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The RpoS Sigma Factor Negatively Regulates Production of IAA and Siderophore in a Biocontrol Rhizobacterium, *Pseudomonas chlororaphis* O6

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The stationary-phase sigma factor, RpoS, influences the expression of factors important in survival of *Pseudomonas chlororaphis* O6 in the rhizosphere. A partial proteomic profile of a *rpoS* mutant in *P. chlororaphis* O6 was conducted to identify proteins under RpoS regulation. Five of 14 differentially regulated proteins had unknown roles. Changes in levels of proteins in *P. chlororaphis* O6 *rpoS* mutant were associated with iron metabolism, and protection against oxidative stress. The *P. chlororaphis* O6 *rpoS* mutant showed increased production of a pyoverdine-like siderophore, indole acetic acid, and altered isozyme patterns for peroxidase, catalase and superoxide dismutase. Consequently, sensitivity to hydrogen peroxide exposure increased in the *P. chlororaphis* O6 *rpoS* mutant, compared with the wild type. Taken together, RpoS exerted regulatory control over factors important for the habitat of *P. chlororaphis* O6 in soil and on root surfaces. The properties of several of the proteins in the RpoS regulon are currently unknown.

Keywords **:** indole acetic acid, iron, oxidative stress, proteomic analysis, sigma factor RpoS

Some rhizobacterial strains have been widely used as biocontrol agents, because they possess beneficial traits, such as the production of plant growth hormones and other secondary metabolites (Kim et al., 2011). An aggressive rhizobacterium, *Pseudomonas chlororaphis* O6 produces secondary metabolites, including phenazines and pyrrolintrin, which directly inhibit the growth of plant pathogenic fungi (Kang et al., 2007; Park et al., 2011). *P. chlororaphis* O6 also produces an iron-chelating pyoverdine-like (PVD) siderophore (Dimka et al., 2011) and the plant growth hormone, indole acetic acid (IAA) (Dimka et al., 2012; Kang et al., 2004), which affects plant growth (Beyeler et al., 1999; Costacurta and Vanderleyden, 1995; Patten and Glick, 2002; Vansuyt et al., 2007). Root colonization of *P. chlororaphis* O6 induces systemic resistance against viral, bacterial, and fungal plant diseases, as well as drought stress (Cho et al., 2008; Han et al., 2006; Kim et al., 2004; Ryu et al., 2007; Spencer et al., 2003).

In plant-associated pseudomonads, the sigma factor RpoS controls the expression of genes promoting survival under stress conditions (Kang et al., 2004; Miller et al., 2001). In *P. chlororaphis* O6, the *rpoS* transcript was influenced by a *ppk* gene encoding a polyphosphate kinase (Kim et al., 2007). The *rpoS* gene in *P. putida* is required for production of oxidative stress related enzymes, such as manganese superoxide dismutase and catalases, and for root colonization (Miller et al., 2001). RpoS also plays an important role in the survival of *P. fluorescens* Pf-5 on plant surfaces (Sarniguet et al., 1995). We used a proteomic approach to initiate a better understanding of the role of RpoS as a regulator in *P. chlororaphis* O6. The previously constructed *rpoS* mutant and its complemented *rpoS* mutant (Oh et al., 2012), as well as a *gacS* and complemented *gacS* mutant (Spencer et al., 2003), were used in this study. Previous studies showed the Gac system in *P. chlororaphis* O6 to regulate expression from *rpoS* (Kang et al., 2004). Strains were preserved in King's B medium (KB) or Luria Bertani (LB) broth containing 15% glycerol (v/v) at –80 °C.

Cell extracts were prepared by sonication of cells grown to stationary phase cells in LB and centrifugation at 15,000 × g for 10 min. Protein concentration was measured using the BioRad Protein Assay kit (BioRad, Hercules, CA, USA). The proteins were separated by isoelectric focusing (IEF) at 20 °C with a pH gradient (4 – 10) using a Multiphor II electrophoresis unit (Amersham Biosciences, Piscataway, NJ, USA). Sodium dodecyl sulfate-PAGE with 10 – 16% gradient gel was performed using a Hoefer DALT 2D system (Amersham Biosciences, Piscataway, NJ, USA) and the gels were silver-stained as described by Oakley et al. (1980) with the omission of glutaraldehyde. Quantitative analysis of the digitized images was carried out using the

