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Ferritins, bacterial virulence and plant defence

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Abstract The enterobacterial pathogen *Erwinia chrysanthemi* causes soft rot diseases on a wide range of plants, including the model plant *Arabidopsis thaliana*. This bacterium proliferates in the host by secreting a set of pectin degrading enzymes responsible for symptom development. In addition, survival of this bacterium *in planta* requires two high-affinity iron acquisition systems mediated by siderophores and protective systems against oxidative damages, suggesting the implication by both partners of accurate mechanisms controlling their iron homeostasis under conditions of infection. In this review, we address this question and we show that ferritins both from the pathogen and the host are subtly implicated in the control of this interplay.

Keywords *Erwinia chrysanthemi* · *Arabidopsis thaliana* · Siderophore · Oxidative stress · Iron homeostasis

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Iron, virulence and immunity

The interaction between a pathogen and a host organism leads to a competitive relationship where both protagonists try to defeat each other. In plant as in animal hosts, a successful pathogen produces virulence factors enabling it to gain access to essential nutriments, to rapidly adapt to new environmental conditions and to overwhelm host defense. One strategy involved by bacterial pathogens to deal with the conditions imposed by the host during infection is the production of specific high-affinity iron transport systems and other factors such as toxins and hydrolytic enzymes, that respond to iron availability (for a review, see Crosa et al. 2004). Indeed, vertebrate hosts activate diverse mechanisms upon pathogen attack aimed at depriving the invader of nutritional iron (for a review, see Ong et al. 2006). For instance, by complexing iron into proteins of the transferrin family and scavenging it into ferritins, the host inhibits the growth of certain bacteria and fungi. The macrophage ferrous iron transporter NRAMP1 (SLC11A1) confers resistance to intracellular bacterial parasites possibly by extruding iron from the phagosomal vacuole to the cytoplasm. Increased expression of the iron regulatory peptide hepcidin during infection and inflammation leads to iron sequestration in enterocytes and macrophages and interrupts the delivery of iron in plasma. NGAL (neutrophil

gelatinase-associated lipocalin) is induced in epithelial cells during inflammation and binds catecholate type siderophores, thus preventing bacteria from acquisition of siderophore-bound iron. These antimicrobial mechanisms constitute the well known iron-withholding strategy and are considered as a part of the vertebrate innate immune system. Similar mechanisms in plant hosts could exist and we are currently investigating this question in *Erwinia chrysanthemi* infected *Arabidopsis* plants.

Importance of iron in *Erwinia chysanthemi* infection

Erwinia chrysanthemi strain 3937 (syn. *Dickeya datantii*, *Pectobacterium chrysanthemi*) is an enterobacterium representative of the pectolytic species which causes soft rot diseases on a wide range of plants, including vegetables and ornementals of economic importance. In natural infections, *E. chrysanthemi* enters the aerial parts of a host plant, through wounds and natural openings. Experimental infections can be performed by inoculation of strain 3937 on diverse plant hosts, including chicory leaves, African violets (*Saintpaulia ionantha*, H. Wendl.) and the model plant, *Arabidopsis thaliana*. Cytological examination of leaf of *Saintpaulia* or *Arabidopsis* plants revealed the occurrence of an asymptomatic phase that may last several days, during which bacterial cells colonize intercellular spaces of the cortical parenchyma and migrate within cell walls without severe injury of cellular structures. The symptomatic phase is consecutive to the bacterial production of pectinases, especially pectate lyases that, by degrading pectic components, progressively dissolve plant cell walls, enabling bacteria to gain access to nutriments and to disseminate within the leaf and petiole. Bacterial cells do not migrate in the vascular tissues. During this invasion process, the bacteria encounter various environmental conditions, of which iron availability and production of reactive oxygen species by the plant are two factors limiting their spread.

