Genetic transfer of amylovoran and stewartan synthesis between *Erwinia amylovora* and *Erwinia stewartii*

Frank Bernhard,^{1†} Dietlinde Schullerus,¹ Peter Bellemann,¹ Manfred Nimtz,² David L. Coplin³ and Klaus Geider^{1‡}

Author for correspondence: Klaus Geider. Tel: +49 6203 106 117 or 120. Fax: +49 6203 106 122.

¹ Max-Planck-Institut für medizinische Forschung, Jahnstrasse 29, D-69120 Heidelberg, Germany

² Gesellschaft für biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Germany

³ Department of Plant Pathology, The Ohio State University, Columbus OH 43210, USA

DNA fragments with *ams* genes of *Erwinia amylovora* and *cps* genes of *Erwinia stewartii* were transferred to exopolysaccharide (EPS)-deficient mutants of the other species. The resulting EPSs were characterized by sensitivity to EPS-dependent bacteriophages, staining with amylovoran-specific fluorescein-isothiocyanate-labelled lectin and chemical techniques, such as determination of the sugar composition and methylation analysis in order to distinguish between amylovoran and stewartan. Degradation by the stewartan-dependent phage ϕ -K9 was used to detect stewartan production, and staining with a lectin from *Abrus precatorius* detected amylovoran capsules. The patterns of sugar linkages were determined by methylation analysis. Stewartan contained a significantly higher glucose to galactose ratio than amylovoran and produced a characteristic signal from 6-linked glucose residues. By these criteria, most *E. stewartii* *cps* mutants displayed exclusively amylovoran synthesis when complemented with the *ams* cluster, and *E. amylovora* *ams* mutants complemented with *E. stewartii* *cps* genes produced stewartan. The complementation to an EPS-positive phenotype may require most genes of the *ams* or the *cps* operon. An exception was an *E. stewartii* *cpsK* mutant that made predominantly stewartan when complemented with the *ams* cosmid. IR spectra showed that both amylovoran and stewartan were acylated when synthesized in *E. amylovora*, but not in *E. stewartii*. The amylovoran-producing *E. stewartii* merodiploids regained virulence to corn seedlings when mucoidy was restored by the *ams* cluster, but the stewartan-producing *E. amylovora* *ams*⁻/*cps*⁺ strains were weakly virulent on pear slices and avirulent on apple seedlings.

Keywords: fireblight, Stewart's wilt, complementation, transgenic bacteria, exopolysaccharide synthesis

INTRODUCTION

Extracellular polysaccharide (EPS) capsules and slime are important to plant-pathogenic bacteria (Leigh & Coplin, 1992). Capsules protect pathogens against recognition by plant defence mechanisms, bind water to keep the bacteria moist, and retain nutrients and ions released from

damaged plant cells. This not only creates a favourable environment for bacterial multiplication in the intracellular spaces and aids the spread of bacteria through plant tissues, but can also cause vascular occlusions and wilt symptoms (Van Alfen, 1989). *Erwinia stewartii* (recently reclassified as *Pantoea stewartii* subsp. *stewartii* by Mergaert *et al.*, 1993) is the causal agent of Stewart's wilt and blight of corn and is transmitted by the corn flea beetle. *Erwinia amylovora* is the causal agent of fireblight on pome fruit trees and other rosaceous plants. The capsular EPSs produced by *E. amylovora* and *E. stewartii* are called amylovoran and stewartan, respectively, and they are important virulence factors for these pathogens.

[†] Present address: Freie Universität Berlin, Institut für Kristallographie, Takustr. 6, D-14195 Berlin, Germany.

[‡] Present address: Max-Planck-Institut für Zellbiologie, Rosenhof, D-68526 Ladenburg, Germany.

Abbreviations: EPS, exopolysaccharide; FITC, fluorescein isothiocyanate.

Amylovoran-deficient mutants of *E. amylovora* are avirulent (Steinberger & Beer, 1988; Bellemann & Geider, 1992; Bernhard *et al.*, 1993b) and EPS-deficient mutants of *E. stewartii* fail to cause wilting and water-soaking, although they can still elicit restricted necrotic lesions (Dolph *et al.*, 1988; Coplin & Majerczak, 1990).

Amylovoran and stewartan are complex heteropolysaccharides, composed of glucose, galactose and glucuronic acid (Smith *et al.*, 1990; Costa, 1991). They are typical of enteric group I polysaccharides, such as the K30-EPS of *Escherichia coli*. As for other EPSs in this group, their synthesis is regulated by a complex mechanism involving transcriptional activation of EPS biosynthesis genes by RcsA and RcsB (Bernhard *et al.*, 1990; Gottesman & Stout, 1991; Poetter & Coplin, 1991). This suggests that gene clusters for the biosynthesis of group I polysaccharide may have a common evolutionary origin, and by analogy to group II biosynthetic clusters they may share a common organization of genes for production of nucleotide sugar precursors, polymerization of subunits, and transport of the polymer across the inner and outer membranes. Genetic comparison of the *ams* gene cluster for amylovoran synthesis and the *cps* gene cluster for stewartan synthesis supports this hypothesis (Bernhard *et al.*, 1993b). Genes involved in amylovoran synthesis have been cloned in cosmid pEA109 (Bernhard *et al.*, 1993b) and genes encoding stewartan synthesis have been cloned in cosmid pES2144 (Dolph *et al.*, 1988). Transposon mutations in each cosmid were introduced into the chromosome and then used in *in trans* complementation tests in order to assign mutations to *ams* and *cps* complementation groups. Both plasmids were able to complement EPS mutants of *E. stewartii* and of *E. amylovora* (Bernhard *et al.*, 1993b). Restoration of EPS synthesis was demonstrated by mucoid appearance, phage typing, and isolation of polysaccharides.

