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Agrobacteria lacking ornithine lipids induce more rapid tumour formation

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Summary

Ornithine lipids (OLs) are phosphorus-free membrane lipids that are widespread among Gram-negative bacteria. Their basic structure consists of a 3-hydroxy fatty acyl group attached in amide linkage to the ϵ -amino group of ornithine and a second fatty acyl group ester-linked to the 3-hydroxy position of the first fatty acid. It has been shown that OLs can be hydroxylated within the amide-linked fatty acyl moiety, the secondary fatty acyl moiety or within the ornithine moiety. These modifications have been related to increased stress tolerance and symbiotic proficiency in different organisms such as *Rhizobium tropici* or *Burkholderia cenocepacia*. Analysing the membrane lipid composition of the plant pathogen *Agrobacterium tumefaciens* we noticed that it forms two different OLs. In the present work we studied if OLs play a role in stress tolerance and pathogenicity in *A. tumefaciens*. Mutants deficient in the OLs biosynthesis genes *olsB* or *olsE* were constructed and characterized. They either completely lack OLs (*olsB*) or only form the unmodified OL (*olsE*). Here we present a characterization of both OL mutants under stress conditions and in a plant transformation assay using potato tuber discs. Surprisingly, the lack of agrobacterial OLs promotes earlier tumour formation on the plant host.

Introduction

Ornithine lipids (OLs) are phosphorus-free membrane lipids that are widespread among Gram-negative bacteria and also present in some Gram-positive bacteria, especially in actinomycetes species, but seem to be absent from Archaea and Eukarya (López-Lara *et al.*, 2003; Geiger *et al.*, 2010; Vences-Guzmán *et al.*, 2012). They contain a 3-hydroxy fatty acyl group that is attached in amide linkage to the ϵ -amino group of ornithine. A second fatty acyl group is ester-linked to the 3-hydroxy position of the first fatty acid. The genes *olsB* and *olsA* encoding the two enzymes essential for OL biosynthesis from ornithine and acyl-ACPs have been first described in *Sinorhizobium meliloti* 1021 (Fig. S1) (Weissenmayer *et al.*, 2002; Gao *et al.*, 2004; Geiger *et al.*, 2010). For a long time it has been known that the ester-linked fatty acid of OLs can be hydroxylated at the C-2 position in some organisms (Asselineau, 1991). Recently, two more OL modifications have been described. In *Burkholderia cenocepacia* the amide-linked fatty acid can be hydroxylated in an unknown position by the hydroxylase OlsD (González-Silva *et al.*, 2011) and in *Rhizobium tropici* the

ornithine headgroup can be hydroxylated in a still unknown position (Vences-Guzmán *et al.*, 2011). All three modifications have been related to increased stress tolerance or symbiotic proficiency (Taylor *et al.*, 1998; Rojas-Jiménez *et al.*, 2005; González-Silva *et al.*, 2011; Vences-Guzmán *et al.*, 2011).

Although OLs are probably found in both membranes of Gram-negative bacteria, they seem to be enriched in the outer membrane (OM) as was shown in the acid-resistant species *Thiobacillus thiooxidans* and *R. tropici* (Dees and Shively, 1982; Vences-Guzmán *et al.*, 2011). It has been speculated that OL hydroxylation may increase hydrogen bonding between neighbouring OL molecules similarly as has been suggested for LpxO-hydroxylated lipid A in *Salmonella* and hydroxylated sphingolipids (Gibbons *et al.*, 2000; Nikaido, 2003; Murata *et al.*, 2007). These additional hydrogen bonds should result in bilayer stabilization and a decrease in membrane permeability which could help to explain the observed stress resistance phenotype related to hydroxylated OLs.

Ornithine lipids together with other phosphorus-free membrane lipids such as diacylglycerol-*N,N,N*-trimethylhomoserine (DGTS) or sulfolipids (SLs) accumulate under phosphate-limiting conditions in several bacterial species while at the same time phospholipids are actively degraded (Benning *et al.*, 1995; López-Lara *et al.*, 2003; Geiger *et al.*, 2010; Zavaleta-Pastor *et al.*, 2010). In *S. meliloti* it has been shown that this remodelling is under the control of the response regulator PhoB (Geiger *et al.*, 1999; Zavaleta-Pastor *et al.*, 2010). As OLs and phosphatidylethanolamine (PE) both are zwitterionic lipids, it has been speculated that OLs might replace PE under these conditions (Benning, 1998), although clear experimental evidence is lacking so far. Some other bacteria such as *Brucella abortus* (Thiele and Schwinn, 1973; Comerci *et al.*, 2006; Bukata *et al.*, 2008), *Mesorhizobium loti* (Devers *et al.*, 2011), *B. cenosepacia* (González-Silva *et al.*, 2011) and *R. tropici* (Rojas-Jiménez *et al.*, 2005; Sohlenkamp *et al.*, 2007; Vences-Guzmán *et al.*, 2011) also form significant amounts of OLs during growth in standard laboratory media such as Luria–Bertani (LB) which contain phosphate in concentrations that are not growth-limiting. It is not clear if OLs biosynthesis is regulated by PhoB in these organisms. We had observed that OLs apparently play an important role in *R. tropici* both under abiotic stress conditions such as heat stress and acid stress but also under symbiotic conditions (Vences-Guzmán *et al.*, 2011).

Agrobacterium tumefaciens is a γ -proteobacterium able to form tumours on many dicotyledonous plant species. Analysing the membrane lipid composition of the plant pathogen *A. tumefaciens* we noticed that it forms two different OLs, corresponding to the unmodified OL S1 and the hydroxylated OL S2 (Fig. S1). In the present work we wanted to study if OLs play a role in stress tolerance and pathogenicity in *A. tumefaciens*.

Agrobacterium tumefaciens mutants deficient in the OLs biosynthesis genes *olsB* or *olsE* were constructed and characterized. They either completely lack OLs (*olsB*) or only form the unmodified OL (*olsE*). Here we present a characterization of both OL mutants under different abiotic stress conditions and in a plant transformation assay using potato tuber discs.

