Manganese transporter protein MntH is required for virulence of *Xylophilus ampelinus*, the causal agent of bacterial necrosis in grapevine

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Abstract

Background and Aims: The aim of this study is to identify proteins involved in the pathogenicity/virulence of *Xylophilus ampelinus*. Characterisation of these proteins could provide new insights into putative targets for controlling bacterial necrosis in grapevines.

Methods and Results: Transposon insertion mutagenesis was used to isolate *X. ampelinus* mutants exhibiting an altered virulence. Characterisation of one of the avirulent mutants revealed the insertion of a transposon into the *mnt*H gene encoding the major manganese transporter. Virulence tests on grapevine leaves clearly showed that the virulence of these mutants was significantly reduced. Phenotypic analysis of an *mnt*H mutant indicated that the MntH protein is a Mn^{++} transporter but that MntH does not play a significant role in the transport of Fe^{++} or Cu^{++}. The MntH mutants exhibited an increased sensitivity to hydrogen peroxide, although catalase and superoxide dismutase activities were not significantly affected.

Conclusion: The MntH protein plays a significant role in the virulence of *X. ampelinus*.

Significance of the Study: This is the first report showing that transposon mutagenesis is an effective strategy for the isolation of *X. ampelinus* mutants. It is also the first report characterising a gene encoding a protein involved in virulence in this grapevine pathogen.

Keywords: *bacterial necrosis*, *grapevine*, *MntH protein*, *virulence*, Xylophilus ampelinus

Introduction

Grapevine bacterial blight is a chronic and destructive vascular disease produced by the Gram-negative bacterium *Xylophilus ampelinus*. This pathology causes serious losses in South African and French vineyards (Grall et al. 2005), although this bacterial blight also occurs in several countries of the Mediterranean region, such as Greece, Italy, Portugal, Spain and Turkey, and even in areas of Asia and South America (EPPO/CABI 1997). This disease is manifest by symptoms, such as reddish-brown to black streaks, cankers and cracks along shoots, brown discoloration of xylem tissues, necrotic lesions on leaves, and finally the death of infected branches and canes that usually leads to complete plant decay (Willens et al. 1987, Dreo et al. 2007). Grapevines appear to be the only host known for *X. ampelinus*. Remarkably, no resistant cultivars have been detected so far, although different degrees of susceptibility have been reported for several cultivars. Thus, in France, cultivars, such as *Vitis vinifera* cvs Ugni Blanc, Clairette and Muscat á Petits Grains, show the greatest susceptibility (Grall and Manceau 2003), whereas in Spain cvs Airén, Garnacha Tintorera, Juan Ibáñez and Vidadillo are the most susceptible (López et al. 1987). Reports indicate that colonisation of young plants using a *gfp*-marked bacterial strain could be initiated after wounding the stem; bacteria were then able to progress down to the crown through the xylem vessels, where they organised into biofilms. When bacterial suspensions were sprayed onto the plants, bacteria progressed in two different directions: both in emerging organs and down to the crown, indicating the importance of epiphytic colonisation in disease development (Grall and Manceau 2003). Some reports show that contamination mainly occurs during pre-pruning, pruning and harvesting activities. Ridé et al. (1983) have suggested that once it has infected the grapevine, *X. ampelinus* can survive inside the plant in a latent state for a few years. Infected plants are therefore an important reservoir of the pathogen, and both old wood and bleeding sap have been reported to be the two main sources of contamination for emerging organs (Grall et al. 2005). In spite of its importance, currently no effective treatment is available to control this disease. Some efforts have been made to develop a rapid detection of the pathogen by using a nested polymerase chain reaction (PCR) approach (Botha et al. 2001) or by real-time PCR (Dreo et al. 2007). Recently, a synthetic D4E1 antimicrobial peptide has shown some in vitro toxicity against *X. ampelinus*, reducing the titre of pathogens in planta when it was transiently expressed in grapevine leaves (Visser et al. 2012). Unfortunately, the knowledge we have about the *X. ampelinus* disease process and virulence mechanisms is almost nonexistent. This fact certainly limits the development of putative control methods. Thus, the main objective of this work was to perform a transposon mutagenesis of *X. ampelinus* to isolate mutants

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showing an altered virulence. Ultimately, it is considered that such an approach will lead to identifying pathogenicity factors and contribute to elucidating the mechanism by which this pathogen causes disease.

Materials and methods

Bacterial strains, growth conditions and plasmids

Xylophilus ampelinus strain CFBP2098 (Collection Française de Bactéries Phytopathogènes, Angers, France) was routinely grown at 24°C on YPGA medium plates [yeast extract, 7 g/L; Bacto-Peptone (BD Biosciences, Madrid, Spain), 7 g/L; glucose, 7 g/L; and agar, 15 g/L; pH 7.2]. Liquid cultures were grown in trypticase soy broth (TSB) (Scharlau, Barcelona, Spain) at 24°C with shaking (220 rpm). *Escherichia coli* strains were cultured at 37°C in Luria–Bertani (LB) medium (Miller 1972). DNA fragments in *E. coli* were cloned with the pBluescript II KS(+) (Stratagene, La Jolla, CA, USA) plasmid vector.

DNA procedures

All basic DNA methods (including hybridisation and Southern blot) were carried out as described by Sambrook and Russell (2001). Total DNA from *X. ampelinus* was isolated from liquid cultures grown in TSB at 24°C with shaking at 220 rpm for 2–3 days. Cells were harvested by centrifugation at 10 000 *g* for 10 min at 4°C, and the pellet was washed with 1 vol of 0.3 mol sucrose. Cells were resuspended in 3 mL of TES solution [20 mmol Tris-HCl (pH 7.5); 25 mmol ethylenediaminetetraacetic acid (EDTA); 75 mmol NaCl,] containing 1 mg/mL lysozyme. Samples were incubated at 37°C for 10 min. Cells were then lysed by vortex for 1 min after adding 4 mL of Kirby solution [2% (w/v) sodium dodecyl sulfate; 12% (w/v) sodium 4-aminosalicylate,; 6% (v/v) neutral phenol; 1 mol Tris-HCl (pH 8.0)]. Crude DNA preparations were cleaned by treatment with 1 vol of neutral phenol-isoamyl chloroform (24:1) followed by extraction with isoamyl-chloroform. Nucleic acids in the supernatant were precipitated with 1 vol of isopropanol and 3 mol sodium acetate (1/10 vol) followed by centrifugation at room temperature and 10 000 *g* for 20 min. The precipitate was resuspended in 1 mL of TE buffer [10 mmol Tris-HCl (pH 8.0); 1 mmol EDTA] and treated with ribonuclease A (RNase) (Sigma-Aldrich, St Louis, MO, USA) to remove RNA. The sample was cleaned by phenolisation, and DNA was precipitated as described above and finally resuspended in 200 µL of TE buffer. DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Hudson, NH, USA). DNA was sequenced by the dideoxynucleotide chain termination method using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA). Probe DNA fragments were labelled with digoxigenin using a random priming kit (DIG DNA labelling mix; Roche Diagnostics GmbH, Mannheim, Germany).

