Alkyl hydroperoxide reductase has a role in oxidative stress resistance and in modulating changes in cell-surface properties in *Azospirillum brasilense* Sp245

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An *ahpC* mutant derivative of *Azospirillum brasilense* Sp245 (strain SK586) that encodes an alkyl hydroperoxide reductase was found to be more sensitive to oxidative stress caused by organic hydroperoxides compared with the wild-type. In addition, the *ahpC* mutant strain had multiple defects in a large array of cellular functions that were consistent with alteration of cell-surface properties, such as cell morphology in stationary phase, Calcofluor White-, Congo Red- and lectin-binding abilities, as well as cell-to-cell aggregation and flocculation. All phenotypes of the *ahpC* mutant were complemented by *in trans* expression of AhpC, and overexpression of AhpC in the wild-type strain was found to affect the same set of phenotypes, suggesting that the pleiotropic effects were caused by the *ahpC* mutation. SK586 was also found to be fully motile, but it lost motility at a higher rate than the wild-type during growth, such that most SK586 cells were non-motile in stationary phase. Despite these defects, the mutant did not differ from the wild-type in short-term colonization of sterile wheat roots when inoculated alone, and in competition with the wild-type strain; this implied that AhpC activity may not endow the cells with a competitive advantage in colonization under these conditions. Although the exact function of AhpC in affecting these phenotypes remains to be determined, changes in cell morphology, surface properties, cell-to-cell aggregation and flocculation are common adaptive responses to various stresses in bacteria, and the data obtained here suggest that AhpC contributes to modulating such stress responses in *A. brasilense*.

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INTRODUCTION

The Gram-negative nitrogen-fixing α -proteobacterium *Azospirillum brasilense* lives in association with the roots of many agriculturally important crops. Upon inoculation of cereals, the bacteria significantly promote plant growth and crop yields (Steenhoudt & Vanderleyden, 2000). The beneficial effect on plant growth has been attributed to the production of phytohormones by the associated bacteria, rather than nitrogen fixation (Steenhoudt & Vanderleyden, 2000). Although much has been learnt about the beneficial effects of inoculated azospirilla on host plants, little is known about the molecular mechanisms underlying the successful establishment of *A. brasilense* in the rhizosphere, probably because of the lack of a distinctive plant phenotype. Several studies have

shown that when inoculated onto sterile wheat roots, azospirilla colonize the root surfaces extensively, as well as the sites of lateral root emergence (Vande Broek *et al.*, 1993; Katupitiya *et al.*, 1995). The attachment of the bacteria to the root surface precedes colonization, and it is proposed to be a two-step process. In the first step, which is dependent on the presence of the polar flagellum, motile *Azospirillum* cells loosely attach to the root surface; in the second step, extracellular polysaccharides (EPS) anchor the cells firmly to the root surface (Michiels *et al.*, 1991). The exact nature of the EPS involved in the anchoring of the bacteria to the roots, and whether other cellular structures are required for the attachment and anchoring, remain to be determined.

Cell aggregation and flocculation in *A. brasilense* are observed under nutritionally stressful conditions, such as an excess of reducing equivalents (high C/N ratios) and prolonged stationary phase, suggesting that this behaviour may represent an adaptive response to such stresses. Recently, Bahat-Samet *et al.* (2004) have shown that EPS

Abbreviations: EPS, extracellular polysaccharide; ROS, reactive oxygen species; TEM, transmission electron microscopy.

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production and flocculation reach a maximum in the stationary phase of growth. Concomitantly, cells change their shape from vibroid and motile to round and non-motile (Sadasivan & Neyra, 1985; Burdman *et al.*, 1998; Pereg-Gerk *et al.*, 1998). Consistent with the hypothesis that multiple signals must be integrated for flocculation to take place, a functional chemotaxis-like signal transduction pathway, Che1, is also required for flocculation in *A. brasilense* strain Sp7 (Bible *et al.*, 2008). Although the exact nature of the signal(s) that can induce cell aggregation and flocculation in *Azospirillum* spp. have not been identified, various nutritional and environmental stresses affect the ability of cells to aggregate and flocculate (Sadasivan & Neyra, 1985; Burdman *et al.*, 1998, 2000; Chowdhury *et al.*, 2006).

The production of EPS has been reported to be essential for cell aggregation and flocculation in *Azospirillum* spp. (Del Gallo *et al.*, 1989; Burdman *et al.*, 1998, 2000). The production of EPS by *Azospirillum* spp. can be observed directly on colonies growing on media supplemented with dyes, such as Congo Red or Calcofluor White, that specifically stain β -linked glucans. Mutants of *Azospirillum* that are impaired in the ability to flocculate also lose the ability to bind the dyes Congo Red and Calcofluor White (Sadasivan & Neyra, 1985; Katupitiya *et al.*, 1995).