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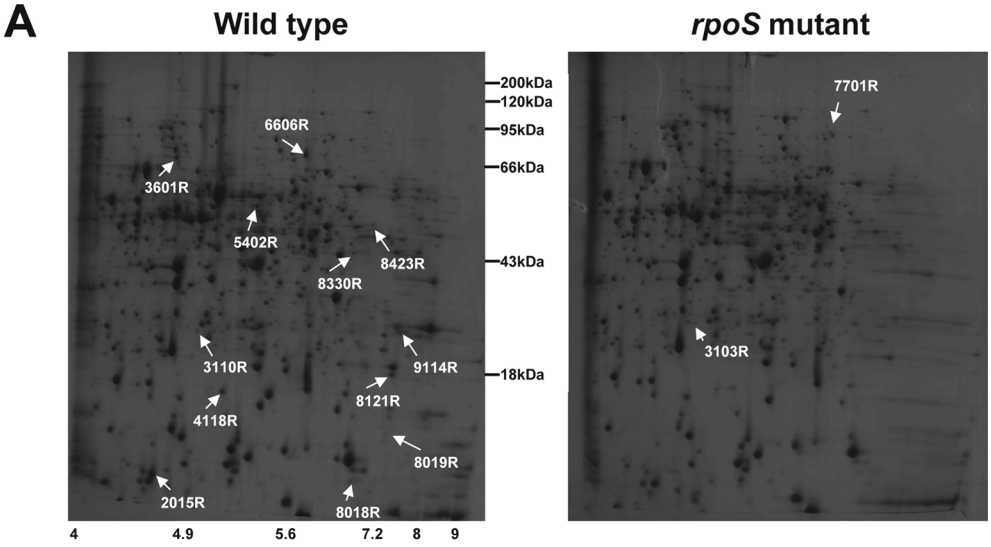
PDQuest (BioRad) software. Three independent 2-D gel analyses were performed, and spots with p-values of < 0.05 were selected for Q-TOF analysis.

The protein spots were digested enzymatically in-gel with 0.2 μg of modified trypsin (Promega, Madison, WI, USA) for 45 min on ice (Shevchenko et al., 1996) and were desalted on columns, 100 – 300 nL of porous reverse-phase R2 material (20 – 30 μm bead size, PerSeptive Biosystems, MA). The peptides were eluted with 1.5 μL of 50% methanol/ 49% H_2O/1% formic acid into a pre-coated borosilicate nano-electrospray needle (Micromass, Manchester, UK) and the MS/MS data obtained with a nano-ESI on a Q-



B

Spot number	MW	pI	wild type	*rpoS*-
2015R	8.26	4.86		
3110R	23.65	5.16		
5402R	53.62	5.56		
6606R	72.00	6.35		
9114R	24.40	8.24		
8330R	41.35	7.15		
8121R	18.14	8.06		
3601R	69.37	5.01		
8423R	47.94	7.59		
4118R	15.57	5.37		
8019R	11.47	7.93		
8018R	8.43	7.29		
3103R	24.64	5.07		
7701R	87.93	6.69		

Fig. 1. Comparison of intensities of proteins spots on two-dimensional gel electrophoresis (2-DE), in which total proteins isolated from stationary cells of *P. chlororaphis* O6 and *rpoS* mutant were loaded. (A) Representative 2-DE maps of *P. chlororaphis* O6 wild type and *rpoS* mutant. Total proteins of wild type and *rpoS* mutant were separated by gradient isoelectric focusing with a pH and SDS-PAGE, and then the gels were stained with silver. (B) The magnified protein spots showed differential expression between *P. chlororaphis* O6 and *rpoS* mutant. Arrows indicate differentially expressed proteins between wild type and *rpoS* mutant in the three independent 2-DE analyses.

TOF2 mass spectrometer (Micromass, Manchester, UK). The MS/MS spectra were searched against the protein sequences from the NCBI databases using the MASCOT search program (www.matrixscience.com).

