The role of *dsbA* in colonization of the wheat rhizosphere by *Pseudomonas fluorescens* Q8r1-96

Olga V. Mavrodi,¹ Dmitri V. Mavrodi,¹ Amanda A. Park,² David M. Weller² and Linda S. Thomashow^{1,2}

Correspondence

Linda S. Thomashow
thomasho@mail.wsu.edu

Department of Plant Pathology¹ and USDA-ARS, Root Disease and Biological Control Research Unit², Washington State University, Pullman, WA, USA

Certain well-conserved genes in fluorescent *Pseudomonas* spp. are involved in pathogenic interactions between the bacteria and evolutionarily diverse hosts including plants, insects and vertebrate animals. One such gene, *dsbA*, encodes a periplasmic disulfide-bond-forming enzyme implicated in the biogenesis of exported proteins and cell surface structures. This study focused on the role of *dsbA* in *Pseudomonas fluorescens* Q8r1-96, a biological control strain that produces the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) and is known for its exceptional ability to colonize the roots of wheat and pea. The deduced DsbA protein from Q8r1-96 is similar to other predicted thiol:disulfide interchange proteins and contains a conserved DsbA catalytic site, a pattern associated with the thioredoxin family active site, and a signal peptide and cleavage site. A *dsbA* mutant of Q8r1-96 exhibited decreased motility and fluorescence, and altered colony morphology; however, it produced more 2,4-DAPG and total phloroglucinol-related compounds and was more inhibitory *in vitro* to the fungal root pathogen *Gaeumannomyces graminis* var. *tritici* than was the parental strain. When introduced separately into a natural soil, Q8r1-96 and the *dsbA* mutant did not differ in their ability to colonize the rhizosphere of wheat in greenhouse experiments lasting 12 weeks. However, when the two strains were co-inoculated, the parental strain consistently out-competed the *dsbA* mutant. It was concluded that *dsbA* does not contribute to the exceptional rhizosphere competence of Q8r1-96, although the *dsbA* mutation reduces competitiveness when the mutant competes with the parental strain in the same niche in the rhizosphere. The results also suggest that exoenzymes and multimeric cell surface structures are unlikely to have a critical role in root colonization by this strain.

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INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are root-associated bacteria from many different genera that can increase the growth of plants when applied to soil, seeds or vegetatively propagated plant parts (Weller & Thomashow, 1994). Despite their potential economic and environmental benefits, efforts to exploit PGPR commercially as biopesticides, biofertilizers or phytostimulants have been restricted by inconsistent results caused in part by poor root colonization. Most introduced rhizobacteria establish high rhizosphere population densities initially, but then the population declines with time and distance from the inoculum source. Densities of introduced rhizobacteria also vary from root

to root and plant to plant, leaving some plants or roots unprotected (Bull *et al.*, 1991; Weller & Thomashow, 1994; Weller, 1988).

Root colonization and ecological fitness of PGPR is a complex phenotype affected by many different traits and by environmental factors (Lugtenberg *et al.*, 2001; Weller & Thomashow, 1994). Among the root colonization determinants shown to contribute to the PGPR–plant root interaction are flagella (de Weger *et al.*, 1987), fimbriae (Camacho Carvajal, 2000), synthesis of the O-antigen of lipopolysaccharide (Dekkers *et al.*, 1998a), and a site-specific *sss* recombinase thought to play a role in regulating the biosynthesis of cell surface components (Dekkers *et al.*, 1998b, 2000). Other root-colonization traits are associated with the ability to synthesize essential compounds (Dekkers *et al.*, 1998c; Simons *et al.*, 1997) and sequester nutrients or tolerate physical stresses (Lugtenberg *et al.*, 2001). In addition, the discovery of type III secretion genes in many PGPR strains

Abbreviations: 2,4-DAPG, 2,4-diacetylphloroglucinol; MAPG, monoacetylphloroglucinol; PGPR, plant growth-promoting rhizobacteria.

The GenBank/EMBL/DBJ accession number for the *dsbA* sequence of *P. fluorescens* Q8r1-96 reported in this paper is AY171618.

(Preston *et al.*, 2001; Rainey, 1999; Mazurier *et al.*, 2004; Rezzonico *et al.*, 2004) suggests that at least some saprophytic rhizobacteria may have a more sophisticated relationship with host tissues than previously was recognized. Evidence of common determinants involved in the pathogenicity of bacteria such as *Pseudomonas aeruginosa* to plants and animals (Lugtenberg *et al.*, 2001; Rahme *et al.*, 2000) further supports this idea.