Results

***Agrobacterium tumefaciens* forms two different ornithine lipids**

When analysing the lipid composition of [¹⁴C]acetate-labelled *A. tumefaciens* C58 by two-dimensional thin layer chromatography (TLC), in addition to the phospholipids phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), dimethyl PE, cardiolipin (CL), and the unmodified OL S1, a lipid migrating like the OL S2

from *R. tropici* CIAT899 (Fig. S1) (Rojas-Jiménez *et al.*, 2005; Vences-Guzmán *et al.*, 2011) can be observed (Fig. 1A). In order to confirm that this unknown lipid is biosynthetically derived from the amino acid ornithine, *A. tumefaciens* C58 cells were labelled with [¹⁴C]ornithine and the lipids were extracted and analysed by TLC. Apparently two ornithine-derived lipids are present in *A. tumefaciens* C58 (Fig. 1B). An identical result was obtained with *A. tumefaciens* strain A208 (data not shown).

The plant pathogen *Agrobacterium tumefaciens* also forms the ornithine lipid S2

Three different hydroxyl group modifications of OLs have been described in bacteria. To identify in which part of the second agrobacterial OL the modification is encountered, lipids were extracted according to Bligh and Dyer from a 1 l culture of *A. tumefaciens* wild-type A208. Both putative OLs were purified from the total lipid extract and analysed by normal phase LC/ESI-MS/MS in the negative ion mode. The [M-H]⁻ molecular ions of OL S1 and S2 were detected at *m/z* 663.6 and 679.6 respectively (Fig. S2). The mass difference of 16 amu suggests the presence of an additional oxygen atom indicating the presence of an additional hydroxyl group in S2. Comparing the fragmentation patterns of both lipids it was determined that a hydroxyl group was present within the ornithine moiety in the case of the unknown OL but not in the fatty acyl chains (Fig. 2). *Agrobacterium tumefaciens* therefore forms the OLs S1 and S2 (Fig. S1). The fragment ion spectra of the [M-H]⁻ ions of S1 and S2 at *m/z* 663.6 and 679.6 respectively, revealed that the amide-linked fatty acid is almost exclusively a C16 (3-OH) fatty acid indicating a substrate specificity of the agrobacterial OlsB for this fatty acid.

Ninhydrin staining of the *A. tumefaciens* membrane lipids separated by two-dimensional TLC confirmed our earlier observation of the OLs from *R. tropici*. S2 reacts with a delay in comparison to S1 and the developed colour by S2 is different. While S1 upon reaction with ninhydrin develops a red-to-purple colour, the reaction of S2 causes the formation of an orange colour (Fig. S3).

Atu0318 is responsible for the synthesis of the second ornithine lipid

The presence of the OL S2 indicates that the *A. tumefaciens* genome should encode an OlsE homologue. One of the closest homologues of OlsE from *R. tropici* CIAT899 is indeed Atu0318 which presents 61% identity and 71% similarity on amino acid level. BLAST searches show (Altschul *et al.*, 1997) that other close OlsE homologues are present in all sequenced *Agrobacterium* strains, *Rhodospirillum centenum* SE, *Azospirillum amazonense*, *Verrucomicrobium spinosum* and *Parvibaculum lavamentivorans* DS-1. Further homologues are present in several actinobacteria, a few α -proteobacteria and a few other β -proteobacteria (Vences-Guzmán *et al.*, 2012). To confirm that Atu0318 serves the same function as OlsE from *R. tropici* it was cloned into the broad host range plasmid pRK404 and subsequently conjugated into *S. meliloti* CS111.pNG25. In this strain a lipid migrating similarly as S2 from *R. tropici* was present that was absent in the negative control strain CS111.pNG25 (Fig. 1C and data not shown).

Lipid composition analysis of *olsE* and *olsB* mutants

We had observed a role for OLs in *R. tropici* stress resistance and symbiosis (Vences-Guzmán *et al.*, 2011) and we therefore considered it a possibility that OLs might play a role in stress resistance and pathogenesis in *A. tumefaciens*. To study the role of OLs in *A. tumefaciens* in more detail, mutants deficient in *olsE* and mutants deficient in *olsB* were constructed. Their lipid compositions were compared with the wild-type strain *A. tumefaciens* A208 (Fig. 3 and Table 1). As expected the *olsE*-deficient mutant MAV07 lacked the OL S2, and the *olsB*-deficient mutant MAV08 lacked S1 and S2. The total amount of OLs, being the sum of S1 and S2, is around 15% in both, the *A. tumefaciens*

wild-type and the *olsE*-deficient mutant MAV07 when grown in complex LB medium at 30°C (Table 1). In the absence of OLs in the mutant MAV08, more PE is formed, whereas no major differences are observed in the relative amounts of the phospholipids, PC, PG and CL between the different strains (Table 1). This suggests that the amounts of PE and OLs are inversely proportional.

Both mutants, MAV07 (*olsE*) and MAV08 (*olsB*), were also complemented. When introducing a functional copy of *olsB in trans* into the mutant MAV08 again formation of S1 and S2 was observed, while when introducing a functional copy of *olsE in trans* into the mutant MAV07 again formation of S2 was observed (data not shown).

Hydroxylated OL S2 accumulates when *A. tumefaciens* is grown at 15°C

We had reported earlier that in *R. tropici* no OlsE-dependent hydroxylation could be observed at 42°C (Vences-Guzmán *et al.*, 2011). We therefore wondered if OlsE might be more active at lower temperatures than at higher temperatures. When comparing the membrane lipid composition of cells grown at 15°C to cells grown at 30°C several differences can be observed (Table 2). The relative amounts of the phospholipids PE and PC seem to increase, whereas CL is drastically reduced and the amount of PG does not change when going from 30°C to 15°C. Interestingly, virtually all OL seems to be in the hydroxylated form at 15°C indicating that OlsE is sufficiently active to convert all OL S1 into hydroxylated S2. When studying the growth of both mutants and the wild-type at 15°C, all strains grow with similar generation times indicating that the presence or absence of the OLs does not affect growth of *A. tumefaciens* under these conditions (data not shown).

Absence of OLs does not affect growth of *A. tumefaciens* under conditions of high osmolarity

Agrobacterium tumefaciens wild-type A208 and both mutants deficient in OL biosynthesis were cultivated in complex 20E medium supplemented with up to 1 M NaCl. Up to 0.8 M NaCl no significant differences in generation time could be observed between the three strains. At 0.9 M NaCl only residual growth was observed for all strains and in the presence of 1.0 M NaCl none of the strains was able to grow. Under all conditions studied both mutants behaved very similar to the wild-type indicating that OLs do not play an important role in protecting the bacteria against osmotic stress (data not shown).