Genomic library construction and screening

Genomic DNA from *X. ampelinus* was partially digested with *Sau*3AI. DNA fragments (17–23 Kb) were purified by ultracentrifugation in a sucrose gradient and ligated to Lambda DASH II *Bam*HI Vector Kit (Stratagene), followed by in vitro packaging. Recombinant bacteriophage plaques of the genomic library were screened by hybridisation using a DNA probe (312 bp) corresponding to an internal region of *mnt*H amplified with mntH1F (5′-CCGCGGCCACGGTTTTCCATGCC-3′) and mntH2R (5′-GGCTGAACACCAGCAGCTTGGCGG-3′) primers.

Preparation of X. ampelinus *electrocompetent cells*

A single colony of *X. ampelinus* was grown in 500 mL of TSB liquid medium at 24°C and 220 rpm until the culture reached an OD_{600nm} of 0.5–0.6. The culture was cooled in an ice bath for 20 min. All of the following steps were performed at 4°C. Cells were harvested by centrifugation at 6000 *g* for 10 min, then gently suspended in 10 mL of sterile 10% glycerol, washed twice in 10% (v/v) glycerol, after which the cells were washed three times with 10 mL of MilliQ (EMD Millipore Corporation, Billerica, MA, USA) water. Finally, the cells were harvested by centrifugation and the pellet resuspended in 1 mL of 10% glycerol. The cell suspension was divided into 50 µL aliquots which were immediately frozen in liquid nitrogen and stored at −80°C until use.

Generation of Xylophilus ampelinus *mutants*

Insertional mutants were generated using the EZ-Tn5 <R6Kγori/KAN-2> Tnp Transposome kit according to the manufacturer's recommendations (Epicentre Bioechnologies, Madison, WI, USA). Electrocompetent *X. ampelinus* cells (50 µL) were transformed with 1 µL of transposome complex (33 ng of DNA) using a 0.2-mm gap cuvette at 2.0 kV, 25 µF and 200 Ω using GenePulser Xcell (Bio-Rad Laboratories, Hercules, CA, USA). Cells were quickly resuspended in 1 mL of ice-cold TSB media supplemented with 20 mmol of glucose and incubated in an orbital shaker at 24°C and 200 rpm for 8 h for expression of antibiotic resistance. Transformants were selected by plating on YPGA plates containing 10 µg/mL of kanamycin at 24°C for 10 days.

Grapevine in vitro cultures

Vine shoots were taken from field-grown mature plants of *V. vinifera* cv. Tempranillo from the Vega Sicilia vineyards (Valbuena de Duero, Valladolid, Spain) in October/November. After treatment with 16% (w/v) copper oxychloride fungicide (Cobre Key-S, Químicas KEY S.A., Lleida, Spain), buds were stimulated to sprout under culture room conditions maintained at 25 ± 2°C with a 16-h photoperiod for 2 months. After that, double-node stem segments were selected as explants (nodal explants). Under aseptic conditions, the explants were surface sterilised by immersing in 70% (v/v) ethanol for 1 min and 0.4% (v/v) sodium hypochlorite solution with four drops of Tween 20 for 2 min, and then rinsed four to five times in sterilised water. Once obtained, the nodal explants were grown on Murashige and Skoog media (Murashige and Skoog 1962) supplemented with 20 g/L sucrose, 1 mg/L benzyl adenine and 8 g/L agar, pH 5.8. Explants were subcultured to fresh medium every 2 months. Rate of proliferation, estimated as number of new shoots per explant, was five.

Virulence tests

Virulence of *X. ampelinus* insertional mutants was checked on leaves from *in vitro* cultures of Tempranillo grapevines in comparison to virulence of wild-type (WT) strain CFBP2098 *X. ampelinus*. Liquid cultures were incubated until they reached an $OD_{600nm} = 1$. Cells from 10 mL of culture were harvested by centrifugation (10 000 *g*) and suspended in 1 mL 20% glycerol, stored at 4°C and used within 20 h of collection. Grapevine leaves from in vitro culture plants (three leaves per mutant) were placed onto the surface of Petri dishes containing water agar media (2% w/v agar) (underside up). A small wound was made in the centre of the leaf with a hypodermic needle and 10 µL of bacterial suspension was applied directly to the injury. A negative control was run in parallel using a 20% glycerol solution; positive controls consisted of inoculating leaves with the WT strain. Petri dishes were sealed with Parafilm and incubated at room temperature (20–24°C) with a 12-h photoperiod until injuries in the positive controls were clearly visible (7–12

days). Initial selection of mutants was visual. Virulence was quantified by an electrolyte leakage assay. Briefly, five 0.2-cm^2 leaf pieces inoculated with every mutant or strain (one fragment per leaf) were obtained from the inoculation point by using a cylindrical metal punch (0.5-cm diameter). The five leaf fragments were transferred to a tube containing 2 mL of double-distilled water. Samples were vortexed for 5 s and then incubated for 10 min at room temperature. This water solution was transferred to another clean tube, and electrolyte leakage was measured with a 522 conductivity meter (Crison, Barcelona, Spain). Mutants were classified as avirulent (Av) when no apparent injuries were observed and the electrolyte leakage value determined was similar to that observed in the negative control with a maximum deviation of 10%. Mutants were classified as hypervirulent (Hv) when the extent of injury was clearly higher than that produced by WT bacteria and the electrolyte leakage value was at least the 2.5 times higher than that estimated for the WT strain. The mutants were classified with data from three independent experiments.