The nature of the EPS produced by the transgenic merodiploids was not determined. The structures of amylovoran and of stewartan (Fig. 1) indicate that genes for certain glycosyltransferases involved in subunit synthesis have diverged between *E. amylovora* and *E. stewartii*. Although the sugars and their linkages appear quite similar, three differences exist: (i) the backbone of the repeating unit of stewartan contains a 6-substituted glucose instead of an identically substituted galactose residue in amylovoran; (ii) the branched galactose residue is substituted in position 6 by a glucose residue to about 90% in stewartan and 10% in amylovoran; and (iii) the side chain of amylovoran is terminated by a pyruvylated galactose, whereas in stewartan another glucose molecule is attached instead of the pyruvate (M. Nimtz, A. Mort, V. Wray, T. Domke, Y. Zhang, F. Qiu, D. Coplin & K. Geider, unpublished). Restoration of mucoidy to EPS mutants of the other species raised the question as to the nature of the EPS produced. In this study, we demonstrate that the ability to synthesize either amylovoran or stewartan can be transferred between *Erwinia* species by cosmid clones containing the large *ams* or *cps* gene clusters. Parts of this work have been presented in the proceedings of various meetings (Bernhard *et al.*, 1993a, 1994).

Amylovoran

Stewartan

* about 10% repeating units branched with (β1→6) Glc (⇒t-G)

Fig. 1. Structure of the repeating units of amylovoran and stewartan as derived from NMR spectra of oligosaccharides and from methylation analysis (M. Nimtz and others, unpublished). The assignments (⇒) refer to the signals listed in Table 3.

METHODS

Strains and bacteriophages. These are listed in Table 1. Phage PEa1(h) (Hartung *et al.*, 1988) was renamed φ-Ea1h in order to avoid confusion with pEA-plasmids and strain Ea1/79. For propagation, the phages were plated on lawns of strains DC283 (for φ-K9) or Ea1/79 (for φ-Ea1h) to obtain confluent lysis. The next day, the phages were extracted with 5 ml phage buffer (0.15 M NaCl, 2 mM MgSO₄, 10 mM Tris/HCl; pH 7.5). A titre of 5×10^9 to 5×10^{11} p.f.u. ml⁻¹ was usually obtained. For degradation experiments phage φ-K9 was purified by precipitation with 10% PEG 6000 and 0.5 M NaCl. The precipitate was resuspended in phage buffer or water and the phages stored at 4 °C.

Isolation and sugar analysis of EPS. *E. amylovora* strains were grown on MM1 agar (Steinberger & Beer, 1988) with sorbitol and *E. stewartii* strains on CPG agar (Bradshaw-Rouse *et al.*, 1981). EPS was prepared as described by Bernhard *et al.* (1993b). The total concentration of EPS was determined by the anthrone method (Spiro, 1966), and galactose was used as a standard. Monosaccharides were analysed according to Chaplin (1982) as methyl glycosides after methanolysis and trimethylsilylation on a Carlo Erba Mega Series gas chromatograph with a 30 m DB1 capillary column connected to a Kratos MS50 mass spectrometer.

Methylation analysis of EPS and identification of the products. The EPSs were methylated according to Hakomori (1964). The permethylated carbohydrate material was purified