In this study, we demonstrate that inactivation of the gene encoding AhpC results in an increased sensitivity to oxidative stress, and in an impaired ability of cells to aggregate and flocculate under nutrient-limiting conditions, but it does not affect wheat root colonization. We also show that an *A. brasilense* mutant lacking *ahpC* (strain SK586) displays pleiotropic phenotypic changes that may be related to alteration in cell-surface properties. Taken together, the data imply that the increased oxidative stress sensitivity, brought about by inactivating *ahpC*, may contribute to modulating several cellular changes, including some changes implicated in inducing cell aggregation and flocculation.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this work were *A. brasilense* strain Sp245 (Baldani *et al.*, 1983) and its mutant derivative, strain SK586 (Scheludko *et al.*, 1998). SK586 was produced by an Omegon-Km insertion, and isolated as a non-motile mutant on semi-soft agar plates (Scheludko *et al.*, 1998). *A. brasilense* cells were grown on minimal MMAB medium (Bible *et al.*, 2008), in rich nutrient broth (NB; Difco) or in tryptone-yeast extract (TY) medium (5 g tryptone l⁻¹, 3 g yeast extract l⁻¹). For growth in low-carbon MMAB medium, the concentration of malate was 0.05 % (w/v). *Escherichia coli* was grown in Luria-Bertani medium. Kanamycin was added to the medium at a concentration of 25 μ g ml⁻¹ for growth of strain SK586. Tetracycline was used at 10 μ g ml⁻¹. Congo Red was added to MMAB plates at a final concentration of 40 μ g ml⁻¹, and Calcofluor White was added at a final concentration of 100 μ g ml⁻¹. Calcofluor White staining of colonies was observed under UV (360 nm) illumination. For observation of cell aggregation and flocculation, cells were grown in TY liquid medium, washed twice in PBS, and then inoculated into a

minimal medium containing 8 mM fructose and 0.5 mM NaNO₃, as previously described (Bible *et al.*, 2008).

Growth curve and survival in stationary phase. The growth and doubling times of the wild-type strain and the mutant were compared by inoculating overnight cultures, adjusted to OD₆₀₀ 0.05, into 100 ml liquid medium. Cultures were incubated at 28 °C with shaking (200 r.p.m.), and growth was monitored by measuring OD₆₀₀, and by plating serial dilutions onto NB agar (solidified with 1.5 % agar). The fraction of cells that remained viable in stationary phase was expressed relative to the maximal number of cells upon entry into stationary phase, as determined by plate counts. Cell morphologies were observed using phase contrast on a Nikon E800 microscope, and by transmission electron microscopy (TEM) on a LEO 906e transmission electron microscope (80 kV). For TEM, cells were grown to stationary phase, washed twice in sterile deionized water, spotted on Formvar-coated nickel grids, and negatively stained with 2 % uranyl acetate or 0.75 % uranyl formate. The TEM images were taken randomly from within a particular grid.

Lectin-binding assay. Pellets from 2 ml stationary-phase cultures in MMAB or NB liquid medium were washed twice in sterile PBS buffer, and resuspended in 200 μ l sterile PBS buffer. A 2 μ l volume of the FITC-labelled lectin to be tested, at a concentration of 10 mg ml⁻¹, was added to the suspension, and incubated in the dark for 20 min. The pellets were washed with sterile PBS, and resuspended in 50 μ l sterile PBS. Aliquots were placed on a 1 % agarose pad, observed with a fluorescent microscope equipped with differential interference contrast (Nikon 80i), and photographed.

DNA manipulations, sequencing and analysis. Subcloning, competent cell preparation, transformation and DNA extraction were carried out according to standard methods (Sambrook *et al.*, 1989). Enzymes were from New England Biolabs and Roche Applied Sciences, and they were used according to the manufacturers' recommendations. The Omegon-Km cassette inserted into strain SK586 possesses efficient transcription and translation terminators of the Omegon interposon at each end, and also carries an *E. coli*-specific origin of replication, which facilitates cloning of flanking DNA fragments into *E. coli* (Fellay *et al.*, 1989). A single Omegon-Km insertion on the chromosome of SK586 has been identified (Scheludko *et al.*, 1998). The DNA sequence flanking the insertion of the Omegon-Km cassette in *A. brasilense* SK586 was determined by inverse PCR, using primers complementary to the 5' and 3' sequences of the Omegon-Km cassette, respectively (omegon-U, 5'-GACAAGATCACCTTCGTCGG-3'; omegon-R, 5'-GGGCAGCAGAGTGTCTT-3'). DNA templates for inverse PCR were generated by digestion with *Xho*I, and by partial restriction of the genomic DNA of strain SK586 (obtained using the Wizard genomic DNA purification kit; Promega) with *Eco*RI or *Bam*HI, followed by self-ligation using T4 DNA ligase (New England Biolabs). The circular DNA generated was used to transform *E. coli* DH5 α competent cells for propagation and primer-walking reactions. The DNA from several clones was isolated in order to obtain various lengths of the flanking DNA region appropriate for DNA sequencing. The Expand High Fidelity PCR system (Roche Applied Sciences) was used according to the manufacturer's instructions. The complete sequence of the DNA region was determined by primer walking on templates generated as described above. All clones yielded similar nucleotide sequences, consistent with a single Omegon-Km insertion, as shown previously (Scheludko *et al.*, 1998). Oligonucleotide primers were synthesized by Sigma Genosys. DNA sequencing was carried out using an ABI prism (MWG Biotech). Computational gene finding was performed using FramePlot 2.3.2 (Ishikawa & Hotta, 1999). Similarity searches were performed by using the BLASTP program (Altschul *et al.*, 1997).

