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Role of the *Dickeya dadantii* Dps protein

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Abstract During infection, the phytopathogenic enterobacterium *Dickeya dadantii* has to cope with iron-limiting conditions and the production of reactive oxygen species by plant cells. A tight control of the bacterial intracellular iron content is necessary for full virulence of *D. dadantii*: previous studies have shown that the ferritin FtnA and the bacterioferrtin Bfr, devoted to iron storage, contribute differentially to the virulence of this species. In this work, we investigated the role of the Dps miniferritin in iron homeostasis in *D. dadantii*. We constructed a Dps-deficient mutant by reverse genetics. This mutant grew like the wild-type stain under iron starvation and showed no decreased iron content. However, the *dps* mutant displayed an increased sensitivity to hydrogen peroxide in comparison to the wild-type strain. This hydrogen peroxide susceptibility only occurs when bacteria are in the stationary phase. Unlike the *bfr* and the *ftnA* mutants, the *dps* mutant is not affected in its pathogenicity on host plants. The *dps* gene expression is induced at the stationary phase of growth. The Sigma S transcriptional factor is necessary for this control. Furthermore, *dps* expression is positively regulated by the oxidative stress response regulator OxyR during the exponential growth phase, after hydrogen peroxide treatment. These results indicate that the Dps miniferritin from *D. dadantii* has a minor role in iron homeostasis, but is important in conferring tolerance to hydrogen peroxide and for survival of cells that enter the stationary phase of growth.

Keywords Miniferritin · Resistance to oxidative stress · Gene regulation · Pathogenicity

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Introduction

Dickeya dadantii 3937 (formerly *Erwinia chrysanthemi*) is a soft rotting enterobacterium that attacks a wide range of plant species, including many vegetables and ornamentals of economic importance. These bacteria are found in soil and on plant surfaces where they may enter via wound sites or through natural openings. During infection, *D. dadantii* first colonizes the intercellular space (apoplast) where they can remain latent until conditions become favourable for the development of the disease (Murdoch et al. 1999). Soft rot, the visible symptom, is mainly due to the

degradation of pectin present in the plant cell wall by pectinases secreted by bacterial cells in response to various stimuli (Kepseu et al. 2010; Franza et al. 2002; Yang et al. 2008). During the invasion process, *D. dadantii* triggers plant defense responses and bacteria have to cope with an oxidative environment due to the production of reactive oxygen species by the plant (Santos et al. 2001; Fagard et al. 2007). Under these conditions, a tight control of the intracellular iron content is important for bacteria, because ferrous iron can exacerbate the oxidative stress through Fenton's reaction, which generates the highly toxic and reactive hydroxyl radical OH°. Several studies of our group and collaborators highlighted the critical question of control of iron homeostasis in planta where bacteria must accurately supply their iron need and cope with changes in redox conditions (Franza et al. 1999; Nachin et al. 2001). Recently, we also showed that the iron storage ferritin and bacterioferritin, FtnA and Bfr respectively, contribute differentially to iron metabolism and virulence of *D. dadantii* (Boughammoura et al. 2008). These proteins belong to the maxiferritin family and owing to their ferroxidase activity, they oxidize excess of ferrous ions and store the ferric form in a bioavailable mineral core (for reviews, see Andrews 2010; Le Brun et al. 2010).

Previous studies analysing intracellular iron distribution in *D. dadantii* demonstrated that the Bfr bacterioferritin is indeed an important target of iron delivery (Expert et al. 2008). These experiments also showed that the Dps protein participates in iron utilization and distribution in *D. dadantii* (Expert et al. 2008). Since their discovery, Dps proteins (DNA-binding proteins from starved cells) were found to protect DNA from oxidative damages by interacting with DNA without apparent sequence specificity (Almirón et al. 1992; Martinez and Kolter 1997). However, Dps proteins possess a ferritin-like function that endows them with iron and hydrogen peroxide detoxification properties (Zhao et al. 2002; for reviews see Calhoun and Kwon 2010; Chiancone and Ceci 2010). Thus, Dps proteins are also called miniferritins, because they are assembled from only 12 identical subunits rather than the 'canonical' 24 subunits (for a review see Haikarainen and Papageorgiou 2010). During stress in *E. coli*, Dps is one of the principally overexpressed proteins and plays a crucial role in protecting cells from various harsh conditions including oxidative stress, toxic electrophiles, high pressure, UV and gamma irradiation, thermal stress, copper toxicity and extreme pH conditions (Wolf et al. 1999; Ferguson et al. 1998; Nair and Finkel 2004; Malone et al. 2006; Thieme and Grass 2010; Choi et al. 2000; Jeong et al. 2008). The global role of Dps in stress resistance has been established in other bacterial species and additionally the involvement of Dps in virulence has been also demonstrated in *Porphyromonas gingivalis*, *Salmonella enterica* serovar *Typhimurium*, *Borrelia burgdorferi* and *Helicobacter pylori* (Ueshima et al. 2003; Halsey et al. 2004; Li et al. 2007; Polenghi et al. 2007). In contrast to the situation in mammalian pathosystems, the importance of the Dps protein in plant pathogenic bacteria is less well documented. Ceci and collaborators have characterized the properties of Dps protein from *Agrobacterium tumefaciens*, but its role in virulence has not been studied (Ceci et al. 2003). However, recently Colburn-Clifford et al. (2010) have demonstrated that the Dps protein from *Ralstonia solanacearum* contributes to oxidative stress tolerance and to colonization of tomato plants. This work was aimed at elucidating the contribution of the miniferritin Dps to stress resistance, control of iron homeostasis and virulence in *D. dadantii* 3937.

