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

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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.

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., 1998b; 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/DDBJ accession number for the dsbA sequence of P. fluorescens Q8r1-96 reported in this paper is AY171618.
Fluorescent Pseudomonas spp. that produce the polyketide antibiotic 2,4-diacytethylphlorogluconol (2,4-DAPG) are highly effective biocontrol agents of soilborne pathogens (Duffy & Défago, 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 of P. fluorescens Q8r1-96 showed a colonizing ability that is superior to that of E. coli S17-1(λ-pir) (Raaijmakers et al., 2001). In this study we identified and characterized the dba ortholog in P. fluorescens Q8r1-96 and evaluated its role in root colonization and the competitiveness of this strain in the wheat rhizosphere. We showed that dba does not contribute to the exceptional rhizosphere competence of Q8r1-96, and that root colonization by a dba 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-Tn7-2gp2 (Validov et al., 2005) was used in competitive colonization experiments. Escherichia coli TOP10 (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 cultivable 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 designed with Oligo 6.65 Software (Molecular Biology Insights).

**Identification of dba 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 dba-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 dba 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 2 min, and a final extension at 68 °C for 5 min. To localize dba within positive clones, cosmid DNA digested with EcoRI, KpnI and SacI was blotted and hybridized with a biotinylated dba probe.

**Transposon mutagenesis, shotgun sequencing and sequence analysis.** To tag dba 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 dba 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 dba gene containing EZ::TN7< Kan-2 > was amplified with DSBA_UP and DSBA_LOW primers using KOD Hot Start DNA polymerase. The cycling programme included a 2 min denaturation at 94 °C.
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics*</th>
<th>Reference of origin</th>
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</thead>
<tbody>
<tr>
<td><strong>P. fluorescens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q8r1-96</td>
<td>DAPG+ Rif&quot;</td>
<td>Landa et al. (2002)</td>
</tr>
<tr>
<td>Q8r1-96Gm</td>
<td>Q8r1-96 tagged with mini-Tn7-gfp2; DAPG+ Rif&quot; Gm&quot;</td>
<td>Validov et al. (2005)</td>
</tr>
<tr>
<td>Q8r1-96dsbA</td>
<td>dsbA::TN &lt;Kan2&gt;; DAPG+ Rif&quot; Kan&quot;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1(λ-pir)</td>
<td>thi pro hsdM recA rpsL RP4-2 (Tet&quot;::Mu) (Kan&quot;::Tn7)</td>
<td>Lab collection</td>
</tr>
<tr>
<td>TOP10</td>
<td>F&quot; mcrA Δ(mrr-hsdRMS-mcrBC) #801aΔΔM15 ΔlacX74 recA1 araΔ139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCPP47</td>
<td>Broad-host-range cosmid derived from pCPP34, tandem cos+, par+, Tet&quot;</td>
<td>Bauer &amp; Collmer (1997)</td>
</tr>
<tr>
<td>pMOB3</td>
<td>Kan&quot; Cam&quot;, oriT sacB</td>
<td>Schweizer (1992)</td>
</tr>
<tr>
<td>pNOT19</td>
<td>ColE1 oriV, Amp&quot;; accessory plasmid</td>
<td>Schweizer (1992)</td>
</tr>
<tr>
<td>pNOT19-dsbA-Kan</td>
<td>pNOT19 containing the 2-2 kb DNA SmaI fragment with sss interrupted by EZ::TN &lt;Kan2&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pNOT19-dsbA-Kan-MOB</td>
<td>pNOT19-dsbA-Kan ligated with 5-8 kb Nof fragment from pMOB3</td>
<td>This study</td>
</tr>
<tr>
<td>pME6010</td>
<td>Broad-host-range plasmid; pVS1 oriV, p15a oriV, Pγ, Tet&quot;</td>
<td>Heeb et al. (2000)</td>
</tr>
<tr>
<td>pME6010-ccdB</td>
<td>Gateway destination vector derived from pME6010 with ccdB-Cam&quot; cassette flanked by attR1 and attR2</td>
<td>This study</td>
</tr>
<tr>
<td>pMK2010</td>
<td>Gateway entry vector; ColE1 oriV, oriT&lt;par&gt;, Kan&quot;, ccdB-Cam&quot; cassette flanked by attP1 and attP2</td>
<td>House et al. (2004)</td>
</tr>
<tr>
<td>pMK2010dsbA</td>
<td>pMK2010 containing the 0-67 kb DNA fragment with dsbA</td>
<td>This study</td>
</tr>
<tr>
<td>pME6010dsbA</td>
<td>pME6010 containing the 0-67 kb DNA fragment with dsbA</td>
<td>This study</td>
</tr>
</tbody>
</table>