Fluorescent *Pseudomonas* spp. that produce the polyketide antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) are highly effective biocontrol agents of soilborne pathogens (Duffy & Dfago, 1997; Keel *et al.*, 1992; Tamietti *et al.*, 1993). Strains of most of the 22 genotypes of DAPG producers described to date are phenotypically very similar (McSpadden Gardener *et al.*, 2000; O. V. Mavrodi *et al.*, 2001), but they differ considerably in their ability to colonize the rhizosphere of certain crop species (Landa *et al.*, 2002b, 2003; Raaijmakers & Weller, 2001). In particular, D-genotype isolates are highly aggressive colonists of wheat and pea and have an affinity for these crops as compared to most other genotypes. D-genotype isolates collected from different locations all display the ability to establish and maintain a large population size in the rhizosphere, even when introduced at densities of only 10–100 c.f.u. (g soil)^{−1} (Landa *et al.*, 2002b, 2003; Raaijmakers & Weller, 2001). Our efforts to understand the molecular basis for the unique root-colonizing ability of *P. fluorescens* Q8r1-96, the most completely described of the D-genotype strains (Landa *et al.*, 2002b; Raaijmakers & Weller, 2001), have focused in part on genes common to saprophytic and pathogenic bacteria and implicated in pathogenesis. Because such genes have a role in an intimate and active molecular dialogue between bacteria and their hosts, we hypothesize that they also may provide insight into the unique affinity of D-genotype strains for their plant hosts. Among these genes is *dsbA*, which encodes a periplasmic protein that catalyses disulfide bond formation in exported proteins of Gram-negative bacteria (Bardwell *et al.*, 1991). While the number and identity of all of the proteins acted upon by DsbA is not known, it has been shown to be essential for the biogenesis of virulence factors and multimeric structures on the bacterial surface including fimbriae and components of the type III secretion apparatus. DsbA has been implicated in the interactions of *P. aeruginosa* and *Pseudomonas syringae* pv. *tomato* with *Arabidopsis* (Rahme *et al.*, 1997; Kloeck *et al.*, 2000), and it is involved in the assembly of a functional type III secretion apparatus in *Shigella flexneri* (Watarai *et al.*, 1995) and *Yersinia pestis* (Jackson & Plano, 1999); in secretion of pectate lyases and cellulase in *Erwinia chrysanthemi* (Shevchik *et al.*, 1995); in formation of toxin-coregulated pili in *Vibrio cholerae* (Hu *et al.*, 1997); and in stabilization of periplasmic lipase in *P. aeruginosa* (Urban *et al.*, 2001). In this study we identified and characterized the *dsbA* orthologue in *P. fluorescens* Q8r1-96 and evaluated its role in root colonization and the competitiveness of this strain in the wheat rhizosphere. We show that *dsbA* does not contribute to the exceptional rhizosphere competence of Q8r1-96, and that root

colonization by a *dsbA* mutant of Q8r1-96 is reduced when the mutant is in competition with the wild-type, but not in the presence of indigenous rhizosphere microflora.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. A rifampicin-resistant derivative of Q8r1-96 (Landa *et al.*, 2002b) was used for gene replacement mutagenesis, while Q8r1-96Gm, a rifampicin-resistant derivative tagged with mini-Tn7-*gfp2* (Validov *et al.*, 2005) was used in competitive colonization experiments. *Escherichia coli* TOP 10 (Invitrogen) was used for cloning experiments and *E. coli* S17-1(λ-pir) was the donor strain in biparental matings with *P. fluorescens* Q8r1-96. *P. fluorescens* and *E. coli* strains were grown at 28 °C and 37 °C, respectively, in Luria–Bertani (LB) medium (Ausubel *et al.*, 1995), *Pseudomonas* agar F (PsF) (Difco), *Pseudomonas* agar P (PsP) (Difco) or King's medium B (KMB) (King *et al.*, 1954). Densities of total culturable heterotrophic bacteria (TCB) were determined in one-tenth-strength Tryptic Soy (TS) broth (Difco). Antibiotic supplements (Sigma) were used at the following concentrations: ampicillin, 100 or 40 µg ml^{−1}; rifampicin, 100 or 90 µg ml^{−1}; tetracycline, 10 or 12.5 µg ml^{−1}; gentamicin, 2 µg ml^{−1}; cycloheximide, 100 µg ml^{−1}; chloramphenicol, 13 or 35 µg ml^{−1}; and kanamycin, 25 or 50 µg ml^{−1}.

DNA manipulations. Standard methods were used for plasmid isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation and transformation (Ausubel *et al.*, 1995). PCR amplification was carried out with *Taq* (Promega) or KOD Hot Start (Novagen) DNA polymerase. The oligonucleotide primers listed in Table 2 were developed with Oligo 6.65 Software (Molecular Biology Insights).

Identification of *dsbA* in *P. fluorescens* Q8r1-96. An arrayed genomic library of *P. fluorescens* Q8r1-96 (Mavrodi, 2004), constructed in the broad-host-range vector pCPP47 (Bauer & Collmer, 1997), was screened by colony hybridization (Birren *et al.*, 1999) with a ³²P-labelled *dsbA*-specific probe amplified by PCR from DNA of *P. fluorescens* SBW25 with primers DSBA_UP and DSBA_LOW (Table 2), which were developed from the *dsbA* sequence of strain SBW25 (http://www.sanger.ac.uk/Projects/P_fluorescens/). Cycling included a 2 min denaturation at 94 °C, followed by 29 cycles of 94 °C for 15 s, 61 °C for 30 s, and 68 °C for 1.2 min, and a final extension at 68 °C for 5 min. To localize *dsbA* within positive clones, cosmid DNA digested with *EcoRI*, *KpnI* and *SacI* was blotted and hybridized with a biotinylated *dsbA* probe.

Transposon mutagenesis, shotgun sequencing and sequence analysis. To tag *dsbA* and adjacent regions of cosmid clones for DNA sequence analysis, purified cosmid DNA was mutagenized *in vitro* by using the EZ::TN <Kan-2> transposition system (Epicentre Technologies). Transposon insertions within DNA fragments containing *dsbA* were identified by restriction mapping and shotgun-sequenced by using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequence data were compiled and analysed with OMIGA 2.0 software (Accelrys). Database searches for similar protein sequences were performed using NCBI's BLAST network service. Searches against PROSITE, Profile, HAMAP and Pfam collections of protein motifs and domains were carried out using the MyHits Internet engine (Pagni *et al.*, 2004), and signal peptide cleavage sites were predicted with SignalP v. 3.0 (Bendtsen *et al.*, 2004).