Analysis of the role of iron in the pathogenicity of *E. chrysanthemi* on *Saintpaulia* plants has shown that during infection, this bacterium requires two high-affinity-iron uptake systems mediated by the siderophores, chrysobactin and achromobactin (for a review, see Expert 2005). Chrysobactin is a monocatecholate-type siderophore while achromobactin is a citrate derived compound that involves carboxylate or/and hydroxyl donor groups for iron binding. Double mutants deficient in achromobactin and chrysobactin production are impaired in symptom initiation because they have reduced survival in intercellular spaces of host tissues (Franza et al. 2005). Interestingly, electron microscopy examination of infected leaf of *Arabidopsis* plants treated with cerium chloride as an indicator of hydrogen peroxide production revealed that this oxidative burst from the plant is more intense after infection with a siderophore negative mutant than with the wild type strain (Fig. 1). This suggests that siderophores are not only required for bacterial iron nutrition but, as iron scavengers, also act as antioxidants. In addition, expression of the genes involved in chrysobactin/achromobactin mediated iron uptake and those encoding pectate lyases important for virulence is controlled by iron, via the Fur sensory/regulatory protein (Franza et al. 2002). Therefore, it seems that in the host, bacteria are able to express their virulence in relation to their iron requirements.

This plant–pathogen interaction illustrates the critical question of control of iron homeostasis in biological situations. Indeed, bacteria must accurately control their intracellular iron utilization and cope with changes in redox conditions (Santos et al. 2001). The question is do bacterial ferritins play a role in this context? The second point concerns the presence of bacterial siderophores *in planta* and their possible interference with the plant iron metabolism. Thus, we investigated whether plants control and modify their iron status in response to infection.

What is the role of the *E. chrysanthemi* ferritin?

Analysis of the genome sequence of *E. chrysanthemi* 3937 (https://asap.ahabs.wisc.edu/annotation/php/ASAP1.htm) enabled us to identify four loci,

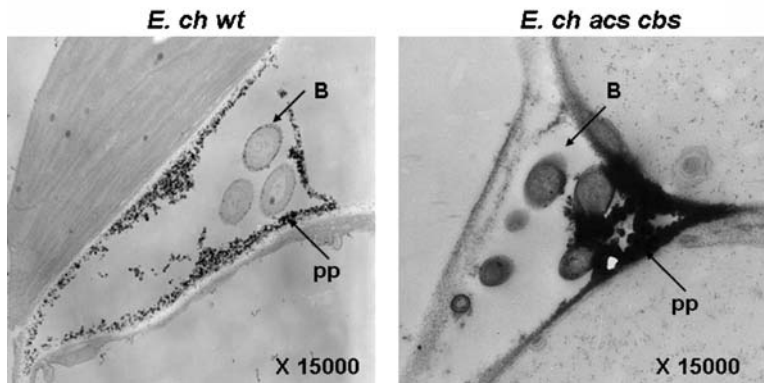

Fig. 1 Localisation of H_2O_2 accumulation in intercellular spaces of Arabidopsis leaf tissues colonized by *E.chrysanthemi* wild type (*E. ch wt*) or achromobactin and chrysobactin negative mutant (*E. ch acs cbs*) cells. Tissue samples were prepared for conventional electron microscopy, 24 h post inoculation. The histochemical assay is based on the reaction of H_2O_2 with cerium chloride to produce electron dense insoluble precipitates of cerium perhydroxides (Bestwick et al. 1997). B: bacterial cell; pp: precipitate

ftnA, *bfr*, *dps1* and *dps2* coding for a ferritin, a haem-containing bacterioferritin and two different DPS proteins (DNA protection during starvation), respectively. Storing iron in a non-reactive form in the cell is the role ascribed to ferritins. Ferritins are organized in a 24-subunit shell surrounding a central cavity, 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 Hintze and Theil 2006; Matzanke 1997). In several bacterial species, ferritins are able to insure protection against the oxidative damage initiated by iron and to serve as an iron reserve. However, there is still little information about the physiological role of bacterial ferritins and their contribution to the virulence of pathogens.

To investigate this question in *E. chrysanthemi*, we cloned the *ftnA* gene and constructed a *ftnA* null mutant by reverse genetics. The *E. chrysanthemi ftnA* gene encodes a protein of 169 amino acid residues that is 78.8% identical to the FtnA ferritin from *Yersinia pestis*. Under iron restricted conditions, the growth of the *ftnA* negative mutant is impaired compared to wild-type strain (Fig. 2), indicating that the intracellular iron level in the mutant is not sufficient to overcome iron deprivation. Growth properties of this mutant are affected in the presence of paraquat, a superoxide generating compound but not in the presence of hydrogen peroxide. The mutant has also an

