4643	YP_347468 (0.0), <i>P. fluorescens</i> PfO-1, Cytosine/purine/uracil/thiamine permease
15.85	<b>CGGCACGAGCTTTGCT</b>	PFLU4734	YP_261571 (1e-100), <i>P. fluorescens</i> Pf-5, Flagella basal body P-ring protein FigA
15.83	<b>CGGCACGCTATTGCT</b>	PFLU4818	YP_261673 (2e-09), <i>P. fluorescens</i> Pf-5, Hypothetical protein
15.79	<b>TGGCACACATCTGCT</b>	PFLU2629	YP_260677 (0.0), <i>P. fluorescens</i> Pf-5, H <sup>+</sup> /citrate symporter
15.77	<b>TGGCACGTAATCTGCT</b>	PFLU4100	BAB53175 (6e-49), <i>Mesorhizobium loti</i> MAFF303099, GntR-type regulator
15.77	<b>TGGCACGTAATCTGCT</b>	PFLU4518	YP_236601 (0.0), <i>P. s. pv. syringae</i> B728a, Cytosine/purine/uracil/thiamine permease
15.76	<b>CGGCACGCCCTTGCT</b>	PFLU1033	YP_273358 (8e-32), <i>P. s. pv. phaseolicola</i> 1448A, Hypothetical protein
15.74	<b>TGGCAAGCCCTTGCT</b>	PFLU0563	YP_346296 (2e-73), <i>P. fluorescens</i> PfO-1, Urease accessory protein UreE
15.68	<b>TGGCACAGAGACTGCA</b>	PFLU2791	YP_349023 (4e-131), <i>P. fluorescens</i> PfO-1, Aliphatic sulfonate ABC transporter
15.63	<b>TGGCACACTCCCTTGCT</b>	PFLU1127	YP_261964 (2e-115), <i>P. fluorescens</i> Pf-5, Phosphoserine phosphatase
15.57	<b>CGGCACAAGTATTGCT</b>	PFLU4442	YP_258761 (8e-157), <i>P. fluorescens</i> Pf-5, Signal transduction histidine kinase, FleS
15.50	<b>TGGCACAAATCACTGCA</b>	PFLU4334	YP_261437 (1e-41), <i>P. fluorescens</i> Pf-5, Hypothetical protein
15.41	<b>TGGCACAGCCAGTGCA</b>	PFLU0709	BAA81928 (3e-37), <i>P. s. pv. broussonetiae</i> , RNA polymerase sigma factor RspL
15.39	<b>CGGCACAGGCTTGCT</b>	PFLU3135	YP_347467 (0.0), <i>P. fluorescens</i> PfO-1, Hypothetical DUF404-DUF407 protein
15.29	<b>TGGCATGAATATGCT</b>	PFLU4306	YP_261412 (1e-108), <i>P. fluorescens</i> Pf-5, GGDEF domain protein
15.18	<b>CGGCACAGCCTTTGCA</b>	PFLU0588	YP_346323 (0.0), <i>P. fluorescens</i> PfO-1, Branched chain amino acid ABC transporter
15.16	<b>TGGCACACTGCCTTGCA</b>	PFLU5248	YP_606531 (6e-17), <i>P. entomophila</i> L48, Secreted lipoprotein (OsmY/BON)
15.12	<b>TGGCATAAGTCTTGCG</b>	PFLU0541	YP_346274 (0.0), <i>P. fluorescens</i> PfO-1, Glutaminase like protein
15.09	<b>TGGCATGTGACTTGCA</b>	PFLU2573	YP_349246 (5e-82), <i>P. fluorescens</i> PfO-1, Hypothetical protein
15.01	<b>TGGCTCAGTATTGCT</b>	PFLU2039	YP_347418 (3e-146), <i>P. fluorescens</i> PfO-1, Fe <sup>3+</sup> ABC transporter
15.01	<b>TGGCCCGTCCCTTGCA</b>	PFLU1122	YP_261959 (3e-139), <i>P. fluorescens</i> Pf-5, Conserved hypothetical protein/arginase
14.91	<b>TGGCCGCTGACTTGCT</b>	PFLU3426	YP_558809 (0.0), <i>Burkholderia xenovorans</i> LB400, Nitrate/sulfite reductase
14.83	<b>TGGCATGCGCTTGCA</b>	PFLU0089	YP_257244 (4e-07), <i>P. fluorescens</i> Pf-5, Hypothetical protein
14.82	<b>TGGCATGGCTTGCTGCA</b>	PFLU5903	ABD64370 (7e-121), <i>Pseudomonas chloraphis</i> , Amino acid ABC transporter
14.65	<b>AGGCATGGCATTGCT</b>	PFLU5678	YP_350964 (0.0), <i>P. fluorescens</i> PfO-1, AraC-family transcriptional regulator
14.41	<b>GGCATTGAAGTTGCT</b>	PFLU2337	ZP_01295658 (1e-37), <i>P. aeruginosa</i> PA7, Nitrate/nitrite sensor protein NarX
14.35	<b>TGGCATTTCTACTTGCA</b>	PFLU1630	YP_276356 (2e-91), <i>P. s. pv. phaseolicola</i> 1448A, LuxR family regulator
14.32	<b>CGGCACGGCTTGCT</b>	PFLU1984	YP_236009 (9e-135), <i>P. s. pv. syringae</i> B728a, NADH:flavin oxidoreductase
14.31	<b>CGGCACGGCTTGCT</b>	PFLU2701	YP_608504 (5e-14), <i>P. entomophila</i> L48, Hypothetical protein
14.30	<b>TGGCAAGGTTTCTGCT</b>	PFLU5953	YP_351234 (7e-40), <i>P. fluorescens</i> PfO-1, Nitrogen regulatory protein P-II
14.14	<b>TGGCATGACCGCTTGCA</b>	PFLU3419	NP_792788 (4e-79), <i>P. s. pv. tomato</i> DC3000, Long chain fatty acid CoA ligase
14.14	<b>TGGCAAGGTTTCC</b>	PFLU2219	ABA24612 (3e-66), <i>Anabaena variabilis</i> ATCC 29413, TPR-domain protein
14.02	<b>TGGCGCATTACTTGCT</b>	PFLU5193	ABA06558 (5e-171), <i>P. fluorescens</i> , AraC family transcriptional activator
14.01	<b>TGGTACAGATCCTTGCT</b>	PFLU4161	AAC33155 (9e-156), <i>P. fluorescens</i> ATCC 13525, Glutaminase-asparaginase

