European Journal of Plant Pathology **107:** 839–844, 2001.

Short communication

Influence of amylovoran production on virulence of *Erwinia amylovora* and a different amylovoran structure in *E. amylovora* isolates from *Rubus*

Martine Maes[1], Kristien Orye[2], Svetoslav Bobev[3], Bart Devreese[4], Jozef Van Beeumen[4], André De Bruyn[5], Roger Busson[6], Piet Herdewijn[6], Kris Morreel[2] and Eric Messens[2,*]
[1]*Department of Crop Protection, Agricultural Research Centre, B-9820 Merelbeke, Belgium;*
[2]*Department of Molecular Genetics, Ghent University, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium;*
[3]*Department of Plant Pathology, Higher Agricultural Institute, 4000 Plovdiv, Bulgaria;*
[4]*Department of Biochemistry, Physiology and Microbiology, Ghent University, B-9000 Gent, Belgium;*
[5]*Department of Organic Chemistry, Ghent University, B-9000 Gent, Belgium;* [6]*Laboratory of Medicinal Chemistry, Rega Institute, Catholic University of Leuven, B-3000 Leuven, Belgium;* [*]*Author for correspondence (Phone: +32 (0)9 2645196; Fax: +32 (0)9 2645349; E-mail: ermes@gengenp.rug.ac.be)*

Accepted 18 June 2001

Key words: artificial inoculation, electrospray mass spectrometry, fire blight, pathogenicity

Abstract

The amylovoran structures of five *Erwinia amylovora* isolates from *Malaceae* sp. and four isolates from *Rubus* sp. host plants were fully established, mainly by NMR. The structural data on one *E. amylovora* isolate from a *Malaceae* sp. host, which had been previously suggested by mass and NMR (Nimtz et al., 1996), were completed. *E. amylovora* strains infective on *Malaceae* sp. host plants had an amylovoran composed of pentasaccharide and 30–40% hexasaccharide repeating-substructures, whereas amylovoran from *E. amylovora* isolates from *Rubus* sp. host plants had only the pentasaccharide substructures. On the other hand, the exopolysaccharide (EPS) production differed in wild-type *E. amylovora* strains. Data on *in vitro* amylovoran production per cell could account for the differences in aggressiveness found in *E. amylovora* strains, as deduced from a pilot test with highly, moderately, and weakly aggressive strains. This correlation was confirmed with several other wild-type *E. amylovora* strains from different origin.

Abbreviations: EPS – exopolysaccharide; NMR – nuclear magnetic resonance.

Fire blight is an important fruit tree disease, which is currently expanding throughout the world. For disease control, new epidemiological understanding is needed. The role of bacterial exopolysaccharide (EPS) in the infection process has been debated for many years (Bennett and Billing, 1978; Ayers et al., 1979; Bennett, 1980; Denny, 1995). EPS is a major pathogenicity factor in *Erwinia amylovora*, essential for the development of disease symptoms (Ayers et al., 1979; Bellemann and Geider, 1992; Bernhard et al., 1993; Tharaud et al., 1994). EPS has been suggested to play a role in disturbance and obstruction of the plant vascular system (Suhayda and Goodman, 1981; Denny, 1995) and to protect the bacterial cells from plant defense reactions (Leigh and Coplin, 1992; Kiraly et al., 1997). The predominant EPS of *E. amylovora* is amylovoran, and amylovoran-defective mutants do not spread in plant tissue (Bellemann and Geider, 1992; Bernhard et al., 1993; Tharaud et al., 1994).

The correct structure of amylovoran was elucidated by Nimtz et al. (1996). Amylovoran consists of a long repetition of heteropolysaccharide units, a combination of pentamers and hexamers. These results were made possible by (i) the availability of an EPS depolymerase enzyme from phage ϕ-Ea1h (Hartung et al., 1988) and (ii) NMR and mass spectrometry studies.