Growth of *A. tumefaciens* under phosphate limitation

Agrobacterium tumefaciens wild-type A208 and mutants deficient in OL biosynthesis were grown in Sherwood minimal medium with high (1.3 mM) or low phosphate (20 µM) concentrations. Under high phosphate conditions the phospholipids PC, PG, PE, dimethyl PE and CL were detected in the wild-type (Table 3). In addition, as observed earlier in cells grown on complex medium the two OLs S1 and S2 were detected. Under low phosphate conditions all phospholipids were reduced and both OLs increased to a total of about 45–50% in the wild-type. In addition, several new lipids were observed in the phosphate-deplete grown cells. In addition to DGTS several unknown lipids were detected. As Benning and colleagues (1995) and Devers and colleagues (2011) had observed the formation of glycolipids in *R. sphaeroides* and *M. loti* under low phosphate conditions we considered it a possibility that the unknown lipids were glycolipids. Naphthol staining of membrane lipids separated by TLC confirmed the presence of at least four different glycolipids. In contrast to *S. meliloti* and *R. sphaeroides* no sulfolipid was detected under phosphate starvation conditions which is consistent with the absence of genes coding for homologues involved in their biosynthesis in the *A. tumefaciens* genome. In the *olsB*-deficient mutant unable to form OLs increased amounts of DGTS can be detected in addition to increased amounts of the unknown lipids (Table 3).

Bioinformatic prediction of the PhoB regulon

A bioinformatic search of the *A. tumefaciens* C58 genome was performed for the presence of Pho boxes to find out if the OL biosynthesis genes are under the control of PhoB and at the same time understand the phosphate starvation response in *A. tumefaciens*. A position-specific scoring matrix (PSSM) was constructed using Pho box sequences from *S. meliloti* 1021 identified in an earlier study (Yuan *et al.*, 2006). The presence of a Pho box was predicted for the *olsB*, but not for the *olsE* promoter from *A. tumefaciens* (data not shown). Additionally, our bioinformatic analysis predicted PhoB-dependent regulation for DGTS biosynthesis as had been shown for *S. meliloti*. Putative Pho boxes were also predicted to precede the genes encoding the putative phosphate transport regulator Atu4634, the putative phospholipase C PlpC (Atu1649), and the putative phosphohydrolase Atu0877. Interestingly, four genes (*Atu0842*, *Atu1808*, *Atu2222*, *Atu2297*) are preceded by putative Pho boxes that might encode glycosyltransferases involved in the biosynthesis of the several unknown and putative glycolipids that accumulate under conditions of phosphate starvation in *A. tumefaciens*.

Phenocopying the OL composition of *R. tropici* in *A. tumefaciens*

We had observed earlier that the presence of the hydroxylase OlsC responsible for the synthesis of P1 was important to confer acid resistance to *R. tropici* (Vences-Guzmán *et al.*, 2011). *Agrobacterium tumefaciens* forms the OLs S1 and S2 and by introducing the gene *olsC* we would phenocopy or mimic the OL composition of *R. tropici*. We speculated that *A. tumefaciens* harbouring *olsC* might show an increase in acid resistance. Wild-type strain A208, mutants deficient in *olsB* or *olsE* and the wild-type expressing *olsC* from *R. tropici* were grown at pH 5.0, pH 5.5 and pH 7.0. Although OLs P1 and P2 could be detected in the strain expressing *olsC*, the presence of OlsC did not improve the growth of *A. tumefaciens* at low pH (Fig. 4, data not shown).

Plant assays of tumours in *A. tumefaciens*

The virulence of *A. tumefaciens* mutants deficient in the formation of OLs was assayed using a potato tuber disc system (Shurvinton and Ream, 1991; Tsai *et al.*, 2009). All strains caused the formation of tumours on potato tuber discs. Interestingly, the mutants deficient in the formation of OLs behaved differently from the wild-type. Tumours appeared within 18 days on discs inoculated with MAV08 or MAV07 while tumours appeared 6 days later on discs inoculated with the wild-type *A. tumefaciens* A208 (Fig. 5 and Table 4). Complementation of both mutants with the respective gene *in trans* restored the wild-type phenotype. The tumours on potato tuber discs inoculated with the mutant strains are also larger than the tumours on tuber discs inoculated with the wild-type. This size difference is probably due to the longer development of the tumours on tuber discs inoculated with the OL-deficient mutant strains.

Discussion

Ornithine lipids are widespread in eubacteria. In addition to the unmodified OL consisting of a 3-hydroxy fatty acid linked by an amide bond to the ϵ -amino group of ornithine and a second fatty acid bound in ester linkage to the first, several hydroxylated forms of OL have been described. The exact function of these modifications is not known yet, but several studies now hint at functions related to abiotic stress resistance and during interactions with eukaryotic hosts (Dees and Shively, 1982; Asselineau, 1991; Taylor *et al.*, 1998; Rojas-Jiménez *et al.*, 2005; González-Silva *et al.*, 2011; Vences-Guzmán *et al.*, 2011).

Here, we wanted to study the role of OLs in the plant pathogen *A. tumefaciens*. We characterized *A. tumefaciens* mutants deficient in OL biosynthesis under a set of abiotic

stress conditions: low growth temperatures, high salt concentrations, low phosphate concentration in the growth medium, and acid stress.

It has been speculated that the 2-hydroxylation of the ester-linked fatty acid in OL may increase hydrogen bonding between neighbouring OL molecules similarly as has been suggested for LpxO-hydroxylated lipid A in *Salmonella* and hydroxylated sphingolipids (Gibbons *et al.*, 2000; Nikaido, 2003; Murata *et al.*, 2007). These additional hydrogen bonds should result in bilayer stabilization and a decrease in membrane permeability. The fact that the OL S2 with OlsE-dependent modification accumulates at low growth temperatures in *A. tumefaciens* indicates that introducing a hydroxyl group into the OL headgroup might have the opposite effect than introducing it into the ester-linked fatty acid.

Agrobacterium tumefaciens forms two OLs even under high phosphate conditions. Together both OLs make up about 15% of the membrane lipids. Under low phosphate conditions we observed an accumulation of OLs to up to 45–50% of total lipids. This is consistent with our bioinformatic analysis indicating that agrobacterial *olsB* is preceded by a Pho box. Apparently although *olsB* is constitutively expressed in *A. tumefaciens*, the response regulator PhoB can further induce the levels of *olsB* transcription under conditions of phosphate limitation. No Pho box was predicted for the *olsE* promoter.