Materials and methods

The bacterial strains, bacteriophage, and plasmids used in this work are described in Table 1. The rich media used were L broth and L agar (Sambrook et al.1989). To determine the effect of iron limitation on the growth of *D.dadantii* cells, ethylenediamine-*N*,*N*'-bis-2-hydroxy-phenylacetic acid (EDDHA) (Sigma-Aldrich) or 2,2′-dipyridyl (Sigma-Aldrich) was added to the media as indicated by Boughammoura et al. (2008). Tris medium was used as the low-iron minimal medium (Franza et al. 1999). For iron-rich conditions, it was supplemented with 40 μM $FeCl_3$ or 40 μM $FeSO_4$. Glucose (2 g/l) was used as the carbon source. For genetic marker exchange by homologous recombination, minimal low-phosphate medium was used (Franza et al. 1999). *Escherichia coli* and *D. dadantii* strains were grown at 37 and 30°C, respectively. Antibacterial agents were added at the following concentrations: 50 μg/ml for ampicillin, 40 μg/ml for spectinomycin, 25 μg/ml for kanamycin, and 25 μg/ml for chloramphenicol. For oxidative growing conditions, cultures grown overnight were diluted 100-fold in L broth and grown under intensive

Table 1 Bacterial strains, bacteriophage and plasmids used in this study

Strains/Plasmids	Relevant characteristics	Sources/References
Strain		
Dickeya dadantii		
3937	Wild-type isolated from African violet	Our collection
PPV49	*dps::Ω-Spec*, Dps^- $Spec^R$	This work
PPV43	*bfr::Ω-Km*, Bfr^- Km^R	Boughammoura et al. 2008
PPV50	*dps::Ω -Spec bfr::Ω-Km* Km^R $Spec^R$	This work
PPV41	*ftnA*::*uidA*, $FtnA^-$ Km^R	Boughammoura et al. 2008
PPV 39	*fur::Ω -Spec* Fur^- $Spec^R$	Franza et al. 1999
A4109	*rpoS::Cm* $RpoS^-$ Cm^R	Boughammoura et al. 2008
BT109	*oxyR::Ω-Spec*, $OxyR^-$ SpecR	Miguel et al. 2000
Escherichia coli K-12		
DH5α	*supE*44 Δ*lac*U169 (f80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi relA*1	Our collection
Phage		
phi-EC2	Generalised transducing phage from *E. chrysanthemi* strain 3690	Résibois et al. 1984
Plasmids		
pGEM-T Easy	3.015 kb vector, pGEM-5Zf derivative, Amp^R	Promega
pBC	3.4 kb vector, pUC19 derivative, Cm^R	Stratagene
pHP45Ω	pBR322 derivative carrying the Ω interposon coding for spectinomycin resistance Amp^R $Spec^R$	Prentki and Krisch 1984
pAB11	1500 bp amplified fragment of the *dps* gene region cloned at the *ApaI and SpeI* sites of pBC Cm^R	This work
pAB12	Interposon Ω-spec cloned into the *EcoRV* site of the *dps* gene from pAB11 Cm^R $Spec^R$	This work

shaking with the following compounds: 0.5 mM hydrogen peroxide (Acros Organics), 6 µM paraquat (Sigma-Aldrich), 2.5 µM streptonigrin (Sigma-Aldrich), and 70 µM spermine NONOate (Acros Organics). Bacterial growth was monitored by reading the optical density at 600 nm (OD_{600}). Sensitivity to H_2O_2 was also determined by bacterial counting. Three millilitre of stationary phase culture or exponentially growing culture in L broth were aliquoted in 1 ml, in which 0, 0.5 and 10 mM of hydrogen peroxide was added. Cells were incubated 60 min under intensive shaking before being diluted and plated on selective solidified growth media for counting.

Construction of Dps-deficient mutants. Genomic fragments from the *dps* locus was amplified by PCR with the following primers 5′-ATTATCGCCTCG CTGGGCA-3′ and 5′-CATCAAAACGTCCTTCCT CT-3′. The PCR amplified fragment was cloned into the pGEM-T Easy vector (Promega). This fragment was subcloned into the pBC plasmid with appropriate restriction enzymes in order to gain a unique *Eco*RV restriction site in the *dps* gene (pAB11 plasmid). The Ω interposon coding for spectinomycin resistance from pHP45-Ω hydrolyzed with *Sma*I was cloned into the *Eco*RV site of the *dps* gene, giving rise to plasmid pAB12. Recombinants were selected as described previously by Boughammoura et al. (2008). The presence of disrupted *dps* gene was confirmed by Southern blot hybridization experiments. Double mutants were constructed by using the generalized transducing phage phi-EC2 (Résibois et al. 1984). Siderophore activities were detected as described previously (Franza et al. 2005).