Identification and analysis of the EZ-Tn5 chromosomal flanking sequences

Genomic sequences flanking the EZ-Tn5 transposon insertion point in mutants were analysed by direct sequencing of chromosomal DNA. Mutant genomic DNA was isolated as reported above. Genomic DNA (1 μg) was digested with *Eco*RI or *Pvu*II (enzymes that do not cut inside the transposon) and then end-repaired (made blunt-ended) and 5′-phosphorylated in order for it to self-ligate using the End-It DNA End-Repair kit (Epicentre Biotechnologies) per the manufacturer's instructions. Digested DNA (1 μg) was self-ligated using 2 U of T4 DNA ligase (MBI Fermentans, Amherst, MA, USA) in 10 μL (final volume) at 15°C for 18 h. Then, 1 μL of the ligation was electroporated into 50 μL TransformMax EC100D *pir*-116 Electrocompetent *E. coli* cells (Epicentre Biotechnologies) following the manufacturer's instructions. Transformants were selected on LB agar plates containing 50 μg/mL kanamycin. Plasmid DNA was isolated using Illustra plasmidPrep Mini Spin kit (GE Healthcare, Little Chalfont, England). The transposon insertion site was mapped by direct sequencing of plasmid DNA using the forward KAN-2 FP-1 (5′-ACCTACAACAAAGCTCTCATCAACC-′) and reverse R6KAN-2 RP1 (5′-CTACCCTGTGGAACACCTACATCT-3′) commercial primers (Epicentre Biotechnologies). Average read length was 600 bp. The BLAST program of the National Center for Biotechnology Information was used (blastx; http://www.ncbi.nlm.nih.gov/BLAST/) to perform sequence similarity searches. To exclude the possibility that the transposon could have inserted itself into the chromosome multiple times, genomic DNA from mutants was isolated and analysed via Southern hybridisation. Extracted DNA was digested with *Eco*RI or *Xho*I and probed with a 660 bp digoxigenin-labelled PCR fragment of the transposon. This fragment was amplified using KAN-2 Fp02 (5′-TTGTTGATGCGCTGGCAGTGTTCCTGC-3′) and KAN-2 Rp01 (5′-GGTTGATGAGAGCTTTGTTGTAGG-3′) primers.

Complementation of mntH mutant

A 1625-bp DNA fragment containing the full *mnt*H gene and including its putative promoter was PCR amplified using the forward MntHCf (5′-CTTAGGATCCGTGTGGCGTGGCGCGCA GCGGT-3′) and reverse MntHCr (5′-CTGTGGATCCGGAAAC GGTCATCGCTACTTTCT-3′) primers and high fidelity *Pfu* DNA polymerase (Stratagene). The PCR product incorporated *Bam*HI recognition sequences at both ends to allow for later cloning. The amplified fragment was sequenced to rule out the presence of any mutation and then, after *Bam*HI digestion, cloned into the broad-host-range vector pHM1 (Huynh et al. 1989), which is a derivative of pRI40 (Innes et al. 1988) that was linearised with *Bam*HI. The recombinant plasmid pHM1/*mnt*H was then electroporated into *X. ampelinus* as described above. Transformants were selected by spectinomycin resistance and confirmed by amplification of an internal fragment of *mnt*H by PCR using the mntH1F and mntH2R primers described above.

Intracellular concentration of metal cations

The intracellular concentration of manganese, iron and copper was estimated by atomic absorption spectrometry in a UNICAM 969 AA Spectrometer (Thermo Scientific) from *X. ampelinus* cultures grown in TSB or TSB supplemented with 0.15 mmol cations. Cells were processed according the method of Li et al. (2011).

Hydrogen peroxide sensitivity assays

The sensitivity of *X. ampelinus* WT, mutant and transformants to H_2O_2 was checked as follows: 200 mL bacterial cultures were grown in triplicate in TSB medium at 25°C until they reached an OD_{600nm} of 1.0. Cells were collected by centrifugation, and the pellet washed with 10 mL of 50 mmol phosphate buffer (pH 7.0). Cells were then resuspended in 5 mL of the same buffer containing 10 mmol H_2O_2 and incubated at 25°C for 15 min. A negative control was run in parallel without H_2O_2. Cells were then collected by centrifugation, washed with 10 mL of TSB and serial tenfold dilutions were made before plating onto YPGA plates. Plates were incubated at 25°C until colonies appeared; colonies were used to calculate the number of viable cells.

Catalase and superoxide dismutase activity

Enzyme activity was estimated in cell-free extracts of *X. ampelinus*. Bacteria were grown in triplicate in 200 mL of TSB liquid media at 25°C with shaking at 220 rpm until the cultures reached an OD_{600nm} of 1.0. Cells were harvested at 4°C by centrifugation at 10 000 *g* for 10 min, and the pellet was washed twice with 50 mL of 50 mmol phosphate buffer (pH 7.0). Cells were preserved frozen at −80°C. Frozen pellets were thawed slowly in an ice bath, adding 3 mL of extraction buffer [50 mmol Tris-HCl (pH 7.5); 10% (w/v) glycerol (0.1 mmol EDTA); 0.1% (v/v) Triton X-100; and 2 mmol dithiothreitol (DTT)]. Cells were disrupted in an ice bath by sonication using the microtip of a 150 W MSE ultrasonic disintegrator (Branson Sonifier, Danbury, CT, USA). Samples were sonicated using ten 5-s bursts with an interval of 3 min between bursts to prevent an excessive increase in temperature. Cellular debris was removed by centrifugation at 40°C and 10 000 *g* for 20 min. Supernatants were collected and used as a crude enzyme preparation. Catalase activity was determined by measuring the initial rates of H_2O_2 decomposition at 240 nm in a solution containing 10.6 mmol H_2O_2 in 50 mmol potassium phosphate buffer (pH 7.0). The assay volume was 1 mL and the reaction was carried out at 25°C for 2 min in a thermostated UviLine 9400 spectrophotometer (VWR, Llinars del Vallés, Spain). Catalase activity, expressed as μmol H_2O_2/(min · mL), was calculated using an extinction coefficient for H_2O_2 of 39.58 mol/cm (del Río et al. 1977). Superoxide dismutase (SOD) activity was estimated using the SOD determination kit (Sigma-Aldrich).

Data analysis

All assays were repeated at least three times. Error bars in graphs indicate standard deviations. The Shapiro–Wilk test was used to test data univariate normality, and then univariate

analysis of variance was performed with the general linear means procedure; $P < 0.05$ was considered significant. When F-ratios were statistically significant, post hoc tests (Tukey's honestly significant difference test) were carried out to determine where the differences between groups lay. R Core Team (3.0.1.) software (http://www.R-project.org/) was used for statistical analysis.

