**Rhizobium etli** HrpW is a pectin-degrading enzyme and differs from phytopathogenic homologues in enzymically crucial tryptophan and glycine residues

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While establishing a nitrogen-fixing symbiosis with leguminous plants, rhizobia are faced with the problem of penetrating the plant cell wall at several stages of the infection process. One of the major components of this barrier is pectin, a heteropolysaccharide composed mainly of galacturonic acid subunits. So far, no enzymes capable of degrading pectin have been isolated from rhizobia. Here, we make an inventory of rhizobial candidate pectinolytic enzymes based on available genome sequence data and present an initial biochemical and functional characterization of a protein selected from this list. **Rhizobium etli** *hrpW* is associated with genes encoding a type III secretion system, a macromolecular structure that allows bacteria to directly inject so-called effector proteins into a eukaryotic host’s cell cytosol and an essential virulence determinant of many Gram-negative pathogenic bacteria. In contrast to harpin HrpW from phytopathogens, *R. etli* HrpW possesses pectate lyase activity and is most active on highly methylated substrates. Through comparative sequence analysis, three amino acid residues crucial for the observed enzymic activity were identified: Trp192, Gly212 and Gly213. Their importance was confirmed by site-directed mutagenesis and biochemical characterization of the resulting proteins, with the tryptophan mutant showing no detectable pectate lyase activity and the double-glycine mutant’s activity reduced by about 80%. Surprisingly, despite *hrpW* expression being induced specifically on the plant root surface, a knockout mutation of the gene does not appear to affect symbiosis with the common bean *Phaseolus vulgaris*.

**INTRODUCTION**

Rhizobia are a group of soil-dwelling, Gram-negative bacteria that can induce and infect specialized organs on the roots of leguminous host plants. Inside these so-called root nodules, the bacteria can fix atmospheric nitrogen for the host’s benefit in exchange for plant-derived carbon sources (Gage, 2004). Upon detection of flavonoids indicative of a compatible host, rhizobia chemotactically migrate towards the plant roots. During root surface colonization, the bacteria secrete Nod factors. These lipochitooligosaccharides cause the root cortex cells to reinitiate cell division, giving rise to nodule primordia, while simultaneously eliciting early Nod factor responses such as calcium spiking and root hair cytoskeleton modification. Root hair curling allows the attached bacteria to settle within the deformation and enter an invagination in the cell membrane, called the infection thread, that grows inwardly toward the base of the root hair and nodule primordium. Once they reach the dividing nodule cells, the bacteria exit from the infection thread by a process resembling endocytosis. They subsequently undergo physiological, structural and morphological changes and differentiate into nitrogen-fixing bacteroids.

During root infection and invasion, there are at least two stages at which the plant cell wall must be breached (Brewin, 2004; Gage, 2004). First, during infection thread initiation. This happens when bacteria become entrapped between two root hair cell walls. Infection thread ingrowth is believed to start after localized cell wall degradation at the site of infection. Secondly, during infection thread exit. As infection threads are bounded by plant cell wall material such as cellulose and pectin, this barrier has to be passed prior to rhizobial endocytosis and symbiosome
development. For decades, a role has been proposed in either of these processes for rhizobial plant cell wall degrading enzymes (e.g. Hubbell et al., 1978; van Spronsen et al., 1994; Mateos et al., 2001). However, bacterial enzymes capable of breaking down plant cell wall material have long eluded identification, and induction of degradative host enzymes for remodelling the plant cell wall seemed just as likely. Only recently, Robledo et al. (2008) succeeded in purifying and characterizing a cell-bound cellulase from *Rhizobium leguminosarum*. This CelC2 enzyme can erode the cell wall of host root hairs and is essential for establishing a symbiosis with the *R. leguminosarum* host plant, white clover (*Trifolium repens*). Furthermore, cellulase activity was detected in a wide range of rhizobia, indicating that the production of plant cell wall degrading enzymes is characteristic of rhizobia in general. As the primary cell walls of dicotyledonous plants are composed not only of (hemi)cellulose, but contain 20–35 % pectin as well (O’Neill & York, 2003), we reasoned that enzymes capable of degrading pectic polysaccharides might also contribute to the symbiotic process. In this study, we set out to identify and characterize the first such rhizobial pectinolytic enzyme.

### METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was routinely grown at 37 °C in Luria–Bertani (LB) medium in the presence of the appropriate antibiotics: ampicillin (Ap, 100 μg ml⁻¹), gentamicin (Gm, 50 μg ml⁻¹), kanamycin (Km, 25 μg ml⁻¹) and tetracycline (Tc, 10 μg ml⁻¹). *Rhizobium etli* was cultured at 30 °C in liquid tryptone yeast (TY) medium supplemented with 7 mM CaCl₂ and stored on yeast extract mannitol (Vincent, 1970) agar plates. The following antibiotics were used: nalidixic acid (Nal, 30 μg ml⁻¹), neomycin (Nm, 40 μg ml⁻¹) and tetracycline (1 μg ml⁻¹).

**Construction of a hrpW mutant.** A *R. etli* hrpW mutant was constructed by amplifying the *hrpW* gene from genomic *R. etli* DNA by PCR (primer sequences available upon request), cloning into pCRII-TOPO and sequencing. The cloned fragment was subcloned into pUC18Not. An interposon fragment conferring kanamycin resistance was isolated from pHP45ΩKm and ligated into the EcoRI site of *hrpW*, orientated in the opposite direction to the *hrpW* ORF. The *hrpW::ΩKm* fragment of this construct was isolated and ligated into pJQ200SK, yielding pCMPG8211. This suicide construct was conjugated to the wild-type *R. etli* strain and, by means of homologous recombination as described previously (D’Hooghe et al., 1997), gave rise to the *hrpW* mutant CMPG8200. Correct recombination was confirmed by Southern hybridization.

#### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOP10</td>
<td>F⁻ mcrA Δ(mrr–hsdRMS–mcrBC) ΔlacX74 deoR recA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>BL21-CodonPlus (DE3)-RP</td>
<td>E. coli B F⁻ ompT hsdS Δ(r6 m1596) dcm T104 galK (ΔE3) endA Hte</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>R. etli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNAF512</td>
<td>NaB² wild-type</td>
<td>Michiels et al. (1998)</td>
</tr>
<tr>
<td>CMPG8200</td>
<td>NaB² Nm² hrpW::ΩKm</td>
<td>This work</td>
</tr>
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<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pCR4Blunt-TOPO</td>
<td>ApR⁷ KmR⁷ cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pET-24(+)</td>
<td>KmR⁷ expression vector with His, tag coding region at 3’ end of polylinker</td>
<td>Novagen</td>
</tr>
<tr>
<td>pCMPG8218</td>
<td>KmR⁷ full-length HrpW cloned into pET24(+)</td>
<td>This work</td>
</tr>
<tr>
<td>pCMPG8219</td>
<td>KmR⁷ HrpW1–103 cloned into pET24(+)</td>
<td>This work</td>
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<td>pCMPG8220</td>
<td>KmR⁷ HrpW104–384 cloned into pET24(+)</td>
<td>This work</td>
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<td>pCMPG8242</td>
<td>KmR⁷ HrpW W192A cloned into pET24(+)</td>
<td>This work</td>
</tr>
<tr>
<td>pCMPG8243</td>
<td>KmR⁷ HrpW G212N G213S cloned into pET24(+)</td>
<td>This work</td>
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<tr>
<td>pFAI1703</td>
<td>ApR⁷ TcR⁷ broad-host-range plasmid with promoterless gusA divergently orientated from polylinker</td>
<td>Dombrecht et al. (2001)</td>
</tr>
<tr>
<td>pCMPG8213</td>
<td>hrpW promoter region (P::hrpW) cloned upstream of gusA into pFAI1703</td>
<td>This work</td>
</tr>
<tr>
<td>pCRII-TOPO</td>
<td>ApR⁷ KmR⁷ cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pUC18Not</td>
<td>ApR⁷ cloning vector</td>
<td>Herrero et al. (1990)</td>
</tr>
<tr>
<td>pHP45ΩKm</td>
<td>ApR⁷ KmR⁷ source of KmR⁷ cassette</td>
<td>Fellay et al. (1987)</td>
</tr>
<tr>
<td>pJQ200SK</td>
<td>GmR⁷ sacB suicide vector</td>
<td>Quandt &amp; Hynes (1993)</td>
</tr>
<tr>
<td>pCMPG8211</td>
<td>GmR⁷ KmR⁷ sacB pJQ200SK carrying RHI-388/389 hrpW fragment with KmR⁷ cassette cloned into EcoRI site of hrpW, opposite orientation of hrpW</td>
<td>This work</td>
</tr>
<tr>
<td>pRK2073</td>
<td>SpR⁷ CoLE1 helper plasmid for triparental conjugation</td>
<td>Figurski &amp; Helinski (1979)</td>
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</table>
Construction of a transcriptional PhrW–gusA fusion. A transcriptional fusion between the putative hrpW promoter region (PhrW) and a promoterless gusA reporter gene was constructed as follows. The region was amplified by PCR with Platinum Pfx polymerase (primer sequences available upon request) from genomic *R. etli* DNA. The fragment was cloned into pCR4Blunt-TOPO, confirmed by sequencing and subcloned into pFAJ1703, resulting in pCMPG8213.