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

Table 2. Oligonucleotides

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

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 SmaI site of pMOB19 (Schweizer, 1992) and the plasmid was then digested with Nof and ligated with a 5 kb Nof fragment of the pMOB3 cassette (Schweizer, 1992) containing sacB and cat genes. The resultant plNOT19-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". 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 AMP1, and 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...
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 Q8r1-96dsbA 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 cyani de detection paper placed on Petri dish lids (Bakker & Schippers, 1987). Observations were made every 24 h and all cultures were screened twice. Poly saccharide 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 PsP 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 PsP 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 replicates were performed.

**Rhizosphere colonization assays.** Bacterial inocula of Q8r1-96dsbA and Q8r1-96Gm, a gentamicin-resistant derivative of the parental strain (Validov et al., 2005), were used to grow in grass. 1 × 10^8 c.f.u. (g soil)^−1 when strains were introduced alone or ~0.5 × 10^8 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 OD_600 > 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. Differ- ences 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 cosmid 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_6, 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 P0–1 (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 PA01 (U84726; 72% identity). Among these, DsbA from P. aeruginosa PA01 has been shown to have thiol:disulfide oxido reductase 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
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).

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

<table>
<thead>
<tr>
<th>Test</th>
<th>Bacterial strain</th>
<th>Q8r1-96</th>
<th>Q8r1-96dsbA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siderophore production*</td>
<td></td>
<td>8.5 a</td>
<td>6.9 b</td>
</tr>
<tr>
<td>Exoprotease production†</td>
<td></td>
<td>6.7 a; 9.8 a</td>
<td>6 a; 10 a</td>
</tr>
<tr>
<td>Motility‡</td>
<td></td>
<td>20-2 a; 32.3 a; 38.7 a</td>
<td>9-33 b; 16.3 b; 19.3 b</td>
</tr>
<tr>
<td>MAPG production§</td>
<td></td>
<td>2.1 x 10⁶ a (100%)</td>
<td>3.0 x 10⁶ a (140%)</td>
</tr>
<tr>
<td>2,4-DAPG production§</td>
<td></td>
<td>9.4 x 10⁶ a (100%)</td>
<td>13.9 x 10⁶ a (147.5%)</td>
</tr>
<tr>
<td>Total phloroglucinol-related compounds §</td>
<td></td>
<td>12.0 x 10⁶ b (100%)</td>
<td>17.6 x 10⁶ a (146.6%)</td>
</tr>
</tbody>
</table>

* 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, z = 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 Q8r1-96dsbA, 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

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**Fig. 1.** Population dynamics of *P. fluorescencs* 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 Q8r1-96dsbA(pME6010dsbA). Each strain was introduced into the soil to give a final density of approximately 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 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 Q8r1-96dsbA 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 exoproteins (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

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**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 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 c.f.u. per g root (fresh weight) after cycle 6 (before storing soil for 10 weeks).

<table>
<thead>
<tr>
<th>Expt</th>
<th>Strain</th>
<th>Mean population density</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Q8r1-96Gm</td>
<td>6.0 a (6-7)</td>
<td>ND</td>
<td>5.4 ab (6-0)</td>
</tr>
<tr>
<td>2</td>
<td>Q8r1-96Gm</td>
<td>6.5 a (5-9)</td>
<td>ND</td>
<td>6.3 a (6-0)</td>
</tr>
<tr>
<td></td>
<td>Q8r1-96Gm</td>
<td>5.7 a (5-7)</td>
<td>5.2 b (5-9)</td>
<td>4.0 c (3-4)</td>
</tr>
</tbody>
</table>
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 FliG, 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 Q8r1-96 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.

ACKNOWLEDGEMENTS

This work was supported by the US Department of Agriculture, National Research Initiative, Competitive Grants Program (grants 2003-35319-13800 and 2003-35107-13777). We thank K. Hansen, M. Young Son and J. Mitchell for technical assistance.
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