Table 1. Bacterial strains, plasmids and bacteriophages

Strain or plasmid	Description	Source or reference
<i>E. amylovora</i>		
CFBP1430	Wild-type	J. Laurent, INRA, Paris
Ea1/79	Wild-type	Falkenstein <i>et al.</i> (1988)
Ea1/79-del100	Deletion of <i>amsA-F</i> and <i>amsJ</i> , substituted by plasmid pfdB14Z'; Cm ^r	Bugert & Geider (1995)
Ea1/79-D4	Nal ^r ; <i>amsE4::Tn5</i>	Bernhard <i>et al.</i> (1993b)
Ea1/79-D41	Nal ^r ; <i>amsE41::Tn5</i>	Bernhard <i>et al.</i> (1993b)
<i>E. stewartii</i>		
449	Wild-type	W. Zeller (from England 1984)
DC283	SW104 (wild-type), Nal ^r	Coplin <i>et al.</i> (1986)
GAL8	DC283 Δ (<i>cpsB-galE</i>)	Dolph <i>et al.</i> (1988)
DM138	DC283 <i>cpsB178::Tn3HoHo1</i>	Coplin & Majerczak (1990)
DM144	DC283 <i>cpsK::Tn3HoHo1*</i>	Coplin & Majerczak (1990)
DM215	DC283 <i>cpsD215::Tn5lac*</i>	D. Coplin & D. Majerczak, unpublished
DM220	DC283 <i>cpsD449::Tn5lac*</i>	Coplin & Majerczak (1990)
PJD1910	DC283 <i>cpsC221::Tn5lac</i>	Coplin & Majerczak (1990)
Plasmids		
pEA109	15.5 kb <i>Hind</i> III fragment with <i>E. amylovora</i> genes <i>amsH-I-A-B-C-D-E-F-J-K-L</i> in pVK100, Tc ^r	Bernhard <i>et al.</i> (1993b)
pES2144	27 kb <i>Hind</i> III partial digestion fragment with <i>E. stewartii</i> genes <i>cpsA</i> to <i>galE</i> in pVK100, Tc ^r	Coplin <i>et al.</i> (1986)
Bacteriophages		
ϕ -K9	Host: <i>E. stewartii</i> with capsules	Bradshaw-Rouse <i>et al.</i> (1981)
ϕ -Ea1h	PEa1(h); host: <i>E. amylovora</i>	Hartung <i>et al.</i> (1988)

* The designation *cpsD'* used in the text refers to the complementation group *cpsD* (Dolph *et al.*, 1988), now known to contain four genes, *cpsD,E,F,G*; the old designation *cpsE* has been renamed *cpsK*.

by chromatography on a Sephadex LH20 column, hydrolysed with 4 M trifluoroacetic acid, reduced by NaBH₄ and peracetylated as previously described (Nimtz *et al.*, 1990). In order to detect hexuronic acids, the permethylated sample was additionally reduced with NaBD₄ and the glucuronic acid converted to glucose with two deuterium atoms incorporated at C6. Analyses were performed using the GC-MS instruments described in the preceding section.

Degradation assays and TLC. EPS (12 mg ml⁻¹; 70 μ l) was mixed with a purified phage ϕ -K9 suspension (1 $\times$ 10¹⁰ p.f.u. ml⁻¹; 5 μ l) and incubated overnight at 42 °C. An aliquot of the reaction mixture was then spotted on a silica gel plate (10 $\times$ 10 cm; 'HPTLC-Fertigplatten Kieselgel 60' without fluorescence indicator; Merck) and developed with n-butanol/H₂O/ethanol (5:4:5, by vol.) for 1.5 h (A. Bermpohl & R. Eichenlaub, personal communication). The plate was air dried, sprayed with 0.3% orcinol (Sigma) in 3 M H₂SO₄ and incubated for 30–45 min in a 100 °C oven.

Acetylation analysis and IR spectroscopy. EPS (0.5 mg) was thoroughly mixed with KBr (150 mg) and pressed into a translucent disk. Spectra were measured with a Perkin Elmer FT-IR 1760X infrared spectrophotometer. Acetate was determined with a commercial test system (Boehringer Mannheim).

Lectin staining. Bacteria were suspended on a glass slide in 5 μ l of a commercial solution (Sigma) of FITC-labelled lectin from *Abrus precatorius* (Jequirity bean). If required, up to 5 μ l of water was added to dilute the bacteria. The cells were visualized with

a Zeiss microscope Axiovert 405M with an oil-immersion lens at 1000 $\times$ magnification and filter BP450–490 FT510 LP520.

Virulence assays. The virulence assays of *E. amylovora* on pear slices were repeated as described previously (Bernhard *et al.*, 1993b) and supplemented by assays with apple seedlings (*Malus communis* cv. Golden Delicious). Ratings for symptoms on slices of immature pears were: 0, no symptoms; 1, little ooze formation and necrosis; 2, moderate amounts of ooze; 3, pear slice almost covered with ooze, necrotic appearance. Six-week-old apple seedlings were inoculated in the centre vein of a leaf with a needle dipped into a bacterial colony. Intermediate stages of symptom formation were not observed in the experiments of this series, which were done in triplicate. Virulence of *E. stewartii* strains on corn seedlings was determined by both a whorl assay for water-soaking (Bernhard *et al.*, 1993b) and a wilting assay. The whorl assay consisted of pipetting 0.1 ml of inoculum into the whorls of 1-week-old sweet corn seedlings (*Zea mays* cv. Earliking) without wounding. Inoculum was prepared at 10⁸ cells ml⁻¹ in 0.01 M potassium phosphate buffer containing 0.2% Tween 40. Symptoms were rated 3 d after inoculation on a continuous scale: 0, no symptoms; 1, scattered water-soaked lesions; 2, numerous water-soaked lesions; 3, extensive lesions with prolonged water-soaking and ooze-forming leaf surfaces. Values reported are means of at least three determinations. To measure wilting ability, seedlings were cut off 2 cm above the soil line, and 10 μ l of inoculum was placed on the cut stump. Wilting symptoms were rated on a scale of 0–3: 0, no symptoms; 1, one or two leaves wilted; 2, most of plant wilted; 3, all of plant wilted and necrotic.

