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Biosynthesis of the repeating units of the exopolysaccharides amylovoran from *Erwinia amylovora* and stewartan from *Pantoea stewartii*

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ABSTRACT

The causative agent of fire blight, *Erwinia amylovora*, synthesizes the exopolysaccharide (EPS) amylovoran, an important pathogenicity factor. Similarly, *Pantoea stewartii*, needs the related exopolysaccharide, stewartan, to cause Stewart's bacterial wilt of sweet maize. These acidic complex EPSs mask the recognition of the pathogens *E. amylovora* and *P. stewartii* by plant defense reactions and promote bacterial growth and movement *in planta*. The *E. amylovora* *ams* gene cluster determines the biosynthesis of the amylovoran EPS repeating unit, which is comprised of four galactose and one glucuronic acid residues. Stewartan is synthesized by the *P. stewartii* *cps* gene products. Functions of Ams proteins were analyzed in an in-vitro system with EDTA-treated cells. Cross-complementation of an *amsD* mutant with the *cpsFG* genes from *P. stewartii* resulted in chimeric EPS with glucose instead of galactose as the second sugar residue in the backbone. AmsG adds the first galactose and AmsD the next one. The ability of CpsF to attach the next galactose residue to the backbone of the chimeric EPS suggests a similar role for AmsE. Evidence for the possible roles of other glycosyltransferases in the assembly of the repeating units of amylovoran and stewartan is presented.

1. Introduction

The Gram-negative enterobacterium *Erwinia amylovora* is the causative agent of fire blight of many rosaceous plants, including apple and pear trees. Typical symptoms are wilting of young shoots, which later turn brown or black and sometimes show droplets of ooze. The exudate from the infected plants contains the exopolysaccharide (EPS) amylovoran, which is strictly required for virulence [1] [2], [3], [4]. The acidic EPS forms a capsule around the cells to prevent recognition by host plant defense mechanisms and it facilitates movement through plant tissue, in addition to aiding survival outside the host plant [6]. Amylovoran is a heteropolysaccharide with pentasaccharide repeating units containing four galactose and one glucuronic acid residues (Fig. 1) [5]. The backbone of the repeating unit consists of three differently linked galactose residues. The major side chain consists of glucuronic acid and a terminal galactose decorated with pyruvyl and acetyl groups. A second side chain is formed when glucose is attached to the branched galactose residue in 50% of the repeating units [7]. Amylovoran biosynthesis is related to the assembly of type 1 capsules of *Escherichia coli* [8]. As shown for other EPSs [9], the repeating units are probably synthesized in the cytoplasm while attached to an undecaprenyl phosphate lipid carrier [10], and then polymerized during transport to the cell surface [11]. The twelve-gene *ams* operon (Fig. 2) provides functions required for the biosynthesis of amylovoran [12]. Earlier sequence database searches in 1995 suggested roles in glycosyl transfer for the *amsG*, *B*, *D*, *E*, *J* and *K* gene products, although homology of these proteins with other characterized glycosyltransferases was relatively low at that time. These comparisons have been updated in this manuscript. Other *ams*-encoded proteins such as AmsH, AmsL and AmsC, may be involved in oligosaccharide transport and assembly, because they display transmembrane domains. AmsA has tyrosine kinase activity [13]. Similar protein kinases are known to be involved in EPS synthesis and have been reported to have an effect on the molecular weight distribution of succinoglycan [14]. The gene preceding *amsA* in the *ams* operon, *amsI*, encodes an acid phosphatase [15]. Its activity could interfere with protein phosphorylation and it seems to be associated with recycling of the diphosphorylated lipid carrier after release of the synthesized repeating unit. AmsF has significant homology with a viral EPS depolymerase [16], which may indicate that it has a role in the processing of the newly synthesized repeating units and/or their polymerization by adding them to an existing amylovoran chain.