Functional complementation. A 1031 bp region of the wild-type strain DNA, containing upstream and downstream regions flanking

the wild-type *ahpC* gene, and including putative regulatory regions, was PCR amplified using the primers pairs: *ahpC*-*XhoI*-F (5'-CCGCTCGAGCATGCACTGCACCAATAATC-3'; the engineered *XhoI* site is underlined) and *ahpC*-*BamHI*-R (5'-GCGGGATCCGAGAGGGCTCCCGAAAGTG-3'; the engineered *BamHI* site is underlined). The PCR fragment was verified by sequencing, and then digested with *BamHI* and *XhoI*, and cloned into similar restriction sites of the broad-host-range low-copy plasmid pRK415 (Keen *et al.*, 1988), yielding pRKAhpC. For functional complementation, the pRK415 and pRKAhpC plasmids were transferred to *A. brasilense* by triparental mating, as previously described (Bible *et al.*, 2008).

Motility. Cell motility was observed in liquid nutrient broth or MMAB, using a compound darkfield microscope (Hobson). The percentage of motile cells in the population was determined by using the Hobson Bactracker, and following the instructions of the manufacturer. Swarm plates, prepared as described previously (Bible *et al.*, 2008), were used to assess motility and chemotaxis.

Resistance to oxidative stress agents in cultures. Aliquots of overnight cultures grown in MMAB and NB liquid media, supplemented with the appropriate antibiotics, were adjusted to an equivalent number of cells (estimated by measuring OD₆₀₀, and by plating serial dilutions onto nutrient agar plates), and about 10⁷ cells ml⁻¹ were inoculated into 5 ml MMAB or NB liquid medium, with 0.005 % menadione, 0.005 % cumene hydroperoxide or 0.001 % hydrogen peroxide, and incubated at 28 °C, with shaking, to late-exponential phase (about 20 h from the time of inoculation). A control culture for each strain was also prepared, and incubated under similar conditions. The viability of the cells after exposure was determined as the number of c.f.u. ml⁻¹ on NB agar supplemented with antibiotics.

Preparation of wheat seed and plant root colonization assays.

Wheat seeds (*Triticum aestivum* cv. Jaegger) were provided by Robert L. Bowden (US Department of Agriculture, Agricultural Research Service, Manhattan, Kansas, USA). Seed surface sterilization and germination, as well as seedling inoculations, were performed essentially as described previously, with minor modifications (Greer-Phillips *et al.*, 2004). *A. brasilense* strains (Sp245 and SK586) were harvested at mid-exponential-growth phase (OD₆₀₀ 0.9–1.0), washed three times in sterile 0.8 % KCl, and adjusted to a concentration of 10⁸ cells ml⁻¹. The density of the cell suspensions was verified by serial dilution and plating onto MMAB agar (supplemented with kanamycin for strain SK586). For each strain, 10⁷ cells were added to 26 mm × 150 mm glass tubes containing 15 ml molten Farhaeus semi-soft agar (4 %, w/v, agar). After the agar had solidified, one sterile germinated seedling was aseptically transferred into each tube. The seedlings were grown with a photoperiod of 16 h light and 8 h dark, and temperatures of 24 °C (light) and 18 °C (dark), in a plant growth chamber (Labline). Ten days after inoculation, the seedlings were washed briefly in sterile 0.8 % KCl to remove excess agar adhering to the roots, blotted on sterile Whatman 3MM filter paper, and weighed. Equal-sized roots from five plants were crushed in 30 ml sterile 0.8 % KCl buffer, using a Waring blender. In order to count colonies of the mutant, serial dilutions were plated on MMAB agar (supplemented with 25 µg kanamycin ml⁻¹), and incubated for 4 days at 28 °C. For competition experiments, washed cultures of Sp245 and SK586, prepared as described above, were inoculated onto sterile wheat seeds in a 1:1 ratio under conditions similar to those used for the single inoculations. The final concentration of each strain in mixed inoculation was about 10⁷ cells ml⁻¹, as determined by plating aliquots of the mixed inoculum on MMAB agar (supplemented with kanamycin for the mutant).

Statistical analysis of data. A *t* test, assuming unequal variances, and with a 0.05 confidence level, was performed to compare root colonization by the wild-type *A. brasilense* strain Sp245 and its SK586 mutant derivative. All experiments were performed in triplicate. All experiments were very reproducible; therefore, the data obtained from only one of the replicates are presented for each experiment.

RESULTS

Identification of the alkyl hydroperoxide reductase subunit C (*ahpC*) gene

The SK586 mutant strain was initially isolated in a screen for non-motile derivatives of *A. brasilense* strain Sp245 generated using an artificial transposon, Omegon-Km (Scheludko *et al.*, 1998). A single Omegon-Km insertion was shown to be located on the chromosome; however, the gene(s) affected in the SK586 strain was not further characterized.

Direct sequencing of the DNA region flanking the Omegon-Km cassette in SK586 revealed that the cassette was inserted at position 470 from the start codon of the gene encoding a homologue of AhpC. *ahpC* encodes an alkyl hydroperoxide reductase, which belongs to a family of antioxidants called the AhpC/TSA (thiol-specific antioxidant) protein family. AhpC converts harmful alkyl hydroperoxides to their corresponding alcohols (Poole, 1996; Poole & Ellis, 1996). Two conserved cysteine residues (Cys46 and Cys165 in the *Salmonella typhimurium* AhpC homologue) involved in forming an active-site disulfide bond are present in the AhpC of *A. brasilense*. Predicted amino acid sequences of the full-length AhpC of *A. brasilense* Sp245 showed 87 % sequence identity to homologous proteins from the closely related α -proteobacterium *Magnetospirillum gryphiswaldense* MSR-1 (gi 144898097).