Allelic replacement in Q8r1-96. The *dsbA* gene containing EZ::TNTM <Kan-2> was amplified with DSBA_UP and DSBA_LOW primers by using KOD Hot Start DNA polymerase. The cycling programme included a 2 min denaturation at 94 °C

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics*	Reference of origin
<i>P. fluorescens</i>		
Q8r1-96	DAPG ⁺ Rif ^r	Landa <i>et al.</i> (2002)
Q8r1-96Gm	Q8r1-96 tagged with mini-Tn7- <i>gfp2</i> ; DAPG ⁺ Rif ^r Gm ^r	Validov <i>et al.</i> (2005)
Q8r1-96 <i>dsbA</i>	<i>dsbA</i> ::TN<Kan2>; DAPG ⁺ Rif ^r Kan ^r	This study
<i>E. coli</i>		
S17-1(λ-pir)	<i>thi pro hsdM recA rpsL</i> RP4-2 (Tet ^r ::Mu) (Kan ^r ::Tn7)	Lab collection
TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 ara</i> Δ139 Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL endA1 nupG</i>	Invitrogen
Plasmids		
pCPP47	Broad-host-range cosmid derived from pCPP34, tandem <i>cos</i> ⁺ , <i>par</i> ⁺ , Tet ^r	Bauer & Collmer (1997)
pMOB3	Kan ^r Cam ^r , <i>oriT sacB</i>	Schweizer (1992)
pNOT19	ColE1 <i>oriV</i> , Amp ^r ; accessory plasmid	Schweizer (1992)
pNOT19- <i>dsbA</i> -Kan	pNOT19 containing the 2.2 kb DNA <i>Sma</i> I fragment with <i>sss</i> interrupted by EZ::TN<Kan2>	This study
pNOT19- <i>dsbA</i> -Kan-MOB	pNOT19- <i>dsbA</i> -Kan ligated with 5.8 kb <i>Not</i> I fragment from pMOB3	This study
pME6010	Broad-host-range plasmid; pVS1 <i>oriV</i> , p15a <i>oriV</i> , P _k , Tet ^r	Heeb <i>et al.</i> (2000)
pME6010- <i>ccdB</i>	Gateway destination vector derived from pME6010 with <i>ccdB</i> -Cam ^r cassette flanked by <i>attR1</i> and <i>attR2</i>	This study
pMK2010	Gateway entry vector; ColE1 <i>oriV</i> , <i>oriT</i> _{RP4} , Kan ^r , <i>ccdB</i> -Cam ^r cassette flanked by <i>attP1</i> and <i>attP2</i>	House <i>et al.</i> (2004)
pMK2010 <i>dsbA</i>	pMK2010 containing the 0.67 kb DNA fragment with <i>dsbA</i>	This study
pME6010 <i>dsbA</i>	pME6010 containing the 0.67 kb DNA fragment with <i>dsbA</i>	This study

*DAPG, strain produces 2,4-diacetylphloroglucinol; Rif^r, rifampicin resistance; Gm^r, gentamicin resistance; Amp^r, ampicillin resistance; Tet^r, tetracycline resistance; Kan^r, kanamycin resistance; Cam^r, chloramphenicol resistance.

followed by 29 cycles of 94 °C for 15 s, 61 °C for 30 s and 68 °C for 1.2 min, and a final extension at 68 °C for 5 min. The amplification product was cloned into the *Sma*I site of pNOT19 (Schweizer, 1992) and the plasmid was then digested with *Not*I and ligated with a 5 kb *Not*I fragment of the pMOB3 cassette (Schweizer, 1992) containing *sacB* and *cat* genes. The resultant pNOT19-*dsbA*-Kan-MOB plasmid was electroporated into *E. coli* S17-1(λ-pir), selected on LB medium supplemented with chloramphenicol and kanamycin, and mobilized by mating into *P. fluorescens* Q8r1-96Rif^r. Mutant clones were selected on LB supplemented with rifampicin, kanamycin and 5 % sucrose. Positive clones were screened for the absence of *sacB*, *bla* and *cat* genes by PCR with primers SAC1 and SAC2, BLA1 and BLA2 (D. V. Mavrodi *et al.*, 2001) and Cm_UP and Cm_LOW (Table 2), respectively. Primers KAN_UP and KAN_LOW (Table 2) and DSBA_UP

and DSBA_LOW were used to detect the kanamycin resistance gene in *dsbA* mutants and to confirm the absence of the wild-type *dsbA* allele. All mutant clones were isogenic and one clone was chosen for further experiments.