Construction of HrpW overexpression plasmids. The full-length hrpW ORF was amplified by PCR with Platinum Pfx polymerase (primer sequences available upon request), cloned into pCR4Blunt-TOPO, confirmed by sequencing and subcloned into pET-24(+) yielding pCMPG8218. Similarly, plasmids expressing HrpW1–103 (pCMPG8219) and HrpW104–303 (pCMPG8220) were constructed.

Expression analysis. *R. etli* cells were grown overnight in TY medium, washed in 10 mM MgSO₄, and resuspended in defined AMS medium (Michiels et al., 1998) in the presence of possible inducers. Cultures were incubated either aerobically or microaerobically in stopped tubes in the presence of an oxygen concentration of 0.3% as described previously (D’Hoooge et al., 1995). GusA expression assays were carried out using p-nitrophenyl β-D-glucuronide as a substrate for β-glucuronidase as described earlier (D’Hoooge et al., 1995). Expression on the plant root surface was assayed as described by Xi et al. (2001).

Plant experiments. Plant culture and bacteroid isolation were carried out as described previously (Dombrecht et al., 2002). Nitrogen fixation activity was determined by an acetylene reduction assay using a Hewlett Packard 5890A gas chromatograph equipped with a PLOT fused silica column.

Protein overexpression and purification. BL21-CodonPlus(DE3)-RP *E. coli* cells were transformed with the pET-24(+) derivatives listed in Table 1. Native recombinant proteins were obtained essentially as described by Dombrecht et al. (2005). However, a standard induction period of 4 h at 37 °C was used prior to harvesting. Supernatant proteins were isolated as described by Xi et al. (2000). Polyclonal antibodies directed against *R. etli* HrpW were raised by immunization of rabbits with purified recombinant HrpW-His6. Antibody production was carried out by Eurogentec using a silica column. Initial immunization was followed by booster injections after 14, 28 and 50 days. Serum was collected 65 days after the first injection.

Site-directed mutagenesis. W192A and G212N G213S mutants of HrpW were obtained by inverse PCR on pCMG8218 with Platinum Pfx DNA polymerase and different sets of mismatch primers (sequences available upon request). The mutations were confirmed by DNA sequencing, after which the mutated ORFs were transferred again into pET-24(+).

Protein analysis. Proteins were separated by SDS-PAGE [10% Bistris NuPage gel (Invitrogen) with 2-(N-morpholino)ethanesulfonic acid buffer as per the manufacturer’s instructions] and analysed by Western hybridization as described by Harlow & Lane (1998) using either monoclonal anti-His6 antibodies (Roche) or polyclonal anti-HrpW antibodies as primary antibodies.

Pectate lyase activity assay. Purified proteins were dialysed against 50 mM Tris/HCl pH 8.0 prior to functional characterization. Pectate lyase activity was measured by monitoring absorbance at 235 nm in a solution composed of a suitable enzyme dilution added to 50 mM Tris/HCl pH 8.0, 0.1% (w/v) pectic substrate and 1 mM CaCl₂, unless stated otherwise. Reactions were carried out at room temperature.

Bioinformatic analyses. Database searches of the sequence data were performed by using the BLAST program (Altschul et al., 1997) on the servers of the National Centre for Biotechnology Information (NCBI).

Phylogenetic analysis was performed using the fragments equivalent to Pfam entry PF03211 of the selected pectate lyases as delineated by CD-Search and the Conserved Domain Database (Marchler-Bauer & Bryant, 2004; Marchler-Bauer et al., 2007). Analyses were conducted using the software package MEGA4 (Tamura et al., 2007). Sequences were aligned with CLUSTAL W (Thompson et al., 1994). Neighbour-joining and minimal-evolution trees were constructed from this alignment using bootstrap testing.

For the identification of amino acids that differ between *R. etli* HrpW and characterized pectate lyases on the one hand and HrpW homologues from phytopathogens on the other, a multiple sequence alignment was constructed. Sequences were aligned using CLUSTAL W (Thompson et al., 1994) and manually edited using GeneDoc (Nicholas et al., 1997).