In order to rule out the possibility that the motility defect in SK586 was due to polar effects on downstream and/or upstream flagellar or motility gene(s), the DNA region was further sequenced in both directions (Fig. 1). This analysis revealed no flagellar gene in this region. A gene encoding a homologue of AhpF was found 230 bp downstream of the *ahpC* gene, and is transcribed in the same direction as *ahpC*. The gene encoding an OxyR homologue was found upstream of *ahpC*, and is transcribed in the opposite direction. A partial ORF, encoding a product with significant similarity to

Fig. 1. Genetic organization of the *ahpC* region in *A. brasilense* Sp245. The genomic region comprises the following genes: *oxyR* (956 bp), *ahpC* (563 bp), *ahpF* (1583 bp) and an incomplete ORF. The black arrowhead represents the insertion of the Omegon-Km cassette in the *ahpC* gene in the SK586 mutant.

oxidoreductases, was identified downstream of *ahpF*, and is transcribed in the opposite direction.

Mutation in *ahpC* results in higher sensitivity to alkyl hydroperoxides

To characterize the SK586 strain, we compared the sensitivity of the wild-type and SK586 strains to organic peroxides, and also tested their sensitivity to hydrogen peroxide, as another oxidative stress agent. We initially used the disk inhibition assay to compare the sensitivity of *A. brasilense* Sp245 and SK586 strains (data not shown) to alkyl peroxides, and confirmed these results by testing the sensitivity of actively growing populations grown in minimal and rich media (Fig. 2a and b). We found that the wild-type strain [Sp245(pRK415)] was more sensitive to hydrogen peroxide and menadione when grown in minimal medium (Fig. 2b) compared with growth in rich medium (Fig. 2a), suggesting that the cells can overcome the oxidative stress caused by these agents more effectively under conditions of faster (less limiting) growth. Interestingly, the wild-type strain was not affected by growth in the presence of cumene hydroperoxide in rich medium. In contrast, growth of the wild-type strain was increased in the presence of cumene hydroperoxide in minimal medium, suggesting that cumene hydroperoxide was metabolized by the cells. The *ahpC* mutant strain [SK586(pRK415)] was more sensitive to all oxidative stress agents tested compared with the wild-type strain, under all conditions, with a more pronounced growth inhibition effect observed when cells were grown in minimal medium (Fig. 2b). Expressing wild-type AhpC in the SK586 strain [SK586(pRKAhpC)] restored resistance to all oxidative stress agents tested to levels similar to or greater than that of the parental strain, including the significant increase in growth seen for the wild-type strain with cumene hydroperoxide. An increase in the ability of the SK586 cells expressing AhpC to grow in the presence of hydrogen peroxide and menadione was also observed in rich, but not in minimal, medium (Fig. 2a and b). Taken together, the data indicate that *ahpC* contributes to the functional alkyl hydroperoxide reductase activity in *A. brasilense* and functions in protecting cells against oxidative stress. Given the observed effect of growth in rich versus minimal medium, these results also imply that *A. brasilense* implements multiple strategies to resist oxidative stress under different nutritional conditions, and that the contribution of AhpC to oxidative stress resistance is greater under conditions of growth in minimal (nutritionally more limiting) medium.

Effect of *ahpC* mutation on motility and chemotaxis

A. brasilense SK586 was initially isolated in a screen for non-motile mutants in the swarm plate assay performed in minimal semi-soft medium (Scheludko *et al.*, 1998). Significant growth of the cells is required in order for a

Fig. 2. The *A. brasilense ahpC* mutant (SK586) is more sensitive to oxidative stress than the wild-type strain (Sp245). pRK415 refers to the vector introduced into the strains as a control; pRKAhpC refers to the plasmid expressing the wild-type *ahpC* gene introduced into the SK586 strain for functional complementation. Survival of cells in the presence of hydrogen peroxide, menadione and cumene hydroperoxide was determined in actively growing cultures in rich (a) and minimal (MMAB) media (b). Cultures containing DMSO or sterile distilled water were used as controls. All control cultures grew to similar population levels ($\sim 10^8$ cells ml^{-1}). Survival of cells was estimated by dilution plating as described in the Methods section. Survival in the presence of the compound tested is estimated as a percentage of surviving cells relative to a similar control culture incubated in the absence of the compound. The dashed lines in panels (a) and (b) represent 100 % survival. The data represent means obtained in two independent experiments. Black bars, Sp245(pRK415); white bars, SK586(pRK415); grey bars, SK586(pRKAhpC).