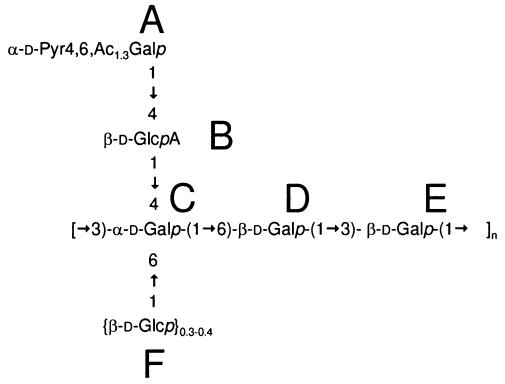

Figure 1. Oligosaccharide-repeating unit of the amylovoran of *E. amylovora* strain Ea1/79 according to Nimtz et al. (1996), with structure and letter code of the monosaccharide residues. Residue A is a non-*O*-acetylated (10%), 2-*O*-acetylated (26%), 3-*O*-acetylated (24%), or 2,3-di-*O*-acetylated (40%) (1 → 4)-α-D-galactopyranosyl residue and bears a 4,6-bound pyruvate residue in the R configuration. Residue B is a (1 → 4)-β-D-glucopyranosyluronic acid residue; C, D, and E are the core (1 → 3 or 1 → 6), -α, or -β, -D-galactopyranosyl-repeating units. F is the additional (1 → 6)-β-D-glucopyranosyl residue.

NMR and mass analysis were possible because the enzymatic digestion of EPS produces homogeneous blocks of the heteropolysaccharide units. The bacterial oligosaccharides were isolated, then purified by molecular sieve chromatography. The pentamer structure was confirmed and NMR data suggested correctly the composition of the hexasaccharide (Figure 1).

Here, the heterogeneity among *E. amylovora* isolates was investigated in respect to EPS properties. From previous experiments it was clear that the four wild-type strains PFB5, PD437, LMG2066, and LMG2085 differ in pathogenicity. On *Pyrus communis*, *Malus sylvestris*, *Crataegus monogyna*, and *Sorbus aria*, each with its typical fire blight susceptibility, the strains behave consequently as highly, moderately, weakly, and non-pathogenic (Table 1), depending on the extent of shoot necrosis induced upon artificial inoculation. In a larger scale experiment (40 plants per strain, type I error of 0.05), this difference in aggressiveness was found to be statistically significant and confirmed previous strain classifications. Not only the number of diseased apple seedlings differed, but also the symptom severity (Figure 2). Extended mass spectrometry and NMR were used for structural analysis of the amylovoran. The oligosaccharide units were obtained upon digestion of amylovoran with phage ϕ-Ea1h depolymerase. ^{1}H and ^{13}C NMR spectra were completely assigned using heteronuclear single-quantum coherence and

Table 1. E. amylovora strains used

Strain numbering[1]	Isolated from	Aggressiveness on *Malaceae* sp.
PD437[2]	*Pyrus communis*	++
LMG2066[2]	*P. communis*	+
PFB5[2]	*Prunus salicina*	+++
LMG2085[2]	*Rubus ideus*	−
LMG2083[3]	*R. ideus*	−
LMG2069[3]	*R. ideus*	−
LMG2084[3]	*R. ideus*	−
LMG1933[3]	*Cotoneaster repens*	++
LMG1976[3]	*Crataegus* sp.	++
BG1[4]	*Cydonia oblonga*	++
BG2[4]	*C. oblonga*	+
BG7[4]	*Pyrus* sp.	++
BG12[4]	*Mespilus germanica*	+
BG16[4]	*Malus* sp.	++++

[1]Strains from the Belgian Coordinated Collections, University Gent (LMG); The Plantenziektenkundige Dienst, Wageningen, The Netherlands (PD); S.K. Mohan, Idaho, USA (PFB5); and S. Bobev, Plovdiv, Bulgaria (BG).
[2]Used for EPS structural analysis, pathogenicity tests, and *in vitro* EPS production measurement.
[3]Used for EPS structural analysis.
[4]Used for pathogenicity tests and *in vitro* EPS production measurement. The strains are classified as not infective (−), weakly (+), moderately (++), highly (+++), and super (++++) aggressive on *Malaceae* host plants.

selective excitated total correlation spectroscopy NMR analysis (Maras et al., 1997; Schraml et al., 1997; Busson et al., 2001). Also EPS production by the different strains was measured. It was monitored during *in vitro* growth in liquid culture, in which the amylovoran forms a loose bacterial slime and is released as free EPS. It is then easily isolated upon centrifugation of the bacterial cells. *E. amylovora* strains were grown in MM2 liquid medium supplemented with 1% sorbitol and free EPS was assayed in a turbidity test, as described by Bellemann et al. (1994). Bacterial titers and EPS production were followed for ten days.