The presence of the OL hydroxylase OlsC has been shown to be important in *R. tropici* to resist conditions of acid stress and high temperature. *Agrobacterium tumefaciens* wild-type grows in complex media at pH 5.2 whereas *R. tropici* is distinctively more acid-resistant and grows at pH 4.0. Expressing *olsC* from *R. tropici* in *A. tumefaciens* would enable *A. tumefaciens* to synthesize the OL P1 which has an important role under these conditions in *R. tropici* (Vences-Guzmán *et al.*, 2011). Although OlsC is clearly active in the *Agrobacterium* strain harbouring the *olsC*-containing plasmid, no effect on acid stress resistance can be observed. This indicates that the stress resistance observed in *R. tropici* and that depended on the presence of OlsC might not be a direct effect of the presence of hydroxylated OL, but rather an indirect effect. The hydroxylated OL might somehow influence the enzyme activity of a protein important in stress resistance present in *R. tropici* but absent in *A. tumefaciens*. An alternative explanation is that OlsC might be able to hydroxylate a second substrate in addition to OLs and that this hydroxylation is responsible for the observed stress resistance.

Potato tuber discs inoculated with the mutant MAV08 (*olsB*) deficient in OL formation or with the mutant MAV07 (*olsE*) deficient in formation of the OL S2, show tumour formation about 1 week earlier than plants inoculated with wild-type bacteria. Complementation of both mutants (MAV07 and MAV08) with the respective genes *in trans* restores the slow tumour growth phenotype. A consequence of the earlier onset of tumour formation is the increased size of the tumours that is observed. Crown gall tumour formation occurs because *A. tumefaciens* can transfer an oncogenic T-DNA from its tumour-inducing (Ti) plasmid into plant cells. After integration of the T-DNA into the plant host genome, T-DNA-encoded proteins cause cytokine and auxin synthesis eventually leading to crown gall formation. T-DNA transfer from *A. tumefaciens* to the plant cell requires activation of the *vir* regulon present on the Ti plasmid (Gelvin, 2006). Salicylic acid and ethylene, two plant signal molecules involved in the regulation of defence-regulated gene expression during plant–microbe interaction (Ecker and Davis, 1987; Prithiviraj *et al.*, 2005) have been shown to inhibit expression of the *vir* regulon (Yuan *et al.*, 2007; Nonaka *et al.*, 2008). One possible hypothesis for the earlier appearance of tumours is that (hydroxylated) OLs can be detected by the plant and that the presence of (hydroxylated) OLs causes some kind of plant defence leading to the production of ethylene or salicylic acid. In the absence of OLs this response is not as strong or absent and the infection process is accelerated. Interestingly, OLs share a 3-

acyl-oxyacylamide structure with lipid A from Gram-negative bacteria, which has been shown to work as an elicitor in plant–microbe interactions (Scheidle *et al.*, 2005; Silipo *et al.*, 2008; 2010; Madala *et al.*, 2011). Both, OLs and lipid A are OM lipids. Several studies have shown that plants can sense lipid A but owing to the limited number of lipid A structures determined from plant-associated bacteria, no clear structure–activity relationship exists as for lipid A effects in animals (Madala *et al.*, 2011). Our observation that the absence of an agrobacterial membrane lipid affects tumour formation is not unprecedented. Wessel and colleagues (2006) had observed that *A. tumefaciens* mutants not able to form PC are deficient in tumour formation because they completely lack the type IV secretion machinery (Wessel *et al.*, 2006).

The exact functions of OLs are still unknown but the present study adds more evidence to an important role of these lipids in bacteria–host interactions.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in the present work and their relevant characteristics are shown in Table S1. *Agrobacterium tumefaciens* strains were grown in complex LB medium at 30°C, in complex 20E medium at 30°C (Werner *et al.*, 1975), or in Sherwood minimal medium (Sherwood, 1970) at 30°C or 15°C. *Escherichia coli* strains were grown in LB medium at 37°C. *Sinorhizobium meliloti* CS111.pNG25 was grown in complex tryptone-yeast extract medium (TY) (Beringer, 1974) supplemented with 10 mM CaCl₂. When needed, antibiotics were added at the following final concentrations (mg ml⁻¹): kanamycin (Km) 50; carbenicillin (Cb) 100; tetracycline (Tc) 8; timentin (Tim) 100, and rifampicin (Rif) 10.

DNA manipulations

Recombinant DNA techniques were performed according to standard protocols (Russell and Sambrook, 2001). Oligonucleotide primer sequences are listed in Table S2.

Expression of the candidate ORF Atu0318 from *Agrobacterium tumefaciens*

The candidate ORF for OlsE (*Atu0318*) was amplified using genomic DNA from *A. tumefaciens* A208 as a template and XL-PCR polymerase (Applied Biosystems). Specific oligonucleotide primers oC58OlsEN and oC58OlsEH incorporating NdeI and HindIII sites into the PCR product were used. After digestion with the respective enzymes, the PCR product was cloned as NdeI/HindIII fragment into pET17b yielding pEMAV27. Subsequently, the plasmid was linearized with HindIII and cloned into the HindIII site of pRK404 yielding pERMAV28. As a negative control pET17b was linearized with HindIII and cloned into the HindIII site of pRK404 yielding pERMAV05.

In vivo labelling of *S. meliloti* and *A. tumefaciens* with [¹⁴C]acetate or [¹⁴C]ornithine and quantitative analysis of lipid extracts

The lipid compositions of bacterial strains were determined following labelling with [1-¹⁴C]acetate (Amersham Biosciences). In the case of *A. tumefaciens*, cells were also labelled with [¹⁴C]ornithine to observe specific incorporation into OLs. Cultures (1 ml) of wild-type and mutant strains were inoculated from precultures grown in the same medium. After addition of 0.5 mCi [¹⁴C]acetate (60 mCi mmol⁻¹) or 1 mCi [¹⁴C]ornithine (56 mCi mmol⁻¹) to each culture, the cultures were incubated for 4 h if not indicated otherwise. Under phosphate starvation conditions cells were labelled for 48 h in Sherwood minimal medium (Sherwood, 1970) containing 20 μM phosphate. Lipid extraction and analysis are described in detail in Vences-Guzmán and colleagues (2011).