More than 700 protein spots were reproducibly detected in each gel, and representative images from *P. chlororaphis* O6 wild type and *rpoS* mutant are shown in Fig. 1. From the gels, we identified 14 spots that had a more than two fold change ($P < 0.05$) in levels in the *rpoS* mutant compared to the wild type (Fig. 1). Of those peptides, no

Table 1. Identities of proteins in proteomic analysis spots showing statistically significant differences in expression levels in *P. chlororaphis* O6 *rpoS* mutant[a]

Spot number	Observed migration			Identified protein[b]			Fold change		
	Mr (kDa)	pI	Protein	Matching sequence	Mr (kDa)	pI	Mean[c]	SE (±)[c]	*p*-value[d]
2015R	8	4.86	No match	NCA-DWPPLMAASG			−69	3	0.006
3110R	24	5.16	No match	QNVVYLEEEGVLQRK			−75	30	0.048
5402R	54	5.56	Type I secretion outer membrane protein, TolC (*P. fluorescens* Pf0-1 ABA73205)	R.TIEVYLEVLK. R.VGVVMNYNLFR.G R.AAYQDQFGLGQR.T	51	5.49	−19.8	2.7	0.037
6606R	72	6.35	Putative Serine protein kinase, PrkA (*P. fluorescens* Pf-5 YP262712)	R.LLSEWYLR.V R.LSEFGGDISQFR.V R.ANQGLMEFVEMFK.A R.QEEFSLQEYLELCK.K K.MFSNTEDLLPVISFNAK.A R.LLLAIGEPELLDTSTNSR.L	74	5.66	−25.5	7.0	0.006
9114R	24	8.24	Peroxidase, AhpC (*P. fluorescens* Pf-5 AAY95129)	TPVCGTTELGYDSR	24	5.87	−16	4.6	0.040
8330R	41	7.15	S-adenosyl-methyltransferase, MraW (*P. fluorescens* YP350409)	R.GISAAEFVNTAPVEEIAR.V	34	6.06	−5.2	1.3	0.047
8121R	18	8.06	Hypothetical protein (*Ralstonia pickettii* 12D ZP02008061)	YAVSTTPYLNR			> −100		0.004
3601R	69	5.01	Tryptophan monooxygenase (amine oxidase, IaaM (*P. syringae* pv. *syringae* AAR06971)	QLVGAGVSGLVA	62	6.51	−14	4.3	0.007
8423R	48	7.59	Polyamine ABC transporter, PotA (*P. fluorescens* Pf-5 AAY94832)	R.NTFVANFIGENNR.L R.LGVTVVYVTHDQGEALTMSDR.V	41	6.15	−25	4.5	0.003
4118R	16	5.37	Glutathione peroxidase, Gpx (*P. fluorescens* Pf-5 YP258072)	K.ALDGQELPLAPFK.G K.CGLTPQYAALENLYQQYK.A	18	5.35	−5	1.6	0.002
8019R	12	7.93	No match	NDPVEYLTLTLK DLLVSSLGTG			> −100		0.004
8018R	8	7.29	No match	LYSAVELFHDWR LAYLDELPH			> −100		0.003
3103R	25	5.07	Heme oxygenase, HemeO (*P. fluorescens* Pf-5 AAY93875)	ALGWLFVSEGSK	22	5.16	12	2.6	0.013
7701R	88	6.69	TonB-dependent outer membrane heme receptor, CirA (*P. syringae* pv. *tomato* T1 EEB60559)	PSTLENYFPFTR TPGYGVLDLT	94	5.98	17	8.71	0.001

[a] The Mr and pI values were estimated on 2-dimentional gels of three independent experiments. Ions score is −10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 49 indicate identity or extensive homology ($p < 0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. Amino acid sequences without ion scores were determined by Q-TOF analysis.

[b] Annotation from NCBI databases using the MASCOT search program (www.matrixscience.com).

[c] The mean and standard error (SE) of fold change of the selected spot was calculated by comparing spot intensities between wild type and *rpoS* mutant of three independent gels using quantitative image analysis (PDQest 2-D analysis Software).

[d] Student's t-test.