With mass spectrometry, molecular mass data of the EPS oligosaccharides of the pathogens on *Malaceae* showed a mixture of components, corresponding to differently acetylated forms of both pentasaccharide and hexasaccharide units as described by Nimtz et al. (1996) (Figure 3a). Strain LMG2085 isolated from *Rubus* produced a mass spectrum with one hexose less (Figure 3b). Because of the high sensitivity of the spectral technique, it could be concluded that this EPS was without any hexasaccharide substructures, consisting

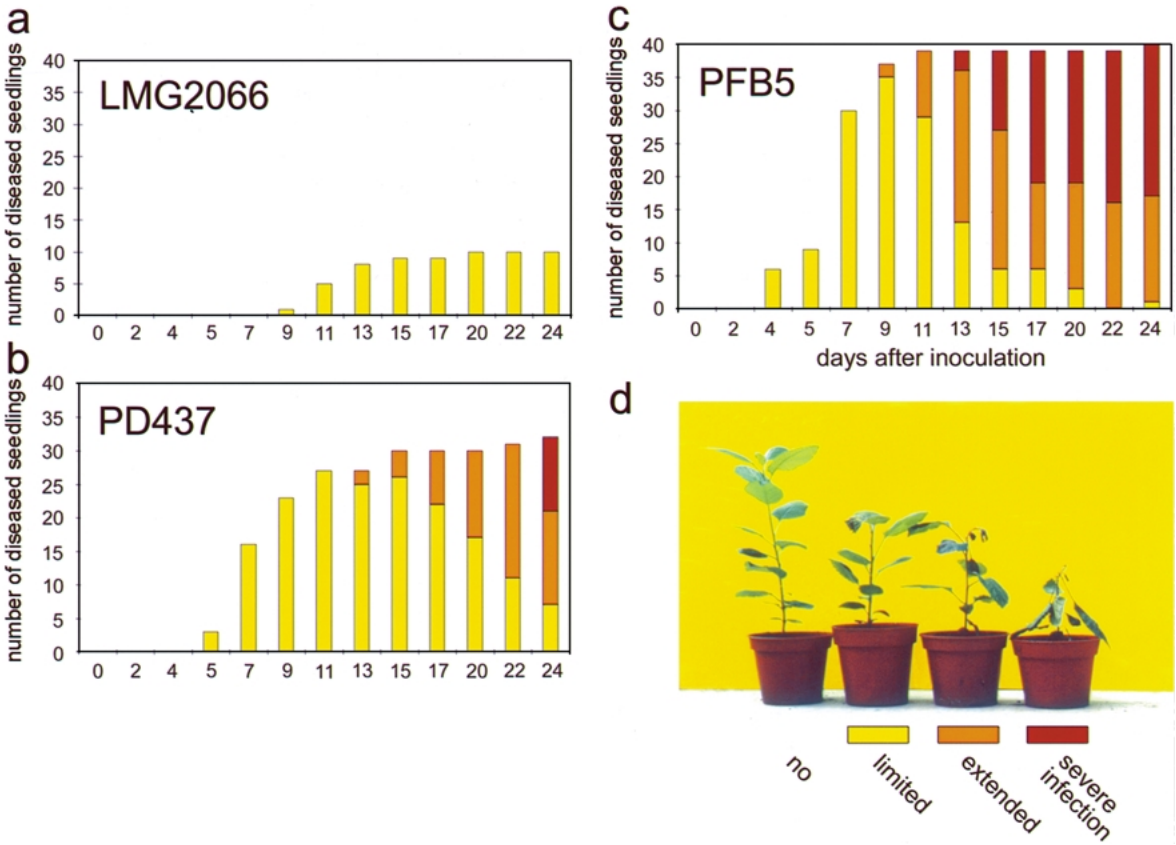

Figure 2. Disease development in apple seedlings artificially inoculated with *E. amylovora* strains LMG2066, PD437, PFB5, and LMG2085. Bacterial cultures were in MM2 liquid medium supplemented with 1% sorbitol (Bellemann et al., 1994) and had titers of approximately 10^8 cfu/ml when used for inoculation. Forty apple seedlings were inoculated per strain in the greenhouse. The third leaf down from the apex was cut and 20 µl of the bacterial suspension was spotted on the wound. Disease development was monitored every 2 days and for 3 weeks. No disease symptoms were scored upon inoculation with LMG2085 isolated from *Rubus* (not shown). Different stages in symptom severity are marked as: yellow bars, seedlings with limited necrosis nearby the inoculation point; red bars, seedlings with extended necrosis; and brown bars, nearly dead seedlings.