Construction of the *olsB* and *olsE* deletion mutants

Oligonucleotide primers oC58OlsEar1 and oC58OlsEar2 were used in a PCR (XL-PCR kit; Applied Biosystems) to amplify about 1.0 kb of genomic DNA upstream of the putative *olsE* (*Atu0318*) gene from *A. tumefaciens*, introducing EcoRI and BamHI sites into the PCR product. Similarly, primers oC58OlsEab1 and oC58OlsEab2 were used to amplify about 1.0 kb of genomic DNA downstream of the putative *olsE* gene from *A. tumefaciens*, introducing BamHI and XbaI sites into the PCR product.

After digestion with the respective enzymes, PCR products were cloned as EcoRI/BamHI or BamHI/XbaI fragments into pUC18 to yield the plasmids pUMAV18 and pUMAV19 respectively. Then, the BamHI/XbaI fragment from pUMAV19 was subcloned into pUMAV18 to yield pUMAV20. Plasmid pUMAV20 was digested with EcoRI and XbaI to subclone the regions usually flanking the *olsE* gene into the suicide vector pK18*mobsacB* (Schäfer *et al.*, 1994) to yield pPMAV21. Via diparental mating using *E. coli* S17-1 as a mobilizing strain, pPMAV21 was introduced into the wild-type strain *A. tumefaciens* A208. Transconjugants were selected on LB medium containing neomycin and rifampicin to select for single recombinants in a first step. The plasmid pK18*mobsacB* contains the *sacB* gene (Selbitschka *et al.*, 1993), which confers sucrose sensitivity to many bacteria. Growth of the single recombinants on high sucrose will therefore select for double recombinants and the loss of the vector backbone of pK18*mobsacB* from the bacterial genome. Single recombinants were grown under non-selective conditions in complex medium for 1 day before being plated on LB medium containing 12% (w/v) sucrose. Several large and small colonies grew after 4 days, and the membrane lipids of eight candidates were analysed by *in vivo* labelling during growth on complex medium with [¹⁴C]acetate and subsequent TLC (data not shown). Southern blot analysis confirmed that the S2-deficient strains were indeed double recombinants in which the gene *olsE* was deleted (data not shown).

Similarly, an *olsB* deletion mutant was constructed. Oligonucleotide primers oC58OlsBar1 and oC58OlsBar2 were used in a PCR (XL-PCR kit; Applied Biosystems) to amplify about 1.0 kb of genomic DNA upstream of the putative *olsB* gene from *A. tumefaciens*, introducing SmaI and BamHI sites into the PCR product. Similarly, primers oC58OlsBab1 and oC58OlsBab2 were used to amplify about 1.0 kb of genomic DNA downstream of the putative *olsB* gene from *A. tumefaciens*, introducing BamHI and HindIII sites into the PCR product. After digestion with the respective enzymes, PCR products were cloned as SmaI/BamHI or BamHI/HindIII fragments into pUC18 to yield the plasmids pUMAV23 and pUMAV24 respectively. The *A. tumefaciens* BamHI/HindIII fragment from pUMAV24 was subcloned into pUMAV23 to yield pUMAV25. Plasmid pUMAV25 was digested with SmaI and HindIII to subclone the regions usually flanking the *olsB* gene into the suicide vector pK18*mobsacB* (Schäfer *et al.*, 1994) to yield pPMAV26. Via diparental mating using *E. coli* S17-1 (Simon *et al.*, 1983) as a mobilizing strain, pPMAV26 was introduced into the wild-type strain *A. tumefaciens*. Subsequent steps were performed as described above for the construction of the *olsE* mutant. Four clones lacking OLs S1 and S2 were identified. Southern blot analysis confirmed that the S1- and S2-deficient strains were indeed double recombinants in which the gene *olsB* was deleted (data not shown).

Complementation of the *A. tumefaciens* mutants MAV07 and MAV08

The *olsE* (*Atu0318*) gene was amplified using genomic DNA from *A. tumefaciens* A208 as template and XL-PCR polymerase (Applied Biosystems). Specific oligonucleotide primers incorporating NdeI and HindIII sites into the final PCR product were used (oC58OlsXN and oC58OlsXH). The digested PCR product was cloned into pET17b to yield the plasmid pEMAV27. Plasmid pEMAV27 was linearized with HindIII and cloned into HindIII-digested pRK404 to yield pERMAV28. As a negative control pET17b was linearized with

HindIII and cloned into the HindIII site of pRK404 yielding pERMAV05. In this construct *olsE* is expressed under control of the T7 promoter. In earlier work we had observed that this promoter causes the constitutive expression of the genes under control in different *Rhizobiaceae* (Gao *et al.*, 2004; Sohlenkamp *et al.*, 2007; Vences-Guzmán *et al.*, 2011).

Similarly, the *olsB*-deficient mutant MAV08 was complemented with an intact copy of *olsB* *in trans*. The gene *olsB* (*Atu0344*) was amplified using genomic DNA from *A. tumefaciens* A208 as template and XL-PCR polymerase (Applied Biosystems). Specific oligonucleotide primers incorporating NdeI and BamHI sites into the final PCR product were used (oC58OlsBN and oC58OlsBB). The digested PCR product was cloned into pET9a to yield the plasmid pEMAV29. Plasmid pEMAV29 was linearized with BamHI and cloned into BamHI-digested pRK404 to yield pERMAV30. As a negative control pET9a was linearized with BamHI and cloned into the BamHI site of pRK404 yielding pERMAV06. In this construct *olsB* is expressed under control of the T7 promoter.

ESI-MS/MS analysis of ornithine lipids

In order to identify in which part of the second OL the modification is encountered, a 1 l culture *A. tumefaciens* was grown to an optical density of 1.2 at 620 nm in LB medium, and lipids were extracted according to Bligh and Dyer (1959). The lipids were separated by one-dimensional TLC and the area of the TLC plate corresponding to the OLs was identified by iodine and ninhydrin staining as described above. Silica containing OLs was scraped from the TLC plates and the OLs were extracted from the silica. OL-containing fractions were dried under N₂ stream and redissolved in methanol/chloroform (1:1, v/v). Normal phase LC-MS/MS of lipids was made as described in detail in Vences-Guzmán and colleagues (2011).