Pantoea stewartii subsp. *stewartii* (formerly *Erwinia stewartii*) causes Stewart's wilt of sweet maize. This bacterium has a *cps* gene cluster for stewartan biosynthesis [3], [18], which is homologous to and colinear with the *ams* region of *E. amylovora*. Stewartan is very similar in structure and properties to amylovoran, except that the second sugar in the backbone of the repeating unit is glucose instead of galactose (Fig. 1) [19] [20]. Stewartan is similarly required for the full virulence of *P. stewartii* in sweet maize [18], where it promotes the

spread of bacteria in intercellular spaces, prolongs watersoaking and bacterial multiplication in leaves, and plugs the xylem vessels to cause systemic wilting. Participation of stewartan and also amylovoran in the formation of biofilms has been shown in planta and was demonstrated in flow cell assays for cell attachment [21], [22].

Preliminary reports of the *ams* and *cps* sequence analysis and a tentative pathway for the assembly of the repeating units of amylovoran were presented at the VIIth and VIIIth Internatl. Workshops on Fire Blight [17], [23]. At that time, the models were based on early data for the characterization of lipid-linked intermediates in amylovoran synthesis. The completed studies presented here for amylovoran assembly have been significantly updated including genetic data for gene complementation and steps for assembly of stewartan.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

E. amylovora and *P. stewartii* cultures were grown in Standard I medium (StI; Merck AG, Darmstadt) or in Luria-Bertani broth (LB) with 1% sorbitol. Ampicillin was supplied at 100 µg/ml, and chloramphenicol at 20 µg/ml. The strains and plasmids used in this study are listed in Table 1. Minimal agar MM2C is MM2 [24] with 1% sorbitol at pH 6.5.

2.2 Genetic methods

To create *galE* mutants, the suicide plasmid pfdA31-*galE* was constructed. The intact *galE* gene was isolated from pEA200 after restriction with *Hind*III and cut in the center with *Eco*RI. The two *galE* fragments were ligated with the "suicide" plasmid pfdA31, which was linearized with *Eco*RI, to create an insertion of pfdA31 within *galE*. pfdA31 can be propagated in *E. coli* cells producing bacteriophage fd gene 2 protein but it cannot replicate in other bacteria. To disrupt the chromosomal *galE* gene, the suicide plasmid pfdA31-*galE* was electroporated into *E. amylovora* wild-type or *ams* mutant strains and stable transformants were selected for ampicillin resistance. To exclude cointegrates, cells from single colonies were checked by PCR for double cross-overs within the chromosomal *galE* using two primers that amplify the *galE* gene (*galE*-L1: 5'-CGATGACGTGGTGATAC; *galE*-R1: 5'-AGTCGGATAGTCATCAC). Plasmid pEA101 was introduced into *E. amylovora* strains by electroporation to increase expression of *rcsA* and thereby enhance incorporation of UDP-sugars.

An *amsG* mutant of Ea1/79 was constructed from Ea7/74-K15 [12] by amplification of an internal part of *amsG*, cloning this fragment into pfdA2, and then electroporation of the suicide plasmid into Ea1/79-*galE* [25]. This resulted in disruption of *amsG* by insertion of pfdA2. A similar approach was used for creation of mutant Ea1/79-K14-*galE*, recreating the same *amsK*::pfdA2 insertion that is present in Ea7/74-K14 [12].

Plasmids pEScpsG and pEScpsFG were created by amplifying *cpsG* and *cpsFG*, respectively, from *P. stewartii* strain DC283 with the primers listed in Table 1 and then cloning the amplicons into the linearized vector pGEMT (Promega).

2.3 Preparation of permeabilized cells

A modification of the method of Reuber and Walker [9] was used to prepare permeabilized cells. Fifty ml StI medium or LB containing 1 % sorbitol was inoculated with 0.5 ml from an overnight bacterial culture and incubated at 28 °C until growth reached an OD₆₀₀ of 0.6-1.0. The *E. amylovora* cells were harvested by centrifugation and washed with 20 ml 70 mM Tris (pH 8.2) and then resuspended in 70 mM Tris/10 mM EDTA (pH 8.2) at OD₆₀₀ = 100. The concentrated cells were frozen three times in liquid nitrogen for 1 min and then thawed for 5 min at 37 °C. The permeabilized cells could then be stored for several months at -80 °C. Attempts to permeabilize *P. stewartii* cells failed, because the treated cells formed sticky aggregates after EDTA treatment.