Whole-cell iron content was analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) at the Service Commun d'Analyze from Centre National de la Recherche Scientifique (Vernaison, France).

^{59}Fe labeling and preparation of whole-cell extracts were prepared as previously described

(Boughammoura et al. 2008). Twenty-five to thirty micrograms of proteins was loaded and run on a 10% polyacrylamide nondenaturing gel. Dried gels were autoradiographed at −80°C for 12–24 h using Kodak X-ray film.

General DNA methods. DNA manipulations (chromosomal DNA isolation, cloning, and electrophoresis) were described previously (Franza et al. 1999). Plasmids were extracted by using the QIAprep Spin Miniprep kit (Qiagen). All cloning experiments were performed in the DH5α strain of *E. coli*. DNA/DNA hybridization analysis was performed by using Denhardt's method as described by Sambrook et al. (1989). Primers used for PCR amplification of the *dps* probe are 5′-AGAAATAAAGAGGATAATACT-3′ and 5′-CCGCCGGTCATCATTGATGA-3′. PCR was performed as described by Boughammoura et al. (2008). PCR products were cloned into the pGEM-T Easy plasmid according to the manufacturer's instructions (Promega). Nucleotide sequencing of PCR products was obtained from Genome Express (Meylan, France). For hybridization, DNA probes were prepared by using the Prime-a-Gene labeling system according to the manufacturer's recommendations (Promega).

RNAs were extracted by using hot phenol as described by Boughammoura et al. (2008). The RNA pellet was washed with 70% ethanol and resuspended in 35 µl of water treated with diethyl pyrocarbonate. Northern blot analysis was performed after electrophoresis: 3 µg of RNA were loaded and run on a 1% Tris–borate–EDTA agarose gel containing 7.2% formaldehyde. After electrophoresis, RNAs were transferred onto a positively charged nylon membrane (N+ Hybond; GE Healthcare), and hybridizations were carried out as described previously (Boughammoura et al. 2008). Membranes were washed twice at 65°C in 5 × SSC (1 × SSC is 0.15 M $NaCl^+$ 0.015 M sodium citrate)–0.5% SDS and in 1 × SSC–0.5% SDS. 16S RNA was used as a control. Membranes were placed against Kodak X-OMAT ray film at −70°C for a few days.

Pathogenicity assays. Pathogenicity tests were performed on chicory leaves and on potted African violets (*Saintpaulia ionantha*) cv. Blue Rhapsody. Bacterial cells were plated onto L agar medium and incubated for 24 h at 30°C. Cells were suspended in an NaCl solution (9 g/l) to give an OD_{600} of 0.4. The resulting suspension (5 µl) was used to inoculate chicory leaves, whereas 100 µl of inoculum was used for one leaf per African violet plant as described previously (Sauvage and Expert 1994). Progression of the symptoms was scored during 3 days for the chicory test and 9 days for the Saintpaulia test.

Results

Genetic organization of the *dps* encoding region

In the genomic sequence of strain 3937, one locus encodes a "typical" miniferritin Dps protein from enterobacteria. The *dps* gene (coding sequence ABF-0018914) is located at position 1993465 on the minus strand of the *D. dadantii 3937* chromosome (Glasner et al. 2011). Sequence analysis of the *dps* locus revealed a putative monocistronic open reading frame of 504 nucleotides encoding a 167-amino-acid polypeptide (Fig. 1). This 18.65 kDa protein is 74% identical to the Dps miniferritin from *Escherichia coli* K-12. Amino acid residues H51, H63, D78, E82 and W52, involved in the formation of the ferroxidase site, are conserved in the *D. dadantii* protein (Chiancone and Ceci 2010). As in the *E. coli* Dps potein, two acidic amino acids (E142 and D146) line the putative pores of the *D. dadantii* miniferritin (Ceci et al. 2011). However, in the N-terminal part, there are only two lysine residues instead of three as it is the case in the *E. coli* protein (K10 is replaced by A10). Several promoters were identified by computational analysis. For confirmation, a primer extension experiment was performed and indicated that there are three transcriptional starts P1, P2 and P3 located 53, 122 and 225 nucleotides upstream of the start codon of the *dps* gene, respectively (Fig. 1). The promoter P1 displays a −10 promoter element with the TGCGTATAAT sequence that matches the consensus sequence identified for the rpoS-encoded alternative sigma factor. A binding sequence for the Fur transcriptional repressor (Fur Box) is present downstream of the P1 transcriptional start (Fig. 1). Another Fur box was identified for the P3 promoter. Furthermore, a putative sequence target (**CTAT**TCC**T**GTA**ATAG**) for the OxyR transcription factor is also present upstream the -35 element of the P1 promoter (Fig. 1) (Zheng et al. 2001).