conclusive phenotype to be observed in this assay. We observed that the SK586 mutant cells inoculated in swarm plates were able to spread from the inoculation point, and form a chemotactic ring (data not shown). However, we found that when the mutant cells were inoculated from colonies or cultures that were in advanced stationary phase (more than 48 h), or when the medium used to grow the inoculum was different from that used for swarm plate assays, the cells grew but did not form a chemotactic ring

in the swarm plate assay. When cells from an exponentially growing culture were used as the inoculum, the motility and chemotaxis of the mutant were indistinguishable from that of the wild-type. Thus, apparent deficiency in motility on swarm plates correlated with the physiological conditions of cells used as inoculum. When observed directly under the microscope, SK586 cells displayed a motility pattern similar to that of the wild-type: cells alternated periods of smooth swimming with brief reversals, indicating that the single polar flagellum rotated in both directions. This observation suggests that the polar flagellum is functional in the SK586 strain, and it argues against a specific defect in flagellar function in this strain. When the motility of *A. brasilense* SK586 was compared with that of *A. brasilense* Sp245 during growth in rich medium, we observed that cells in the SK586 population lost motility at a higher rate compared with cells from the wild-type population (Table 1). As a result, most of the SK586 cells were non-motile upon entry into stationary phase, whereas a large fraction of the wild-type cells were fully motile (Table 1). Expression of *ahpC* from a plasmid in the SK586 mutant restored the wild-type swimming motility phenotype. These observations are consistent with the hypothesis that the premature loss of motility in the mutant strain results from the lack of functional AhpC rather than a specific defect in flagellar structure and function. The failure of the *ahpC* mutant strain to maintain motility during prolonged growth further suggests that cells are impaired in the ability to adequately cope with, and respond to, increasing stress(es) that are likely to occur during growth, and prompted us to characterize further the growth of this strain, and its survival in stationary phase.

Mutation in *ahpC* does not affect growth rates, but it impairs survival in prolonged stationary phase under nutritionally limiting growth conditions

We compared the growth of the wild-type and the mutant in rich medium and minimal medium (MMAB), and found that the two strains had similar doubling times in

each medium. When grown in rich medium (Fig. 3b) or in MMAB minimal medium (data not shown), there was no difference in the ability of the wild-type strain and the SK586 strain to survive in the stationary phase of growth. We hypothesized that no difference was observed because the nutritional stress experienced in minimal medium was not sufficient. Thus, we compared survival of cells grown in a minimal medium in which the concentration of carbon was significantly reduced (MMAB 0.05 % malate) as a way to increase corresponding nutritional stress. The doubling times of cells under these conditions were similar to those in standard MMAB (Fig. 3a). A defect in the ability of *A. brasilense* SK586 to survive prolonged periods during stationary phase was detected only when cells were grown in minimal medium with low carbon concentrations (MMAB 0.05 %) (Fig. 3c), suggesting that a functional AhpC may be critical under these conditions. These results also indicate that unless the nutritional stress experienced by the cells is enhanced by growth under limiting conditions (growth in MMAB 0.05 % versus standard MMAB), the lack of AhpC does not significantly affect the ability of cells to survive under prolonged stationary phase.

Defective AhpC function is concomitant with changes in cell-surface properties

Upon observation of cells in stationary phase under the bright-field microscope, we noticed an apparent difference in the morphology of the wild-type and the SK586 cells. We used TEM to analyse the morphology of cells, and found that while the morphology of stationary-phase cells of the wild-type and the complemented SK586 strain were heterogeneous in size, with numerous occurrences of abnormally elongated cells, the SK586 cells were comparatively smaller and of a more homogeneous size distribution (Fig. 4a). To confirm that the changes in the morphology of stationary-phase cells were caused by AhpC, we over-expressed AhpC from a plasmid in the wild-type strain [Sp245(pRKahpC)], and found that cells were smaller in stationary phase, similar to cells of the mutant strain (Fig. 4b).

Changes in the cell morphology in *A. brasilense* have been previously observed during flocculation, which is a differentiation process induced under specific growth conditions (Sadasivan & Neyra, 1985; Burdman *et al.*, 1998; Pereg-Gerk *et al.*, 1998; Bahat-Samet *et al.*, 2004). Therefore, we compared Sp245 and SK586 strains for the ability to flocculate. Under conditions conducive to cell aggregation and flocculation (culture under high C/N ratios), the wild-type strain flocculated, but the SK586 strain did not (Fig. 5a). Longer incubation times (up to 2 weeks) did not yield any differentiated cells or visible flocs in the culture of the SK586 strain. The ability to flocculate was restored in the SK586 strain by complementation with a plasmid expressing the wild-type *ahpC* gene (Fig. 5a). We also made the serendipitous observation that overexpression of AhpC in the wild-type cells, but not

Table 1. Motility of *A. brasilense* Sp245 and its *ahpC* mutant derivative, SK586

Growth phase (OD ₆₀₀)*	Motile cells (%)†	
	Sp245	SK586
Early exponential (0.1–0.4)	92 ± 5	89 ± 8
Late exponential (0.5–0.7)	96 ± 4	50 ± 9
Early stationary (0.8–1.0)	92 ± 4	33 ± 5
Late stationary (1.0)	81 ± 6	26 ± 4

*Cells were grown in 50 ml NB broth, and the growth was followed by measuring OD₆₀₀.

†The percentage of motile cells was determined for a total of 100 cells by calculating the number of motile cells in the field of view in groups of 20 cells.

Fig. 3. Growth characteristics of *A. brasilense* Sp245 and SK586, its *ahpC* mutant derivative. (a) Growth of *A. brasilense* Sp245 (■) and SK586 (◇) in low-carbon MMAB medium (MMAB 0.05 % malate as the sole carbon source). The volume of each culture was 100 ml. (b and c) Survival in stationary phase of the wild-type strain Sp245 (■) compared with the SK586 strain (◇) in nutrient broth (b), and in low-carbon MMAB (MMAB 0.05 %) (c). The fraction of surviving cells in the stationary phase of growth is expressed relative to the maximum number of cells in the culture at the beginning of the stationary phase. Cells of SK586 survived at the same level as the wild-type when grown in MMAB (not shown). The experiments were performed in triplicate, and similar results were obtained for each experiment. Representative results are shown.

in any of the other strains, caused flocculation in all media maintained in prolonged stationary phase (about 48 h) (Fig. 5b), and this further implicated AhpC in this process.