a. The high scoring promoters for PFLU2216 and PFLU2218 lie within a small hypothetical ORF, PFLU2217, which shows no similarity to other genes, and which may not be a genuine ORF.

b. The RpoN binding site lies upstream of a small ORF, PFLU5238, which is only similar to a small ORF from *P. fluorescens* Pf-5, and which may not be a genuine ORF. The BLASTP result for PFLU5239 is shown.

Candidate RpoN binding sites were identified by HMM analysis as described in *Experimental procedures*. Column 1 lists the score for each binding site based on a HMM matrix constructed from 186 proteobacterial sequences (Barrios *et al.*, 1999). Binding sites have been selected for inclusion based on the moderately stringent cut-off value of 14.0. Further information on sites scoring  $\geq 14.0$  is available in Table S5, including operon predictions and IHF and EBP binding site predictions. The majority of intragenic sites have been excluded. However, high-scoring intragenic sites predicted to correspond to promoters to intergenic predictions due to misannotated start codons are included. The identifiers for the corresponding ORFs are listed in italics in column 3. Column 2 lists the candidate binding site sequence, with the highly conserved GG and GC residues highlighted in bold. Column 3 lists the SBW25 protein identifier for the ORF immediately adjacent to each predicted binding site ([http://www.sanger.ac.uk/Projects/P\\_fluorescens](http://www.sanger.ac.uk/Projects/P_fluorescens)). Column 4 lists the GenBank identifier for the highest BLASTP hit to each ORF followed by the corresponding BLASTP score in parentheses, the source organism and the predicted function.

It is important to emphasize that bioinformatic predictions of RpoN binding sites cannot give a comprehensive list of all the genes in SBW25 that are transcriptionally dependent on RpoN, as Table 5 and Table S5 list several putative RpoN-regulated transcriptional regulators that link RpoN to an extended regulatory network. For example, the RpoN-regulated sigma factor RspL has been shown to positively regulate several operons linked to type III protein secretion in SBW25 (Jackson *et al.*, 2005). Some of the regulatory proteins listed in Table 5 and Table S5 are situated close to putative nutrient assimilation genes for which they could be cognate regulators. PFLU5678 is located upstream of a putative L-serine deaminase gene; PFLU1630 is adjacent to an L-amino acid amidase and peptide ABC transporter; PFLU3204 (Table S5) is adjacent to aldoxime and phenol utilization genes, and PFLU6060 (Table S5) is in an operon containing a polyamine transport gene and an acetyl polyamine aminohydrolase. We also identified a high-scoring binding site upstream of an operon encoding the mRNA-modifying enzyme poly(A) polymerase (PcnB, PFLU5239) and the folate biosynthesis enzyme FolK (PFLU5240). RpoN-dependent regulation of poly(A) polymerase activity in SBW25 could have a global regulatory effect on mRNA stability and gene expression.