Complementation of the *dsbA* mutant strain. The full-length *dsbA* gene was cloned into the stable broad-host-range plasmid pME6010 (Heeb *et al.*, 2000) using Gateway Technology (Invitrogen) in conjunction with nested PCR as described by House *et al.* (2004). Briefly, *dsbA* was amplified with primers *dsbAF*-*dsbAR* (Table 2) and *attB* sequences were introduced by reamplification with primers 2F-2R (House *et al.*, 2004). The resultant PCR fragment was cloned into the entry plasmid pMK2010 (House *et al.*, 2004) with BP Clonase II (Invitrogen), single-pass sequenced to confirm its integrity, and

Table 2. Oligonucleotides

Primer name	Sequence*
DSBA_UP	5'-GAT ACC AAG CCG ATG CAG A-3'
DSBA_LOW	5'-GGA CAT GAC ATG ATG CTC GT-3'
KAN_UP	5'-TGG CAA GAT CCT GGT ATC GGT-3'
KAN_LOW	5'-GAA ACA TGG CAA AGG TAG CGT-3'
Cm_UP	5'-ATC CCA ATG GCA TCG TAA AGA-3'
Cm_LOW	5'-AAG CAT TCT GCC GAC AT-3'
<i>dsbAF</i>	5'-GGA GGC TCT TCA ATG CGT AAT CTG ATC ATC AG-3'
<i>dsbAR</i>	5'-AGC TGG GTT CTA GTT GGC AGC AGC CTT GGT GG-3'

*Oligonucleotides were designed by using Oligo 6.65 Primer Analysis Software.

transferred with LR Clonase II (Invitrogen) into pME6010, which had been converted into a Gateway destination vector by introduction of a *ccdB*- and *attR*-containing cassette (Invitrogen) (Table 1). The resultant plasmid was electroporated into Q8r1-96*dsbA* as described by Enderle & Farwell (1998).

Phenotypic analysis *in vitro*. For motility assays, colony diameter was measured 24, 48 and 72 h after inoculation. Exoprotease production was assessed on skim milk agar (Sacherer *et al.*, 1994) as a cleared zone surrounding bacterial growth after incubation for 48 and 72 h at 28 °C. Hydrogen cyanide production was monitored by using cyanide detection paper placed on Petri dish lids (Bakker & Schippers, 1987). Observations were made every 24 h and all cultures were screened twice. Polysaccharide production was scored after 3 days of growth on PsP agar on a scale of 0–5, where 0 indicates a non-mucoid isolate and 5 indicates a moderately mucoid culture. Siderophore production was determined by measuring orange haloes after 2 days of growth at 28 °C on CAS agar (Schwyn & Neilands, 1987). Experiments were repeated twice with four replicates per strain. Inhibition of *G. graminis* var. *tritici* by *P. fluorescens* Q8r1-96Gm and its mutants was assayed on PsF agar as described earlier (McSpadden Gardener & Weller, 2001). Assays were repeated twice, with six replicates per strain. Carbon substrate and nitrogen utilization profiles were generated by using Biolog SF-N2 and PM3 MicroPlates, respectively. Four independent repetitions were performed with each strain. M9 minimal media supplemented with 0.4% D-galactose as a carbon source or with 10 mM of nitrogen sources was used for validation of Biolog assays. Phloroglucinol compounds were extracted with ethyl acetate from bacterial cultures grown for 48 h at 27 °C in PsF broth. Extracts were fractionated on a Waters NOVA-PAK C₁₈ Radial-PAK cartridge (4 µm, 8 × 100 mm) as described earlier (Bonsall *et al.*, 1997). Two independent experiments with five replications were performed.

Rhizosphere colonization assays. Bacterial inocula of Q8r1-96*dsbA* and Q8r1-96Gm, a gentamicin-resistant derivative of the parental strain (Validov *et al.*, 2005), were prepared to give approx. 1×10^4 c.f.u. (g soil)⁻¹ when strains were introduced alone or $\sim 0.5 \times 10^4$ c.f.u. (g soil)⁻¹ in 1:1 mixtures. Actual strain densities were determined by assaying the inoculated soil as described by Landa *et al.* (2002a). The control treatment consisted of soil amended with a 1% methylcellulose suspension. Seeds of pre-germinated spring wheat (*Triticum aestivum* L.) cv. Penawawa were sown in square pots (6.5 cm high × 7 cm wide) filled with 200 g Quincy virgin soil inoculated with one or both bacterial strains. Seedlings were grown for 2 weeks (one cycle) in a controlled-environment chamber and the population size of the introduced bacteria was determined after each of six cycles (Landa *et al.*, 2003). Each treatment was replicated six times, with one pot serving as replicate, and the experiment was conducted twice. After the sixth cycle, soil from pots of the same treatment was decanted into plastic bags and stored at 20 °C for 10 weeks before again growing wheat for two cycles in order to assess the ability of the strains to survive in the absence of roots.

Population densities of the introduced strains were determined by the dilution end-point method (McSpadden Gardener *et al.*, 2001) as modified by Validov *et al.* (2005). Briefly, soil (0.5 g) or root samples in 10 ml sterile distilled water were vortexed, sonicated, serially diluted in 96-well microtitre plates pre-filled with sterile distilled water, and then each dilution was transferred to another plate containing one-third-strength KMB broth supplemented with rifampicin, cycloheximide, ampicillin and chloramphenicol (1/3 × KMB⁺⁺⁺Rif) (McSpadden Gardener *et al.*, 2001). After 3 days these cultures subsequently were replicated to fresh 1/3 × KMB⁺⁺⁺Rif plates amended with kanamycin or gentamicin to distinguish between strains from mixed inoculations.

Bacterial growth was assessed after 72 h with an OD₆₀₀ ≥ 0.07 scored as positive (McSpadden Gardener *et al.*, 2001). In some cases, results were verified by PCR.

Densities of total culturable heterotrophic bacteria were determined by the terminal dilution end point assay in one-tenth-strength TS broth supplemented with cycloheximide (McSpadden Gardener *et al.*, 2001).