Identification of potential Pho boxes in the *Agrobacterium tumefaciens* genome

To study if OL biosynthesis and/or modification are under control of PhoB the genome sequence of *A. tumefaciens* C58 was searched for the presence of putative Pho boxes. A PSSM was constructed using Pho box sequences reported previously for *S. meliloti* 1021 (Yuan *et al.*, 2006). *Escherichia coli* sequences were excluded for matrix construction. Sequences used for matrix construction are listed in Table S3. The program Info-Gibbs (Defrance and van Helden, 2009) was used for matrix construction. One motif per sequence was expected and the desired matrix size was fixed to 18 bp. As background model the regions upstream of *A. tumefaciens* C58 genes were employed. The resulting matrix has a maximum score for putative Pho boxes of 12.4, and the threshold used for the search was set to 7. The program Matrix-scan (Turatsinze *et al.*, 2008) was used to search the *A. tumefaciens* C58 genome for the presence of Pho boxes. The Markov order used was 0. Again the upstream regions of genes from *A. tumefaciens* C58 were employed as the background model. The search was performed scanning the upstream regions of predicted genes in 18 bp windows and comparing them with the PSSM. This search was performed for both strands. The list of genes presenting putative upstream Pho boxes was analysed for the presence of OL biosynthesis genes *olsB*, *olsE* and genes coding for glycosyltransferases (Cantarel *et al.*, 2009).

Tumour assays using potato tuber discs

The tumour assays were performed according to Tsai and colleagues (2009) (Shurvinton and Ream, 1991; Tsai *et al.*, 2009). Potato tubers were peeled and then immersed for 5 min in 1.05% sodium hypochlorite (17.5% bleach) solution. Tubers were rinsed three times with sterile water. A sterile cork borer was used to cut 1.0 cm diameter cylinders of potato tubers. About 1 cm pieces were removed from each end of a cylinder and the remaining cores were cut into 2–3 mm thick discs and placed on water agar plates. Each disc was inoculated on one side with 10⁶ cells suspended in 100 µl PBS. Discs were placed on 1% water agar plates

and incubated for 2 days in a controlled growth chamber at 28°C with a 15 h day/9 h night cycle. Potato discs were treated with timentin to kill all bacteria and then transferred to 1% (w/v) water agar plates containing timentin. Potato discs were incubated for 48 days under the conditions described above. Discs were scored every 3 days for the presence of tumours. After 48 days images were taken of the potato discs using a stereomicroscope equipped with a digital camera.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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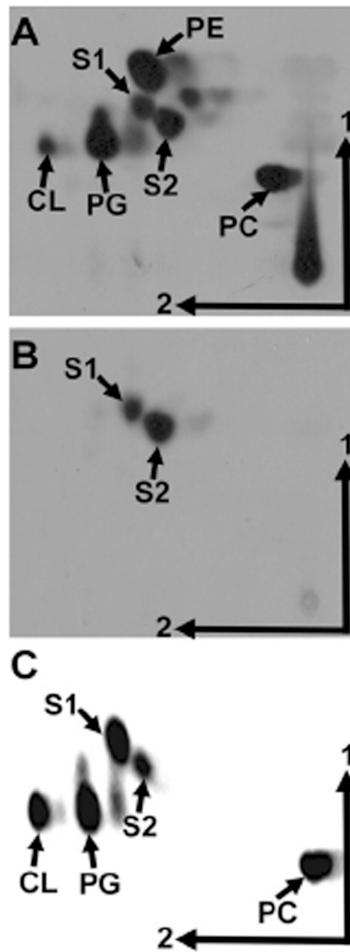


Fig. 1. *Agrobacterium tumefaciens* forms an OL migrating similarly as S2 from *R. tropici*. [¹⁴C]acetate- (A) and [¹⁴C]ornithine- (B) labelled lipids from *A. tumefaciens* wild-type C58 grown at 30°C in 20E medium and [¹⁴C]acetate-labelled lipids from *S. meliloti* CS111.pNG25.pERMAV14 grown in TY medium (C) were separated by two-dimensional TLC. The phospholipids PE, monomethyl PE (MMPE), PC, phosphatidylglycerol (PG), cardiolipin (CL), and the OLs S1 and S2 are indicated.

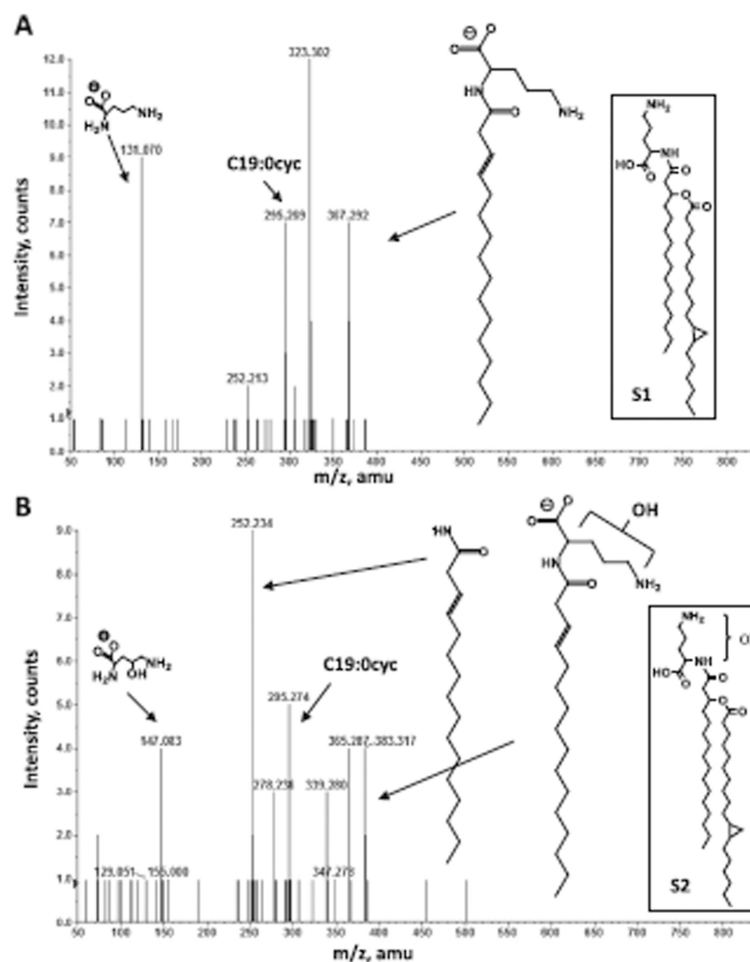


Fig. 2. Collision-induced dissociation mass spectra of OL S1 and S2 detected in lipid extract of *A. tumefaciens*. Negative ion collision induced dissociation mass spectra of $[M-H]^-$ ions at m/z 663.6 (A) obtained from OL S1 and m/z 679.6 (B) obtained from OL S2. The structures of major fragment ions are indicated. The structures of the parental lipids are shown in the boxes.