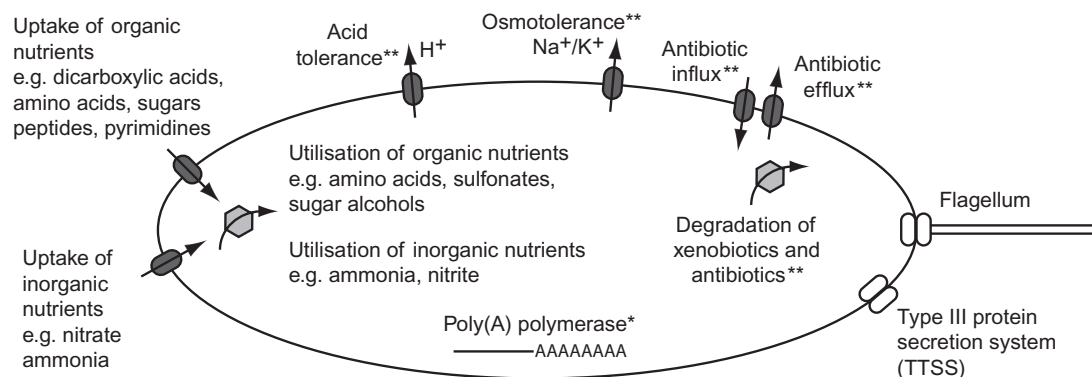
## Discussion

The alternative sigma factor RpoN is a key regulator of traits involved in rhizosphere colonization by *P. fluorescens* SBW25. An *rpoN* deletion mutant of SBW25 is non-motile, unable to assimilate a wide variety of C and N sources known to be present in root exudates, such as dicarboxylic acids and amino acids, and shows reduced tolerance to acidic pH and a wide range of anti-

microbial chemicals. However, SBW25  $\Delta rpoN$  performed as well as wild-type bacteria in some phenotypic assays, and shows increased resistance to RNR inhibitors when compared with SBW25. Bioinformatic analyses of RpoN binding sites demonstrate a close correspondence between the predicted functions of candidate RpoN-regulated genes and RpoN-dependent traits, particularly with respect to motility and nutrient assimilation, and have identified new targets for RpoN that may account for some of the novel RpoN-dependent traits described in this study. RpoN-dependent traits predicted or shown to be present in SBW25 are summarized in Fig. 5.

### Functional and bioinformatic analysis of RpoN-dependent genes

Expression of RpoN-regulated genes depends on signal transduction by RpoN-activating EBPs. Many RpoN-regulated genes are only expressed in the presence of specific inducing chemicals, or in response to physiological signals such as nitrogen starvation, which poses a significant challenge for researchers trying to identify the components and functions of the RpoN regulatory network. We have addressed this challenge in three ways, first by performing targeted phenotypic analyses based on previous studies of RpoN regulated traits, second by comparing the metabolic activity of SBW25 and SBW25  $\Delta rpoN$  in more than 1700 different environments, using the Biolog PhenoType Microarray™ system, and third by performing bioinformatic analyses to identify putative RpoN-regulated genes in the genome of SBW25. Many, although probably not all, RpoN-regulated genes are likely to be transcribed or provide a useful function in at least one phenotypic assay, which may be observed in terms of differences in the relative performance of SBW25



**Fig. 5.** Overview of predicted and experimentally demonstrated RpoN-dependent traits in *Pseudomonas fluorescens* SBW25. \*Bioinformatic evidence only. \*\*Experimental analyses of SBW25 and SBW25  $\Delta rpoN$  identified both gain and loss of function changes with respect to chemosensitivity and stress tolerance, while bioinformatic analyses identified numerous membrane transport proteins and enzymes that are likely to be regulated by RpoN. Gain of function changes in SBW25  $\Delta rpoN$  may be associated with loss of antibiotic uptake mechanisms or targets. Loss of function changes may be associated with defects in membrane efflux or antibiotic detoxification mechanisms. Further analyses are needed to link stress tolerance and antibiotic resistance results with specific RpoN-regulated genes and traits.

and SBW25  $\Delta rpoN$ . RpoN-regulated genes that do not have a detectable phenotypic effect will still be identified in bioinformatic predictions of RpoN-binding motifs.

One challenge encountered in analysing phenoarray data was to identify specific roles for RpoN, against a general loss of fitness in a wide range of environments. Normalization of results allowed us to identify assays in which the relative performance of SBW25  $\Delta rpoN$  differed significantly from that of SBW25. The strong correspondence observed between normalized results, validated results and results from previous studies clearly demonstrates that researchers can benefit from analysing phenoarray data in terms of both normalized signal strength and raw values.

Bioinformatic analyses generated a comprehensive list of putative RpoN-regulated genes in SBW25, based on the presence of high-scoring RpoN binding sites (Table 5 and Table S5). Many of these genes show a high degree of similarity to previously characterized genes, and we can predict their functions with some degree of confidence. Others encode hypothetical proteins and proteins with poorly defined functions, which may be involved in some of the novel traits observed in phenoarray assays. The moderately stringent cut-off value of 14.0 gives 67 binding sites that are in the correct orientation and location to regulate the expression of adjacent genes. This is broadly consistent with previous estimates of the total number of RpoN-regulated genes in other species of *Pseudomonas* (~55 promoters in *P. putida*; Cases *et al.*, 2003). The combination of phenoarray analyses and bioinformatic analyses provides detailed insight into the role of RpoN in SBW25, and a strong foundation for further hypothesis-driven research into the regulation and role of RpoN-dependent traits.