Results

Isolation of mutants with altered virulence

Electroporation of electrocompetent *X. ampelinus* CFBP2098 strain cells with 1 µL (33 ng) of EZ-Tn5 <R6Kγ*ori*/KAN-2>Tnp Transposome in two independent experiments led to the isolation of 923 and 1395 kanamycin-resistant mutants. Efficiency of mutation corresponded to an average insertion rate of 3.51×10^4 cfu/µg of DNA. Thirty-five clones were randomly selected and tested for their virulence on grapevine leaves. Two (clones 16 and 23) out of the 35 clones analysed had greatly reduced virulence (Table 1), and in fact, in leaf tests, they behaved as if they were avirulent (Figure 1). Indeed, the electrolyte leakage assay for the WT strain indicated a 217.0 µS/cm conductivity value compared with the 40.0 µS/cm conductivity measured for the 16 µS/cm clone and 43.0 µS/cm for the 23 clone (these differences were significantly different at $P = 0.05$). Accordingly, they were named clones Av16 and Av23. Two other clones (3 and 32) were classified as hypervirulent (Hv clones) (clones Hv3 and Hv14) according to data from both the test on the leaves and the electrolyte leakage test. Compare the 217.0 µS/cm conductivity value of the WT strain with the 877.0 µS/cm conductivity value measured for clone 3 and 674.0 µS/cm for clone 32 (Figure 1). Again these differences were statistically significant at $P = 0.05$. An analysis of the transposon insertion point in every mutant was carried out. The sequence (765 nt) of the chromosomal DNA region to the right of the insertion point in the Av23 mutant revealed that the interrupted gene encoded an open reading frame (ORF) with high similarity (*E* value 5×10^{-81}; 80% amino acid identity) to a Mn^{++} and Fe^{++} transporter of the NRAMP family from *Magnetospirillum magnetotacticum* (accession number WP_009867038.1). On the left side of the insertion point, the sequence obtained (792 nt) showed great similarity (*E* value 3×10^{-138}; 82% amino acid identity) to a manganese transporter from *Pseudomonas psychrotolerans* (accession number WP_007160634.1). From these data, we concluded that in the Av23 mutant, the transposon had inserted (Figure 2) into a gene that appeared to encode for a MntH-like protein belonging to the NRAMP family of transporters that may facilitate Mn^{++}/Fe^{++} transport. A similar analysis showed that in the Av16 mutant, the transposon had inserted into a gene encoding a protein with a high similarity (73% amino acid identity) to a tyrosine phosphatase from *Xanthomonas campestris* (Table 1). Among the

Table 1. Identification of the transposon insertion point in the genome of *Xylophilus ampelinus* in the avirulent (Av) and hypervirulent (Hv) mutants isolated.

Xylophilus ampelinus mutant	Site of insertion	Similarity to†/Amino acid identity (%)‡/ GenBank accession§	GenBank accession number¶
Av16	5′-AGGCCCGAACCCGCGTATGG-transposon sequence-CGCGTATGGTGATTTGAACAA-3′	*Xanthomonas campestris*: tyrosine phosphatase/73%/ YP_361664.1	HG531361
Av23	5′-GCTTGCGCTGTTCGTCAACGC-transposon sequence-GGCCATCCTGATCACCGCGGC-3′	*Pseudomonas psychrotolerans*: Mn^{++} and Fe^{++} transporter of the NRAMP family MntH/82%/ WP_007160634.1	HG531364
Hv3	5′-TTCTCGAAGGCCGCGGTGCCG-transposon sequence-CTGGCGCTTTCCAAGGCCAGC3′	*Variovorax* sp.: LysR family transcriptional regulator/ 68%/WP_007829610.1	HG531362
Hv32	5′-CACGCTGTCGTAGGTGTAGCC-transposon sequence-GTGTAGCCCAGAACGCTACCG-3′	*Delftia acidovorans*: tyrosine-aspartate (YD)-repeat-containing protein/43%/YP_001564737.1	HG531363

†Sequences were analysed by nucleotide query translated database alignments (blastx, http://www.ncbi.nlm.nih.gov/BLAST/). ‡Identities refer to the gene fragments obtained. §GenBank accession numbers of proteins showing similarity to *Xylophilus ampelinus* proteins encoded by genes interrupted by transposon insertion. ¶Sequences deposited in GenBank under the accession number provided.

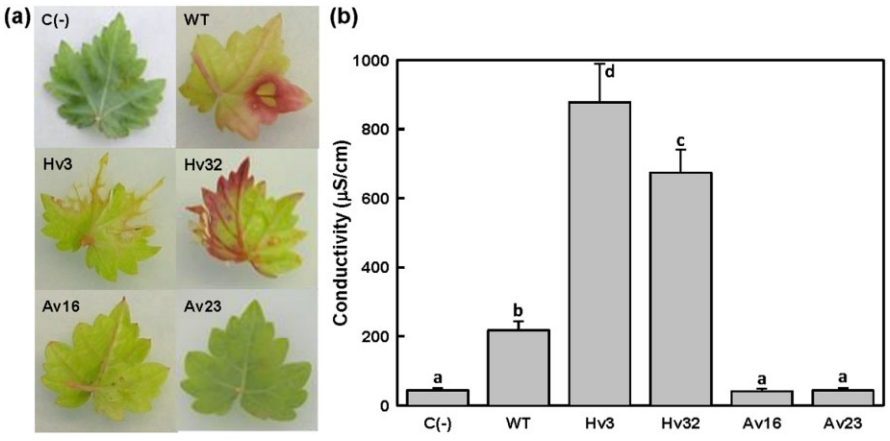

Figure 1. Injuries and necrotic activity of *Xylophilus ampelinus* wild-type (WT) and different hypervirulent (Hv) and avirulent (Av) mutants visualised (a) by lesion formation on leaves and (b) by quantification of necrosis by an electrolyte leakage assay. Data shown represent the mean ± standard deviation (SD) from three independent experiments. Bars marked with the same letter do not differ at $P = 0.05$.