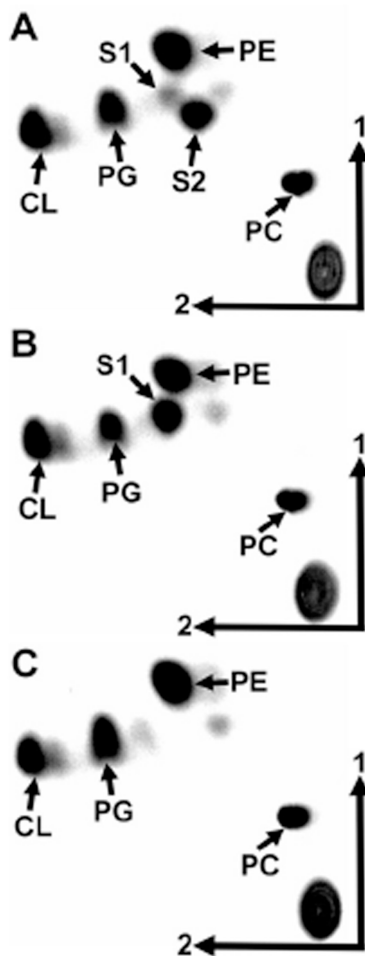


Fig. 3. Analysis of membrane lipid composition of *A. tumefaciens* wild-type A208 (A), *olsE*-deficient mutant MAV07 (B) and *olsB*-deficient mutant MAV08 (C). Lipids were labelled with [^{14}C]acetate during growth in 20E medium at 30°C and separated using two-dimensional TLC. The phospholipids phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL) and the OLS S1 and S2 are indicated.

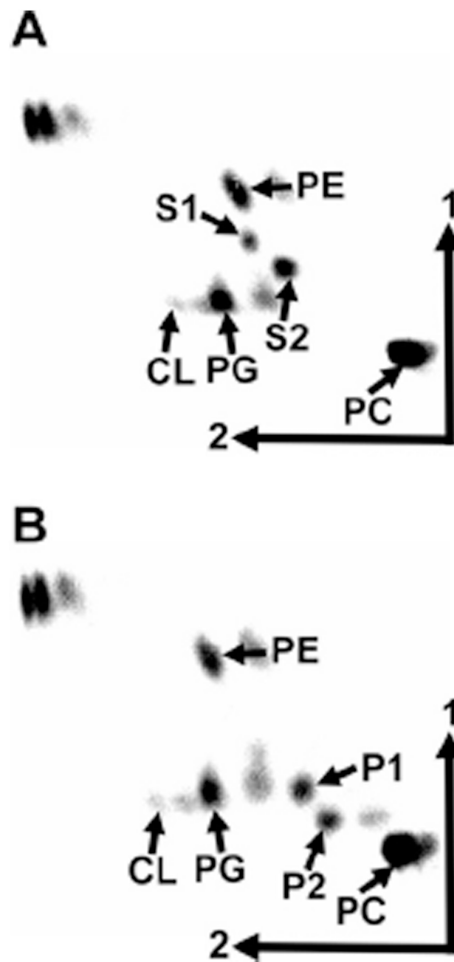


Fig. 4. Heterologous expression of *olsC* from *Rhizobium tropici* in *Agrobacterium tumefaciens* A208. Lipids of the wild-type *A. tumefaciens* A208 harbouring the empty plasmid pERMAV06 (A) or the *olsC*-containing plasmid pERMAV15 (B) were labelled with [^{14}C]acetate for 48 h during growth in LB medium at 30°C and separated using two-dimensional TLC. The phospholipids phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL) and the Ols S1, and S2 are indicated. Ols P1 and P2 derived from OlsC-dependent hydroxylation of S1 and S2 are also indicated.