RESULTS

Survey of rhizobial genomes for candidate enzymes

Pectinolytic enzymes can degrade pectic substances either through hydrolysis (hydrolases) or trans-elimination (lyases) (Jayani et al., 2005). To estimate the genetic potential of fully sequenced rhizobia to encode such proteins, the carbohydrate-active enzymes (CAZY) database (Cantarel et al., 2009) was searched for relevant matches. A total of 15 deduced protein sequences were retrieved from CAZY families glycosyl hydrolase (GH) 28 and polysaccharide lyase (PL) 1, 2, 3, 9 and 10 (Table 2). *R. etli* CNF42, *R. etli* CIAT652, *Rhizobium* sp. NGR234 and *R. leguminosarum* biovars trifolii and viciae are all predicted to encode related polygalacturonases. In addition, both the *R. etli* CIAT652 and *R. leguminosarum* biovars trifolii genomes contain genes encoding similar haemolysin-type calcium-binding proteins classified as PL9 family members. Another PL9 member is *Mesorhizobium* sp. BNC1 meso_0099. Both *Sinorhizobium melloti* and *Sinorhizobium medicae* encode predicted pectate lyases similar to proteins found in phylogenetically distant bacteria. The *S. medicae* genome also contains a gene similar to *Pectobacterium chrysanthemi* pectate lyase peLE, while other proteins with sequence similarity to pectate lyase proteins from *Pectobacterium* spp., PehZ and PehA, respectively, are found in *R. etli* CIAT652 and *Bradyrhizobium japonicum*. Interestingly, the corresponding *B. japonicum* gene bbl1993 was earlier reported to be inducible by genistein, suggesting a role during early symbiosis (Caldelari Baumberger et al., 2003). Finally, the genomes of *R. etli* CNF42 and CIAT652 contain genes encoding proteins homologous to PL3 pectate lyases from bacteria, nematodes and fungi, and HrpW from plant-pathogenic bacteria such as *Pectobacterium carotovorum* and *Pseudomonas syringae*. Conceptual translation of publicly available nucleotide sequences from 10 geographically diverse *R. etli* strains (Flores et al., 2005) revealed a strong conservation of HrpW, with 93–100%
identical amino acid residues compared to the type strain CFN42. Moreover, *R. etli hrpW* is part of a genomic locus predicted to encode a type III secretion system (T3SS), a protein secretion system specifically used in host–microbe interactions and previously shown to contribute to symbiotic nitrogen fixation (Marie et al., 2001; Fauvart & Michiels, 2008). We therefore selected *R. etli* HrpW for further investigation.

### Biochemical characterization

HrpW is modular in nature, with a pectate lyase domain located at the carboxy-terminus. Full-length HrpW, the amino-terminal fragment HrpW^{1–103} and the carboxy-terminal part HrpW^{104–304} were individually overexpressed and purified. Clear overexpression bands were obtained for all three constructs (data not shown).

The carboxy-terminally located domain shares similarity with pectate lyases belonging to PL family 3. Generally, pectate lyases (EC 4.2.2.2) degrade polygalacturonic acid (PGA) through a Ca^{2+}-dependent β-elimination mechanism, producing unsaturated oligogalacturonates in the process (Benen & Visser, 2003). These reaction products exhibit strong absorbance at 230–235 nm, allowing the identification and quantification of β-eliminative cleavage of pectic substances.

Purified and dialysed proteins were used for enzymic characterization. As reported pH optima of pectate lyases vary between 7.5 and 10.5, an intermediate value was arbitrarily chosen for initial characterization. Substrates were added at a concentration of 0.1 % (w/v), Ca^{2+} was provided in the form of 1 mM CaCl\textsubscript{2}. To detect enzymic activity, absorbance was measured at 235 nm at various time intervals.

Full-length HrpW, HrpW^{1–103} and HrpW^{104–304} were assayed for their ability to degrade PGA. In contrast to HrpW of *Erwinia amylovora* and *Ps. syringae* (Charkowski et al., 1998; Kim & Beer, 1998), *R. etli* HrpW gave rise to detectable pectate lyase activity. As expected, this activity was also found in the carboxy-terminal HrpW fragment containing the pectate lyase domain, whereas the amino-terminal part tested negative (data not shown). Next, the substrate specificity was studied. HrpW showed increased activity with an increasing degree of substrate esterification, with degradation of 93 % esterified pectin resulting in a threefold higher absorbance at 235 nm compared to PGA (Fig. 1A). HrpW^{104–304} produced similar results, while HrpW^{1–103} failed to degrade any of the substrates (data not shown). Subsequent tests were carried out with 93 % esterified pectin as the substrate.