#### *RpoN-dependent nutrient utilization and plant colonization by SBW25*

Phenotypic and bioinformatic analyses have identified numerous ways in which SBW25  $\Delta rpoN$  differs from SBW25. But which of the RpoN-dependent genes and traits identified in this study account for the poor performance of SBW25  $\Delta rpoN$  in rhizosphere colonization assays? We have previously shown that RspL (PFLU0709) is a positive regulator of type III protein secretion (TTSS) genes in SBW25 and that RspL is regulated by the EBP RspR (Jackson *et al.*, 2005). Furthermore, RspL and RspR mutants are impaired in rhizosphere competence (Jackson *et al.*, 2005). However, regulation of TTSS activity is unlikely to be the only explanation for poor rhizosphere colonization by SBW25  $\Delta rpoN$ , as the defect in rhizosphere colonization shown by SBW25  $\Delta rpoN$  is much greater than that observed for TTSS mutants.

Several studies have shown that motility towards root exudates is important for root colonization by *Pseudomonas* (Broek and Vanderleyden, 1995; Turnbull *et al.*, 2001; de Weert *et al.*, 2002). SBW25  $\Delta rpoN$  is clearly impaired in motility and chemotaxis due to the loss of RpoN-dependent flagellar biosynthesis genes. SBW25  $\Delta rpoN$  is also impaired in the ability to assimilate a large number of diverse carbon and nitrogen sources, including chemicals such as amino acids and dicarboxylic acids, which have been shown to be present in root exudates. Mutations affecting assimilation of single, or even multiple metabolites have frequently been observed to have little impact on bacterial fitness in complex environments such as the rhizosphere (Lugtenberg *et al.*, 1999; Zhang *et al.*, 2006). However, SBW25  $\Delta rpoN$  is impaired in so many nutrient assimilation pathways that it seems highly unlikely that loss of nutrient assimilation does not contribute to the reduced rhizosphere competence of this strain.

SBW25  $\Delta rpoN$  is not a glutamine or amino acid auxotroph, as has been reported for some *rpoN* mutants of *Pseudomonas* (Totten *et al.*, 1990; Alarcon-Chaidez *et al.*, 2003; Pechy-Tarr *et al.*, 2005). The lack of glutamine or amino acid auxotrophy in SBW25  $\Delta rpoN$  is not unprecedented, as *rpoN* mutants of several strains of *Pseudomonas*, including *P. aeruginosa* PA14 and PAK (Hendrickson *et al.*, 2001), *P. syringae* pv. *maculicola* ES4326 (Hendrickson *et al.*, 2000b) and *P. putida* KT2440 (Köhler *et al.*, 1989), have all been reported to grow in M9 in the absence of glutamine. However, this is the first study to describe a *P. fluorescens* strain in which *rpoN* is not required for ammonia assimilation and glutamine synthesis.

#### *RpoN-dependent stress tolerance and antibiotic resistance mechanisms in SBW25*

The rhizosphere is not only a nutrient-limited environment, but is also a stressful environment, in which bacteria must be able to tolerate, exclude or detoxify abiotic stressors and exogenous and autogenous antimicrobial chemicals. SBW25  $\Delta rpoN$  showed increased sensitivity to acid pH and to a wide range of antimicrobial chemicals, and was particularly sensitive to tetracycline antibiotics and other inhibitors of protein synthesis, demonstrating that RpoN-regulated genes contribute to stress tolerance and antibiotic resistance in SBW25. RpoN mutants have not previously been reported to show increased sensitivity to tetracycline and aminoglycosides, although Zhou and colleagues (2003) reported that an NtrC mutant of *E. coli* showed increased susceptibility to aminoglycosides.

Chemosensitivity and stress assays are particularly challenging to interpret in terms of individual RpoN-regulated genes and further experiments are needed to determine whether the RpoN-dependent changes in

stress tolerance and chemosensitivity assays observed in this study can be linked to specific RpoN-regulated genes and processes. However, one set of genes that could be linked to some of the changes observed in SBW25  $\Delta rpoN$  are the flagellar biosynthesis genes, as flagellar biosynthesis mutants of *P. putida* have previously been reported to show increased sensitivity to organic solvents (Segura *et al.*, 2001).