only of pentasaccharide oligomers. In NMR, the structure proposed by Nimtz et al. (1996) was confirmed. However, in contrast with the proposed 10% occurrence of the hexasaccharide, we found that the hexasaccharide (with residue F) constituted 30–40% of the amylovoran in the strains infective on *Malaceae*. In the spectrum with the Rubus-type LMG2085, NMR revealed the absence of residue F ((1 → 6)-β-D-glucopyranosyl on compound C) (Figure 1) and confirmed the mass spectrometry results. Three additional isolates from *Rubus* sp. were analyzed and confirmed the homogeneous pentasaccharide polymeric structure of their EPS, which we then considered as typical for *E. amylovora* strains isolated from *Rubus*. EPS analysis of two more isolates from the *Malaceae* hosts *Cotoneaster* and *Crataegus* confirmed the 30–40% presence of the hexasaccharide in the polymer. Genome typing was performed on three Rubus-type strains to check for independence of the isolates. AFLP analysis was performed according to Janssen et al. (1996). Optimal band distribution was obtained with the enzyme combination *Taq* I-*Eco* RI and the PCR primer set T01-E01 (T and E stand for *Taq* I and *Eco* RI, respectively, and 01 stands for adenine as selective base at the 3′ end). The Rubus-type strains LMG2085 and LMG2069 exhibited identical AFLP profiles, while LMG2084 differed by the presence/absence of five distinct bands. This AFLP heterogeneity was higher than that found among the AFLP patterns of 30 Malaceae-type isolates from