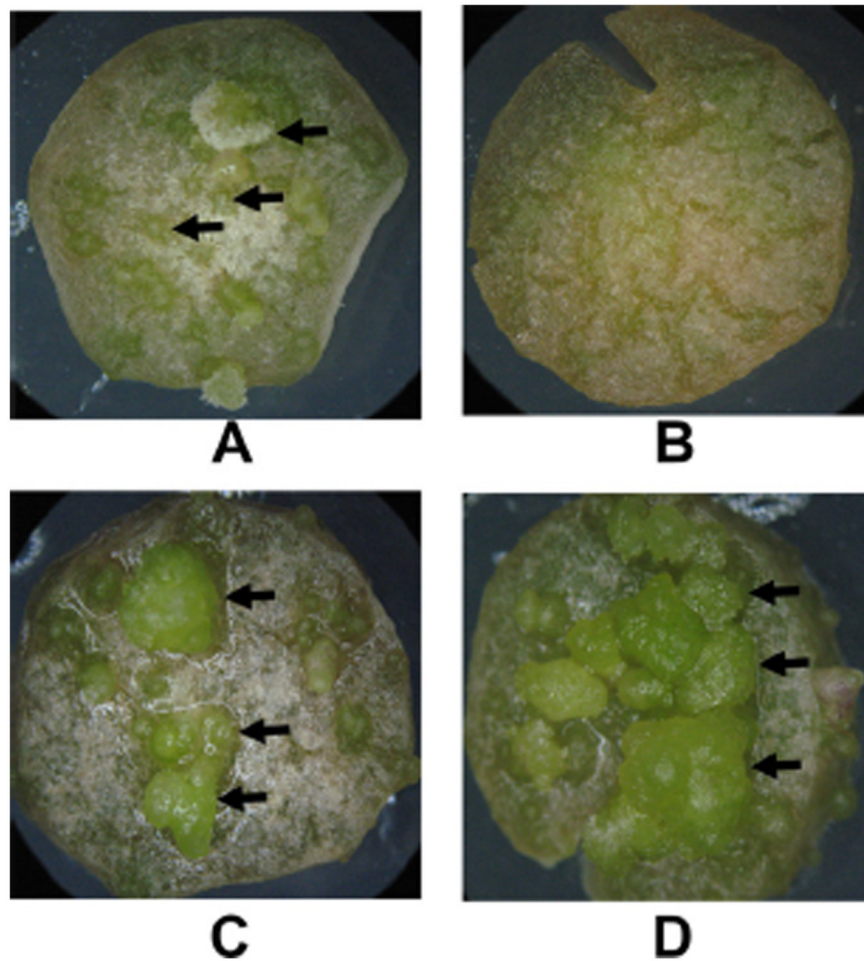


Fig. 5. Assay of tumour formation by *Agrobacterium tumefaciens* using potato tuber discs. Potato discs were inoculated with (A) *A. tumefaciens* A208, (B) no bacteria, (C) OlsE-deficient mutant MAV07, (D) OlsB-deficient mutant MAV08. Potato tuber discs were scored every 3 days for tumour formation. The tuber discs have a diameter of 1 cm. Images were taken 48 days after inoculation. Tuber discs were incubated at 28°C. The arrows highlight some tumours on the potato discs.

Table 1

Membrane lipid composition of *A. tumefaciens* wild-type A208, *olsE*-deficient mutant MAV07 and *olsB*-deficient mutant MAV08 after growth on complex LB medium at 30°C.

Lipid	Composition (% of total ¹⁴ C)		
	A208	MAV07 (<i>olsE</i>)	MAV08 (<i>olsB</i>)
PC	21.3 ± 0.5	26.0 ± 1.2	24.2 ± 0.5
S1	5.8 ± 0.2	15.9 ± 0.3	n.d.
S2	9.5 ± 0.2	n.d.	n.d.
PE	41.5 ± 0.4	35.7 ± 1.3	53.2 ± 0.2
PG	18.8 ± 0.4	20.5 ± 0.2	20.2 ± 0.9
CL	3.1 ± 0.5	1.9 ± 0.1	2.4 ± 0.2

The values shown are mean values ± standard deviation derived from at least three independent experiments. S1: unmodified ornithine lipid; S2: hydroxylated ornithine lipid; n.d.: not detected.

Table 2

Membrane lipid composition of *A. tumefaciens* wild-type, *olsE*-deficient mutant MAV07 and *olsB*-deficient mutant MAV08 after labelling for 48 h with [¹⁴C]acetate in Sherwood minimal medium at 30°C or 15°C.

Lipid	Composition (% of total ¹⁴ C)					
	30°C			15°C		
	A208	MAV07	MAV08	A208	MAV07	MAV08
PC	13.6	14.7	19.3	28.4	34.1	33.3
S1	6.8	20.2	n.d.	n.d.	16.0	n.d.
S2	14.3	n.d.	n.d.	16.8	n.d.	n.d.
PE	29.7	30.8	43.0	39.0	31.3	52.6
PG	12.2	11.5	18.9	11.6	15.0	11.7
CL	23.4	22.8	18.8	4.2	3.6	2.4

The values shown are mean values derived from at least two independent experiments.
S1: unmodified ornithine lipid; S2: hydroxylated ornithine lipid; n.d.: not detected.

Table 3

Analysis of *A. tumefaciens* strains grown in Sherwood medium containing 20 μ M or 1.3 mM phosphate.

	Composition (% of total 14 C)					
	High phosphate			Low phosphate		
	A208	MAV07	MAV08	A208	MAV07	MAV08
PC	13.6 \pm 0.5	14.7 \pm 0.5	19.3 \pm 0.7	7.3 \pm 0.6	9.5 \pm 0.5	10.0 \pm 1.0
PG	12.2 \pm 0.2	11.5 \pm 0.5	18.9 \pm 0.7	3.6 \pm 0.6	4.5 \pm 0.1	5.3 \pm 0.6
PE	29.7 \pm 0.6	30.8 \pm 1.4	43.0 \pm 1.2	2.7 \pm 0.4	2.0 \pm 0.6	6.6 \pm 0.5
CL	23.5 \pm 1.0	22.8 \pm 1.1	18.8 \pm 0.9	3.9 \pm 0.9	5.9 \pm 0.1	7.0 \pm 0.1
S1 + GL4	6.8 \pm 1.0	20.2 \pm 0.3	n.d.	21.8 \pm 0.8	42.9 \pm 0.9	6.4 \pm 0.5
S2	14.2 \pm 0.3	n.d.	n.d.	30.0 \pm 0.1	n.d.	n.d.
GL1	n.d.	n.d.	n.d.	12.9 \pm 0.9	20.9 \pm 0.5	17.1 \pm 0.3
GL2	n.d.	n.d.	n.d.	2.6 \pm 0.5	2.9 \pm 0.3	11.3 \pm 0.8
GL3	n.d.	n.d.	n.d.	1.5 \pm 0.4	2.5 \pm 0.1	2.6 \pm 0.5
DGTS	n.d.	n.d.	n.d.	13.7 \pm 0.5	8.9 \pm 0.5	33.7 \pm 1.4

A208 – wild-type, MAV07 – *olsE* mutant, MAV08 – *olsB* mutant. The values shown are mean values \pm standard deviation derived from at least three independent experiments. The bacterial strains were labelled for 48 h with [14 C]acetate.

S1: unmodified ornithine lipid; S2: hydroxylated ornithine lipid; GL1, GL2, GL3, GL4: unknown glycolipids; n.d.: not detected.

Table 4

Kinetics of tumour formation by *A. tumefaciens* on potato tuber discs.

Strain	Genotype	Days after inoculation															
		3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
No bacteria		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MA V07	<i>olsE</i> ⁻	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
MA V07.pERMA V05	<i>olsE</i> ⁻	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
MA V07.pERMA V28	<i>olsE</i> ⁺	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
MA V08	<i>olsB</i> ⁻	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
MA V08.pERMA V06	<i>olsB</i> ⁻	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
MA V08.pERMA V30	<i>olsB</i> ⁺	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
<i>A. tumefaciens</i> A208	Wild-type	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+

Discs were inoculated with 10⁸ bacteria on day 0 and bacteria were killed by timentin treatment after 48 h. Discs were scored for tumour formation every 3–4 days. The experiment was repeated independently three times. Each experiment contained 30 replicates of each strain.