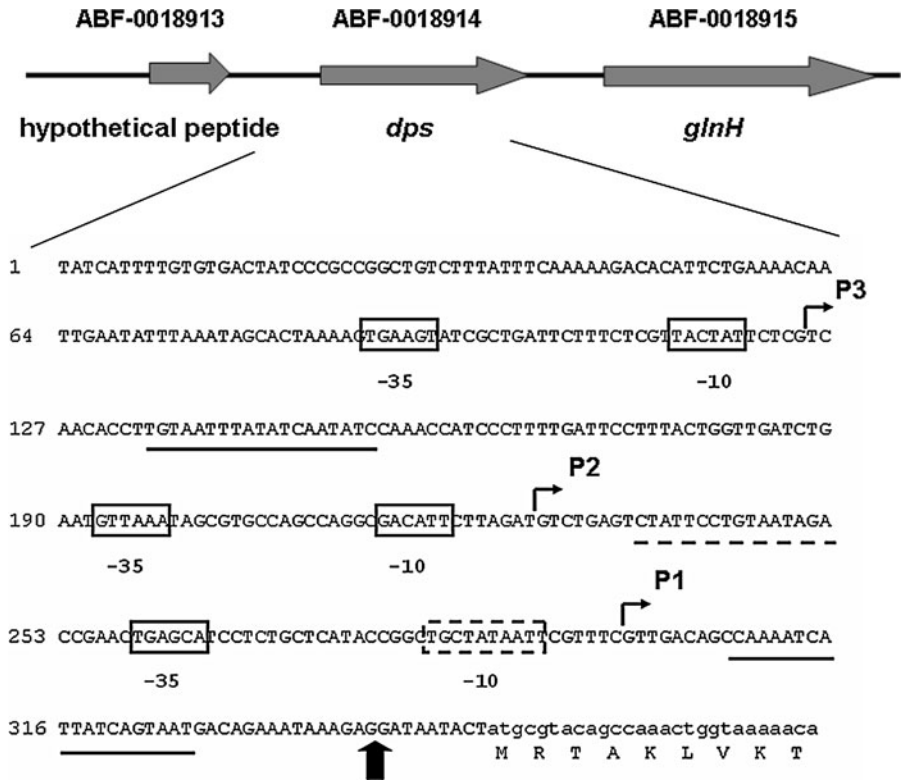

Fig. 1 Genetic organization of the locus encoding the miniferritin Dps of *D. dadantii* 3937. The −35 and −10 promoter elements are boxed. The three transcriptional starts identified by primer extension experiments are indicated by an *arrow*. The P1 promoter element TGCTATAAT that is similar to the RpoS factor recognition consensus sequence is boxed by a *dotted line*. Possible Fur binding sites are *underlined*. The putative OxyR *box* is indicated by a *dotted line*. The Dps ATG start codon is preceded by an AGGA ribosome binding site indicated by an *bold arrow*. The *glnH* gene encodes the periplasmic binding component of the glutamine ABC transporter

Construction of the *dps* and *dps bfr* mutants

We constructed *dps* and *dps bfr* mutants and we checked the absence of production of the corresponding encoded proteins by labeling exponentially growing cells with $^{59}FeCl_3$. Crude cell extracts from the wild-type strain and the *dps* and *dps bfr* mutants were analyzed by native polyacrylamide gel electrophoresis (Fig. 2). *D. dadantii* wild-type extracts showed two major bands corresponding to iron-labeled protein species (Fig. 2). The upper band was previously identified as the Bfr protein (Boughammoura et al. 2008). The lower band was missing in the extracts of the *dps* and *dps bfr* mutant, indicating that this band corresponds to the Dps protein. We were not able to transduce the *dps* mutation in the FtnA ferritin-deficient mutant and reciprocally. Thus, the simultaneous absence of the FtnA ferritin and the Dps protein seems to be lethal in *D. dadantii* under our experimental conditions.

Growth characteristics and iron metabolism in the *dps* and *dps bfr D. dadantii* mutants

We checked the growth properties of the miniferritin-deficient mutants in L broth and in the low-iron minimal Tris medium amended or not with iron or iron chelators. The growth capacity of the *dps* and *dps bfr* mutants was identical to that of the wild-type strain under all the tested conditions (data not shown). Growth kinetics and survival of the *dps* mutant on L agar plate were similar to those of the wild-type strain (data not shown). The absence of functional bacterioferritin and Dps protein can change the intracellular iron content. Therefore, we analyzed the production of siderophores, chrysobactin and achromobactin (Münzinger et al. 2000; Persmark