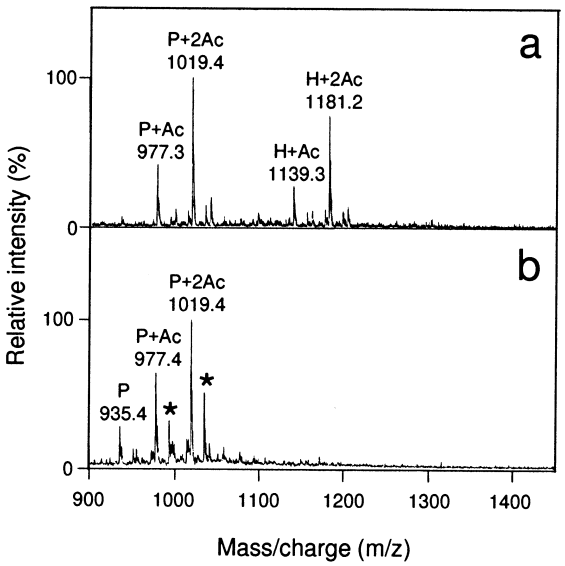

Figure 3. Electrospray mass spectra of the oligosaccharides obtained from *E. amylovora* infective on *Malaceae* sp. (a) and *Rubus* sp. (b). MS analyses were performed on a VG Bio-Q triple quadrupole MS (Micromass, Manchester, UK) with classical flow injection at 5 µl/min of 10 µl. Spectra were obtained in the positive ion mode using 3.4 kV on the capillary and scanning the analyzer from 550 to 1550 Da in 10 s. The molecular mass of the different EPS corresponds to the Na adduct of the components, except for those labeled with an asterisk, which are K adducts. H and P correspond to the molecular mass of the hexasaccharide-repeating unit of EPS and pentasaccharide unit lacking the F residue, respectively (Figure 1). Differently acetylated forms are indicated. The non-*O*-acetylated hexasaccharide unit (H) has the formula $C_{39}H_{62}O_{34}$ and a theoretical molecular mass of 1074.3123 Da.

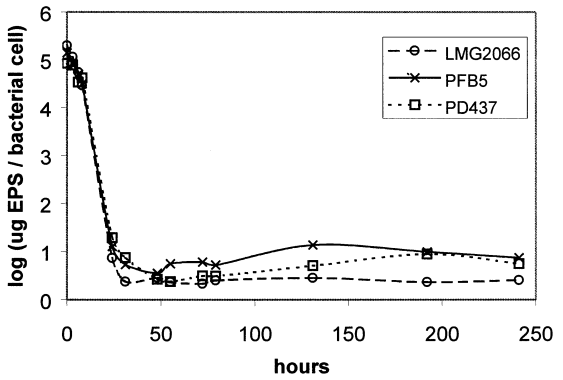

Figure 4. Plot of EPS concentrations per viable cell in MM2 liquid cultures supplemented with 1% sorbitol (Bellemann et al., 1994) produced by the highly (PFB5), moderately (PD437), and weakly (LMG2066) aggressive *E. amylovora* strains. Cell titers were monitored by serial dilution plating on LB medium. EPS concentrations in culture were determined in a turbidity test, according to Bellemann et al. (1994). Our standardization of the turbidity function ($y = a - b^x$) was $y = 1.03776 - 0.98712^x$ with y = absorption at 600 nm and x = (µg EPS/bacterial cell).

different locations and hosts, where only one AFLP band diverged. McManus and Jones (1995) reported similar results. RFLP analysis of the 16S-23S rDNA spacer region with the enzymes *Alu* I and *Cfo* I produced identical patterns for LMG2085 and LMG2069, and a single band difference for LMG2084. By both AFLP and rDNA-RFLP, the Rubus-type strains were clearly differentiated from Malaceae-type strains.

During growth of the strains in liquid MM2 medium, the most aggressive strain, PFB5, produced two- and three-fold more EPS per cell than the moderately and weakly aggressive strains, respectively, measured in stationary growth phase (Figure 4; Table 2). EPS production of the Rubus-type strain LMG2085 was comparable to the moderately aggressive strain PD437 (data not shown). The pathogenicity of this strain was tested by inoculation on *Rubus ideus*, where it induced leaf and stem blight. Five additional *E. amylovora* isolates were classified according to *in vitro* EPS production (Table 1). Their aggressiveness was evaluated by the mean lesion length produced on shoots of *C. monogyna* and *Prunus spinosa*, which is a less susceptible fire blight host. EPS production and aggressiveness were determined relative to PFB5 and LMG2066, which were included as reference strains in all growth and inoculation tests. For all strains the correlation between EPS production rate *in vitro* and aggressiveness was confirmed. Strain BG16 produced four times more EPS per cell than the aggressive PFB5 strain. Eighteen days after inoculation, PFB5 produced an average shoot blight zone (3 plants per test) of 10 (±3) and 0.6 (±1.4) cm on *C. monogyna* and *P. spinosa*, respectively, while BG16 produced an average shoot blight zone of 16.6 (±3.4) cm on both hosts.