RESULTS

Properties of *E. amylovora* and *E. stewartii* EPS-dependent bacteriophages

Cloned chromosomal fragments with *ams* genes of *E. amylovora* in cosmid pEA109 and with *cps* genes of *E. stewartii* in cosmid pES2144 (Fig. 2) were able to complement mutations in EPS genes of the other organism to a mucoid phenotype (Bernhard *et al.*, 1993b). In

this study, we analysed the type of EPS synthesized after heterologous complementation. For that purpose, two EPS-dependent bacteriophages, ϕ -Ea1h and ϕ -K9, were characterized for their ability to distinguish amylovoran and stewartan capsules. Phage ϕ -Ea1h grew well on *E. amylovora* wild-type strain Ea1/79, but was unable to lyse the EPS-deficient mutant Ea1/79-del100 [Δ *amsA-amsF*] (Table 2). In spot tests on a lawn of *E. stewartii* wild-type strain DC283, ϕ -Ea1h produced partial clearing and

Fig. 2. Comparison of the EPS genes cloned in pES2144 and pEA109. Complementation groups of the *E. stewartii* *cps* region (Dolph *et al.*, 1988) and the *E. amylovora* central *ams* region are indicated (Bernhard *et al.*, 1993b). The arrows assign regions of equivalent functions obtained by complementation analysis and from partial nucleotide sequences.

Table 2. Phage sensitivity of *Erwinia* strains and their virulence on corn seedlings or pear slices and apple seedlings

Strain	Spot test* with phage		Virulence on corn (WS/WI)†	Virulence on pear slices/apple seedlings‡
	ϕ-K9	ϕ-Ea1h		
<i>E. stewartii</i>				
DC283	3	1	2.9/2.9	
GAL8	0	0	0/-	
DC283(pEA109)	3	1	3.0/-	
DM138, DM215, DM220, PJD1910	0	0	0-1.3/0	
Same with pEA109	0	1	2.3-3.0/2.0-2.7	0/-
DM144	0	0	0/0	
DM144(pEA109)	2	1	3.0/2.7	0/-
<i>E. amylovora</i>				
Ea 1/79	0	2		3/3
Ea1/79-del100	0	0		0/-
Ea1/79(pES2144)	2	3		1.5/-
Ea1/79-D4, -D41	0	0		0/0
Same with pES2144	3	3		0-0.5/0

* From a phage solution of about 10^8 p.f.u. ml⁻¹, 10 μ l was applied to the agar surface with bacteria of the strains indicated. Rating of spot tests with phages: 0, no visible reaction; 1, barely visible halo; 2, turbid halo; 3, clear lysis.

† WS, whorl assay to determine water soaking ability; WI, cut stump assay to determine virulence from wilting symptoms. -, Experiment not done.

‡ Symptoms on apple seedlings at two weeks after inoculation (0, no symptoms; 3, wilting and necrosis). -, Experiment not done.

Fig. 3. Separation of phage ϕ -K9 cleavage products on silica gel. Lanes: M, marker oligosaccharides (predominantly pentamers and tetramers of glucose; Merck); 1, amylovoran from Ea1/79; 2, amylovoran from Ea1/79 with phage; 3, stewartan from DC283; 4, stewartan from DC283 with phage; 5, EPS from DM144(pEA109); 6, EPS from DM144(pEA109) with phage; 7, EPS from DM220(pEA109); 8, EPS from DM220(pEA109) with phage; 9, EPS from PJD1910(pEA109); 10, EPS from PJD1910(pEA109) with phage.

scattered plaques in the zone of phage application. The clearing observed on lawns of each species suggests that the ϕ -Ea1h depolymerase can degrade both amylovoran and stewartan. In another set of experiments we used phage ϕ -K9, isolated from *E. stewartii*, to characterize EPS degradation. In spot tests, ϕ -K9 lysed only wild-type capsulated cells of *E. stewartii*, and not *E. amylovora* strain Ea1/79. *E. stewartii* *cps* mutants were not permissive for phage ϕ -K9. Interestingly, plaque formation of ϕ -K9 phages on wild-type *E. amylovora* carrying plasmid pES2144 indicated that stewartan production was the only factor limiting phage infection.

Characterization of EPS synthesis using *Erwinia* phages

When phage ϕ -Ea1h was applied in a spot assay to a lawn of *E. stewartii* *cps* mutants DM138 (mutated in *cpsB*), DM144 (*cpsK*), DM215 (*cpsD'*), DM220 (*cpsD'*) and PJD1910 (*cpsC*) carrying plasmid pEA109, turbid zones were observed (Table 2). Considering phage dependence on a capsule, the results confirmed complementation of the mutants by pEA109. Complementation of *E. amylovora* *ams* mutants with pES2144 also restored lysis by phage ϕ -Ea1h.