In addition to changes in the cell morphology, we also noticed that on rich or minimal medium, the colonies of SK586 were smooth with regular edges, whereas the colonies of the wild-type strain appeared irregular, and the surface was striated, suggesting changes in EPS production (data not shown). Consistent with this hypothesis, we observed that colonies of the SK586 strain did not bind Congo Red, and that they bound Calcofluor White very weakly compared with colonies of the wild-type strain (data not shown). To test this hypothesis further, the ability of stationary-phase cells to bind lectins was compared (Table 2, Fig. 6). We found that cells of the wild-type strain and the SK586 strain differed in the ability to bind several lectins. In addition, SK586 cells appeared to bind some of the lectins to an extent that was significantly less than the wild-type strain. Expressing wild-type AhpC from a plasmid complemented the defects observed in SK586 (Table 2, Fig. 6). Collectively, these results are consistent with the hypothesis that mutation in *ahpC* results in quantitative and/or qualitative changes in the ability of cells to produce EPS.

Root colonization abilities of the SK586 strain are not impaired

Given the pleiotropic defects of the *ahpC* mutant strain, including bacterial determinants shown to be important for root colonization (Burdman *et al.*, 2000), we wondered whether AhpC played any role in plant root colonization in *A. brasilense*. We compared the ability of the wild-type and SK586 strains to colonize sterile wheat seedlings in single and co-inoculation assays. When the strains were inocu-

lated singly into sterile wheat seedlings at similar levels, no significant difference in the ability of the strains to colonize the roots of sterile wheat plantlets was observed between the wild-type strain [3.1×10^7 c.f.u. (g root)⁻¹] and the SK586 strain [1.9×10^7 c.f.u. (g root)⁻¹]. When co-inoculated in a 1:1 ratio at levels similar to that used for single inoculations, both strains colonized the root surface, but the total population levels established in the rhizosphere were significantly lower [total bacterial population recovered from roots was 1.9×10^6 c.f.u. (g root)⁻¹], suggesting that the two bacterial strains compete for root surface colonization. However, when individual populations (from the co-inoculation assay) were compared, we found that both strains established similar population levels in the rhizosphere [9.3×10^5 c.f.u. (g root)⁻¹ for Sp245, and 1.0×10^6 c.f.u. (g root)⁻¹ for SK586; not statistically different at the $P < 0.05$ level], indicating that the competition between the two strains does not result in the exclusion of one of the populations. Together, the results indicate that functional Ahp activity is not required for short-term (10 days) colonization of sterile wheat roots. Furthermore, impaired Ahp activity does not affect competitive colonization of root surfaces under these conditions.

DISCUSSION

In order to cope with oxidative stress that results from normal respiratory metabolism or exposure to oxidants, bacteria have evolved multiple, and often redundant, defence systems against reactive oxygen species (ROS); these systems include superoxide dismutase, catalase and alkyl hydroperoxide reductase (Storz & Zheng, 2000). In the present study, we have characterized a mutant of *A. brasilense* deficient in the alkyl hydroperoxide reductase

Fig. 4. Effect of AhpC on the morphology of stationary-phase cells. pRK415 refers to the vector introduced into the strains as a control; pRKAhpC refers to the plasmid expressing the wild-type *ahpC* gene introduced into the SK586 strain for functional complementation, or into Sp245 for overexpression. (a) Morphology of cells in stationary phase. The cells were observed by TEM. (b) Morphology of cells in stationary phase as observed by differential interference contrast microscopy. All experiments were performed in triplicate, and similar results were obtained for each experiment. Representative results are shown.

subunit C (AhpC), which is one of the key components in the defence against ROS in bacteria. Interestingly, a defect in AhpC function also resulted in pleiotropic phenotypic changes, including morphology and cell-surface properties, consistent with an effect on the adaptive response of *A. brasilense* to various stresses.

Azospirilla possess an oxidative type of metabolism, and have a complex branched electron transport system (Alexandre *et al.*, 1999) that is likely to generate ROS during normal metabolic function. Azospirilla grow best under conditions of low oxygen concentrations, which they seek primarily by aerotaxis (Barak *et al.*, 1982; Zhulin *et al.*, 1996). At high oxygen concentrations, superoxide dismutase activity increases in *A. brasilense* Cd (Nur *et al.*, 1982; Clara & Knowles, 1984), while induction of catalase activity seems to be more complex, with the activity increasing as oxygen tension decreases (Nur *et al.*, 1982; Clara & Knowles, 1984). In addition, azospirilla aggregate

Fig. 5. AhpC affects the ability of cells to flocculate. (a) Flocculation in cultures of the wild-type strain Sp245(pRK415), its *ahpC* mutant derivative strain SK586 [SK586(pRK415)], and SK586 complemented with the wild-type *ahpC* gene [SK586(pRKAhpC)]. Flocculated liquid cultures were poured into Petri dishes prior to taking the photographs. The ability to flocculate was restored in SK586 upon complementation with a wild-type *ahpC* gene. (b) Overexpression of AhpC in the wild-type strain caused flocculation after prolonged incubation in rich and minimal media. The photograph represents a typical 48 h culture in rich medium.