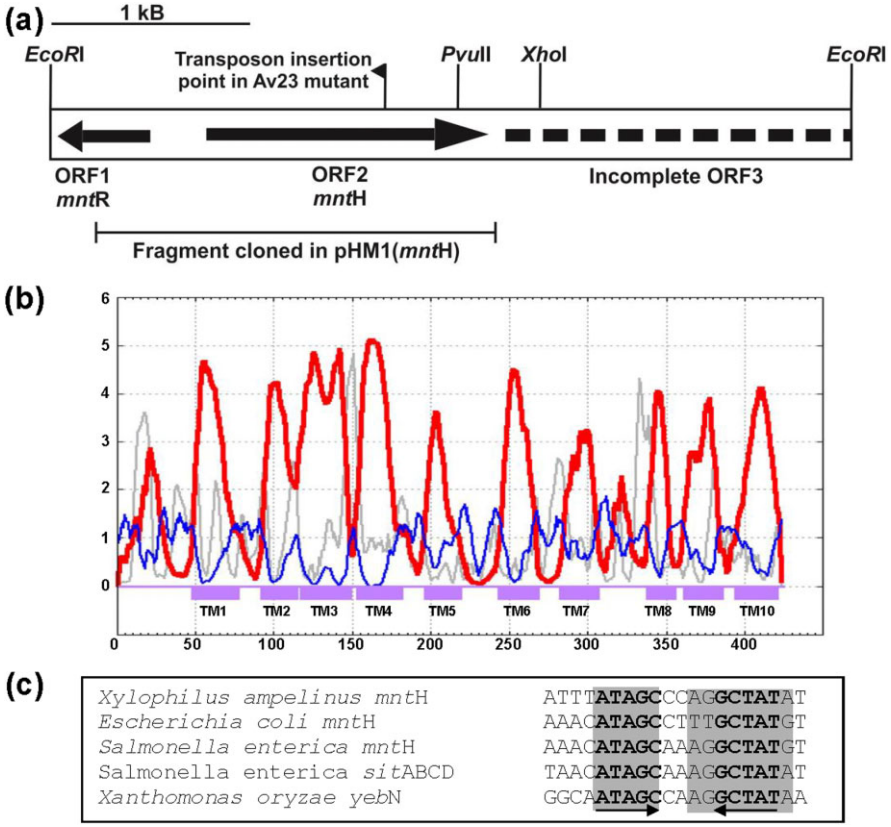

Figure 2. (a) Partial restriction endonuclease map of the DNA region containing the *mnt*H gene [open reading frame (ORF) 2] from *Xylophilus ampelinus*. Also shown are ORFs corresponding to the putative *mnt*R gene (ORF1) and an incomplete ORF3. The DNA fragment subcloned in the pHM1 vector to complement the Av23 mutant is indicated by a solid line in the lower part of the figure. (b) Secondary protein structure for detection of transmembrane domain (TM) was predicted using Split-4.0 software (http://split4.pmfst.hr/split/4/). MntH protein contains 10 predicted TMs (■) indicated as TM1 to TM10. Transmembrane domains were predicted according to transmembrane helix preference (THM index) (—); beta preference (BET index) (—) and modified hydrophobic moment index (INDA index) (—). (c) Alignment of the putative MntR binding site detected in the intergenic region between *mnt*R and *mnt*H genes from *X. ampelinus* with MntR binding sequences from *Escherichia coli mnt*H (Patzer and Hantke 2001), *Salmonella enterica mnt*H and *sit*ABCD promoters (Ikeda et al. 2005) and *Xanthomonas oryzae yeb*N promoter (Li et al. 2011). Residues conserved in all the sequences are highlighted. The two divergent arrows indicate the putative palindromic sequence recognised by the MntR protein.

hypervirulent mutants, the transposon inserted into a gene encoding a putative transcriptional regulator of the LysR family in the Hv3 clone, whereas the gene interrupted by the transposon insertion in Hv32 mutant encoded a protein with a high similarity to several tyrosine-aspartate (YD)-repeat-containing proteins (Table 1). To ensure that the transposon had not inserted itself into the chromosome multiple times, genomic DNA from these mutants was isolated and analysed via Southern hybridisation. Results confirmed that the transposon had inserted itself only once per mutant (data not shown).

Cloning and characterisation of mntH gene and the deduced polypeptide

An internal fragment of 0.31 kb from the *mnt*H gene was amplified by PCR from the sequence obtained from characterisation of the transposon insertion point in the avirulent Av23 mutant. This DNA fragment was used as a probe to screen a genomic library of *X. ampelinus* made in the vector λ-DASH II. Screening permitted the isolation and purification of a recombinant phage, λ-XAMntHI, which contained a 4.0-kb *Eco*RI fragment that hybridised with the probe (data not shown). The entire 4.0-kb *Eco*RI fragment was sequenced (accession number HG531364). It contained two full ORFs (ORF1 and ORF2 shown in Figure 2a) and an incomplete ORF (ORF3). ORF1 was 477 nt (nt 105–581) and encoded a protein of 158 amino acids with a deduced molecular mass of 16 541 Da and a theoretical isoelectric point of 9.729. The deduced polypeptide showed a high similarity to many proteins recorded in databases as manganese transport transcriptional regulators, MntR; the greatest similarity was to a MntR protein (accession number WP_003474595.1) from *Xanthomonas translucens* (*E* value = 6×10^{-45}; 61% amino acid identity). ORF2 (nt 910-2184) stretched upstream of ORF1 but in the opposite orientation (Figure 2a), with a size of 1275 nt. It encodes a protein of 424 amino acids with a deduced molecular mass of 45 227 Da and a theoretical isoelectric point of 9.036. The deduced polypeptide showed high similarity to many proteins recorded in the GenBank database as manganese/iron transporters from the NRAMP family or as MntH proteins. The highest similarity (82% identical amino acids) was to a MntH protein from *P. psychrotolerans* (accession number WP_007160634.1). Analysis of the ORF2-encoded protein secondary structure was predicted to contain ten transmembrane domains (TM1 to TM10 in Figure 2b), indicating that it could be classified as an integral membrane protein. Both data strongly suggested that ORF2 encoded a typical MntH protein, and accordingly this ORF was renamed as a *mnt*H gene. Downstream of ORF2 (nt 2270–4025) an incomplete ORF3 was located. The deduced polypeptide exhibited a high similarity to many bacterial proteins recorded in databases as outer membrane (OM) TonB-dependent receptors. TonB-dependent