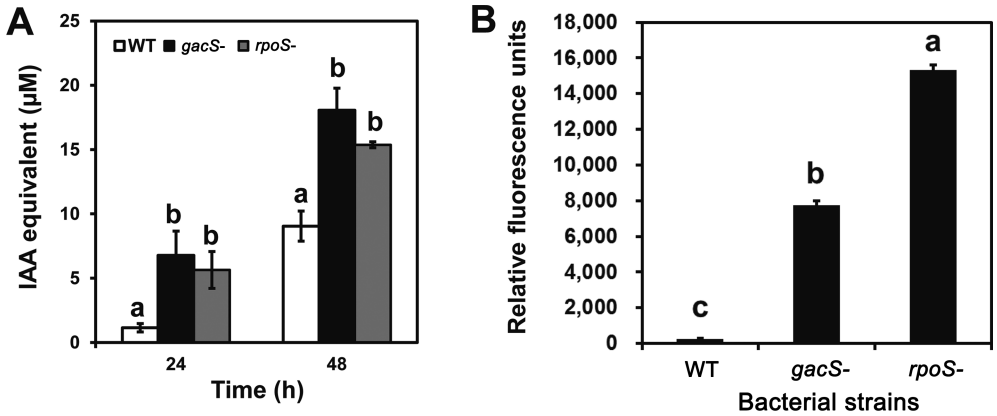


Fig. 2. Mutations in *rpoS* and *gacS* in *P. chlororaphis* O6 increased IAA production (A) and PVD-like fluorescent siderophore production (B) over that of the wild type strain (WT). (A) IAA was detected in supernatants generated after growth for 24 h, and 48 h: authentic IAA was used as the standard in the Salkowski assay. (B) Siderophore levels were assessed by the characteristic fluorescence of the PVD-like siderophore with excitation at 398 nm and emission at 460 nm. The data for the relative fluorescent units are means from two studies, each with two replicates; standard deviation is shown. Different letters represent significant differences at each time point ($P < 0.05$) based on at least two separate studies.

matches were detected for four proteins (Table 1) and another corresponded to a hypothetical protein found in a *Ralstonia* isolate. A tryptophan monooxygenase (IaaM), outer membrane protein (TolC), which is potentially involved in export, other proteins involved in oxidative stress [peroxidase (AphC) and glutathione peroxidase (Gpx)], and at growth [polyamine transporter (PotA)], were signiticantly decreased in the *rpoS* mutant. Two proteins with the potential to function in cell signaling, S-adenoysyl methyltransferase (MraW) and a putative serine protein kinase (PrkA), were down-regulated in the *rpoS* mutant. In contrast, two proteins involved in the cellular utilization of iron from heme sources, heme oxygenase (HemeO) and a TonB-dependent membrane heme receptor (CirA) were detected at higher levels in the mutant (Table 1). RpoS regulated genes and proteins were identified in *Geobacter sulfurreducens* and *Burkholderia pseudomallei* (Nunez et al., 2006; Osiriphun et al., 2009). As expected, most of the RpoS regulated genes and proteins are related to stress responses, but many of the RpoS regulated genes and proteins are involved in the expression of diverse physiological traits, such as general metabolism, transcription, translation, cell motility and secretions (Nunez et al., 2006; Osiriphun et al., 2009).

To understand the significance of these changes, the production of the plant hormone IAA was compared between the *rpoS* mutant and the wild type strains. Cells were grown for 48 h in the low-iron medium described by Dimkpa et al. (2012) amended with 200 μM tryptophan for the IAA studies. IAA was detected in cell free culture filtrates by the Salkowski colorimetric assay (Patten and Glick, 2002), using the authentic compound as the standard and was authenticated by HPLC as described in Dimkpa et al. (2012). Production of IAA was increased in the *rpoS* mutant. The highest level was similar to those observed from cultures of the *gacS* mutant (Fig. 2A) suggesting that RpoS was the major regulator. Although *P. chlororaphis* O6 is reported to produce IAA in the presence of tryptophan (Kang et al., 2006), the pathway was not elucidated clearly. A recent study indicated that the indole-3-acetamide pathway is involved for IAA production in *P. chlororaphis* O6 (Dimkpa et al., 2012). In this study, it was unexpected that transcripts from *iaaM*, encoding the first enzyme, tryptophan monooxygenase, potentially involved in conversion of tryptophan to indole-3-acetamide was down regulated in the *rpoS* mutant (Table 1). We are currently constructing the *iaaM* defective mutant and transcriptional analysis of the *iaaM* transcript in wild type and *rpoS* mutant to resolve the uncertainty between the proteomic result and phenotype in IAA production.