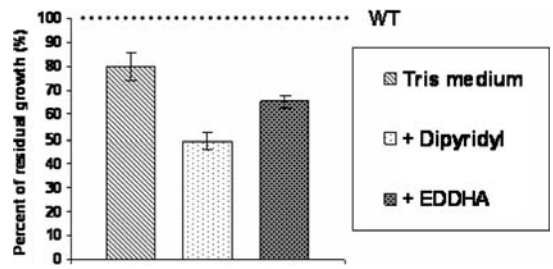

Fig. 2 Growth of the ftnA negative mutant under iron restricted conditions, compared to that of wild type strain. Tris medium is a low-phosphate minimal medium, that was supplemented with EDDHA or 2, 2′-dipyridyl as indicated, to give a final concentration of 80 μM or 100 μM respectively (Franza et al. 2005). Results correspond to the stationary phase of growth as measured by the absorbance at 600 nm. Experiments were carried out in triplicate and standard deviations are shown

increased sensitivity to streptonigrin that produces radicals in the presence of metal and oxygen and to nitric oxide-generating compounds. Interestingly, we found by Mössbauer spectroscopy, that the intracellular ferrous iron pool in the mutant is twice higher than in the wild-type (data not shown). Thus, as in *Campylobacter jejuni* (Wai et al. 1996), the FtnA ferritin in *E. chrysanthemi* can protect the cell against oxidative stress, which is not the case in *Escherichia coli* or *Helicobacter pylori* (Abdul-Tehrani et al. 1999; Bereswill et al. 1998).

Another point of interest is the positive regulation of the *E. chrysanthemi ftnA* gene by iron and Fur. This control is mediated by a small antisens RNA (RyhB-like, Gottesman 2005) able to pair with the 5'end of *ftnA* RNA thereby blocking *ftnA* gene translation/transcription (Fig. 3). Indeed, we observed that a transcriptional *ftnA::uidA* fusion is upregulated by addition of iron (Fig. 4), and in a *fur* background where RyhB RNA accumulates, the fusion is shut off (data not shown). In a Δ*ryhB* mutant, *ftnA* gene transcripts accumulate to high levels compared to wild type strain (Fig. 4). Therefore, this situation seems to differ from that described in *E. coli* since the *E. coli ftnA* gene is not RyhB post-transcriptionally regulated. In *E. coli*, RyhB controls the expression of a number iron containing proteins including bacterioferritin (Bfr) and SodB (Massé et al. 2005).

We analysed the pathogenic behavior of the *ftnA* mutant on chicory leaves and potted African violets, in comparison with wild type strain. On these two plants, the mutant displayed a delay in the appearance of symptoms. However, once the symptoms had appeared, their progression was the same for both strains. This delay is consistent with the fact that the mutant has reduced iron stores and is more susceptible to oxidative conditions. Therefore, it seems that the ferritin confers on *E. chrysanthemi* cells, the capability to adapt to the conditions encountered in the host at the onset of infection. In *Helicobacter pylori*, the Pfr ferritin is essential for survival in the gastric mucosa, likely because it enables the bacterium to overcome toxicity of iron at low pH (Waidner et al. 2002).

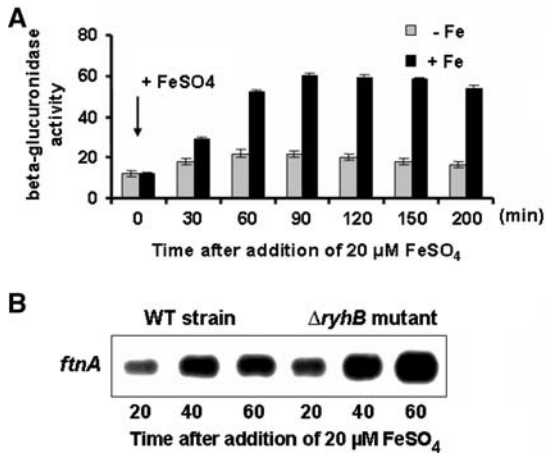

Fig. 3 Transcriptional expression of the *ftnA* gene is regulated by iron and the small RNA RyhB. A: Time course expression of a chromosomal *ftnA::uidA* fusion in *E. chrysanthemi* cells grown in LB medium, supplemented with iron (+Fe), or not (–Fe). B: Northern blot analysis showing *ftnA* (transcripts) accumulation in the wild type strain and in a ΔryhB mutant, at different times (in mn) after supplementation by iron, as indicated

Arabidopsis response to *E. chrysanthemi* infection: the role of Atfer1 ferritin in plant immunity