by cell-to-cell interactions (clumping) in the presence of increasing oxygen tensions (Nur *et al.*, 1982). Changes in cell morphology from vibroid to coccoid shapes are concomitant with cell-to-cell aggregation, perhaps to reduce the surface-to-volume ratio, and hence oxygen diffusion to the cells (Bible *et al.*, 2008). Various stresses, including nutritional stresses, also cause flocculation (visible large aggregates of cells embedded in a dense extracellular matrix) in *Azospirillum* spp. Interestingly, cell-to-cell aggregation, followed by flocculation, is a common response of diverse bacteria to oxidative and/or nutritional stresses (for example, Nachin *et al.*, 2005; Tree *et al.*, 2007). Consistent with these data, we found that the *A. brasilense* strain carrying a mutation in *ahpC* (SK586) was sensitive to oxidative stress caused by hydrogen peroxide, cumene hydroperoxide and menadione, and that it also displayed other defects encompassing cell morphology, cell-to-cell aggregation and flocculation. While expression of AhpC in the mutant strain restored the wild-type behaviours, overexpression of AhpC in the wild-type strain affected the morphology, and the ability of cells to flocculate. Thus, the pleiotropic phenotypes of the SK586 strain, ranging from sensitivity to oxidative-stress-generating agents to cell morphology and flocculation, are consistent with a defect in AhpC that results in the inability to mount an adaptive response to these stresses. This defect in the adaptive response to stress may also explain the observation that the *ahpC* mutant cells lost motility at higher rates in the

Table 2. Lectin binding to stationary-phase cells of *A. brasilense* Sp245 and SK586 in minimal medium

FITC-labelled lectin	Specificity*	Lectin binding†	
		Sp245	SK586
Peanut	Galactose/ <i>N</i> -acetylgalactosamine	—	—
Lentil	Mannose	++	—
Wheat germ agglutinin	Sialic acid/ <i>N</i> -acetylglucosamine	+	+
Tomato	<i>N</i> -acetylglucosamine	+	+

*Lectin-binding specificity was provided by the manufacturer.

†Binding was visualized by fluorescence microscopy, as described in Methods: —, no fluorescence; +, some cells fluorescent; ++, most cells fluorescent.

Fig. 6. Representative images obtained in the lectin-binding assay. The summary of the results is detailed in Table 2. WGA, wheat germ agglutinin.

stationary phase of growth. While we can not rule out a direct role of AhpC in mediating these responses, it is likely that the effect of lacking a functional AhpC in SK586 is amplified and/or overlaps with other stresses experienced by the cells. This hypothesis is also consistent with the observation that reduced survival of the *ahpC* mutant strain in prolonged stationary phase of growth was observed only when cells were grown under conditions of nutritional stress. Indeed, interconnection of adaptive responses to various stresses, and overlapping effects of different environmental stresses, have been demonstrated in several bacteria, including in response to oxidative, acid or alkaline, and envelope stresses that result in pleiotropic phenotypic changes (Foster, 2000; Maurer *et al.*, 2005; Nachin *et al.*, 2005; Tree *et al.*, 2007).

The genetic organization of the *oxyR*–*ahpC*–*ahpF* gene cluster in *A. brasilense* is similar to that in several distantly related bacteria, such as *Bacillus subtilis* and *E. coli* (Storz *et al.*, 1989; Antelmann *et al.*, 1996). In diverse bacteria, the *ahpC* and *ahpF* genes may be transcribed in the same

direction, although not always as an operon, and the orientation of *oxyR* relative to *ahpCF* is variable. For example, the *ahpC* and *ahpF* genes form an operon in *E. coli* and *S. typhimurium* (Storz *et al.*, 1989), as well as in *Bacteroides fragilis* (Rocha & Smith, 1999). However, in *B. fragilis*, *ahpC* is also transcribed as a monocistronic mRNA, while in *Xanthomonas campestris*, *ahpC* and *ahpF* do not form an operon, and *ahpC* is transcribed as a monocistronic mRNA (Loprasert *et al.*, 1997; Mongkolsuk *et al.*, 1997). Thus, significant variation is likely to exist in the regulation of the expression of this gene. Furthermore, the genome of some bacterial species possesses an *ahpC* homologue, but lacks an *ahpF* homologue (Alm *et al.*, 1999; Parkhill *et al.*, 2000). In *Helicobacter pylori*, the AhpC homologue has been shown to restore alkyl hydroperoxide resistance to an *E. coli* *ahpC* mutant, suggesting that this homologue is functional (Lundström & Bölin, 2000). In the present study, we successfully complemented the defect in the *ahpC* mutant strain (SK586) by expressing AhpC from a plasmid. While this does not rule out the possibility