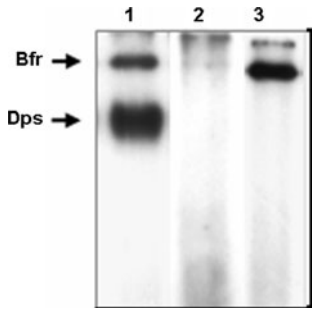

Fig. 2 Analysis of ^{59}Fe-labeled *D. dadantii* proteins from soluble cell extracts after electrophoresis on a native 10% polyacrylamide gel. Bacteria were grown in L broth and $^{59}FeCl_3$ was added at a final concentration of 1 μM, when the optical density at 600 nm reached 0.5. Samples were collected 80 min after iron addition. *Lane 1*: wild-type strain; *lane 2*: *dps bfr* mutant; *lane 3*: *dps* mutant. A diverse range of ^{59}Fe signals can be visualized as a continuum of faint bands and intense bands after longer exposure (Expert et al. 2008). The faint signal present in *lane 2* is likely to correspond to another iron binding protein migrating in the same range as Bfr

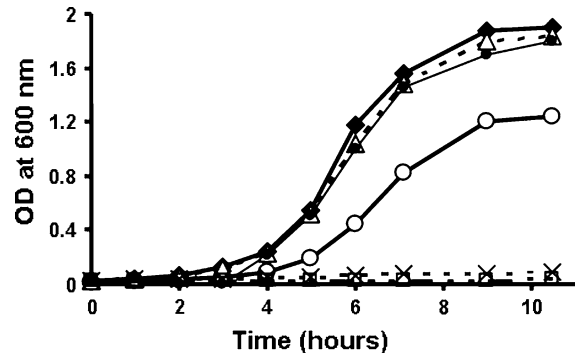

Fig. 3 Growth of the wild-type strain and the ferritin deficient mutants of *D. dadantii* in the presence of hydrogen peroxide. Cells were grown in L medium with intensive shaking in the presence of 0.5 mM of H_2O_2. *Filled diamonds*: wild-type strain; *open triangles*: *bfr* mutant; *filled circles*: *dps* mutant harboring the pAB11 plasmid, *open circles*: *ftnA* mutant; *open squares*: *dps* mutant; cross: *dps bfr* mutant. Experiments were performed five times and the data reported are the mean of three independent experiments with standard deviations corresponding to <5%

et al. 1989), in the *dps* and *dps bfr* mutants grown in Tris medium. Kinetics and levels of achromobactin and chrysobactin production were almost identical for the wild-type strain and these ferritin-deficient mutants (data not shown). The iron storage property of the wild-type strain and the mutants was checked by analyzing whole-cell iron content by ICP-AES. There was no significant difference in the amount of iron present in the wild-type strain, the *dps* and *dps bfr* mutants grown in L broth (60 ± 4, 62 ± 3, 61 ± 3 ppm, respectively) or in L broth with added iron (140 ± 5, 137 ± 4, 135 ± 6 ppm, respectively).

Sensitivity of the miniferritin mutants to oxidative stress

Our previous work indicated that the intracellular iron pool could contribute to oxidative stress (Boughammoura et al. 2008). Thus, we checked the sensitivities of the *dps* and *dps bfr* mutants to compounds generating the superoxide ion O_2^- (paraquat), radicals (streptonigrin), and nitric oxide (spermine NONOate). These mutants displayed no increased sensitivity to all these compounds in comparison to that of the wild-type strain (data not shown). We also tested hydrogen peroxide. For this purpose, overnight grown cultures were hundred-fold diluted in L broth and 0.5 mM H_2O_2 was added to the medium at different times of growth (i.e. from the early to the late exponential phase of growth). Under these conditions, a similar slight growth delay was observed for the wild-type stain and the mutants (data not shown). However, when H_2O_2 was added at the beginning of the culture, the *dps* and the *dps bfr* mutants were unable to grow (Fig. 3). The *bfr* mutant behaved like the wild-type strain, whereas the *ftnA* mutant displayed an intermediate growth phenotype (Fig. 3). The introduction of plasmid pAB11, containing the wild-type *dps* gene, enabled the *dps* mutant to grow in the presence of H_2O_2 (Fig. 3). Susceptibility to hydrogen peroxide was also monitored by colony counting. Bacterial cells were incubated in L broth for 60 min in the presence of 0.5 and 10 mM of H_2O_2 under intensive shaking. Exponentially growing cells showed no sensitivity to these concentrations of H_2O_2 (data not shown). However, late stationary phase cells of the Dps deficient mutants displayed 50 and 90% decrease in survival in comparison to the wild type-strain, after incubation with 0.5 and 10 mM of H_2O_2, respectively (Fig. 4). The FtnA ferritin deficient mutant showed an intermediate susceptibility to these H_2O_2 concentrations (Fig. 4). For shorter times of incubation, these concentrations of H_2O_2 had no effect on bacterial survival (data not shown).