2.4 Isolation of lipid-linked intermediates

The method of Reuber and Walker [9] was modified to isolate lipid-linked intermediates. For incorporation of labeled EPS-precursors, a 100 µl reaction mixture contained: 70 µl permeabilized cells and final concentrations of 5 µM UDP-galactose, 100 µM UDP-glucose, 100 µM UDP-glucuronic acid, 100 µM acetyl-coenzyme A, 100 µM succinyl-coenzyme A, 12 mM MgCl₂, 100 µM phosphoenolpyruvate, and 1 µCi UDP-[³H]galactose galactose-4,5-³H(N) (from NEN-Perkin Elmer). Alternatively, 1 µCi UDP-[³H]glucuronic acid glucuronic acid-4,5-³H(N)] was added to the mixture instead of labeled galactose. In this reaction, unlabeled UDP-glucose was added and non-radioactive UDP-glucuronic acid was omitted. The mixture was incubated for 30 min at 8 °C and then stopped by adding cold 0.5 ml 70 mM Tris/10 mM EDTA (pH 8.2). The reaction mixture was centrifuged for 10 min at 12,000 X g. The cell pellet was then washed twice with 0.3 ml 70 mM Tris (pH 8.2) and extracted two times with 100 µl chloroform/methanol/water (1:2:0.3) to recover the labeled lipid-sugar intermediates. To determine the rate of incorporation, 20 µl of the extract were measured in a scintillation counter. When the amount of incorporated radioactivity was sufficient for further analysis, the rest of the extract was dried in a Speed-Vac centrifuge. For the hydrolysis of the intermediates from the lipid-carrier, the pellet was incubated in 200 µl 0.01 N trifluoroacetic acid at 90 °C for 20 min. After cooling on ice, the sample was neutralized by addition of 2 µl 1 M ammonia and dephosphorylated overnight at 37 °C in 10 mM glycine-NaOH (pH 10), 0.5 mM MgCl₂ with 8 U alkaline phosphatase (Type VII-S, Sigma). The intermediates were separated from the lipid-carrier by extraction with 500 µl chloroform/methanol (2:1). The aqueous phase was dried in a Speed-Vac centrifuge. For

analysis by HPLC, the pellet was resuspended in 0.1 M acetic acid containing 5 mg/ml cellodextrin, 2.5 mg/ml galactose and 2.5 mg/ml BSA as size markers.

2.5 HPLC analysis

A gel column (100 x 1 cm; Toyopearl HW-40S; particle size 30 micron; Tosohaas) in 0.1 M acetic acid was used for HPLC analysis (Beckman, System Gold). About 90 µl of the sample were applied to the column. The running time was 140 min at 0.4 ml/min, samples of 0.4 ml were collected from 55 min until 135 min, and radioactivity was measured in a scintillation counter. The comparison of the elution times of size markers with the peak fractions containing the labeled oligosaccharides gave standardized results. Partition coefficients (K) were calculated using the elution times (elution volumes) according to Reuber & Walker [9] as $K = (V_e - V_o) / (V_s - V_o)$ V_e : elution volume of the peak fraction, V_o : elution volume of BSA (exclusion volume), V_s : elution volume of galactose (salt volume). To assign precise molecular weights to individual peaks, partition coefficients were calculated that were based on the elution times for the markers BSA, galactose, tetraose, and pentaose for intermediate sizes of oligosaccharides.

3.6 Gene complementation and methylation analysis

Plasmids pEScpsFG and pEScpsG were transformed into the *E. amylovora* mutant Ea1/79-D50 (*amsD*) by electroporation. Ampicillin-resistant colonies were screened on minimal agar MM2C [24] and on slices from immature pears [26] for restoration of EPS synthesis and virulence. Ooze formation on pear slices indicated complementation of the *amsD* mutant by *cpsFG*. The cells were removed by centrifugation and the supernatant with EPS was dialyzed against water and freeze-dried. Methylation analysis was performed as described before [26].