To determine the type of EPS produced, we used the stewartan-dependent phage ϕ -K9. All *E. amylovora* *ams* mutants carrying pES2144 formed cleared zones in spot tests with ϕ -K9 (Table 2), similar to *E. stewartii* DC283 and DM144(pEA109). This was in contrast to mutants DM138, DM215, DM220 and PJD1910 with pEA109 (Table 2), which were ϕ -K9 resistant. We assume that the complemented *E. stewartii* mutants synthesized amylovoran, which is not degraded by phage ϕ -K9. On the other hand, *E. amylovora* mutants and wild-type strain Ea1/79 with pES2144 were permissive to phage ϕ -K9 (Table 2). Results of the spot tests were confirmed by

plaque formation (data not shown), which showed not only degradation of stewartan but also propagation of the phages in these bacteria.

Degradation of EPSs by phage ϕ -K9 depolymerase

The ability of phage ϕ -K9 to specifically degrade stewartan was exploited to further analyse the EPS obtained from *ams/cps* merodiploids. EPS preparations were digested with partially purified phage particles, and the cleavage products were analysed by TLC. The size distribution of degraded stewartan was heterogeneous, but easily distinguishable from intact amylovoran. The main cleavage product of stewartan migrated in the range of heptasaccharides (Fig. 3). In this assay, EPS from DM144(pEA109) had a pattern almost identical to stewartan (compare lanes 6 and 4 in Fig. 3), whereas EPS from strains DM220(pEA109), PJD1910(pEA109) (Fig. 3, lanes 8 and 10) and DM215(pEA109) (not shown) was not cleaved by the EPS depolymerase in phage ϕ -K9 particles. We assume that depolymerase cleaves a bond within the backbone of stewartan, because it reduces the molecular mass of the polymer. Since this enzyme does not affect amylovoran, it is likely that it cleaves adjacent to the glucose in the backbone. These results suggest that the EPSs synthesized after heterologous complementation were similar in structure to either amylovoran or stewartan.

Sugar composition and methylation analysis of EPS from *ams/cps* merodiploids

The repeating units of amylovoran and stewartan contain the same sugars, but differ in the amount of glucose (Fig. 1). EPS from the transgenic strains Ea1/79-D4(pES2144) and DM144(pEA109) was similar in sugar composition to EPS from *E. stewartii* wild-type strain DC283. However, complementation of *E. stewartii* mutants DM215 (*cpsD'*), PJD1910 (*cpsC*), and DM220 (*cpsD'*) with pEA109 resulted in a substantial decrease in the ratio of glucose to galactose, resembling the sugar composition of amylovoran (data not shown).

A more precise way to distinguish between amylovoran and stewartan-like EPS was by methylation analysis (Table 3). Since only stewartan contains 6-substituted glucose residues in its repeating unit (Fig. 1), the detection of the respective derivative by methylation analysis offers an easy way of classifying amylovoran and stewartan. Additionally, pyruvylated terminal galactose detected as the respective 4,6-disubstituted derivative was typical of amylovoran. Furthermore, amylovoran contains predominantly 3,4-disubstituted core galactose, whereas in stewartan this residue is almost completely substituted in position 6 by a glucose residue, indicated by the detection of di- or trisubstituted galactose, respectively. For the wild-type EPSs, all of the observed methylated derivatives were consistent with the structures proposed in Fig. 1. The quantity of each derivative depended on the susceptibility of the respective bond to hydrolysis: therefore

Table 3. Methylation analysis of EPS preparations from wild-type and complemented *Erwinia* strains

The signals were assigned to sugar residues in Fig. 1 as indicated in the last column: P1 to P6; pyr, pyruval group; t-G, terminal glucose, this compound could be derived in part from sources different from EPS (LPS and branched glucan in the case of *E. amylovora* strains). The most significant signal for distinction of amylovoran and stewartan is shown in bold print.

Peracylated derivative of	Subst. in posn	Ea1/79*	Ea1/79-D41* (pES2144)	DC283	DM144 (pEA109)	DM215 (pEA109)	DM220 (pEA109)	
Galactitol								
2,4,6-Tri-O-methyl-	3	++	++	++	++	++	++	/P1
2,3,4-Tri-O-methyl-	6	+++	+	++	++	+++	+++	/P2
2,6-Di-O-methyl-	3; 4	+	+	—	(+)	+	+	/P3
2,3-Di-O-methyl-	4; 6	+	++	—	+	+	+	/pyr
2-Mono-O-methyl-	3; 4; 6	+	(+)	+	+	+	+	/P4
Glucitol								
2,3,4,6-Tetra-O-methyl-	—	+	++	+++	+++	++	++	/t-G
2,3,4-Tri-O-methyl-	6	—	++	++	++	—	—	/P5
2,3-Di-O-methyl-(6-D ₂)-†	4	+	+	+	+	+	+	/P6

* From *E. amylovora* cultures, a signal for 3,4,6-tri-O-methylglucitol was detected, which probably originates from contaminating glucan.

† Obtained by additional NaBD₄ reduction of the permethylated sample.