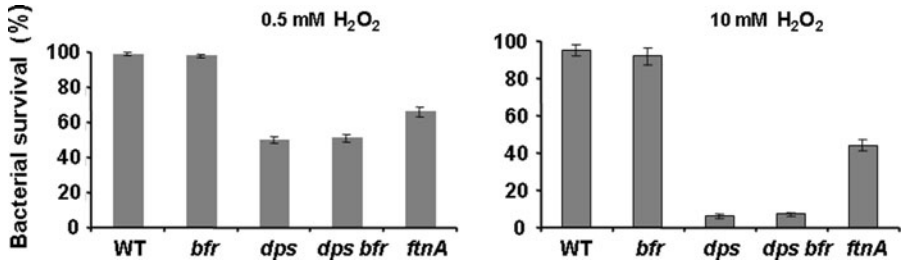

Fig. 4 Sensitivity of the wild-type strain and the ferritin deficient mutants of *D. dadantii* to hydrogen peroxide. Susceptibility to hydrogen peroxide was monitored by colony counting after 1 h of incubation in the presence of 0.5 and 10 mM of H_2O_2 under intensive shaking. The data reported are the mean of five independent experiments with standard deviations

Pathogenicity of the ferritin mutants

First, we examined the behaviors of the *bfr*, *dps* and *dps bfr* mutants of strain 3937 after inoculation onto leaves of potted African violets (*Saintpaulia ionantha*). One week after inoculation, the numbers of systemic responses for the wild-type strain and those of all the mutants were identical indicating that these mutants were as virulent as the wild-type strain on African violets (data not shown). Pathogenicity tests were also performed on chicory leaves. The *dps* mutant exhibited the same maceration capacity on chicory leaves as the wild-type strain (Fig. 5). As previously reported by Boughammoura et al. (2008), the *bfr* mutant displayed a delay in the appearance of symptoms of maceration (Fig. 5). However, once symptoms started, the kinetics of maceration caused by the *bfr* mutant was similar to that caused by the wild-type strain. Inactivation of the *dps* gene had no additional effect on the virulence in a *bfr* genetical background, i.e. the *bfr* and *dps bfr* mutants have the same behaviour on chicory leaves (Fig. 5). Thus, the

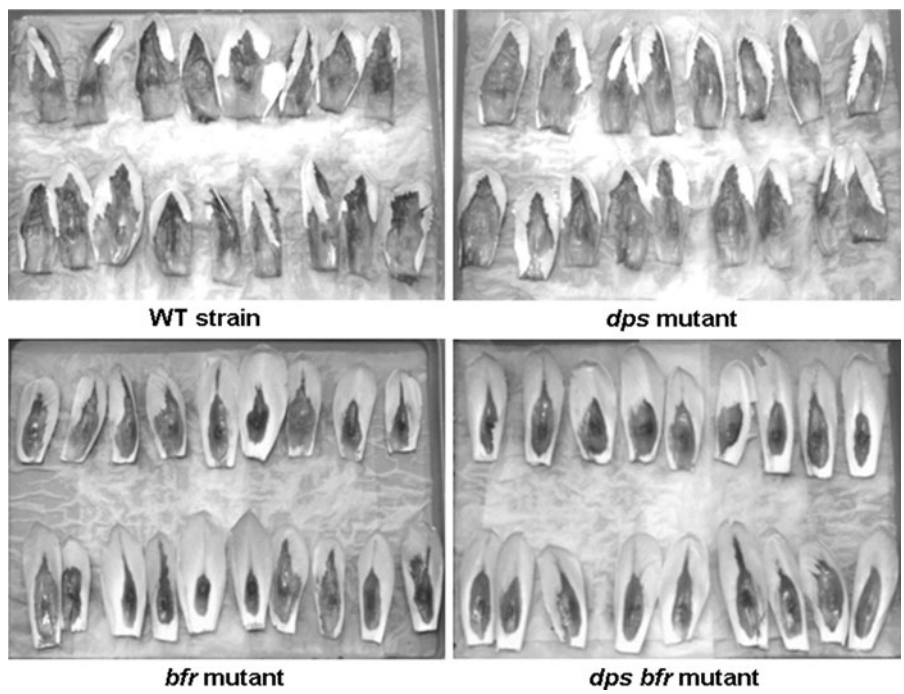

Fig. 5 Pathogenicity of the wild-type strain and its ferritin negative mutants. Symptoms caused by the wild type strain of *D. dadantii,* the *dps*, *bfr* and *dps bfr* mutants on chicory leaves 36 h post-inoculation. Tests were performed five times and one typical experiment is presented

dps mutation has no effect on the pathogenicity of *D. dadantii* under the conditions tested.