3. Results

3.1. Comparison of the *E. amylovora ams* and *P. stewartii cps* gene clusters

The major difference between amylovoran and stewartan is that the second sugar residue in the repeating unit of stewartan is glucose instead of galactose and the corresponding gene products (Figs. 1, 2) are different. Consequently, comparative sequence analysis was done so that biochemical and genetic approaches to the synthesis in either *E. amylovora* or *P. stewartii* could be applied to models for the synthesis of both EPSs. We note that in most cases similarly located genes in each cluster have >58% amino acid identity, which suggests that they have similar catalytic functions. Colinear map order was found at the left side of the clusters for AmsG/CpsA, AmsH/CpsB, AmsI/CpsI, and AmsA/CpsC and on the right side for AmsF/CpsH, AmsJ/CpsJ, and AmsK/CpsK, and AmsL/CpsL. In the central part of the clusters, four genes,

which presumably encode glycosyltransferases [12] that specify the structure of the EPS, are arranged differently according to their amino acid similarity (Fig. 2). These findings are supported by similar length for the putative corresponding proteins ($\pm < 5\%$). The sizes for AmsC/CpsD are 375/368 amino acids (aa), for AmsB/CpsE 301/314 aa, for AmsE/CpsF 266/252 aa, and for AmsD/CpsG 354/344 aa, respectively. AmsB and CpsE are 78% similar; AmsC/CpsD or AmsD/CpsG less related to each other with only 53% and 45% similarity, respectively; and AmsE/CpsF are quite dissimilar with 34% similarity. This suggests that AmsE and CpsF could have different substrate specificities and/or functions.

3.2 *A mutation in galE and overexpression of RcsA increases incorporation of labeled galactose into EPS*

In initial experiments to synthesize labeled amylovoran repeating units in EDTA-permeabilized *E. amylovora* cells, the incorporation of radioactivity from labeled precursors into the lipid-linked intermediates was low and this made their HPLC analysis impossible. To improve incorporation, *galE* mutants were constructed. The *galE* gene encodes UDP-galactose-4-epimerase that interconverts UDP-galactose and UDP-glucose. We predicted that loss of this function should lead to better and more specific incorporation of UDP-[³H]galactose into EPS. Table 2 shows representative data for [³H]galactose incorporation into lipid-linked intermediates in EDTA-treated cells of strains with and without a *galE* mutation. In all cases, the incorporation of radioactivity into the *galE* strains was increased 3 to 5 fold. We also attempted to increase precursor incorporation by overexpression of *rcsA*⁺ (on plasmid pEA101), which encodes a transcriptional activator of the *ams* operon [28]. As shown in Table 2, overexpression of *rcsA* increased incorporation of UDP-[³H]galactose in the *amsC*, *D* and *E* mutants and *ams*⁺ strain, but decreased it in the *amsB*, *F* and *K* mutants when compared to the strain without pEA101. RcsA overexpression was used in the experiments below when it increased incorporation of label from UDP-[³H]galactose in a particular *ams* mutant.

3.3 *Lipid carrier-glycosyl intermediates are formed by the E. amylovora wild-type strain but not by an amsG mutant*

The HPLC elution profiles from wild-type cells resulted in several peaks, which represent intermediates in the synthesis of amylovoran. They ranged from two glycosyl residues attached to the lipid carrier up to the entire 5-sugar repeating unit (Fig. 3A). However, permeabilized cells of the *amsG* mutant, Ea1/79-G10-*galE*, did not incorporate a detectable amount of labeled precursors. The recovered label was below the amounts of all other mutants (Table 2) and did not produce a peak in the HPLC elution profile. AmsG shows homology to RfbP (now WbaP), which is responsible for the addition of the first sugar residue to the lipid-carrier in O-antigen synthesis of *Salmonella typhimurium* [11], [29]. The protein contains the LbetaH-domain with three imperfect tandem repeats connected to

acyltransferase activity. Further details of its functional domains can be found in Genbank accession number CAA54879. The *E. amylovora* *amsG* gene also shows homology to *exoY* from *Sinorhizobium meliloti*, which together with *exoF* encodes proteins necessary for incorporation of the first sugar in succinoglycan synthesis [9]. WcaJ from *Escherichia coli* is also homologous to AmsG and this protein is involved in the biosynthesis of colanic acid, probably by adding the first glucose residue to the endogenous acceptor [29]. Based on the failure of an *amsG* mutant to incorporate galactose into an amylovoran precursor and its homology to known enzymes, we propose that *amsG* encodes a galactosyl-1-phosphate-undecaprenyl-phosphate-transferase that adds the first galactose molecule to the lipid carrier.