To understand altered iron metabolism, the production of the fluorescent siderophore was compared between the *rpoS* mutant and the wild type strains. Cells were grown for 48 h in low-iron medium (Dimkpa et al., 2011) with and without 100 μM Fe^{3+}. Siderophore levels were assessed in the cell free culture by excitation at 398 nm and measuring fluorescence emission at 460 nm (Dimkpa et al., 2011). Production of the PVD-like siderophore was greatly elevated in the *rpoS* mutant compared to that in wild type strain, with the increased production of the *rpoS* mutant being significantly greater than that of the *gacS* mutant (Fig. 2B). Growth of the strains in medium amended with 100 μM Fe^{3+} eliminated the characteristic PVD fluorescence, as anticipated from iron-repression of siderophore production.

Proteins in cells extracts were separated by non-denaturing electrophoresis and were stained for activities of peroxidase, catalase, and superoxide distmutase as described previously (Katsuwon and Anderson, 1990; MacAdam et al., 1992). Activity staining of extracts from late- and stationary-phase cells revealed isozymes with both catalase and peroxidase activities (Fig. 3A). Extracts from the *rpoS* and *gacS* mutants, lacked the same isozymes, whereas these activities were restored in the complemented mutants. The hydrogen peroxide-sensitive iron superoxide dismutase isozyme was produced constitutively by each strain (Fig. 3A). However, the *rpoS* and *gacS* mutants over-expressed a manganese superoxide dismutase isozyme, which was not inhibited by hydrogen peroxide. Expression of the Mn-isozyme was suppressed in the complemented strains (Fig. 3A).

To measure hydrogen peroxide sensitivity, cells were grown in liquid KB medium at 28 ºC to late-log phase (OD_{600nm} = 1.8) when 4.5 mM hydrogen peroxide was added. Serial dilutions of the culture were made and were plated onto LB agar plates containing kanamycin (50 μg/mL) for the *rpoS* mutant and kanamycin and gentamycin (12.5 μg/mL) for the complemented mutant at the defined times. Colonies were counted after 2 days of incubation at 28 ºC. Three independent experiments were evaluated for each strain. Recovery of cells exposed to hydrogen peroxide was lowest for the *rpoS* mutant and was restored to the wild type level by *rpoS* complementation (Fig. 3B).

These proteomic and metabolic studies demonstrated that RpoS regulated several proteins in *P. chlororaphis* O6. The proteins under RpoS control included specific isozymes of catalase, peroxidases and superoxide dismutase involved in protection against oxidative stress. The greater sensitivity to hydrogen peroxide displayed by the *rpoS* mutant showed the functionality of these and other changes, such as the polyamine transporter PotA (Jung and Kim, 2003; Tkacheko et al., 2001), the AhpC peroxidase (Hishinuma et al., 2006), and the Gpx glutathione peroxidase (Lan et al., 2010). These findings of altered levels of proteins involved in oxidative stress and increased sensitivity agree with observations of *rpoS* mutants of *P. aeruginosa* and *P. fluorescens* (Heeb et al., 2005; Suh et al., 1999). The role of RpoS in generating resistance to oxidative stress could be important in counteracting the oxidative stress imposed as the bacterium interacts with the root surface (Kang et al., 2004; Katsuwon and Anderson, 1990; Kim et al., 2000, 2004; Miller et al., 2001).

Maintenance of cellular Fe levels is essential in the control of oxidative stress. Effects of the *rpoS* mutation on iron homeostasis is probable because the proteomic analysis revealed accumulations of a heme-uptake receptor and a heme oxygenase and the mutant displayed enhanced sec-