In the course of a cDNA differential screening between *Arabidopsis* infected or not by *E. chrysanthemi*, we pulled out a cDNA encoding a ferritin (*Atfer1*), indicating an upregulation of the *Atfer1* gene in response to infection. Ferritins are encoded by a four-member gene family in *Arabidopsis* (*Atfer1–4*) and are regulated at the transcriptional level (Petit et al. 2001a, b). *Atfer1* gene expression is stimulated in the presence of excess of iron, hydrogen peroxide or nitric oxide (Gaymard et al. 1996; Murgia et al. 2002; Petit et al. 2001a, b). To investigate whether *Atfer1* gene expression is a part of an iron-withholding defense system induced in response to bacterial invasion, we used an *Arabidopsis* T-DNA insertion mutant line abolishing *Atfer1* gene expression. By comparing the susceptibility to infection of this line with the wild-type ecotype Col-0, we found that spreading of the symptoms is faster and the number of systemic infections is higher in the *atfer1* mutant line. This result indicates that the *Atfer1* gene is involved in a basal level of resistance of *Arabidopsis* to *E. chrysanthemi* (Dellagi et al. 2005).

Analysis of the *Atfer1* gene transcriptional activity after infection showed that this gene is highly expressed at 24 h post inoculation and this event is independent of the presence of IDRS (iron-dependent regulatory sequence), a cis acting element required for the *Atfer1* gene response to iron excess and nitric oxide. Interestingly, this upregulation does not occur after inoculation with an *E. chrysanthemi* siderophore negative mutant,

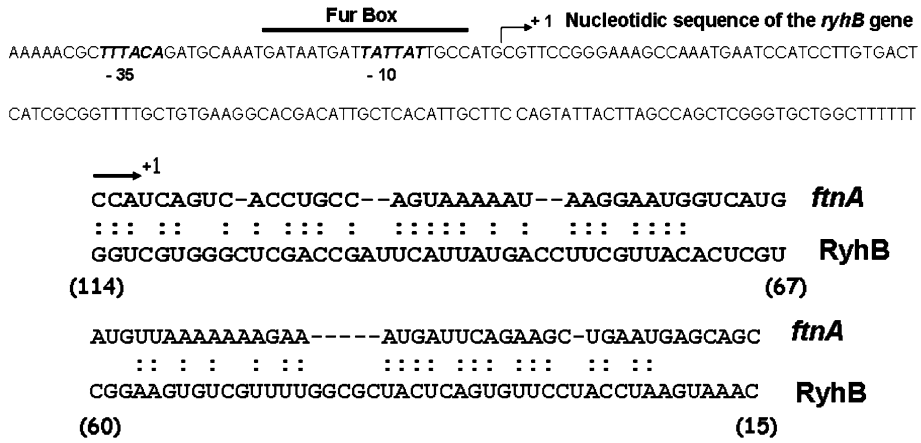

Fig. 4 Promoter region of the *E. chrysanthemi ryhB* gene and sequence complementarity between the *ftnA* transcript and the small RyhB RNA. The *ryhB* gene is regulated by the Fur repressor. The non-coding 115 bp RNA is able to pair with the 5' end of the *ftnA* RNA and can block its translation/transcription. *RyhB* and *ftnA* gene transcriptional start sites were determined experimentally (data not shown)

suggesting a role of siderophores in the *Atfer1* gene induction (Fig. 5). Indeed, infiltration of chrysobactin increased *Atfer1* gene transcription and this effect was not observed with the iron-loaded form of this siderophore (Fig. 5). Similar data were obtained by using desferrioxamine and its ferric complex. A pharmacological approach indicated that neither oxidative stress nor nitric oxide is involved in the response to chrysobactin (Dellagi et al. 2005). Therefore, the mechanism by which siderophores upregulates *Arabidopsis* ferritin gene expression is an open question. The molecular mechanisms that enable plants to control their iron homeostasis are not still understood (Arnaud et al. 2006), but however it is conceivable that a severe iron depletion in leaf tissues caused by the siderophore enhances an afflux of iron resulting from a redistribution of intracellular iron and/or activation of iron acquisition systems of the cell. For instance, iron stored in the vacuole might be remobilized to the cytosol by specific ferrous ions transporters of the NRAMP family (Lanquar et al. 2005). Another interpretation would be that the siderophore is recognised as a signaling molecule inducing a regulatory cascade leading to elevated levels of *Atfer1* gene transcripts. Both chrysobactin (Persmark et al. 1989) and desferrioxamine (Kachadourian et al. 1996) are bacterial products that could have been at the origin of a perception system in the host, like the lipocalin system in mammalian cells (Flo et al. 2004). Any of these possibilities are mutually exclusive and they are currently being investigated.