that *ahpC* and *ahpF* form an operon in *A. brasilense*, it strongly suggests that AhpC may function, at least in part, independently from AhpF in oxidative stress resistance. This assumption is further supported by the fact that phenotypic defects in SK586 were complemented by expression of AhpC only. Similarly, expression of AhpC alone restores resistance to organic peroxides in *X. campestris* (Loprasert *et al.*, 1997), *Corynebacterium diphtheriae* (Tai & Zhu, 1995) and *Porphyromonas gingivalis* (Johnson *et al.*, 2004). In *E. coli* and *S. typhimurium*, AhpC is required in order to detoxify alkyl hydroperoxides (Jacobson *et al.*, 1989; Storz *et al.*, 1989; Poole, 1996; Poole & Ellis, 1996). While the AhpCF complex is required in order to reduce damaging organic peroxides into the corresponding alcohols, using NADH or NADPH as electron donors (Poole, 1996; Poole & Ellis, 1996), AhpC alone is considered to be responsible for scavenging most of the peroxides, including hydrogen peroxide, generated by metabolic activities in bacteria; this finding is also consistent with the observation that many bacteria possess AhpC, but lack an AhpF homologue (Alm *et al.*, 1999; Parkhill *et al.*, 2000; Lundström & Bölin, 2000; Seaver & Imlay, 2001; Charoenlap *et al.*, 2005; LeBlanc *et al.*, 2006). Noticeably, AhpC was also found to be a more efficient scavenger than catalase of low levels of hydrogen peroxide (produced endogenously); catalase represents the primary scavenger of hydrogen peroxide at high levels (Seaver & Imlay, 2001). The SK586 strain, in which AhpC was expressed from a plasmid, also showed an increased resistance to hydrogen peroxide relative to the wild-type strain when tested in actively growing cultures in rich medium; this finding is consistent with a similar role for AhpC in *A. brasilense*. A similar increase in growth of the complemented mutant was also observed in rich medium containing the superoxide generator menadione. Interestingly, we observed that when growing in minimal medium in the presence of cumene hydroperoxide, wild-type cells and complemented mutant cells expressing AhpC grew to greater cell density, suggesting that cumene hydroperoxide was metabolized under these conditions. Analysis of the ongoing complete genome sequence of *A. brasilense* Sp245 (<http://genome.ornl.gov/microbial/abra/19sep08/>) indicates the presence of several homologues of enzymes shown to be involved in the metabolism of aromatic compounds. Together with the observation that the inhibitory effects of all chemicals tested were greater when cells were grown in minimal media, these data suggest that the cells experience different types and/or intensity of stresses (perhaps via an effect of a non-functional AhpC in amplifying other stresses) during growth in rich versus minimal medium, and that the contribution of AhpC to oxidative stress adaptation depends on the growth conditions.

Strain SK586 lost motility at a higher rate than the wild-type during growth, so that most SK586 cells were non-motile in stationary phase. Thus, it is likely that the loss of motility observed here, or by Scheludko *et al.* (1998),

resulted, at least in part, from an impaired ability of cells to overcome stress(es) generated during growth in the absence of functional AhpC. In addition to this possibility, loss of motility under adverse environmental conditions, such as that encountered in the stationary phase of growth or upon exposure to oxidative, pH or envelope stresses, has been observed in several bacterial species, and may result directly or indirectly (for example, via overlapping effects of responses to different stresses) from changes in cell physiology caused by the stress agent (Amsler *et al.*, 1993; Li *et al.*, 1993; Soutourina *et al.*, 2001; Maurer *et al.*, 2005; Nachin *et al.*, 2005; Tree *et al.*, 2007). Interestingly, other changes that were best observed in stationary-phase-grown SK586 cells, including alterations in morphology, and in the ability to bind lectins, Congo Red and Calcofluor White, as well as in flocculation, are consistent with alterations in the cell-surface properties. Cell aggregation and flocculation are induced in response to various environmental stresses in *A. brasilense* (Sadasivan & Neyra, 1985; Burdman *et al.*, 1998; Pereg-Gerk *et al.*, 1998; Burdman *et al.*, 2000; Bahat-Samet *et al.*, 2004). Thus, the pleiotropic phenotypes of strain SK586 are likely to reflect an impaired ability to mount the appropriate set of cellular responses under conditions of stress. The lack of functional AhpC in the SK586 strain may amplify other stresses encountered by the cells under these conditions. Therefore, the set of phenotypes altered in the SK586 strain may result from a direct effect of AhpC on the cell physiology, as well as from indirect effects of other systems that normally function in mounting the adaptive stress response in the wild-type *A. brasilense* Sp245.

Despite its altered cell-surface properties and oxidative stress resistance, the *ahpC* mutant of *A. brasilense* was not found to be impaired in its ability to colonize the surface of sterile wheat roots. Similarly, wild-type and *ahpC* mutant strains competed for root colonization, and both colonized the roots, albeit at lower cell densities. However, neither of the strains became dominant, indicating that AhpC might not provide the cells with a competitive advantage in root colonization, at least under the optimal conditions of our experiment (absence of indigenous microflora, optimal temperature for plant growth, etc.). Using similar colonization and competition assays, we have previously found a dramatic difference between the wild-type of *A. brasilense* Sp7 and its mutant impaired in energy taxis (Greer-Phillips *et al.*, 2004). Therefore, we are confident in the sensitivity of the methods used. These results are not unexpected since, in contrast to intracellular micro-organisms, cells that colonize the surface of roots are not likely to experience oxidative stress. We can not rule out the possibility that the function of AhpC may be important under conditions of field experiments where competing indigenous rhizospheric microflora are present, and plant growth rates are altered as a result of various environmental stresses.

In conclusion, we show that, in addition to its role in mediating resistance to peroxide stress in *A. brasilense* Sp245, AhpC has an effect on the ability of cells to adapt to

stationary-phase conditions, and to grow under limiting nutrient conditions (minimal medium with limiting carbon or flocculation conditions), thus implicating AhpC in mounting a response to these stresses.

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