3.4. Analysis of the accumulated glycosyl intermediates in other *ams* mutants

An *amsD* mutant was able to incorporate a significant amount of labeled galactose into material found in the organic phase that contained the lipid carrier. The HPLC profile of the *amsD* mutant D50-galE(pEA101) showed one peak with $K = 1.010$ in the position of a monosaccharide (Fig. 3B). Accordingly, we propose that AmsD attaches the second galactose residue to the growing repeating unit.

The putative glycosyltransferase mutants Ea1/79-D41-galE(pEA101) (*amsE*), Ea1/79-D44-galE (*amsB*), Ea1/79-K14-galE (*amsK*) each produced small peaks in their HPLC profiles at the positions of a mono- and a disaccharide (data not shown). It is possible that trisaccharide formation is a rare event, which restricts sequential growth of chains, so that larger intermediates, expected to accumulate in some mutants, were not made in our system. In some experiments *amsC* mutant, Ea1/79-D12-galE(pEA101), exhibited peaks at similar positions. Although AmsC is presumably not a glycosyltransferase and shows weak homology to polysaccharide polymerases (as to ZP_03390646), it may have a pivotal role in processing the growing repeating units by flipping them through the inner membrane so that they can be polymerized to high molecular weight amylovoran.

3.5. Complementation of an *amsD* mutant by *cps* genes from *P. stewartii*

The *E. amylovora* mutant Ea1/79-D50 (*amsD*) was transformed with plasmid pEScpsFG, carrying the *P. stewartii* genes *cpsF* and *cpsG*. On plates with minimal MM2C agar, the complemented mutant made very little EPS, but it produced ooze and necrosis on slices of immature pears [3]. The EPS from pear slices was analyzed for sugar composition and linkages. Methylation analysis revealed that a glucose residue had replaced the second galactose of the repeating unit in the EPS produced by the *cpsFG*-complemented *amsD* mutant (Fig. 4, P5). This product was not obtained by complementation with plasmid pEScpsG, which carries only *cpsG*, indicating that both *cpsF* and *cpsG* are necessary to complete the polysaccharide. In

homology studies, CpsG corresponds to AmsD, which is the glycosyltransferase that adds the second galactose to the growing repeating unit of amylovoran (Figs 2 and 5). CpsF is related to AmsE and this protein from *E. amylovora* might attach the third galactose in the repeating unit of amylovoran. CpsF would then be needed to add the third galactose, since the next transferase in the amylovoran pathway (AmsE) would only recognize a galactose residue as a substrate. The elution profile for methylation analysis also showed termination of the side chain by a pyruvyl residue (Fig. 4, pyr). This structure represents a chimeric repeating unit with the backbone of stewartan and the side chain of amylovoran. It is significant that the complemented *amsD* mutant was virulent on pear slices indicating that the chimeric EPS was still functional *in planta*.

4. Discussion

The repeating unit of amylovoran consists of five sugar residues and a single glucose side chain on about half of the subunits [5], [6], [26]. The biosynthetic enzymes are encoded by the *ams*-operon (Fig. 1), which contains twelve genes that are transcribed as one polycistronic mRNA [12]. Previous genetic complementation analysis of the *ams* region indicated that Tn5-insertions in any of the *ams* genes behave as if they are non-polar [3]. This is due to the presence of secondary promoters in front of the individual genes. Therefore, these Tn5 mutants were suitable for systematically blocking a single step in the amylovoran biosynthetic pathway and then characterizing the intermediates that accumulated in each mutant.