Conclusion

Ferritins have been the focus of abundant research for many years, from a structural, functional and physiological standpoint, particularly in mammalian organisms where they play a central role in controlling iron homeostasis during inflammatory processes and pathogenesis of diseases. As reported by E. Theil, ''ferritin evolved as the only protein able to solve the problem of iron/oxygen chemistry and metabolism'' (2003) and it may not be surprising that these proteins are involved in many complex regulatory circuits that link iron, oxygen and antioxidant metabolism. It is also noteworthy, that bacteria are equipped with a rich arsenal of ferritins (ferritin-like protein, haem-containing bacterioferritin and mini-ferritins of the DPS family) and determining their role in control of bacterial iron acquisition/distribution and physiology are important issues. From a number of published studies (Smith 2004), it appears that the individual role and gene regulation of ferritins and DPS are related to the bacterial lifestyle and the diversity of ecological niches that the bacteria

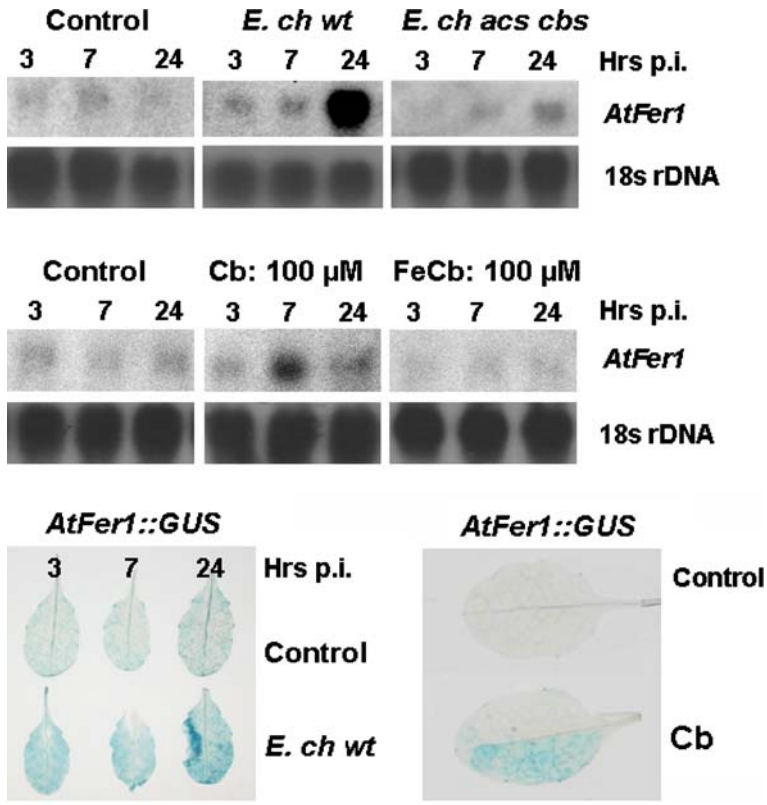

Fig. 5 Transcriptional regulation of the *Atfer1* gene after infection of *Arabidopsis* by *E. chrysanthemi* wild type (*E. ch wt*) compared to achromobactin and chrysobactin negative mutant (*E. ch acs cbs*) cells or after infiltration with chrysobactin (Cb) compared to ferric chrysobactin (FeCb). Transcript accumulation was monitored by Northern blot analysis, using *Atfer1* gene as a radioactive probe (top and middle panels). *Atfer1* promoter activity in response to wild type bacterial infection or to chrysobactin infiltration was observed in an Arabidopsis transgenic line containing 1400 bp of *Atfer1* promoter fused to the glucuronidase reporter gene (*Atfer1::GUS*) as indicated. Methods were described previously (Dellagi et al. 2005)

occupy. However, the role of bacterioferritin as a transient iron pool is still enigmatic. Associated to chloroplasts and mitochondria, ferritins in plants also play a pivotal role in iron and oxygen metabolism as highlighted by the number of environmental signals (iron, nitric oxide, hydrogen peroxide, the stress hormone abscissic acid, photoinhibition) to which ferritin encoding genes respond. We know that ferritin gene expression is also regulated by pathogen attack and elucidating the mechanisms by which this regulation takes place deserves our attention.

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