Chemosensitivity assays also generated some of the most novel results obtained in this study, including the observation that SBW25  $\Delta rpoN$  showed significantly increased tolerance to hydroxyurea and Guanazole. Hydroxyurea and Guanazole both inhibit the dNTP synthase enzyme RNR (Nordlund and Reichard, 2006), which is involved in the synthesis of deoxyribonucleotides and in regulation of DNA replication initiation (Herrick and Sclavi, 2007). Genome sequenced strains of *P. aeruginosa* contain all three known classes of RNR, but many *Pseudomonas*, including SBW25, only contain homologues of NrdA and NrdB, which comprise the class I enzyme that is specifically targeted by hydroxyurea (Lundin *et al.*, 2005; Torrents *et al.*, 2005; Nordlund and Reichard, 2006). Interestingly, sublethal concentrations of hydroxyurea induced visible flocculation in SBW25, but not in SBW25  $\Delta rpoN$ . Klebensberger and colleagues (2006) noted that *P. aeruginosa* PAO1 formed aggregates in response to detergent-related stress, but that aggregate formation could be blocked by anoxia, CCCP or KCN. Aggregation of SBW25, but not SBW25  $\Delta rpoN$ , could therefore be linked to the direct effects of hydroxyurea on SBW25 and to low energy status in SBW25  $\Delta rpoN$ . Although we cannot offer a conclusive explanation as to why SBW25  $\Delta rpoN$  displayed increased tolerance to hydroxyurea and Guanazole, one plausible explanation is that resistance is due to reduced uptake of these toxic chemicals due to loss of one or more of the nutrient uptake systems listed in Table 5 and Table S5. Loss of antibiotic uptake systems or targets may also help to explain the relatively high tolerance of SBW25  $\Delta rpoN$  to other toxic chemicals, such as boric acid.

#### *RpoN, RpoS and PcnB – cross-talk between global regulatory mechanisms?*

One candidate RpoN binding site is located upstream of *pcnB*, which encodes poly(A) polymerase. PcnB governs polyadenylation at the 3' ends of mRNAs, thereby modulating mRNA stability and possibly mRNA translation (Jasiecki and Wegrzyn, 2003; Deutscher, 2006; Santos *et al.*, 2006). Putative RpoN binding sites are also present upstream of *pcnB* in other *Pseudomonas*, including *P. aeruginosa* PAO1, *P. putida* KT2440 and *P. syringae* pv. *tomato* DC3000 (<http://www.promscan.uklinux.net/>). Polyadenylation occurs in the mRNAs of all the major

functional classes of genes in *P. aeruginosa* (Saravanan *et al.*, 2004), and has been linked to stress and stationary-phase adaptation in *P. putida* and *E. coli* (Jasiecki and Wegrzyn, 2003; Reva *et al.*, 2006). Polyadenylation is likely to occur at high levels in rhizosphere-colonizing bacteria, which inhabit a nutrient and stressor limited environment. However, the extent of polyadenylation in rhizosphere-colonizing bacteria, and the effect of polyadenylation on rhizosphere competence are unknown. PcnB mutants have been shown to display higher levels of RpoS proteolysis, with concomitant effects on RpoS-dependent genes (Santos *et al.*, 2006). The presence of a putative RpoN binding site upstream of *pcnB*, and possible regulatory links between PcnB and RpoS introduces additional global regulatory factors into the RpoN regulatory network that could have a significant impact on experimental results (King *et al.*, 2004), and highlights an important area for future research.

#### *Conclusion*

RpoN regulates a wide range of traits in SBW25, many of which are likely to have important roles in rhizosphere colonization. However, it is interesting to note that the results of our rhizosphere colonization experiments differ from those reported by Pechy-Tarr and colleagues (2005), in which *P. fluorescens* CHA0 *rpoN*<sup>-</sup> shows relatively little difference in rhizosphere competence compared with wild-type bacteria, despite showing defects in growth, stress tolerance and motility *in vitro*. This difference could be explained by strain-specific differences with respect to RpoN-regulated traits, or by assay-specific differences in the performance of RpoN mutants. The relatively high performance of SBW25  $\Delta rpoN$  in some assays confirms that *P. fluorescens* could encounter nutrient or stressor-limited niches in which RpoN-independent genes can be used to assimilate plant metabolites, in which motility is of limited importance, and in which the performance of wild-type bacteria and  $\Delta rpoN$  mutants would be relatively similar. However, in most assays the performance of *rpoN* mutants of *P. fluorescens* is significantly impaired relative to wild-type bacteria, and we predict that *rpoN* mutants are likely to be impaired in fitness in most natural habitats colonized by *P. fluorescens*. The use of phenoarrays to identify conditions in which bacteria grow well is a relatively unexplored application of phenoarray technology, which could be used more widely in the future to optimize growth conditions for mutated, recombinant or fastidious strains.