The EPS coat participates in the first and intimate contact between the bacterium and host plant. Presence of an extra glucose branch in 30–40% of the coat polysaccharide moieties undoubtly influences the three-dimensional structure and the physiological properties of amylovoran. We have no data on differences in other physical properties between Malaceae- and Rubus-type EPS. EPS coat production is surely an important factor for epiphytic and endophytic spread of wild-type *E. amylovora*. Strains with less slime

Table 2. Two-way ANOVA[1] on EPS concentrations during stationary phase

Source		Sum of squares	df	Mean squares	*F*	Sig.
Two-way ANOVA[1]						
Model		26.792	21	1.276	37.201	<0.001
Time		0.861	6	0.143	4.184	0.002
Strain		1.956	2	0.978	28.513	<0.001
Time × strain		0.627	12	0.052	1.523	0.154
Error		1.440	42	0.034		
Total		28.232	63			
Strain *i*	Strain *j*	Mean difference	Std. error	Sig.	95% confidence interval	
					Lower bound	Upper bound
LSD[2]						
LMG2066	PFB5	−0.431	0.057	<0.001	−0.576	−0.286
LMG2066	PD437	−0.203	0.057	0.004	−0.348	−0.058
PFB5	PD437	0.229	0.057	0.001	0.083	0.374

[1]Two-way ANOVA was performed on the values of log(μg EPS / bacterial cell) obtained during the stationary phase of the bacterial growth curve (from 48 hours to 241 hours).
[2]Because strain PD437 is expected to be intermediate between LMG2066 and PFB5, Least Significant Difference (LSD) was chosen as *post hoc* test. For the ANOVA and the *post hoc* test the significance threshold was set to 0.05.

formation are sometimes found as surviving forms in cankers or dead wood tissue (J. Van Vaerenbergh, personal communication), but also in these cases fire blight probably did not spread with 'dry' cells. Genetic work shows that EPS synthesis, however, is not sufficient for pathogenicity. Other genetic determinants including *hrp-dsp* signalization (Tharaud et al., 1994; Barny, 1995) and to a lesser extent levan synthesis (Denny, 1995; Bereswill et al., 1997) are needed. Bellemann et al. (1994) concluded that the fire blight pathogen depends strongly on the EPS coat during all growth stages in order to escape plant defense reactions. They noticed a lower EPS content per cell in mid-log phase than at the beginning of stationary phase. Here, we confirm this observation, but by comparing a set of different wild-type strains, we can conclude additionally that this EPS content is strain dependent and important for strain-dependent virulence.

The present results are based only on correlations among chemical, biological, and phytopathological data. The precise relationship between amylovoran structure and host-specific virulence can be better defined with availability of more *E. amylovora* isolates from *Rubus* and genetic experiments. It is possible that the missing glucose (residue F) in Rubus-type EPS, for example, represents a phylogenetic trait with no consequence for host-specific pathogenicity. Rubus-type strains have to be complemented with Malaceae-type amylovoran and inoculated on *Malaceae* plants. The demonstration that Malaceae-type amylovorans extend the host range of genetically transformed Rubus-type bacteria would provide strong evidence for their role in host-range determination. To our knowledge, the gene for addition of the glucose side chain (residue F) has not been identified, however. The full *ams* operon, eventually together with any area connected to *ams* functions, would have to be introduced and transformants tested for EPS structure and host range. Meanwhile, EPS production measurement in culture is used regularly and has been found reliable for determining wild-type strain aggressiveness. It is a practical alternative to time-consuming plant inoculation tests. To obtain additional genetic evidence for correlation of EPS production and strain aggressiveness, *ams* overexpression can eventually be obtained by introduction of activator genes *rcsA* or *rcsB*.

Acknowledgements

We thank Klaus Geider for fruitful discussion, J.S. Hartung for providing the ϕ-Ea1h depolymerase clone, S.K. Mohan for strain PFB5, S. Baeyen for excellent technical assistance, and J. Strommer for revision of the English. This work was granted by the Belgian Ministry of Small Enterprises and Agriculture

(to M.M.) and the Fund for Scientific Research (Flanders) ('Krediet aan Navorsers' nr 1.5.515.98N to E.M.). B.D. is a postdoctoral fellow of the Fund for Scientific Research (Flanders).