Table 4. FITC-lectin staining of capsules of *Erwinia* wild-type strains and heterologously complemented mutants

Host cells	Fluorescence (%)*			
	Bright	Medium	Weak	None
<i>E. stewartii</i>				
DC283	0	0	0	100
DC283(pEA109)	0	0	100	0
DM144(pEA109)	0	0	100	0
DM138(pEA109), DM215(pEA109)	0	70	0	30
DM220(pEA109), PJD1910(pEA109)	0	100	0	0
<i>E. amylovora</i>				
Ea1/79	10	80	0	10
Ea1/79(pES2144)	0	10	0	90
Ea1/79-D41(pES2144)	0	0	1	99
<i>E. amylovora</i> from pear ooze†				
Ea1/79	0	20	0	80
Ea1/79(pES2144)	0	1	0	99
Ea1/79-D41(pES2144)	0	0	1	99

* The proportion of cells staining at different intensities is given as the percentage of total cells visualized by transmitted light; about 200–500 cells were evaluated in one experiment.

† Diluted 1:20 in distilled water. For confirmation the experiments were repeated at least twice.

the data listed in Table 3 are not quantitative. The complemented *E. stewartii* mutant DM144(pEA109) and the *E. amylovora* mutant Ea1/79-D41(pES2144) showed the expected amount of 6-substituted glucose, indicating the presence of a stewartan-type backbone in these EPSs. The side chains of the polysaccharides, however, could

contain a mixture of stewartan and amylovoran structural motifs. Especially, the glucosylation at position 6 of the side chain galactose apparently competes with the pyruvylation, as is indicated by the detection of varying amounts of the respective galactose derivatives in different mutants.

E. stewartii mutants DM215 and DM220 complemented with pEA109 produced a polysaccharide very similar to native amylovoran, except that the incidence of glucose at position 6 of the branched galactose residue was slightly higher, as indicated by the ratio of the 3,4- and 3,4,6-substituted galactose derivatives.

The detection of 2- and 2,6-substituted glucose in addition to high values of terminal glucose in the EPSs of *E. amylovora* strains Ea1/79 and Ea1/79(pES2144) could be due to contaminating glucan, since a low-molecular-mass EPS type containing only glucose is also found in polysaccharide preparations of *E. amylovora* mutants deficient in amylovoran synthesis (unpublished results; Smith *et al.*, 1995). The presence of branched 1,2-glucan could explain most of the terminal glucose found in EPS isolated from strains Ea1/79 and Ea1/79-D41(pEA109) (Table 3).

Identification of capsular amylovoran by staining with FITC-labelled lectin

Lectin from *Abrus precatorius* has a strong affinity for amylovoran (Bellemann *et al.*, 1994), but does not bind to stewartan. We used this property to distinguish between the two EPS types in capsules of *cps/ams* merodiploids (Table 4). When pEA109 was introduced into *E. stewartii* wild-type strain DC283, the cells were faintly stained by the lectin, whereas the parent strain was completely unreactive. Conversely, only 10% of cells from strain Ea1/79(pES2144) were intermediately stained, in contrast to 80% of the cells from the parent strain without this plasmid (Table 4). These results further demonstrated altered EPS in wild-type strains carrying the heterologous EPS gene cluster. Cells of the complemented *ams* mutant Ea1/79-D41(pES2144) were only weakly stained with lectin, indicating that this complemented mutant synthesized mostly stewartan. When the *cpsB*, *cpsC* and *cpsD'* *E. stewartii* mutants were complemented with pEA109, they bound the lectin and stained with medium fluorescence. On the other hand, the *cpsK* mutant DM144 with plasmid pEA109 was weakly stained, similar to DC283(pEA109). This is evidence that DM144(pEA109) produced predominantly stewartan, but that amylovoran was additionally synthesized, at a low level.

IR spectroscopy

The IR spectra of various EPS preparations from *E. amylovora* and *E. stewartii* wild-type strains and from *ams/cps* merodiploids were measured. The IR profile for amylovoran was identical for strain Ea1/79 and for a derivative of strain CFBP1430, isolated in France (not shown). Identity was also found for the spectra of stewartan, when prepared from *E. stewartii* strains DC283 or 449. Typical differences between amylovoran and stewartan were observed in absorption peaks at 1734, 1375 and 1254 cm^{-1} (Fig. 4). The absorption profile at other wavenumbers was similar for amylovoran and stewartan. A band at 1609 cm^{-1} was slightly shifted to 1647 cm^{-1} for the EPS preparations and not considered to be significant. When amylovoran was treated with alkali

Fig. 4. Comparison of IR spectra of (a) amylovoran from wild-type *E. amylovora* strain Ea1/79 and (b) stewartan from wild-type *E. stewartii* strain DC283. Only the significant range from 1800–1500 cm^{-1} differing for the two EPS species is shown.

Table 5. Typical signals of IR spectra for various EPS preparations

1/ ν refers to the wavenumber in cm^{-1} .