This study shows how bioinformatic and phenotypic analyses can be combined to generate a detailed model of a complex regulatory network, and to generate new hypotheses regarding the function and regulation of this network. Further deconstruction of the RpoN regulatory

**Table 6.** Bacterial strains and plasmids used in this study.

Strain	Relevant characteristics	Source/reference
<i>Pseudomonas fluorescens</i>		
SBW25	Wild-type strain isolated from phyllosphere of sugar beet plant	Bailey <i>et al.</i> (1995)
SBW25 $\Delta$ rpoN	Unmarked deletion of <i>rpoN</i>	This study
<i>E. coli</i>		
DH5 $\alpha$	F <sup>-</sup> , <i>recA</i> , $\Delta$ <i>lacU169</i> ( $\Phi$ 80 <i>lacZ</i> $\Delta$ M15), <i>endA</i> , <i>hsdR</i> , <i>gyrA</i>	Gibco-BRL
DH5 $\alpha$ $\lambda$ <i>pir</i>	F <sup>-</sup> , <i>recA</i> , $\Delta$ <i>lacU169</i> ( $\Phi$ 80 <i>lacZ</i> $\Delta$ M15), <i>endA</i> , <i>hsdR</i> , <i>gyrA</i> , $\lambda$ <i>pir</i> lysogen	M. Mahan
S17-1 $\lambda$ <i>pir</i>	Tr <sup>+</sup> , Sm <sup>r</sup> , <i>recA</i> , <i>thi</i> , <i>hsdR</i> -M <sup>+</sup> , RP4::2-Tc::Mu::Km::Tn7, $\lambda$ <i>pir</i> lysogen	Simon <i>et al.</i> (1983)
Plasmids		
pUIC3	Tc <sup>r</sup> , Tra <sup>-</sup> , Mob <sup>+</sup> , R6K replicon	Rainey (1999)
pBroadgate	Gateway <sup>®</sup> (Invitrogen) derivative of pBBR1MCS5	R. Thwaites and J. Mansfield
pBroadgate-D	pBroadgate vector control constructed by replacing the <i>ccdB</i> cartridge with a small non-coding DNA fragment	C. Moon, S. Giddens and R. Jackson
pJJP1	pUIC3 with a 3 kb insert in BglIII site containing <i>rpoN</i> flanking DNA	This study
pJJP6	<i>rpoN</i> gene of <i>P. fluorescens</i> SBW25 cloned into pBroadgate	This study

network through mutagenesis, expression analyses, DNA–protein binding assays, mRNA polyadenylation assays and phenotypic analyses will confirm which genes are regulated directly or indirectly by RpoN, which EBPs connect RpoN to specific genes and traits, and show which of the genes and traits identified in this study contribute to plant colonization and survival in the rhizosphere.

## Experimental procedures

### Bacterial strains and media

Bacterial strains and plasmids are listed in Table 6. *Escherichia coli* strains were grown at 37°C overnight on LB (Difco; Sambrook and Russell, 2001) agar or broth. SBW25 was grown at 28°C on LB agar, King's medium B (King *et al.*, 1954) agar or in LB broth, unless otherwise noted. LB, M9 (Sambrook and Russell, 2001) and modified LB and M9 containing 20 mM of selected C and N sources were used in growth assays. Antibiotics and supplements were used to final concentrations of: ampicillin (50  $\mu$ g ml<sup>-1</sup>), kanamycin (25  $\mu$ g ml<sup>-1</sup>), tetracycline (12.5  $\mu$ g ml<sup>-1</sup>), CFC (0.5X) (Oxoid), 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) (40  $\mu$ g ml<sup>-1</sup>) and isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) (40  $\mu$ g ml<sup>-1</sup>). Plasmids were introduced into competent *E. coli* cells by heat shock and into SBW25 and SBW25  $\Delta$ *rpoN* by electroporation using a BIO RAD Electroporator or triparental mating using the helper plasmid pRK2103.

### Construction and complementation of an *rpoN* mutant of *P. fluorescens* SBW25

An *rpoN* deletion mutant of SBW25 was constructed using overlap extension (OE)-polymerase chain reaction (PCR) (Horton *et al.*, 1989) in conjunction with a two-step allelic exchange strategy (Gal *et al.*, 2003; Zhang *et al.*, 2006). All PCR amplifications were performed using TaKaRa Pyrobest™ (TaKaRa Bio). Polymerase chain reaction products representing the 5'-flanking [primers rpoNLFS\_bg (5'-GAAGATCTCTCCCTATTTGCTCGGTCTGG-3') and

rpoNLRs (5'-CAAACGCATCCCAGGACGCGCAGGGGCTTAACACCTTATTC-3')] and 3'-flanking [primers rpoNRFs (5'-GAATAAGGTGTTAAGCCCCTGCGCGTTCCGGGATGCGTTTGG-3') and rpoNRRS\_bg (5'-GAAGATCTCAATAAGGGTTGGCAGGCATC-3')] regions of *rpoN* were amplified. Primers rpoNLRs and rpoNRFs are complementary. The resulting PCR products were purified with QIAquick PCR purification kit (Qiagen), mixed and used as template for a second-round PCR with primers rpoNLFS\_bg and rpoNRRS\_bg. The resulting 3 kb product was gel extracted, digested with BglIII (New England Biolabs), purified and cloned into the BglIII site of the suicide vector pUIC3 (Rainey, 1999), resulting in pUIC3-rpoNF (pJJP1). The sequence and orientation of the insert were confirmed by sequencing using the primers listed above and pBla (Gal *et al.*, 2003).