References

Ayers AR, Ayers SB and Goodman RN (1979) Extracellular polysaccharide of *Erwinia amylovora*: a correlation with virulence. Applied and Environmental Microbiology 38: 659–666

Barny MA (1995) *Erwinia amylovora hrp*N mutants, blocked in harpin synthesis, express a reduced virulence on host plants and elicit variable hypersensitive reactions on tobacco. European Journal of Plant Pathology 101: 333–340

Bellemann P, Bereswill S, Berger S and Geider K (1994) Visualization of capsule formation by *Erwinia amylovora* and assays to determine amylovoran synthesis. International Journal of Biological Macromolecules 16: 290–296

Bellemann P and Geider K (1992) Localization of transposon insertions in pathogenicity mutants of *Erwinia amylovora* and their biochemical characterization. Journal of General Microbiology 138: 931–940

Bennett RA (1980) Evidence for two virulence determinants in the fireblight pathogen *Erwinia amylovora*. Journal of General Microbiology 116: 351–356

Bennett RA and Billing E (1978) Capsulation and virulence in *Erwinia amylovora*. Annals of Applied Biology 89: 41–45

Bereswill S, Jock S, Aldridge P, Janse JD and Geider K (1997) Molecular characterization of natural *Erwinia amylovora* strains deficient in levan synthesis. Physiological and Molecular Plant Pathology 51: 215–225

Bernhard F, Coplin DL and Geider K (1993) A gene cluster for amylovoran synthesis in *Erwinia amylovora*: characterization and relationship to *cps* genes in *Erwinia stewartii*. Molecular and General Genetics 239: 158–168

Busson R, De Bruyn A, Herdewijn P, Maes M and Messens E (2001) Identification of the exopolysaccharide amylovoran by NMR. Journal of Carbohydrate Chemistry 20: 109–120

Denny TP (1995) Involvement of bacterial polysaccharides in plant pathogenesis. Annual Review of Phytopathology 33: 173–197

Hartung JS, Fulbright DW and Klos EJ (1988) Cloning of a bacteriophage polysaccharide depolymerase gene and its expression in *Erwinia amylovora*. Molecular Plant–Microbe Interactions 1: 87–93

Janssen P, Coopman R, Huys G, Swings J, Bleeker M, Vos P, Zabeau M and Kersters K (1996) Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology 142: 1881–1893

Kiraly Z, El-Zahaby HM and Klement Z (1997) Role of extracellular polysaccharide (EPS) slime of plant pathogenic bacteria in protecting cells to reactive oxygen species. Journal of Phytopathology 145: 59–68

Leigh JA and Coplin DL (1992) Exopolysaccharides in plant–bacterial interactions. Annual Review of Microbiology 46: 307–346

Maras M, De Bruyn A, Schraml J, Herdewijn P, Claeyssens M, Fiers W and Contreras R (1997) Structural characterization of N-linked oligosaccharides from cellobiohydrolase I secreted by the filamentaous fungus *Trichoderma reesei* RUTC 30. European Journal of Biochemistry 245: 617–625

McManus PS and Jones AL (1995) Genetic fingerprinting of *Erwinia amylovora* strains isolated from tree-fruit crops and *Rubus* spp. Phytopathology 85: 1547–1553

Nimtz M, Mort A, Domke T, Wray V, Zhang Y, Qiu F, Coplin D and Geider K (1996) Structure of amylovoran, the capsular exopolysaccharide from the fire blight pathogen *Erwinia amylovora*. Carbohydrate Research 287: 59–76

Schraml J, De Bruyn A, Contreras R and Herdewijn P (1997) A versatile NMR technique for the identification of phosphorylation sites in oligosaccharides. Journal of Carbohydrate Chemistry 16: 165–170

Suhayda CG and Goodman RN (1981) Early proliferation and migration and subsequent xylem occlusion by *Erwinia amylovora* and the fate of its extracellular polysaccharide (EPS) in apple shoots. Phytopathology 71: 697–707

Tharaud M, Menggad M, Paulin J-P and Laurent J (1994) Virulence, growth and surface characteristics of *Erwinia amylovora* mutants with altered pathogenicity. Microbiology 140: 659–669