In contrast to pectin lyases, pectate lyases exhibit an absolute Ca^{2+} requirement for enzymic activity (Benen & Visser, 2003). This feature was verified by adding 2 mM EDTA, a calcium-chelating agent. No reaction was detected in the absence of Ca^{2+}, confirming the classification of HrpW as a pectate lyase (data not shown). The influence of Ca^{2+} ions was further investigated by determining enzymic activity at varying concentrations of CaCl\textsubscript{2} (Fig. 1B). Under the conditions used, the optimal Ca^{2+} concentration is approximately 2 mM, but over 90 % of maximal activity was

### Table 2. Overview of rhizobial genes encoding possible pectinolytic enzymes

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene name/locus tag</th>
<th>Accession no.</th>
<th>CAZy family</th>
<th>Predicted function</th>
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</thead>
<tbody>
<tr>
<td><em>Bradyrhizobium japonicum</em></td>
<td>bbr1993</td>
<td>NP_768633</td>
<td>GH28</td>
<td>Endopolygalacturonase</td>
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<td>USDA110</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Mesorhizobium</em> sp. BNC1</td>
<td>meso_0099</td>
<td>YP_672669</td>
<td>PL9</td>
<td>Pectate lyase/exopolygalacturonate lyase</td>
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<tr>
<td><em>Rhizobium leguminosarum</em></td>
<td>pRL120688</td>
<td>YP_765192</td>
<td>GH28</td>
<td>Pectate lyase</td>
</tr>
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<td>NP_659975</td>
<td>PL3</td>
<td>Polygalacturonase</td>
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<tr>
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<td>YP_472911</td>
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<td>Polygalacturonase</td>
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<td>RHECIAT_CH0001980</td>
<td>YP_001978119</td>
<td>PL9</td>
<td>Haemolysin-type calcium-binding protein</td>
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<tr>
<td><em>Rhizobium leguminosarum</em></td>
<td>RHECIAT_PB0000094</td>
<td>YP_00198457</td>
<td>PL3</td>
<td>Pectate lyase, similar to T3SS substrate HrpW</td>
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<td>Pectate lyase</td>
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M. Fauvart and others

Microbiology 155
retained at concentrations up to 5 mM. Residual activity was detected at 0 mM, probably due to the presence of traces of Ca\(^{2+}\) in other components used, such as the pectin.

In order to determine the optimal pH for pectin degradation by HrpW, enzymic activity was monitored in suitably buffered solutions covering pH 3–12. The highest activity was detected in a narrow pH range around pH 8.5 (Fig. 1C).

Comparative sequence analysis and site-directed mutagenesis

The unexpected finding of an enzymically active HrpW homologue prompted us to carry out an in silico analysis of the *R. etli* HrpW pectate lyase fragment. As indicated earlier, HrpW proteins are typified by a domain that shares similarity with PL family 3. This family contains pectate lyases from bacteria, fungi and nematodes. The CAZy database lists 181 sequences under PL3 (last updated December 17, 2008). Sequences of biochemically characterized proteins were extracted after an extensive literature search and merged with selected HrpW sequences retrieved from the NCBI protein database, totalling 16 sequences. Each sequence was individually aligned with Pfam entry PF03211 (‘pectate_lyase’) to delineate the domain of interest for further analysis; surrounding amino acid residues were discarded. The cured sequences were aligned with CLUSTAL W and a phylogenetic tree was constructed (Fig. 2A). Neighbour-joining and minimal-evolution methods produced nearly identical topologies, with sequences from bacteria, nematodes and fungi clustering separately except for *Erwinia chrysanthemi* Pell and *Pectobacterium carotovorum* PelB and Pel-3, which together form a fourth clade. *R. etli* HrpW groups together with homologues of phytopathogenic bacteria (42–48% sequence identity). This suggests a common ancestry, although it does not explain the discrepancy in biochemical properties. To pinpoint individual amino acids that could be responsible for this difference, the multiple sequence alignment was studied in detail (Fig. 2B). Three amino acids are conserved between characterized pectate lyases and *R. etli* HrpW but differ in HrpW of *E. amylovora* and *Ps. syringae* Trp105, Gly125 and Gly126 (Bacillus sp. KSM-P15 full-length Pel-15 nomenclature is used throughout the text). In addition, 10 fully conserved residues were identified among all sequences, six of which were previously implicated in the enzymic activity of Pel-15: His93, Glu110, Asp111, Asp133, Lys134 and Arg159 (Hatada et al., 2000; Akita et al., 2001). Other catalytically important residues include Glu65 (not shown), Asp90, Lys116 and Lys156. While the former three are conserved within the bacterial PL3 cluster, a K156T substitution is found in HrpW of *R. etli* as well as that of *E. amylovora* and *Ps. syringae*. Hence, none of the three amino acids proposed here to be essential for the observed enzymic activity have been implicated in such a role before.