pJJP1 was transferred from *E. coli* DH5 $\alpha$   $\lambda$ *pir* into SBW25 by triparental mating. Recombinant strains arising from a single homologous recombination event were selected by plating on LB agar containing tetracycline and X-Gal. Blue, tetracycline-resistant colonies were grown without selection in M9 broth for 12 h and plated on LB agar containing X-Gal. Approximately 2% of the colonies were white and tetracycline sensitive. Deletion of the *rpoN* gene was confirmed by PCR, and by Western blotting using a monoclonal antibody against the RpoN protein of *P. putida* fused to an M13 phage (kindly supplied by De Lorenzo) (Jurado *et al.*, 2003). Phagemid rescue and Western blotting procedures were performed as previously described (Fraile *et al.*, 2001).

A plasmid that constitutively expresses *rpoN* was constructed by amplifying the *rpoN* gene from SBW25 using primers rpoNF (5'-CACCGGTACCATTGCTTATGTTTGGC GCTCAC-3') and rpoNR (5'-CGAGCTCATAACGCGAC GGGTAGGAATAC-3') and cloning the PCR product into pBroadgate (a Gateway derivative of pBBR1-MCS5) to create pJJP6. The sequence and orientation of the insert was confirmed by sequencing. pJJP6 was introduced into SBW25 and SBW25  $\Delta$ *rpoN* by electroporation.

### Plant colonization

Sugar beet seeds (*Beta vulgaris* cv. Amethyst) were inoculated as described by Rainey (1999) and Gal and colleagues

(2003). Seeds were planted in non-sterile vermiculite in 5 ml scintillation vials and incubated in a temperature-controlled, illuminated growth chamber at 21°C under a 12:12 h dark:light illumination cycle. Plants were harvested after 7 and 14 days by transferring the contents of the vials to 5 ml of Ringers solution in a 15 ml Falcon tube, adding approximately 20 glass beads and vortexing for 1 min. Serial dilutions were plated onto LB supplemented with 0.5× CFC and incubated overnight at 28°C.

#### Motility and adhesion analyses

Bacterial cultures were grown overnight in LB at 28°C, 200 r.p.m. Cultures were adjusted to an OD<sub>600</sub> of 0.5 and stabbed into the centre of 1/10 LB, 0.3% agar plates. Plates were incubated at room temperature and measured and photographed at 24, 48 and 72 h post inoculation. Adhesion assays were performed using the method described by O'Toole and colleagues (1999).

#### Electron microscopy

Carbon-coated EM grids were inverted onto an aqueous suspension of bacterial cells taken directly from LB agar plates (OD<sub>600</sub> ~0.1). Grids were exposed to bacteria for 30 s, and then directly stained with 2% uranyl acetate for 2 min. Grids were air-dried and bacteria were examined using a JEOL 1200 EXII transmission microscope (JEOL, Tokyo, Japan) operating at 120 kV.

#### Analysis of Phenotype MicroArray™ data

Phenotype MicroArray™ data were generated by Biolog according to standard protocols, with the single modification that glucose was substituted for succinic acid as a C source in the minimal media used in N source plates. Two sets of plates were analysed for both SBW25 and SBW25  $\Delta rpoN$ . A baseline correction was made separately for each plate by subtracting the value for the relevant negative control. For assays comprising a single well within a 96-well plate, corrected OD values were normalized to the total for the plate, and the values for the two genotypes compared by a *t*-test, followed by False Discovery Rate (FDR) correction for multiple testing (Benjamini and Hochberg, 1995), to give the reported *P*-values. In all cases normality of residuals from the *t*-test was checked using a Shapiro–Wilk test, but no evidence for non-normality was found ( $P > 0.02$  in each case, before FDR correction). Assays where neither strain grew (average OD < 0.05) were then ignored. For assays testing growth on different C and N sources, the null hypothesis used in the *t*-test was of no difference between the genotypes. For assays testing the effect of additives under stressful conditions, the null hypothesis tested was that the difference between strains should be that observed between the strains in the stressful conditions without the additive. For assays using more than one well to test a condition (testing different levels of an additive or of pH), growth was calculated as a proportion of the maximum growth in wells on a particular plate testing a particular condition. These proportions were arcsine transformed, the average for each condition on each

plate was taken and these values were compared, using a *t*-test to test the null hypothesis of no difference between the two strains as above. All analyses were carried out in R 2.1.1 (Ihaka and Gentleman, 1996; R Development Core Team 2007), FDR correction was performed using the Multtest package (Gentleman *et al.*, 2005).