Data analysis. Treatments in competitive colonization experiments were arranged in a complete randomized design. Data were analysed with STATISTIX 8.0 software (Analytical Software). Population data were converted to log c.f.u. (g soil)⁻¹ or fresh root weight. Differences in population densities among treatments were determined by standard analysis of variance, and mean comparisons among treatments were performed by using Fisher's protected least significant difference test ($P=0.05$) or by the Kruskal–Wallis test ($P=0.05$). Data from phenotypic analyses *in vitro* were compared by using a two-sample *t* test or Wilcoxon rank sum test ($P=0.05$).

RESULTS

Identification, cloning and characterization of the *dsbA* gene

Clones containing the *dsbA* gene from *P. fluorescens* Q8r1-96 were identified in an ordered genomic library of 1536 clones by colony hybridization with a *dsbA*-specific probe from *P. fluorescens* SBW25. An initial round of hybridization yielded eight positive clones, from which cosmid DNA was isolated, digested with restriction endonucleases *EcoRI*, *KpnI* or *SacI*, blotted, and again hybridized with a *dsbA* probe in order to localize the gene. One clone, 3B12, in which *dsbA* mapped to a 4.6 kb *EcoRI* fragment, was then mutagenized *in vitro* with EZ::Tn<Kan2>. DNA from 200 kanamycin-resistant clones was digested with *EcoRI* and cosmids with shifts in the electrophoretic mobility of the target 4.6 kb fragment were sequenced with transposon-based primers.

The sequence data were assembled into a 2962 bp contig containing five potential open reading frames, one of which encoded a product similar to bacterial thiol:disulfide exchange proteins. This gene, designated *dsbA*, was preceded by a well-conserved ribosome-binding site, AGGAG, and encodes a predicted 213 aa protein of molecular mass 23 080 Da. The contig also contained two putative genes encoding precursors of cytochromes *c*₅ and *c*₄, and two genes for conserved hypothetical proteins, one of which was located immediately downstream of *dsbA*.

The deduced DsbA protein is highly similar to predicted thiol:disulfide interchange DsbA proteins from other pseudomonads, including *P. fluorescens* Pf0-1 (NCBI accession number ZP_00266803; 91% identity), *P. syringae* pv. *syringae* DC3000 (AA053886; 79% identity), *Pseudomonas putida* KT2440 (NP_742297; 77% identity), *P. fluorescens* Pf-5 (YP_257237; 74% identity) and *P. aeruginosa* PAO1 (U84726; 72% identity). Among these, DsbA from *P. aeruginosa* PAO1 has been shown to have thiol:disulfide oxidoreductase activity in the same range as that of DsbA from *E. coli* (Urban *et al.*, 2001). The consensus DsbA catalytic site, Cys-Pro-His-Cys (residues 56–60), a conserved

PROSITE pattern associated with the thioredoxin family active site (residues 47–65), and a Pfam DsbA-like thioredoxin domain (residues 46–181) also are present in DsbA from *P. fluorescens* Q8r1-96. Results of SignalP-HMM (Bendtsen *et al.*, 2004) predictions revealed a well-conserved signal peptide and a potential cleavage site between residues 22 and 23. In addition, based on analysis of the complete genome sequence of other pseudomonads, *P. fluorescens* Q8r1-96 has one copy of *dsbA*.

Phenotypic effects of the *dsbA* mutation

The *dsbA* gene of strain Q8r1-96 was mutated as described in Methods. The resulting mutant exhibited reduced motility, reduced fluorescence and changes in colony colour. Altered motility was most apparent on 0.3 % agar, on which migration of the mutant was on average 2.2 and 2.0 times less than that of the wild-type after 24 and 48 h, respectively (Table 3). Neither the mutant nor the wild-type exhibited swarming behaviour at higher agar concentrations.

P. fluorescens produces water-soluble siderophores that strongly bind ferric iron under iron-limiting conditions. Siderophore excretion as estimated on CAS agar was significantly reduced in the *dsbA* mutant compared to wild-type Q8r1-96 (Table 3). Differences also were observed in the appearance of colonies of the parental and mutant strains: those of the *dsbA* mutant were less mucoid on PsP agar (data not shown) than those of the wild-type. On LB medium supplemented with glucose, colonies of the *dsbA* mutant were consistently orange-tan in colour whereas colonies of Q8r1-96 were greyish-brownish in dense areas and isolated colonies were beige with a darker centre.

As compared to Q8r1-96, the *dsbA* mutant grew more slowly on D-galactose as a source of carbon and on D-serine as a source of nitrogen when grown in Biolog SF-N2 and PM3 MicroPlates. These differences were validated in studies conducted with appropriately supplemented cultures grown on M9 medium (data not shown).

The *dsbA* mutant was not impaired in the accumulation of extracellular protease (Table 3) or the production of hydrogen cyanide (data not shown).

Effect of the *dsbA* mutation on phloroglucinol production and inhibition of *G. graminis* var. *tritici* *in vitro*

Both wild-type *P. fluorescens* Q8r1-96 and the *dsbA* mutant produced 2,4-DAPG as well as monoacetylphloroglucinol (MAPG) and three other uncharacterized phloroglucinol-related compounds (data not shown). Because MAPG is a precursor for 2,4-DAPG (Bangera & Thomashow, 1999; Shanahan *et al.*, 1992) and the uncharacterized phloroglucinols detected in these analyses may include condensation products of either or both compounds (R. F. Bonsall, O. V. Mavrodi, D. V. Mavrodi & D. M. Weller, unpublished data), we quantified all three classes of phloroglucinol-related compounds (Table 3). The *dsbA* mutant produced approximately 50 % more 2,4-DAPG and total phloroglucinol-related compounds than did wild-type Q8r1-96.