To increase incorporation of radioactively labeled galactose into oligosaccharide intermediates, a *galE* mutation was introduced into each *ams* mutant background. GalE is an epimerase, which reversibly converts UDP-glucose into UDP-galactose, the main precursor for biosynthesis of amylovoran and stewartan. Loss of this enzyme prevents the conversion of labeled UDP-galactose to UDP-glucose. Several transcriptional regulators of amylovoran synthesis have been described for *E. amylovora*. The activators RcsA [28] and RcsB [30] substantially enhance EPS production, so we attempted to increase EPS synthesis in our labeling studies by overexpressing RcsA. This approach was successful in the *amsC*, *D* and *E* mutants and the *ams*⁺ strain, but it decreased labeled UDP-galactose incorporation in the *amsB*, *F* and *K* mutants when compared to the strain without the *rcaA*⁺ plasmid. In the latter mutants, increased activity of the blocked amylovoran pathway may have led to the accumulation of toxic intermediates that eventually interfered with the vitality of the cells or down-regulated the pathway. Larger chains attached to the lipid carrier, which would be expected to accumulate in the *amsB* and *amsK* mutants, may affect growth more than mono- or disaccharide intermediates, which would be expected to accumulate in the *amsD* and *amsE* mutants. A similar situation has been reported for *S. meliloti* where the lack of the negative

regulator ExoR is often lethal in combination with other *exo*-mutations, possibly due to accumulation of lipid-linked intermediates [31].

Due to the absence of labeled galactose incorporation and a detectable intermediate, AmsG is likely to add the first galactose residue to the lipid-carrier (Fig. 5). Since a monosaccharide intermediate accumulated in the *amsD* mutant, AmsD probably adds the second galactose residue. This function has been genetically confirmed by cross-complementation of corresponding *ams* and *cps* mutants of *E. amylovora* and *P. stewartii* using cosmid clones of the two gene clusters [26]. In this study, *cpsG*, which is homologous to *amsD*, complemented the *amsD* mutant Ea1/79-D50 only in the presence of *cpsF*, which is homologous to *amsE*. In the complemented strain, the second galactose residue in the repeating unit of amylovoran was replaced by a glucose, as found in the backbone of stewartan. The reason that this complementation also required *cpsF* is that AmsE probably cannot recognize Gal-Glc as a substrate for the addition of the next galactose. Consequently, this finding also suggests that AmsE and CpsF add the third galactose residue to their respective repeating units. In the next steps, the glucuronic acid molecule may be added to the trisaccharide by AmsB, because in preliminary experiments, an *amsB* mutant, unlike an *amsK* mutant, was unable to incorporate labeled glucuronic acid (data not shown). Finally the pentameric sugar chain is most likely completed by the addition of the last galactose residue by AmsK, which is the remaining glycosyltransferase in the gene cluster. Based on sequence similarity and map order, CpsE would probably function the same as AmsB and CpsK the same as AmsK in the synthesis of stewartan. Finally, the pyruvyl group might be attached by AmsJ due to its close homology to pyruvyl transferases. Similarly CpsJ could also function in the addition of pyruvate to stewartan, but our structural studies of stewartan [19], [20] did not detect pyruvate. It may be that expression of this gene is dependent on growth conditions or strain differences since Darus [32] reported 1% pyruvate in preparations of stewartan from the type-strain of *P. stewartii*.

At this point, there is no evidence that an enzyme encoded by a gene within the *ams* region can add the optional glucose residue as the second side chain. This argument is based on studies with *E. pyrifoliae*, which produces an EPS similar to amylovoran except that it lacks this glucose side chain [6], even though its *cps* gene cluster contains all of the genes present in the *ams* gene cluster. When an EPS⁻ *E. pyrifoliae* *cpsB* mutant was complemented with the entire *ams* gene cluster, the resulting EPS still lacked the glucose side chain (M. Schollmeyer, M. Nimtz & K. Geider, unpublished). Similarly, acetylation of the repeating unit is probably also a function of a gene located outside of the *ams* gene cluster. This is based on the finding that when amylovoran synthesis is directed by a plasmid-borne *ams* cluster in a Δ *cps* mutant of *P. stewartii*, the resulting amylovoran is not acetylated [26].

After the repeating unit is synthesized in the cytoplasm, it has to be transported to the periplasm and then polymerized during transport to the cell surface. The pentasaccharide

repeating unit is presumably synthesized as a linear chain (Fig. 5) and then the main side chain (Gal-GlcA-Gal) is formed when two subunits are polymerized by attaching the third galactose residue of one chain to the first galactose residue of an existing chain. A similar mechanism has been proposed for xanthan biosynthesis [33]. The polymerization of amylovoran repeating units into EPS could be accomplished by AmsC and AmsF. Both proteins have a low homology to possible polysaccharide polymerases. AmsF is highly homologous to CpsH of *P. stewartii* [17] and resembles ExoP from *S. meliloti*, which acts in polymerization of succinoglycan subunits [8], [14], [33].