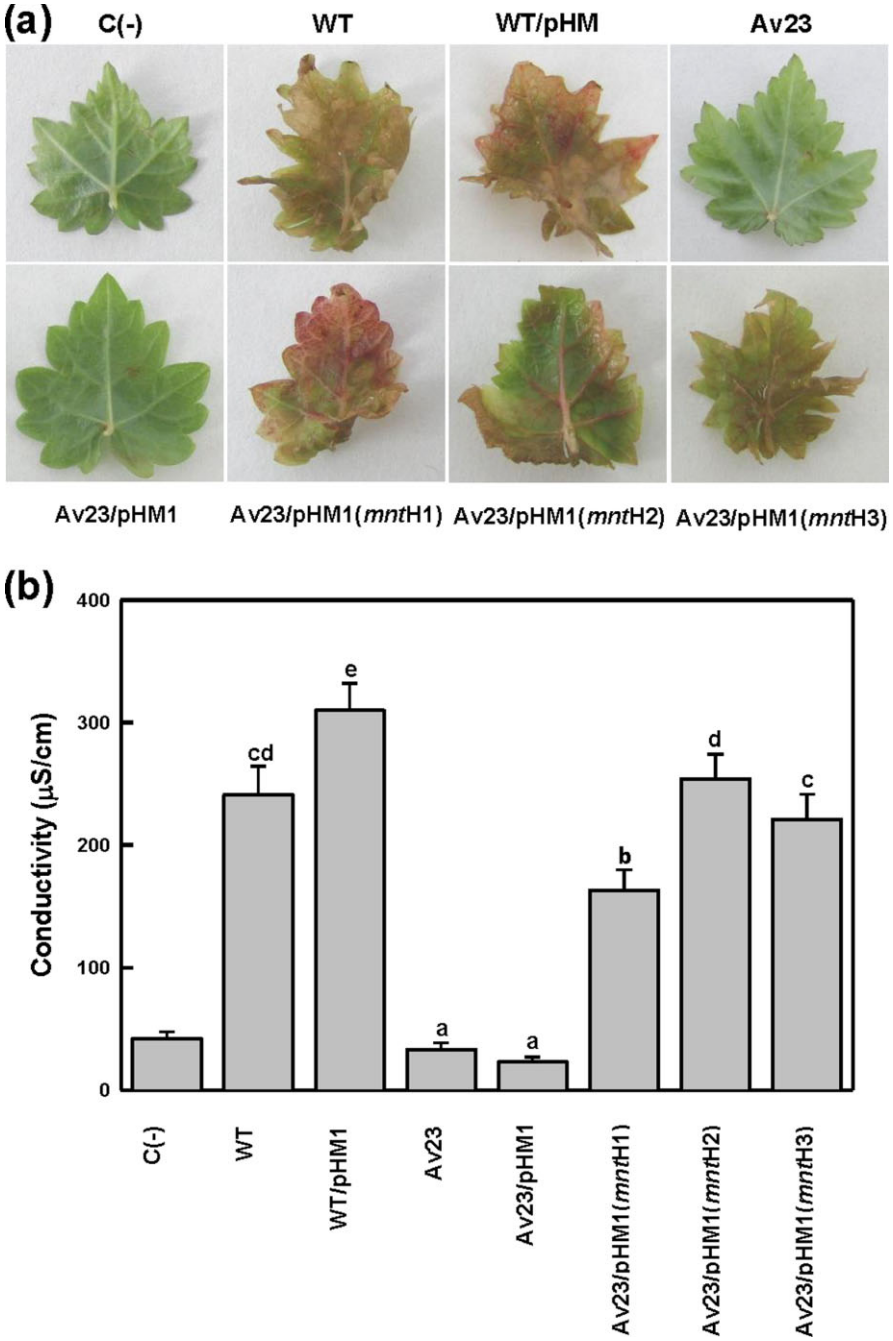

Figure 3. Injuries produced on leaves by *Xylophilus ampelinus* wild-type (WT), the avirulent mutant Av23, their respective transformants with pHM1 vector (WT/pHM1 and Av23/pHM1) and the *mnt*H gene-complemented strains Av23/pHM1(*mnt*H1), Av23/pHM1(*mnt*H2) and Av23/pHM1(*mnt*H3) as (a) visible lesion formation on leaves and (b) quantification of the necrosis by electrolyte leakage. Data shown correspond to mean ± standard deviation (SD) from three independent experiments. Bars marked with the same letter do not differ at $P = 0.05$.

receptors are bacterial OM proteins that bind and transport ferric chelates, called siderophores, as well as vitamin B12, nickel complexes and also some carbohydrates. The greatest similarity was exhibited to a TonB-dependent receptor (accession number YP_001355379.1) of *Janthinobacterium* sp. (60% amino acid identity). Bioinformatic analysis of the intergenic region between ORF1 (putative *mnt*R) and ORF2 (putative *mnt*H) revealed the existence of a palindromic sequence with high similarity to putative MntR binding sites (MntR box) located in several promoters of Mn^{++}-regulated genes (Figure 2c).

Reduced virulence of Av23 mutant is reversed by complementation of mntH gene

In order to confirm the validity of the preliminary data from the Av23 mutant suggesting that inactivation of the *mnt*H gene led to reduced virulence, the Av23 mutant was complemented by introduction of an *X. ampelinus* DNA fragment containing the full *mnt*H gene. The gene was cloned into broad-host range vector pHM1. Three different transformants [*X. ampelinus* Av23/pHM1(*mnt*H1), Av23/pHM1(*mnt*H2) and Av23/pHM1(*mnt*H3)] were randomly selected and their virulence tested. As shown in Figure 3, introduction of a copy of the *mnt*H gene into the Av23 mutant resulted in a clear recovery of the virulence capability ranging from an average of 67.6% [clone Av23/pHM1(*mnt*H1)], 105.4% [clone Av23/pHM1(*mnt*H2)] and 91.7% [clone Av23/pHM1(*mnt*H3)] compared with the WT strain, according to electrolyte leakage tests. The significant data obtained ($P = 0.05$) strongly supported the role of the MntH protein as a pathogenicity factor in *X. ampelinus* and its involvement in virulence.

MntH protein is involved in Mn^{++} *uptake in* Xylophilus ampelinus

Sequence similarity data strongly suggested that ORF2 might be the *mnt*H gene from *X. ampelinus* encoding an Mn/Fe