These data correlated with the results of fungal inhibition assays, which showed that the *dsbA* mutant was more inhibitory *in vitro* to *G. graminis* var. *tritici* than was the wild-type. Calculated hyphal inhibition indices for the mutant and the

Table 3. Phenotypic effects of the *dsbA* mutation in *P. fluorescens* Q8r1-96

Test	Bacterial strain	
	Q8r1-96	Q8r1-96 <i>dsbA</i>
Siderophore production*	8.5 a	6.9 b
Exoprotease production†	6.7 a; 9.8 a	6 a; 10 a
Motility‡	20.2 a; 32.3 a; 38.7 a	9.33 b; 16.3 b; 19.3 b
MAPG production§	2.1 × 10 ⁶ a (100 %)	3.0 × 10 ⁶ all (140 %)
2,4-DAPG production§	9.4 × 10 ⁶ a (100 %)	13.9 × 10 ⁶ a (147.5 %)
Total phloroglucinol-related compounds§	12.0 × 10 ⁶ b (100 %)	17.6 × 10 ⁶ a (146.6 %)

*Siderophore production was determined by measuring orange haloes after 2 days growth at 28 °C on CAS agar. Values are means of four replicate plates. Values followed by the same letter are not significantly different according to the two-sample *t* test.

†Zone of casein degradation on milk agar plates in mm, after 48 and 72 h bacterial growth, respectively. Values are means of three replicate plates. Values followed by the same letter are not significantly different according to the two-sample *t* test.

‡Diameter of bacterial spread in mm on 0.3 % LB. Mean values of six replicate plates after 24, 48 and 72 h bacterial growth. Values followed by the same letter are not significantly different according to the two-sample *t* test.

§Peak area/OD. Values in parentheses represent percentages. Values are means of five replicate extractions. Values followed by the same letter are not significantly different according to the two-sample *t* test or the Wilcoxon rank sum test, $\alpha=0.05$.

wild-type strain at 4 days after inoculation were 0.41 and 0.36, respectively. After 6 and 7 days, inhibition indices were 0.22 and 0.13 for the mutant and 0.16 and 0.07 for the parental strain.

Effect of the *dsbA* mutation on rhizosphere colonization by Q8r1-96

Rhizosphere colonization studies were conducted using Q8r1-96*dsbA*, which is kanamycin-resistant, and Q8r1-96Gm, a gentamicin-resistant derivative tagged with mini-Tn7-*gfp2* (Validov *et al.*, 2005). This allowed the wild-type and mutant strains to be distinguished in mixed inoculation experiments. The growth kinetics of the wild-type and mini-Tn7-*gfp2* tagged strains did not differ in 1/3 × KMB and MMP minimal media, and the growth rates of Q8r1-96Gm and the *dsbA* mutant also were indistinguishable in LB medium and minimal M9 medium supplemented with glycerol (data not shown).

The rhizosphere competence of the *dsbA* mutant was compared to that of Q8r1-96Gm in single and mixed (1:1 ratio) inoculation studies on roots of wheat grown in a natural Quincy virgin soil. Fig. 1 shows the population dynamics for the two strains in the rhizosphere during six 2 week growth cycles. The population densities established in the soil were similar for both strains at the beginning of each experiment (cycle 0). In both the individual and the mixed inoculations, population sizes of both strains had increased by four orders of magnitude after one cycle and then the densities slowly declined over the next five cycles.

When the strains were introduced separately, the population dynamics of Q8r1-96Gm and the *dsbA* mutant did not differ consistently in the wheat rhizosphere. In experiment 1, the population sizes of the two strains were comparable through cycle 4, but in cycles 5 and 6 the population size of Q8r1-96Gm was significantly ($P=0.05$) greater than that of the mutant (Fig. 1a). However, in experiment 2, the population sizes of the two strains did not differ in cycles 1, 2, 4 and 6; the population size of Q8r1-96Gm was greater than that of the mutant in cycle 5; and the population size of the mutant was greater than that of the wild-type strain in cycle 3 (Fig. 1b).

When introduced together into the soil, the *dsbA* mutant consistently colonized the wheat rhizosphere to a lesser extent than did Q8r1-96Gm. In experiment 1, the population sizes of both strains were similar throughout cycles 1, 2 and 3, but the population size of Q8r1-96Gm was significantly greater than that of the mutant in cycles 4, 5 and 6 (Fig. 1a). These differences were consistent and occurred to a greater extent in experiment 2, in which the population size of the mutant was significantly less than that of the wild-type in all cycles (Fig. 1b).

Population densities of total culturable aerobic bacteria in the wheat rhizosphere in all four bacterial treatments and in

Fig. 1. Population dynamics of *P. fluorescens* Q8r1-96Gm and its *dsbA* mutant on the roots of wheat (cv. Penawawa) grown in Quincy virgin soil for six consecutive 2 week cycles. (a, b) Results of two independent experiments. (c) Results of a similar experiment with Q8r1-96Gm(pME6010) and the complemented mutant Q8r1-96*dsbA*(pME6010*dsbA*). Each strain was introduced into the soil to give a final density of approximately $\log 4$ c.f.u. (g soil) $^{-1}$ (cycle 0) in single inoculations and $\sim 0.5 \times 10^4$ c.f.u. (g soil) $^{-1}$ of each strain in mixed inoculations as described in Methods. Mean values and standard deviations are presented. Means followed by the same letter are not significantly different ($P=0.05$) according to Fisher's protected least significant difference test or the Kruskal-Wallis test ($P=0.05$) (for cycles marked by an asterisk).

the control were above $\log 8.6 \text{ c.f.u. (g root)}^{-1}$ in all six cycles, and population sizes did not differ (data not shown).