EPS source	1/ ν = 1734	1/ ν = 1375	1/ ν = 1254
Ea1/79	+	+	+
Ea1/79 (KOH-treated)	—	—	—
Ea1/79-D4(pEA109)	+	+	+
Ea1/79-D4(pES2144)	+	+	+
DC283	—	—	—
DM220(pEA109)	—	—	—
PJD1910(pEA109)	—	—	—

(20 mM KOH overnight at room temperature) the typical bands at 1734, 1375 and 1254 cm^{-1} disappeared. No change was seen when stewartan was treated with alkali. The absence of significant amounts of acetyl residues in stewartan was confirmed with a commercial test for acetic acid. When applied to amylovoran, the test was positive, and the acetate content was estimated to be about 7%. No acetate could be detected enzymically after KOH treatment and dialysis of amylovoran. We concluded that the IR absorption bands differing for the two EPSs were derived from acyl residues, which were removed by alkaline hydrolysis of amylovoran. Although stewartan is not normally acylated, the absorption pattern changed when it was synthesized in *E. amylovora* strain Ea1/79-D4(pES2144) (Table 5). The presence of the three peaks suggested acylation of stewartan by the *E. amylovora*

merodiploid. On the other hand, no acyl groups were found in IR spectra of EPS from strains DM220(pEA109) and PJD1910(pEA109) (Table 5). It appears that *E. stewartii* strains derived from DC283 were unable to decorate any EPS with acyl residues. Stewartan from *E. stewartii* strain 449 was also not acylated.

Virulence of *ams/cps* merodiploids

In previous studies, mutants with transposon insertions in *cps* genes of *E. stewartii* showed a greatly reduced ability to damage corn seedlings. The water-soaking ability of different *cps* mutants varied and was probably affected by relative humidity (Coplin & Majerczak, 1990). Virulence with the whorl assay (Bernhard *et al.*, 1993b) was confirmed for transgenic *E. stewartii* *cps* mutants with pEA109 by application of bacteria into the pseudostem (whorl) of the seedlings. *E. stewartii* *cps* mutants complemented with pEA109 regained the ability to cause water-soaked lesions and to cause wilt (Table 2). The virulence of wild-type strain DC283 was unaffected by pEA109 carriage. None of the *E. stewartii* strains carrying pEA109 was pathogenic on immature pears.

In contrast to the heterologously complemented *cps* mutants on corn seedlings, the *E. amylovora* *ams* mutants carrying pES2144, were barely virulent on pear slices (Table 2). Even wild-type strain Ea1/79 carrying pES2144 showed reduced virulence. When ooze from inoculated pear slices was diluted and capsulated bacteria were stained with FITC-labelled lectin, the proportion of positively staining capsules of Ea1/79(pES2144) and Ea1/79-D41(pES2144) was found to be much lower *in planta* than in culture, as already found for the wild-type *E. amylovora* strain Ea1/79 (Table 4). *E. amylovora* strain Ea1/79-D4(pES2144) did not produce any symptoms when assayed on apple seedlings (Table 2).

DISCUSSION

The heteropolysaccharides amylovoran and stewartan are related in sugar composition (Fig. 1) but different in structure. In the case of amylovoran, the backbone of the repeating unit contains only galactose residues. A galactose and a glucuronic acid residue form the side chain. Stewartan contains glucuronic acid, galactose and glucose in a ratio of 1:3:3, and glucose residues could be located in the backbone and in the side chain (Costa, 1991; M. Nimtz and others, unpublished). An oligosaccharide of about seven residues was observed after cleavage of stewartan by ϕ -K9 depolymerase. Differences in EPS structure may be important for the interactions of these two plant pathogens with their hosts.

In previous studies, *E. stewartii* *cps* mutants with the cloned *ams* gene cluster and *E. amylovora* *ams* mutants with the cloned *cps* gene cluster regained mucoidy (Bernhard *et al.*, 1993b), suggesting functional equivalence of some of the EPS genes. The *ams* and *cps* clusters have recently been compared by sequence analysis. The *ams* operon contains 12 genes (Bugert & Geider, 1995), and sequence data

from *E. stewartii* (D. Coplin & D. Majerczak, unpublished) suggest that the *cps* cluster has a similar organization, including many genes homologous to *ams* genes.

Our finding that FITC-labelled lectin from *A. precatorius* specifically binds to amylovoran, but not to stewartan, allowed the distinction of the two EPS capsules by fluorescence microscopy. Differences in capsule staining within a population of merodiploids might reflect individual levels of amylovoran synthesis. However, the brightness of stained capsules and their frequency in a cell population is not a completely reliable way to determine amylovoran synthesis in individual bacteria, due to loose association of capsules with the *E. amylovora* cells (Politis & Goodman, 1980). To further distinguish between amylovoran and stewartan production by the interspecific transconjugants, we used spot tests with EPS-specific phages, and sugar composition and methylation analysis of the EPSs from the complemented mutants. Phage ϕ -Ea1h from *E. amylovora* (Hartung *et al.*, 1988) reacted only with capsulated cells of *E. amylovora* and *E. stewartii*. Bacteriophage ϕ -K9 from *E. stewartii* (Bradshaw-Rouse *et al.*, 1981) was strictly dependent on the presence of stewartan, presumably by its recognition of the glucose residue in the backbone. The results of phage sensitivity tests were confirmed by using the depolymerase activity present in purified preparations of ϕ -K9 virions to break down stewartan, which was analysed by TLC on silica gel. Degradation of EPS from DM144(pEA109) and Ea1/79-D41(pES2144) by ϕ -K9 depolymerase indicated that the EPS synthesized was stewartan. A unique signal for 1 $\rightarrow$ 6-linked glucose was obtained by methylation analysis for the repeating unit of stewartan. The sugar with this property was absent in amylovoran, and the signal could be used to exclude low quantities of stewartan in EPS from the heterologously complemented *cps* mutants DM215(pEA109) and DM220(pEA109).