#### Validation of Phenotype MicroArray™ data

Carbon and N source assimilation was assayed in M9 minimal media containing either: (i) the selected C source (20 mM) and ammonium (20 mM) as the sole N source to confirm differences seen in plates PM01 and PM02A, or (ii) the selected N source (20 mM) and glucose (20 mM) as the sole N source to confirm differences seen in plates PM03B and PM6–PM8. Chemical sensitivity was assayed in LB. Strains were grown overnight in LB media at 28°C, 200 r.p.m. and then washed: (i) in 1/5 LB for C source metabolism studies, (ii) in M9 minimal media containing glucose (20 mM) for nitrogen studies and (iii) in LB for chemical sensitivity studies. The washed bacteria were diluted to an OD<sub>600</sub> of 0.5 in the media used for washing and 10  $\mu$ l of this suspension was added to 90  $\mu$ l of the appropriate medium in a 96-well plate giving a starting OD<sub>600</sub> of ~0.05. Plates were incubated at 28°C, 250 r.p.m. for 48 h, during which time the OD<sub>600</sub> was measured at regular intervals.

#### Software and databases

Computational analyses were automated using custom Bioperl scripts (Stajich *et al.*, 2002). Scripts are available on request. The complete genome sequence of SBW25 and the coordinates of predicted genes were produced by the Pathogen Sequencing Unit at the Wellcome Trust Sanger Institute and were downloaded from <ftp://ftp.sanger.ac.uk/pub/pathogens/pf/>. HMMER version 1.8.5 was used for DNA sequence analysis and was downloaded from <http://hmm.janelia.org> (Durbin *et al.*, 1998). For protein sequence analysis we used HMMER version 2.3.2, also from <http://hmm.janelia.org>. The PFAMSCAN Perl wrapper script for HMMER by Sam Griffith-Jones was downloaded from <ftp://ftp.sanger.ac.uk/pub/databases/Pfam/Tools/>. Version 19.0 of the Pfam database was downloaded from Wellcome Trust Sanger Institute (<ftp://ftp.sanger.ac.uk/pub/databases/Pfam/>) (Finn *et al.*, 2006). We performed BLASTP searches for each SBW25 sequence against GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>; Altschul *et al.*, 1990), and UniProt (<http://www.ebi.uniprot.org/>; The Uniprot Consortium, 2007).

#### Computational identification of candidate RpoN, IHF and EBP binding sites

We constructed a profile HMM representing the *rpoN* promoter sequence based on 186 sequences from a wide range of *Proteobacteria* (Barrios *et al.*, 1999) using the HMMB program from HMMER (Eddy, 1995). We searched the complete SBW25 and *R. solanacearum* GMI1000 genome sequences against this profile HMM using the HMMER HMMLLS program (Eddy, 1995). Multiple sequence alignments

of IHF and NtrC binding sites from *E. coli* were downloaded from RegulonDB (Salgado *et al.*, 2006). Alignments for FleQ and NorR binding sites were taken from Jyot and colleagues (2002; FleQ) and Tucker and colleagues (2005; NorR). We built a profile HMM for each alignment using the HMMB program from HMMER. We then searched the complete SBW25 genome sequence against this profile HMM using the HMMER hmmls program. For each match, we determined whether it fell within a predicted gene or a predicted intergenic region using the downloaded gene coordinates, and its orientation with respect to predicted genes.

#### Automated annotation of SBW25 gene products and operons

We translated each of the predicted genes into proteins using TRANSYS from the EMBOSS software suite (Rice *et al.*, 2000). We searched each predicted protein sequence against each profile HMM from the Pfam database of conserved domains using the PFAMSCAN wrapper script for HMMER and the Pfam-specified score thresholds. We defined a predicted operon in SBW25 as a series of adjacent predicted genes, all encoded on the same strand, and separated by no more than 75 nucleotides.

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### Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** Validation of C source utilization data. Growth assays were performed as described under *Experimental*

*procedures*. Data shown are the average of six replicates  $\pm$  standard deviation, recorded at 12 and 24 h after inoculation into test media. SBW25 (12 h, grey; 24 h black); SBW25  $\Delta rpoN$  (12 h, yellow; 24 h, red); SBW25  $\Delta rpoN$  (pJJP6) (12 h, pale blue; 24 h, dark blue).

**Fig. S2.** Validation of N source utilization data. Growth assays were performed as described under *Experimental procedures*. Data shown are the average of six replicates  $\pm$  standard deviation, recorded at 12 and 24 h after inoculation into test media. SBW25 (12 h, grey; 24 h black); SBW25  $\Delta rpoN$  (12 h, yellow; 24 h, red); SBW25  $\Delta rpoN$  (pJJP6) (12 h, pale blue; 24 h, dark blue).

**Table S1.** Carbon source data

**Table S2.** Nitrogen source data

**Table S3.** Stress tolerance data (single wells)

**Table S4.** Chemosensitivity and stress tolerance data (multiple wells)

**Table S5.** RpoN binding sites in SBW25

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