In order to assess the survival of the mutant in the absence of roots, soils after the final cycle of each colonization experiment were stored at 20°C for 10 weeks, and then wheat was sown again. The mean population densities for two consecutive cycles are presented in Table 4. Densities of the *dsbA* mutant were significantly less than those of the wild-type in the rhizosphere of wheat sown in soil that had either single or mixed bacterial inoculations, suggesting that the mutant did not survive as well as the wild-type.

We complemented the Q8r1-96*dsbA* mutant with a wild-type copy of the gene in the low-copy-number plasmid pME6010, which is stably maintained in *P. fluorescens* CHAO in the absence of selection in the sugarbeet rhizosphere (Heeb *et al.*, 2000). Our own experiments indicated that pME6010 is retained in Q8r1-96Gm colonizing the rhizosphere of wheat for at least 4 months (data not shown). Results of the rhizosphere colonization assays (Fig. 1c) and subsequent statistical analysis of mean colonization values after six cycles (data not shown) indicated that introduction of functional gene copies *in trans* indeed complemented the mutation in *dsbA*.

DISCUSSION

Successful root-colonizing rhizobacteria must establish and maintain themselves in the presence of a large, metabolically active microbial population supported by metabolites provided by the root. Considering the complexity of these interactions, it is not surprising that diverse bacterial traits have been implicated in root colonization by PGPR of different genera and species. These traits range from motility (de Weger *et al.*, 1987), chemotaxis (Lugtenberg *et al.*, 2001) and the presence of cell surface polysaccharides (Dekkers *et al.*, 1998a), which may facilitate interactions with root surfaces,

to prototrophy (Simons *et al.*, 1997; de Weert *et al.*, 2002), required for growth in the absence of preformed cellular building blocks; and the ability to sequester resources such as iron and oxygen (Camacho Carvajal, 2000), which may provide a competitive advantage over other rhizosphere micro-organisms. More recently, evidence of type III secretion genes in many *Pseudomonas* strains (Preston *et al.*, 2001; Rainey, 1999; Mazurier *et al.*, 2004; Rezzonico *et al.*, 2004, 2005) and the preferential colonization of wheat and pea by different strains of DAPG-producing *P. fluorescens* (Landa *et al.*, 2002b) led us to speculate that some PGPR may participate in more specialized interactions with the host, and that genes involved in these interactions may be among those broadly implicated in pathogen–host interactions. One such gene is *dsbA*, which influences interactions between *P. aeruginosa* and human, animal and plant hosts (Rahme *et al.*, 1997, 2000) and contributes to pathogenicity in *P. syringae* pv. *tomato* and *Erwinia chrysanthemi* (Kloek *et al.*, 2000; Shevchik *et al.*, 1995). DsbA catalyses disulfide bond formation in the periplasm of Gram-negative bacteria, and *dsbA* mutants exhibit a pleiotropic phenotype because the correct folding of many proteins is affected (Collet & Bardwell, 2002). Among the effects of *dsbA* mutations are deficiencies in pathogenicity and competitiveness associated with loss of motility and the inability to produce fimbriae and secrete exoproducts (Kloek *et al.*, 2000; Shevchik *et al.*, 1995; Dailey & Berg, 1993). The fact that some of these traits are also associated with the ability of saprophytic pseudomonads to colonize and persist in the plant rhizosphere prompted us to investigate the role of *dsbA* in root colonization.

We used a *dsbA* probe from *P. fluorescens* SBW25 to identify *dsbA*-containing clones in a Q8r1-96 gene library. DNA sequence analysis revealed that the putative *dsbA* gene from Q8r1-96 encodes a member of the thioredoxin superfamily containing the conserved dithiol-active site consensus motif C-P-H-C. The enzyme has a molecular mass similar to that

Table 4. Population densities of introduced wild-type and mutant strains in the rhizosphere of wheat sown in soil after 10 weeks storage

The survival of the strains was determined as the mean population density on roots of wheat plants after two cycles of growth following a 10 week fallow period as described in Methods. Mean population densities in $\log \text{ c.f.u. per g root}$ (fresh weight) across two cycles except cycle 0 are presented; ND, none detected. Different lower-case letters indicate a statistically significant difference according to Kruskal–Wallis all-pairwise comparisons test ($P=0.05$). Numbers in parentheses represent population density in $\log \text{ c.f.u. per g root}$ (fresh weight) after cycle 6 (before storing soil for 10 weeks).

Expt	Strain	Mean population density		
		Q8r1-96Gm	Mutant	Q8r1-96Gm and mutant at 1:1
1	Q8r1-96Gm	6.0 a (6.7)	ND	5.4 ab (6.0)
	Q8r1-96 <i>dsbA</i>	ND	5.0 bc (5.7)	4.3 c (5.5)
2	Q8r1-96Gm	6.5 a (5.9)	ND	6.3 a (6.0)
	Q8r1-96 <i>dsbA</i>	ND	5.2 b (5.9)	4.0 c (3.4)

of DsbA from other bacteria (approx. 20 kDa), has a well-conserved leader peptide for secretion via the Sec pathway, and is highly conserved (>70% identity at the amino acid level) relative to DsbA from *P. syringae*, *P. putida*, *P. fluorescens* and *P. aeruginosa*. Taken collectively, these results strongly suggest that the gene identified in strain Q8r1-96 is a true orthologue of *dsbA* from *E. coli*.