Regulation of the *dps* gene

We first examined the expression of the *dps* gene during bacterial growth in L medium. Amount of *dps* RNA increased in late exponential growth phase with high abundance at the beginning of the stationary phase (Fig. 6). Since the identified P1 promoter of *dps* contains an RpoS consensus sequence, we monitored transcription of the *dps* gene in an *rpoS* mutant. In an *rpoS* genotype, the transcription of the *dps* gene was reduced in comparison to that of the wild-type strain (Fig. 7). Thus, in *D. dadantii* the sigmaS transcription factor is involved in the regulation of the *dps* gene. The presence of binding sites for the Fur repressor located in the promoter region of the *dps* locus led us to analyze *dps* gene expression after the addition of iron. Cells were grown in L broth, and $FeSO_4$ was added to a final concentration of 20 μM at an optical density of 1. Exogenous iron addition had no effect on transcription of the *dps* locus (Fig. 8a). We thus examined the role of the Fur repressor in the expression of the *dps* gene. In a *fur* genotype, there was a decreased amount of *dps* RNA when the optical density reached 1.5 (Fig. 8b). This result indicates that the control of *dps* gene expression involves the transcriptional repressor Fur. Sequence analysis of the *D. dadantii dps* promoter region revealed a putative OxyR box. In *E. coli*, the OxyR transcriptional regulator, that is a member of the LysR family of transcriptional activators, induces the expression of antioxidant defense genes that include *dps*, *katG*, and *ahpCF*, the latter encoding hydroperoxidase I and an alkyl hydroperoxide reductase, respectively (Toledano et al. 1994). Indeed, this redox-sensitive regulator controls *dps* gene expression during the exponential phase of growth in response to hydrogen peroxide stress (Altuvia et al. 1994). The sensitivity to hydrogen peroxide of the *D. dadantii dps* mutant led us to monitor transcription of the corresponding gene during the exponential growth phase in response to H_2O_2. Bacteria were grown in L medium and 0.2 mM of H_2O_2 was added at an optical density of 0.5. Unlike the wild-type strain, the *dps* gene transcription was not induced after H_2O_2 treatment in the *oxyR* mutant (Fig. 9). Thus, in *D. dadantii* OxyR

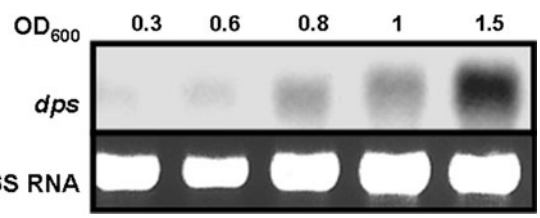

Fig. 6 Northern blot analysis of the *D. dadantii dps* RNA messenger accumulation in the wild-type strain. Total RNAs were extracted from cells collected at the indicated OD_{600} (optical density at 600 nm) and 3 μg of RNA were blotted

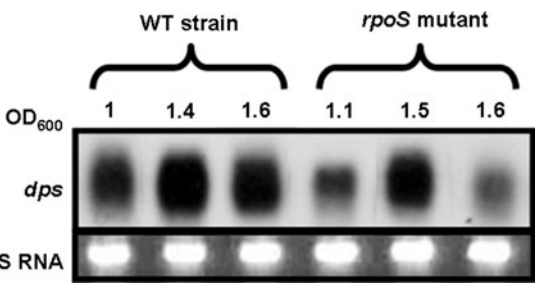

Fig. 7 Effect of an *rpoS* mutation on the expression of the *D. dadantii dps* gene. RNA accumulation was analysed in the wild-type strain and its derivative *rpoS* mutant. Cells were grown in L medium and samples were collected at the indicated OD_{600}

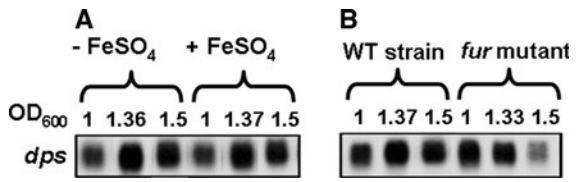

Fig. 8 Northern blot analyses of *dps* gene expression in *D. dadantii*. **a** the wild-type strain was grown in L broth, and $FeSO_4$ was added to a final concentration of 40 μM at an optical density of 1. Samples were collected every 25 min. **b** Dps RNA accumulation in the wild-type strain of *D. dandantii* and its Fur^- derivative. Cells were grown in L broth and samples were collected at the indicated OD_{600}

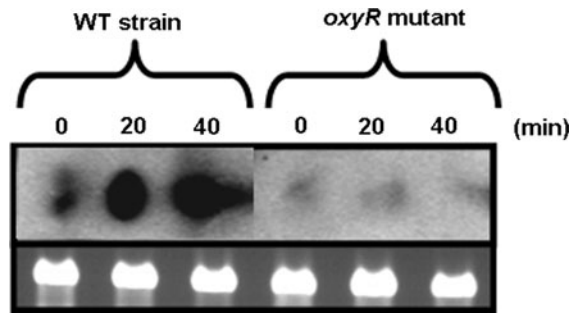

Fig. 9 Effect of an *oxyR* mutation on the expression of the *D. dadantii dps* gene. Cells were grown in L broth and 0.2 mM of H_2O_2 was added at an optical density of 0.5. Samples were collected every 20 min

regulates *dps* gene expression in response to hydrogen peroxide exposure.