By these criteria, *E. amylovora* strains with mutations in *amsA* through *amsF* carrying pES2144 synthesized EPS with properties of stewartan and, conversely, the *E. stewartii* strains with mutations in *cpsB* through *cpsD'* carrying pEA109 produced EPS with properties of amylovoran. Therefore, EPS production in these strains was due to substitution of a major part of the biosynthetic pathway, rather than complementation of single genes. An exception was the *E. stewartii* *cpsK* mutant, which behaved like the wild-type strain when complemented with pEA109 and synthesized a small amount of amylovoran in addition to near-normal amounts of stewartan. In this case, it appears that *cpsK* has a similar function in both *Erwinia* species. This could be a step either in synthesis of nucleotide-sugar precursors or in assembly and transport of the repeating unit.

The ability of the *ams* and the *cps* genes carried in pEA109 and pES2144 to direct synthesis of foreign EPS in another species allows us to form several hypotheses about the functions of the genes in these clusters. The *ams* region on pEA109 and the *cps* genes on pES2144 encode the genetic information for most of the glycosyltransferases needed to form entire repeating units. The cloned fragments may

not contain all the genes needed for EPS synthesis, since there are mutations of *E. amylovora* (Bellemann & Geider, 1992) and *E. stewartii* (D. Coplin & D. Majerczak, unpublished) that are apparently unlinked to the *ams* and *cps* clusters. Since synthesis of amylovoran or stewartan has not been observed in *Escherichia coli* K12 strains harbouring plasmids pEA109 or pES2144, it is possible that these unlinked genes encode factors specific to EPS synthesis in *Erwinia*, rather than common factors such as *rcs* regulatory proteins. Common steps in EPS biosynthesis between the two *Erwinia* species may also include a similar undecaprenol lipid carrier and synthesis of the same UDP-sugar precursors. It is unlikely that the cloned *ams* and *cps* regions contain genes for undecaprenol synthesis, since deletion of these regions does not affect LPS synthesis. The EPSs from *E. amylovora* and *E. stewartii* differ in acylation. The acyltransferases of *E. amylovora* can apparently modify stewartan, since the EPS from D4(pES2144) was acylated, but the transacylase genes may not be a part of the *ams* cluster, because EPS from *E. stewartii* strains carrying pEA109 was not acylated. The clusters do, however, contain a similar region for synthesis of nucleotide sugars, which includes *galE* (Metzger *et al.*, 1994) and an ORF with homology to *galU* (L. Kolb & K. Geider, unpublished) that is located to the right of the *cps* region in *E. stewartii* (Dolph *et al.*, 1988; D. Coplin & D. Majerczak, unpublished), and to the right of the *ams* operon in *E. amylovora* (P. Bugert, S. Bereswill & K. Geider, unpublished).

For O-antigens, the serotype can depend on the acetylation pattern (Yao & Valvano, 1994). Despite its acylation, stewartan synthesized in *E. amylovora* strains did not substitute for amylovoran in pathogenesis, since pES2144 barely restored virulence of *ams* mutants assayed on pear slices and had no effect on virulence of these strains when assayed on apple seedlings. The inability of stewartan to restore virulence to *ams* mutants could be due to a failure to block host defence reactions by harpin. This protein is an important factor for induction of the hypersensitive reaction by *E. amylovora* (Wei *et al.*, 1992). On the other hand, the vascular pathogen *E. stewartii* appears to be less dependent on the type of EPS produced, since the virulence of *cps* mutants to corn can be restored by pEA109. This may reflect the lack of an induced resistance response in corn to non-capsulated *E. stewartii*, so that there is no need for a particular capsule to protect the bacterium against host defences. It is also interesting that the production of amylovoran by *E. stewartii* did not extend its host range to pears. On the other hand, the results of this study show that there are no inherent barriers to the expression of heterologous EPS genes within the *Erwinias*. In comparable experiments, the expression of *Klebsiella* K2 capsular polysaccharide in *Escherichia coli* and in various *Klebsiella* serotypes was obtained after transfer of the cloned *cps* gene cluster (Arakawa *et al.*, 1991). For *Escherichia coli*, a single glycosyltransferase gene could complement in pathways for LPS synthesis, resulting in hybrid O-antigens (Kogan *et al.*, 1993). For *E. amylovora* and *E. stewartii*, more information about the functions and expression of the *ams*

and the *cps* genes is required to achieve gene-to-gene complementation.

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