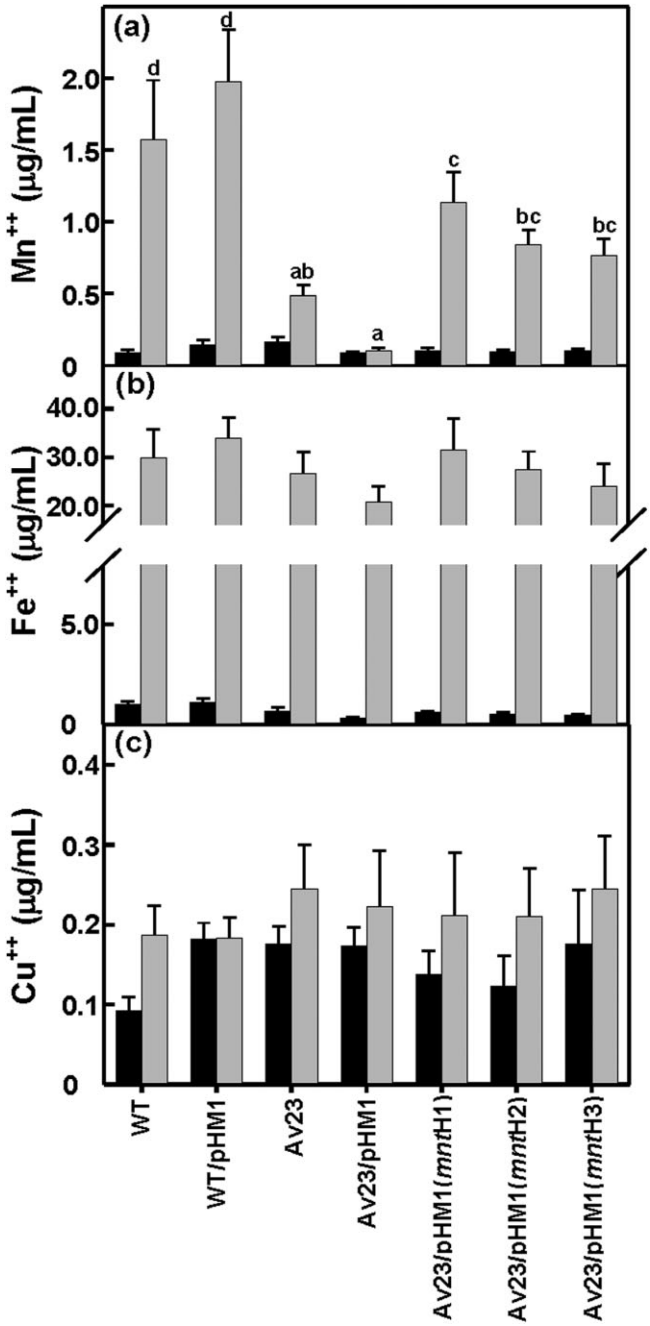

Figure 4. Intracellular content of Mn^{++}, Fe^{++} and Cu^{++} in *Xylophilus ampelinus* wild-type (WT), the avirulent Av23 mutant and their different transformants. Strains were grown in trypticase soy broth (TSB) (■) or TSB supplemented with either (a) 0.15 mmol Mn^{++}, (b) Fe^{++}, or (c) Cu^{++} (■). Data shown correspond to mean ± standard deviation (SD) from three independent experiments. Bars marked with the same letter do not differ at $P = 0.05$.

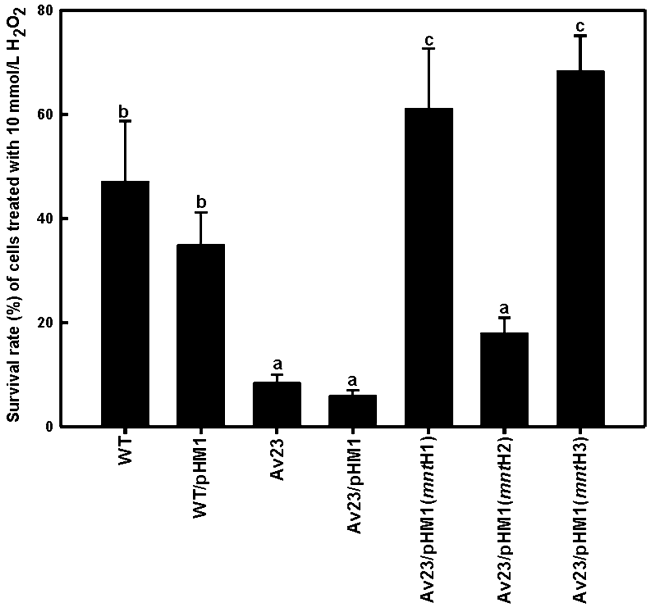

Figure 5. Viability of *Xylophilus ampelinus* wild-type (WT), the avirulent Av23 mutant and their different transformants under oxidative stress (treatment with 10 mmol H_2O_2). Data represented correspond to mean ± standard deviation (SD) from three independent experiments. Bars marked with the same letter do not differ at $P = 0.05$.

transporter. To confirm this, we analysed the ability of the Av23 mutant and the three complemented transformants to import Mn^{++}, Fe^{++} and Cu^{++}, and we compared it with the ability of the WT strain to accumulate these cations in the cytoplasm. The data presented in Figure 4a clearly showed that when the mutant Av23 strain was grown in the presence of 0.15 mmol Mn^{++}, the intracellular concentration of this cation was significantly ($P = 0.05$) reduced (68.9% reduction) compared with that detected in the WT strain. The significant ($P = 0.05$) reduction observed was higher (94.6%) when the WT and Av23 strains containing the pHM1 vector were compared (Figure 4a). The ability to import Mn^{++} was partially restored when a copy of the *mnt*H gene was introduced into the complemented transformants Av23/pHM1(*mnt*H1), Av23/pHM1(*mnt*H2) and Av23/pHM1(*mnt*H3). No clear differences, however, were observed in the intracellular concentration of Fe^{++} (Figure 4b) and Cu^{++} (Figure 4c) in the different strains analysed. These results are logical because although MntH can act as an iron transporter, its depletion can be counteracted by other major iron transporters existing in the cell. As expected, our data indicated that MntH does not have a known role in Cu^{++} transport.

Reduced survival of Av23 mutant in the presence of hydrogen peroxide even though catalase and SOD activities are not affected

Manganese is known to play a significant role in resistance to oxidative stress in many bacteria (Jakubovics et al. 2002, Anjem et al. 2009, Li et al. 2011). Consequently, we speculated that a deficiency in MntH protein, which diminishes the cytoplasmic concentration of Mn^{++}, could make the cells more sensitive to oxidative stress. Compared with WT, the Av23 mutant showed a significant ($P = 0.05$) decrease (82.4%) in cell viability in response to H_2O_2 treatment (Figure 5). Cell viability was increased in the complemented mutants Av23/pHM1(*mnt*H1) and Av23/pHM1(*mnt*H3) to a level ($P = 0.05$) higher than that measured for WT, whereas it was partially restored in transformant Av23/pHm1(*mnt*H2) (Figure 5). These data clearly showed that the MntH protein is somehow important to ensure viability in cells exposed to oxidative stress. When the activitiy of catalase and SOD, however, was measured in all the strains tested, no significant difference was obtained (data not shown).

Discussion

In spite of the great threat that *X. ampelinus* represents to vineyards worldwide, our current knowledge about how this bacteria develops its pathological process is limited. This scarce

knowledge is partly due to: (i) the absence of a fast and reliable test to check its virulence; and (ii) the limited tools available for genetic modification of this pathogen. Our work has attempted to overcome both limitations.

First, we developed a virulence test based on the inoculation of leaves from in vitro grapevine cultures. After inoculation, virulent strains were able to produce necrotic injuries, frequently seen as discoloured and wilted areas on the leaves. Using this methodology, injuries are clearly observed 7–12 days after inoculation. The extent of the necrotic areas was quantified easily by an electrolyte leakage assay (Ottman et al. 2009). We also tested this methodology on grapevine leaves of adult plants, but unfortunately, the development of lesions did not appear to follow a regular pattern, and so this material was not used in pathogenicity tests. Leaves from in vitro grapevine cultures, however, have another important advantage, in that suitable testing material is available at any time of the year.