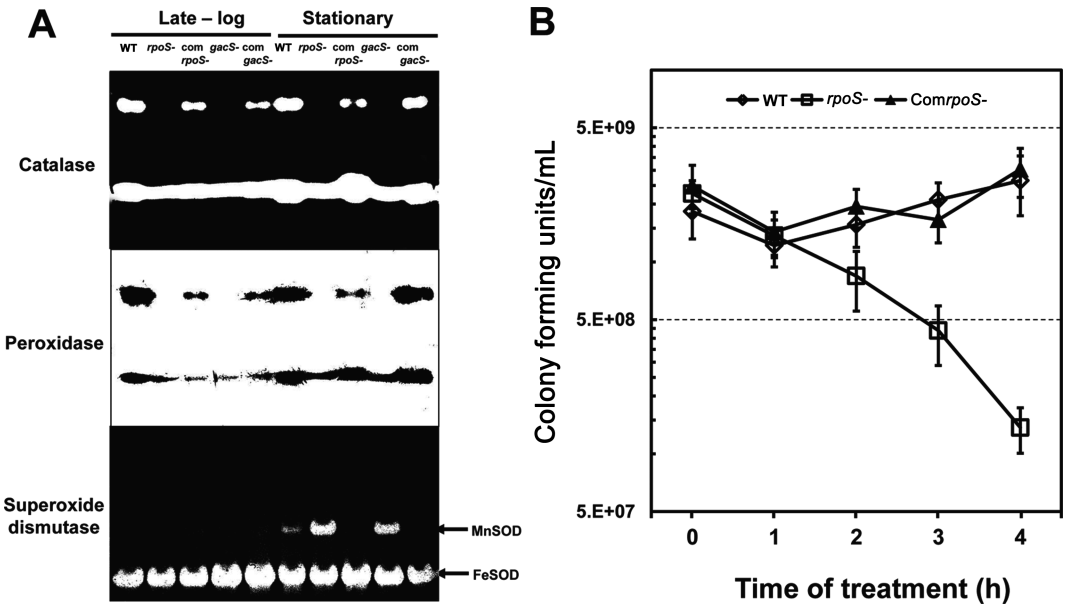


Fig. 3. Effect of mutation in *rpoS* and *gacS* genes in *P. chlororaphis* O6 on catalase, peroxidase, and superoxide dismutase (SOD) isozyme patterns (A) and hydrogen peroxide sensitivity (B). Enzyme activities were detected after non-denaturing polyacrylamide gel electrophoresis of 100 μg of protein from extracts of cells grown in KB broth to the late log-phase (OD_{600nm} = 1.8), or stationary-phase (OD_{600nm} = 2.4). The results are typical of three independent experiments. (B) Hydrogen peroxide (4.5 mM final concentration) was added to *P. chlororaphis* O6 strains grown to the late log-phase (OD_{600nm} = 1.8) in liquid KB cultures. Serial dilutions were performed with the culture and were plated to determine cell culturability at defined times. Error bars represent the standard deviations of three independent experiments. Com*rpoS*⁻, *rpoS*⁻ mutant complemented with *rpoS* gene.

retion of a Fe(III)-binding fluorescent siderophore. Earlier, Suh et al. (1999) also found that an *rpoS* mutant in *P. aeruginosa* had increased siderophore production.

RpoS regulation of other factors involved in rhizosphere colonization is extended to IAA synthesis; IAA production was increased in the *rpoS* mutant. This finding agreed with our previous findings that production of IAA in *P. chlororaphis* O6 was negatively regulated by a two component sensor kinase, GacS (Kang et al., 2004; 2006). However, this finding did not correlate with the observation that transcript accumulation from *iaaM*, encoding the monooxygenase involved in transformation of tryptophan to IAA, was down-regulated according to the proteomic finding. One possibility is that the *rpoS* mutant prematurely down regulated *iaaM* transcription compared with the wild type where stationary phase repression has been noted (Dimkpa et al., 2012). Another possibility is that there is an alternate mechanism for IAA synthesis. Three bacterial pathways for IAA production from tryptophan are reported, namely the indole-3-pyruvate (IPyA), the tryptophan-side chain oxidase pathway, and the *IaaM* pathway (Beyeler et al., 1999; Costacurta and Vanderleyden, 1995; Patten and Glick, 2002).

Our observations showed that whereas RpoS was the major regulator for IAA formation in *P. chlororaphis* O6, both RpoS and the Gac sytem regulated siderophore production. Regulatory control by both RpoS and GacS for IAA production was found for *Enterobacter cloacae* (Saleh and Glick, 2001). Likewise, RpoS was a key regulator for IAA and siderophore production in the endophytic plant growth-stimulating *Burkholderia phytofirmans* (Sun et al., 2009).

These findings illustrate the pivotal role of RpoS in the regulation of factors involved in root colonization and interaction for *P. chlororaphis* O6. Like other microbes the RpoS-regulated proteins include those involved in oxidative stress and iron homeostasis. However, other as yet uncharacterized protein products were also RpoS-regulated and their function is currently under analysis.

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