The results of the above analysis were put to the test by constructing and overexpressing the corresponding *R. etli* HrpW mutants (W192A and G212N G213S, respectively). Biochemical analysis showed HrpW G212N G213S to possess about 20% of wild-type activity, while the W192A mutant did not give rise to any detectable pectate lyase activity at all (Fig. 2B), thus confirming the predictions from the in silico analysis.

Expression analysis

*R. etli* hrpW is the distal ORF of a predicted operon containing the structural T3SS gene *rhcV* (homologous to
the FlhA/YscV T3SS inner-membrane basal component) downstream of a gene encoding a hypothetical protein. To study hrpW expression, a transcriptional gusA fusion was constructed with the intergenic region upstream of this operon. Free-living growth in minimal medium resulted in approximately 150 Miller units of expression, indicating a basal level of transcription. No significant induction could be observed either in the presence of flavonoids, a known inducer of other rhizobial T3SS genes (Marie et al., 2001), under microaerobic conditions, such as found inside root nodules, or in the presence of pectic substances or galacturonic acid (data not shown). In planta hrpW gene

**Fig. 2.** (A) Phylogenetic analysis of members of polysaccharide lyase family 3. The neighbour-joining method was used to construct the tree; bootstrap values of 1000 replicates are shown at the nodes. Sequences from bacteria, nematodes and fungi cluster separately as indicated. (B) Multiple sequence alignment of the sequences shown in (A). Fully conserved residues are highlighted in black; residues that are conserved between characterized pectate lyases and *R. etli* HrpW but not HrpW of *E. amylovora* or *P. syringae* are highlighted in grey. The effect of mutating these amino acid residues on *R. etli* HrpW pectate lyase activity is shown below the alignment. Black triangles indicate residues that have been shown to be critical for enzymic activity in *Bacillus* sp. KSM-P15 Pel-15. Only residues equivalent to Bs_Pel-15 are shown. Source organisms and accession numbers are as follows. Fs_PelA, Fs_PelB, Fs_PelC and Fs_PelD: *Fusarium solani* f. sp. *pisi*, AAA33338, AAA87383, AAA87382 and AAC49420; Gr_Pel2: *Globodera rostochiensis*, AAM21970; Bx_Pel1: *Bursaphelenchus xylophilus*, BAE48369; Ec_Pell: *Erwinia chrysanthemi*, CAA73784; Pc_PelB and Pc_Pel-3: *Pectobacterium carotovorum*, CA955814 and AAA57140; Pb_PelA: *Paenibacillus barcinonensis*, CAB40884; Re_HrpW: *Rhzobium etli*, AAM54988; Aa_HrpW, *Acidovorax avenae*, BAE60672; Ea_HrpW: *Erwinia amylovora*, AAC62314; Ps_HrpW: *Pseudomonas syringae*, AA054895.
expression was evaluated qualitatively on the root surface of inoculated bean plants by staining with X-GlcA as a substrate. The wild-type strain carrying a promoterless gusA gene was used as a negative control. Microscopic examination allowed the observation of blue spots that grew increasingly dark 1–3 days after inoculation for the PhrpW construct, while no coloration was apparent for the empty gusA vector (Fig. 3), evidencing specific induction on the root surface. Finally, expression was determined in bacteroids isolated from the nodules of 21-day-old bean plants and found to be about twofold higher compared to values obtained using cells from free-living cultures (data not shown).