Discussion

During infection, *D. dadantii* 3937 cells must overwhelm host defenses and adapt to new environmental conditions that are continually changing in terms of iron availability and redox conditions. Several studies have demonstrated the importance of a perfect control of iron homeostasis in this enterobacterium involving a connection between iron metabolism and tolerance to oxidative stress (Franza et al. 1999; Nachin et al. 2001). Proper control of iron storage by ferritins is also involved in pathogenicity of strain 3937 (Boughammoura et al. 2008). In this work, we investigated the physiological role of the miniferritin protein Dps from *D. dadantii* 3937. We constructed a *dps* mutant by reverse genetics and we characterized its phenotype. No phenotype could be assigned to this mutation under normal growth conditions: the mutant behaved like the wild-type strain under conditions of iron deficiency or iron excess in minimal or rich medium. Whole cell iron contents were similar in the *dps* mutant and the wild- type strain indicating that the miniferritin Dps is not a main iron storage component. This is in agreement with our previous data which showed that the iron charge in Dps increased only when the Bfr bacterioferritin was missing or when iron uptake was not regulated in a *fur* mutant (Expert et al. 2008). The miniferritin deficient mutant was found as sensitive as the wild-type strain to superoxide and nitrosative stresses. However, the *dps* mutant displayed an increased sensitivity to hydrogen peroxide in comparison to the wild-type strain. We observed this effect only when bacteria reach the stationary phase of growth, the time at which the gene is expressed at a high level (see below). Despite this susceptibility to H_2O_2, the *dps* mutant was not affected in its pathogenicity on African violet plants or chicory leaves. These data are in contrast to those demonstrating that the FtnA maxiferritin and the Bfr bacterioferritin contribute to the virulence of *D. dadantii* (Boughammoura et al. 2008). One explanation is that *D. dadantii* possess several systems of protection against hydrogen peroxide, like catalase and alkylhydroperoxidase, and a functional FtnA ferritin scavenging iron, that enable the *dps* mutant to cope with the oxidative conditions from the host plant.

The control of the *dps* gene expression in *D. dadantii* was investigated: a high level of transcription of this gene was observed at the beginning of the stationary phase. The transcription factor sigma S, encoded by *rpoS*, that controls the expression of a large number of genes involved in the transition to stationary phase mediates this response. Indeed, transcription of the *dps* gene was reduced in a *rpoS* mutant mainly at a high cellular density. This result is in agreement with the presence of a RpoS factor recognition consensus sequence identified in the P1 promoter of this gene. It remains to be elucidated whether this regulation involves the histone-like IHF protein that it is required for RpoS-mediated induction of *dps* as it is the case in *E. coli* (Altuvia et al. 1994). A putative OxyR binding site was found to be present upstream the P1 promoter. Accordingly, we showed that in an *oxyR* mutant *dps* transcription was no more induced by H_2O_2 treatment as it occurs in the wild-type strain. Thus, in *D. dadantii* the transcriptional activator OxyR is involved in the upregulation of the *dps* gene during the exponential phase of growth. We also identified two putative Fur binding sites located downstream the P1 and P3 promoters. However, expression of the *dps* gene was not found to be iron regulated. On the other hand, in a *fur* mutant *dps* transcription was decreased at the beginning of the stationary phase. Interestingly, we previously showed that there was a delay in the accumulation of the *rpoS* transcript in a *fur* mutant (Boughammoura et al. 2008). Thus, this lower *rpoS* transcript level could account for the reduced expression of the *dps* gene in the *fur* mutant at high cell density. Whether the Fur-regulated small antisense RNA RyhB is involved in this control remains to be determined (Boughammoura et al. 2008).

This work completed our knowledge on iron homeostasis in *D. dadantii*. We now have a picture of the precise role of the mini- and maxiferritins in the physiology of this bacterial species. The FtnA ferritin is involved in long term iron storage and contributes to the resistance to oxidative and nitrosative stresses (Boughammoura et al. 2008). The Bfr bacterioferritin with the Bfd bacterioferredoxin participates in optimized intracellular distribution and utilization of iron that can be beneficial to *D. dadantii* cells for growth in

fluctuating environments (Expert et al. 2008). The *D. dadantii* Dps protein is able to store iron. However, it seems that this property occurs only under certain conditions (Expert et al. 2008). One main function of Dps may be to confer tolerance to hydrogen peroxide on *D. dadantii* cells that enter the stationary phase of growth. This effect could be due to the capacity of the Dps proteins to detoxify ferrous iron and H_2O_2 by the ferroxidation reaction thus preventing the production of the highly toxic hydroxyl radical OH° through the Fenton's reaction (Zhao et al. 2002). The role in degradation of hydrogen peroxide by Dps may be important when cells become metabolically inactive and start to accumulate free iron from degraded metalloproteins. Absence of a functional Dps protein can also be critical when the FtnA ferritin is also missing. This may be the reason why our attempts to construct a double *ftnA dps* mutant were unsuccessful. In conclusion, the Dps protein could be important for the survival of *D. dadantii* in the various stressful environments that this bacterium encounters during its saprophytic life, such as soil water and a variety of plant materials and debris associated to agricultural practices.

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