The inactivation of *dsbA* in *P. fluorescens* Q8r1-96 resulted in a phenotype similar to that described for *dsbA* mutants in other bacterial species. Like *dsbA* mutants of *E. coli* (Dailey & Berg, 1993) and *P. syringae* pv. *tomato* (Kloek *et al.*, 2000), the *dsbA* mutant of Q8r1-96 exhibited reduced motility and did not swarm. This is probably due to defective disulfide bond formation in FlgI, a component of the flagellar motor (Collet & Bardwell, 2002; Dailey & Berg, 1993). Like the *dsbA* mutant of *P. syringae* pv. *tomato* (Kloek *et al.*, 2000), that of Q8r1-96 also was less fluorescent than the wild-type on siderophore-inducing PsF agar (Table 3). In contrast to the *dsbA* mutant of *P. aeruginosa*, which produced reduced clearing zones on skim milk agar (Malhotra *et al.*, 2000), the Q8r1-96 mutant was unimpaired in the accumulation of extracellular protease and hydrogen cyanide. The latter indicates that the GacA/GacS regulatory circuitry, which coordinately regulates the production of secondary metabolites and exoprotease (Whistler *et al.*, 1998), has not been disturbed in the *dsbA* mutant. However, the Q8r1-96 mutant also produced elevated amounts of 2,4-DAPG and total phloroglucinol-related compounds and, as a result, was more inhibitory *in vitro* to *G. graminis* var. *tritici* than the wild-type. These and other changes, including those affecting colony morphology and nutrient utilization, presumably resulted from inactivation or functional alterations in periplasmic enzymes involved in secretion, nutrient uptake and the synthesis of cell envelope constituents. Whether the pleiotropic phenotype of the *dsbA* mutation impacts on the ability of strain Q8r1-96 to function as a biological control agent in natural soil currently is under investigation.

We evaluated the contribution of *dsbA* to the unique root-colonizing ability of strain Q8r1-96Gm over several months in a natural field soil to which bacteria were added only once, after which a succession of wheat crops was grown. This approach allows for extended studies under controlled conditions in the presence of indigenous microbial populations. *P. fluorescens* Q8r1-96Gm and the *dsbA* mutant were introduced into the soil either alone or in combination. When introduced separately, both strains colonized the rhizosphere similarly, whereas when the two were mixed, the mutant colonized the rhizosphere significantly less. We subscribe to the definition of root colonization proposed by Weller & Thomashow (1994): 'the process whereby rhizobacteria introduced on seeds, vegetatively propagated plant parts or into the soil become distributed along roots growing in raw soil, multiply, and then survive for several weeks in the presence of indigenous soil microflora.' Based on this definition and our findings, we conclude that DsbA does not significantly contribute to the unique root-colonizing ability

of Q8r1-96 even though the mutant was less fit in co-inoculation studies. Indeed, it is not surprising that the mutant was less competitive in the presence of the wild-type because the wild-type is highly adapted to the root environment and almost any change would likely be disadvantageous. Apparently, however, the magnitude of the loss was not sufficient to have an impact on the mutant's ability to compete with the indigenous microflora.

Efforts over the past 25 years to establish the importance of chemotaxis and motility in root colonization have provided conflicting results. Motility has been found to contribute to the ability of bacteria to attach or become distributed over the root surface (de Weger *et al.*, 1987; Catlow *et al.*, 1990; Turnbull *et al.*, 2001), particularly when studies are conducted in the absence of percolating water. On the other hand, the importance of active motility in soil has been questioned because flagella-mediated movement requires the presence of water films that become too thin to support motility when soil is drier than -50 kPa (Griffin & Quail, 1968). Passive movement of bacteria on elongating roots was described by Howie *et al.* (1987), but downward percolation of water was shown to be more important for bacterial dispersal through the rhizosphere (Bahme & Schroth, 1987). Our finding that the *dsbA* mutant in this study was not impaired in root colonization may be due to our experimental conditions, which included regular watering of the plants. The water flow presumably contributed to the distribution of the bacteria along growing wheat roots, thus neutralizing the absence of motility.

Considering the range of exported proteins known to be acted upon by DsbA, and because a *dsbA* mutation typically causes the same phenotype as a mutation in the gene encoding the protein acted upon, our results indicate that complex surface structures including flagella and fimbriae, previously studied in relation to root colonization, as well as the type III secretory apparatus, the importance of which is yet to be directly assessed, are unlikely to contribute significantly to the exceptional rhizosphere competence of *P. fluorescens* Q8r1-96. More generally, the relatively minor impact of the *dsbA* mutation on root colonization by Q8r1-96 suggests that the unique host affinity of this strain does not depend on certain of the highly conserved virulence-related factors utilized by bacterial pathogens in their interactions with evolutionarily divergent hosts. Nonetheless, strain Q8r1-96 must, like pathogens, interact with and exploit nutrient resources provided by its host, and it remains to be determined whether the functional similarities associated with these processes will share a common underlying genetic basis.

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