**Secretion and role in symbiosis**

HrpW is abundantly secreted into the culture medium by phytopathogenic bacteria grown under T3SS-inducing conditions (Charkowski et al., 1998; Kim & Beer, 1998). Therefore, secretion of _R. etli_ HrpW was probed by extracting proteins from the culture supernatant after up to 48 h of free-living growth in minimal medium. Proteins isolated from wild-type cultures and from a _hrpW_ mutant were separated by SDS-PAGE and visualized by Coomassie staining as well as Western detection using polyclonal antibodies raised against recombinant _R. etli_ HrpW. No secretion could be detected (data not shown). This procedure was repeated using intracellular proteins, similarly without successful HrpW detection (data not shown).

Next, a possible role for HrpW in the nitrogen-fixing symbiosis between _R. etli_ and its natural host, the common bean, was investigated. _Phaseolus vulgaris_ ’Limburgse vroege’ seedlings were inoculated with the wild-type strain or a _hrpW_ mutant and assayed for nitrogen fixation capacity 21 days post-inoculation. In addition, the nodule number and nodule mass were determined for each plant. Surprisingly, no statistically significant differences (P<0.05) were observed for any of these parameters (data not shown).

**DISCUSSION**

This report details the characterization of a rhizobial enzyme with pectinolytic activity. Bacterial enzymes capable of degrading plant cell wall components have long been suggested to play a crucial role in establishing the nitrogen-fixing _Rhizobium–legume_ symbiosis. Recently, Robledo _et al._ (2008) were the first to support this hypothesis with molecular and genetic evidence by identifying a cellulase gene from _R. leguminosarum_. CelC2 can erode the cell wall of host root hair tips and is essential during the initial stages of symbiosis. Inspired by this example, we compiled an inventory of rhizobial proteins possibly involved in the breakdown of pectin, which like cellulose is one of the main components of cell walls of dicotyledonous plants. _R. etli_ HrpW was selected because of its strong conservation among diverse _R. etli_ strains, a clear similarity with members of the well-characterized PL3 family and its association with a T3SS supposedly involved in the interaction with a host organism.

In contrast to previously characterized HrpW homologues from phytopathogenic bacteria (Charkowski _et al._, 1998; Kim & Beer, 1998), the _R. etli_ protein was found to be enzymatically active. In accordance with sequence similarity data, this activity was only observed for the carboxy-terminal fragment. Substrate specificity was examined by using pectin with various degrees of methyl esterification in addition to PGA. All substrates were successfully degraded by HrpW and the highest activity was found when using 93% esterified pectin. To the best of our knowledge, a similar substrate preference profile has not been reported previously for a pectate lyase. The lack of activity of other pectate lyases on highly methylated pectic substrates has been explained by the presence of methylesterases, which can lower the degree of substrate esterification and thus complement the enzymic deficiency. However, genome sequence data show no pectinesterases to be produced by _R. etli_. It is therefore likely that HrpW should be able to degrade the naturally highly esterified pectic plant cell wall components in the absence of auxiliary enzymes. Inspection of the CAZy database yields only one rhizobial candidate pectinesterase, encoded by a _B. japonicum_ gene downstream of a predicted polygalacturonase. This suggests that other rhizobial pectin-degrading enzymes might have a substrate preference similar to that of _R. etli_ HrpW.

Further characterization of HrpW showed a dependence on Ca$^{2+}$ for enzymic activity, confirming its classification as a

**Fig. 3.** In planta expression analysis of _R. etli_ _hrpW_ gene expression. _P. vulgaris_ ’Limburgse vroege’ plants were inoculated with _R. etli_ carrying either (A) a PhrpW–gusA fusion, or (B) the empty vector pFAJ1703, then grown for 2 days and stained by a β-glucuronidase assay using X-GlcA as substrate.
pectate lyase. The substrate degradation rate was maximal in the presence of 2 mM calcium and at a pH of 8.5. These values are within the range usually associated with pectate lyases (Benen & Visser, 2003).