Second, we demonstrated that the commercial mutagenesis system used, based on a transposome (Goryshin et al. 2000), can efficiently generate transposon mutants of *X. ampelinus*, and thus is a valuable tool for isolating mutant strains. This system was previously used to generate mutants in other plant pathogens, such as *Xanthomonas oryzae* and *X. campestris* (Sun et al. 2003), *X. citri* (Yan and Wang 2011) or even the grapevine pathogen *Xylella fastidiosa* (Guilhabert et al. 2001), among others. The main advantages of this mutagenesis system are that the transposon insertion occurs at random sites in the bacterial genome and that because the transposon used lacks the transposase gene, it can no longer transpose once inserted into the chromosome, which results in genetically stable mutants (Goryshin et al. 2000). The ability to insert itself in a random way led us to identify several genes whose inactivation produced different kinds of mutants. The hypervirulent Hv3 mutant resulted from transposon insertion into a gene encoding a transcriptional regulator of the LysR family. This protein family represents the most abundant type of transcriptional regulator in the prokaryotic kingdom. Despite considerable conservation, both structurally and functionally, LysR-type transcriptional regulators regulate a wide and diverse set of genes, including those involved in virulence, metabolism, quorum sensing and motility (Maddocks and Oyston 2008). Because inactivation of a gene encoding a LysR transcriptional regulator in *X. ampelinus* resulted in a hypervirulent phenotype, it can be hypothesised that this gene acts as a repressor of a pathway involved in the pathology process. The inactivation of a gene encoding a putative YD repeat-containing protein, which resulted in a hypervirulent phenotype (mutant Hv32) in our assay, was intriguing; YD repeat-containing proteins belong to a family of proteins characterised by tandem repetitions of the tyrosine-aspartic acid dipeptide. In spite of the fact that these proteins are widely distributed in bacteria and eukaryotes, their biological functions are poorly understood. Recently, YD repeat-containing proteins from diverse bacteria were reported to share a common function in contact-dependent growth inhibition (Koskiniemi et al. 2013). Inactivation of a gene encoding a YD repeat-containing protein in Hv32 might have resulted in loss of the growth inhibition mechanism leading to an increased ability of the mutant to overgrow and colonise vegetal tissues. Conversely, inactivation of a gene encoding a putative tyrosine phosphatase (mutant Av16) resulted in an avirulent phenotype. The role of tyrosine phosphatases in virulence of phytopathogenic bacteria is not well understood, although it could be exerted by controlling the biological activity of type III secretion system effectors (Block and Alfano 2011).

The avirulent mutant Av23 was generated by transposon insertion into a gene encoding the Mn^{++} transporter protein MntH. Acquisition of transition metal ions is critical for normal cell metabolism and also plays an important role in pathogen virulence. Among metals, Mn^{++} is an important cofactor for several enzymes, especially enzymes (like SOD) involved in protection against oxidative stress, and is required for virulence in many pathogens infecting animal cells (Papp-Wallace and Maguire 2006). The putative role of Mn^{++}, however, in the virulence of phytopathogens has remained elusive until recently when Li et al. (2011) showed that a novel manganese efflux system (YebN) is required for virulence in *X. oryzae*. Our work confirms the significant role of Mn^{++} in the virulence of *X. ampelinus*. Transposon-mediated inactivation of the *mnt*H gene resulted in a significant loss of virulence, which was restored when a copy of the WT *mnt*H gene was reintroduced into the mutant strain. Interruption of the *mnt*H gene also produced a marked decrease in the ability of the mutant strain to accumulate intracellular Mn^{++}. These data, together with the high homology of the encoded protein to members of the NRAMP H^{+}- Mn^{++} transporter family, confirmed that the transposon-interrupted ORF2 is the *mnt*H gene of *X. ampelinus* and encoded a major Mn^{++} transporter.

The Av23 mutant also demonstrated a significant decrease in cell viability after exposure to H_2O_2. This is an important and remarkable finding because it could imply a higher sensitivity of the Av23 mutant to reactive oxygen species (ROS) produced by grapevine cells upon infection. Recently, Anjem et al. (2009) confirmed that *E. coli* cannot tolerate peroxide stress when the *mnt*H gene is inactivated. For a long time, it was thought that manganese might act inside the cell as a chemical scavenger of ROS: superoxide, hydrogen peroxide or both (Seib et al. 2004). More recent results, however, have confirmed that manganese is not an efficient scavenger of ROS and instead is involved in the OxyR response to H_2O_2 in *E. coli* (Anjem et al. 2009). According to our data, the intracellular level of SOD and catalase activities was not affected in Av23 mutants. This suggested that loss in cell viability could not be related to detoxification of ROS forms, as would be expected, because cellular activities were not affected. In fact, as shown in Figure 4, the Av23 mutant strain is able to accumulate a basal level of Mn^{++} that could be sufficient to maintain a normal cellular level of the activity of both enzymes. These data were intriguing especially because SOD activity relies heavily on Mn^{++}-dependent superoxide dismutase (SodA). The importance of SodA in virulence has been investigated in several bacterial species, but results have been contradictory. Superoxide dismutase is essential for virulence in several human pathogens, such as *Yersinia enterocolitica*, *Streptococcus pneumoniae* and the phytopathogen *Erwinia chrysanthemi*. But it appears to have no effect on virulence in other bacteria, such as *E. coli*, *Pseudomonas aeruginosa* or *Staphylococcus aureus* (Papp-Wallace and Maguire 2006). This led us to consider how this metal might influence cell viability. Li et al. (2011) recently postulated that manganese and manganese transporters may regulate membrane stability. This hypothesis is based on evidence that integral membrane proteins (like MntH) affect membrane stability (Van Dort et al. 2001). Also, manganese is involved in the regulation of some enzymes catalysing the biosynthesis of polysaccharides and phospholipids, which are essential components of the cell envelope (Kehres and Maguire 2003). Putting all theses data together, Li et al. (2011) reported that the intracellular level of manganese could be important in regulating the bacterial hypo-osmotic response in the intercellular spaces of plants.

Finally, we would like to point out that the characterisation of pathogenicity factors could provide new insights into putative targets for controlling bacterial necrosis in grapevines. We have shown that MntH protein is required for virulence in *X. ampelinus*. Because the Av23 mutant showed a low intracellular Mn^{++} concentration and an increased sensitivity to H_2O_2, we can speculate if a restriction in the Mn^{++} concentration inside the plant, especially in the vascular tissue, where the bacteria mainly develops (Grall and Manceau 2003), might result in a low survival rate. Further studies are required to know how to proceed in order to drastically reduce the Mn^{++} concentration in the vascular tissue, without compromising plant activity, for example, by providing the plant at the end of the growing season, a foliar fertiliser comprising a Mn^{++} chelating agent.

Conclusions

A mutant with an interrupted *mnt*H gene (Av23 mutant) showed a reduced ability to accumulate intracellular Mn^{++}. Consequently we concluded that the *mnt*H gene of *X. ampelinus* encodes the major transporter for manganese uptake. MntH protein plays a significant role in the virulence of *X. ampelinus*. Indeed, its inactivation resulted in an avirulent phenotype. Virulence, however, was restored when a copy of the *mnt*H gene was reintroduced into the Av23 mutant strain. A deficiency in the MntH protein also resulted in higher sensitivity of the mutant to H_2O_2 even though the cellular activity of SOD and catalase was not affected.

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