To better understand the discordance in enzymic activity between HrpW homologues of *R. etli* and phytopathogenic bacteria, a multiple sequence alignment was constructed of the pectate lyase domains of all characterized PL3 members, together with those of a number of HrpW homologues from various plant pathogens. This allowed speculation about which amino acid residues could be responsible for the observed difference in biochemical properties. As such, three residues were identified that might account for the observations presented in this report. Their role in enzymic activity was subsequently investigated by site-directed mutagenesis. Gly125 and Gly126 (*Bacillus* sp. KSM-P15 full-length Pel-15 nomenclature) are conserved in all active PL3 pectate lyases characterized to date, but not in any of the HrpW homologues of phytopathogens. This makes for an attractive option as it offers the possibility of linking biochemical properties to biological background in a straightforward way. Indeed, the *R. etli* HrpW G212N G213S mutant protein showed a reduction in pectate lyase activity of about 80%. This is striking, as no glycine residues in the G212N mutant proteins are fully active, while mutations converting methionine to arginine were previously reported to be important for pectate lyase activity of about 80%. This is striking, as no glycine residues were previously reported to be important for pectate lyase activity, either by mutational analysis or by structure determination (Akita et al., 2001). A third possible culprit is Trp105. This residue is conserved in all PL3 family members of non-pathogenic bacteria and in HrpW of *Acidovorax avenae*. In addition, in PL3 pectate lyases of fungi and nematodes this position is occupied by either a tryptophan or a phenylalanine residue. Most importantly, Hatada et al. (2000) showed that W105F and W105Y Pel-15 mutant proteins are fully active, while mutations converting tryptophan to residues such as those found in HrpW of *E. amylovora* and *Ps. syringae* (i.e. W105V and W105A, respectively) did not result in enzymically active proteins. A role for a single, unspecified tryptophan residue in pectate lyase activity was suggested previously by Rao et al. (1996), while aromatic amino acid residues such as tryptophan and phenylalanine are also found in the substrate-binding cleft of pectin lyases (Benen & Visser, 2003). Recently, aromatic residues were shown to be involved in the recognition of oligogalacturonides by a periplasmic binding protein involved in pectin metabolism of *Yersinia enterocolitica* (Abbott & Boraston, 2007). In accordance with these earlier findings, a *R. etli* HrpW W192A mutant showed no detectable pectate lyase activity. In line with this, we predict *A. avenae* HrpW to be enzymically active, unlike other phytopathogenic *HrpW* homologues.

Despite *R. etli* HrpW being enzymically active, a *hrpW* mutant strain did not display altered symbiotic properties compared to the wild-type strain. Nitrogen fixation capacity, nodule number and nodule mass were unaffected when assessed 21 days post-inoculation on *P. vulgaris* ‘Limburgse vroege’ plants. However, expression analysis showed *hrpW* to be induced during the early stages of root infection and inside nitrogen-fixing nodules. This induction is probably due to some kind of plant-specific recognition event, as no clear expression could be obtained under free-living conditions. Accordingly, no secreted HrpW could be detected upon probing of proteins isolated from culture supernatant.

The lack of a symbiotic phenotype is reminiscent of the findings originally described for HrpW from *E. amylovora* and *Ps. syringae*, as it was reported that virulence of *hrpW* mutants in these bacteria was at wild-type level (Charkowski et al., 1998; Kim & Beer, 1998). Only recently, Kvitko et al. (2007) succeeded in shedding light on the biological role of HrpW. They did so by constructing a polymutant that is severely affected in its capacity to direct effector proteins through the host cell membrane, and by studying complementation with individual proteins supposedly involved in this activity. HrpW tested positive, albeit with only moderate efficiency. This indicates functional redundancy and a minor role for HrpW in *Ps. syringae* T3SS-dependent virulence, and could also apply to the *R. etli* homologue. Furthermore, mutations in the rhizobial T3SS and its constituents are notorious for producing host-specific effects (Marie et al., 2001; Fauvart & Michiels, 2008), and a role for *R. etli* HrpW might only become apparent in a limited set of host–microsymbiont combinations. Finally, it has been proposed that the host plant could also contribute to the remodelling of its own cell walls during symbiosis. Cell wall degradation is essential to processes such as root hair initiation, fruit ripening, pollen tube elongation and leaf abscission. In addition, symbiosis-specific expression of polygalacturonases and pectin methyl esterases was observed during the early steps of the *S. meliloti–Medicago* interaction (Munoz et al., 1998; Rodriguez-Llorente et al., 2004). So, instead of degrading plant cell walls themselves, rhizobia might induce the expression of degradative host enzymes to the same effect, and a deficiency such as that caused by mutation of *hrpW* could be masked.

In summary, the data obtained in the course of this study show for the first time that rhizobia are capable of producing pectinolytic enzymes, exemplified by *R. etli* HrpW. Although expression analysis indicates the involvement of this pectate lyase in establishing a nitrogen-fixing symbiosis with leguminous plants, no phenotypic evidence could be obtained in support of this. Further investigation into a biological role for HrpW is ongoing, while the identification of candidate pectinolytic enzymes in numerous other rhizobial genome sequences shows that much more work needs to be done in understanding the bacterial contribution to host plant cell wall penetration during the symbiotic nitrogen fixation process.

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