AmsH appears to be localized to the cell membrane. We have found that fusion of AmsH to a signal-peptideless β -lactamase protein results in ampicillin resistance, which could only occur if the fusion product was secreted or localized to the outer surface of the cell membrane (H. Ullrich & K. Geider, unpublished). Homology studies revealed that AmsA contains an ATP binding site and acts as a kinase because it has homology to tyrosine kinases, it cross-reacts with antibodies against an *E. coli* tyrosine kinase, and it exhibits kinase activity [13].

The *amsI* gene is adjacent to *amsA* and it has been shown to encode an acid phosphatase [15] that could also be involved in dephosphorylation of the substrate for the AmsA kinase. AmsI might also play a role in recycling the lipid carrier by removing its terminal phosphate and converting it from the diphosphate to monophosphate form [15]. An *amsI* mutant is deficient in amylovoran synthesis and overexpression of *amsI* diminishes EPS synthesis [15]. Similar functions might be ascribed to *P. stewartii* CpsB, I and C, which are homologous to AmsH, I and A, respectively. CpsB and CpsC also have been shown by use of *phoA* protein fusions to be localized in the cell membrane and CpsI confers acid phosphatase activity (D. Coplin, unpublished). Two additional chromosomal regions of *P. stewartii* have been recently identified [34] that participate in attachment of the terminal glucose in the side chain of stewartan (Fig. 6). The responsible protein may be encoded by the gene *wceO* supported by *wzx2*.

Studies on the adaptation of EPS to particular hosts was made possible by the functional transfer of genes for EPS synthesis between *E. amylovora* and *P. stewartii* [26]. This revealed that in pears *E. amylovora* was much more virulent when it produced amylovoran than stewartan. The results of this study therefore demonstrate how a bacterial EPS gene cluster in two closely related pathogens has become adapted to very different host plants by undergoing relatively few gene changes that affect polysaccharide structure.

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Table 1

Bacterial strains, plasmids and PCR primers.

Genotype		Source
Bacterial strains		
Ea1/79	wild-type, isolate from <i>Malus</i> sp., Germany [35]	
-D41	Ea1/79N, <i>amsE</i> ::Tn5, avirulent, EPS-	[3]
-D12	Ea1/79N, <i>amsC</i> ::Tn5, avirulent, EPS-	[3]
Ea1/79-galE	Ea1/79, <i>galE</i> -fdA31	This study
-D12-galE	D12, <i>amsC</i> ::Tn5, <i>galE</i> ::fdA31	[3], this study
-D26-galE	D26, <i>amsF</i> ::Tn5, <i>galE</i> ::fdA31	[3], this study
-D41-galE	D41, <i>amsE</i> ::Tn5, <i>galE</i> ::fdA31	[3], this study
-D44-galE	D44, <i>amsB</i> ::Tn5, <i>galE</i> ::fdA31	[3], this study
-D50-galE	D50, <i>amsD</i> ::Tn5, <i>galE</i> ::fdA31	[3], this study
-G10-galE	Ea1/79, <i>amsG</i> ::fdA2, <i>galE</i> ::fdA31	This study*
-K14-galE	Ea1/79, <i>amsK</i> ::fdA2, <i>galE</i> ::fdA31	This study
Plasmids		
pEA101	3.6 kb <i>HindIII</i> -fragment with the <i>rcaA</i> -gene [3] of <i>E. amylovora</i> in pSUP106, Cm ^r	
pEA200	2 kb <i>HindIII</i> -fragment with <i>galE</i> in pUC19, [36] Ap ^r	
pfdA2	suicide plasmid, phage fd ori, Km ^r	[37]
pfdA31	suicide plasmid, phage fd ori, Ap ^r	[37]
pfdA31-galE	suicide plasmid, <i>galE</i> gene disrupted by pfdA31, Ap ^r	This study
pEScpsFG	1.9 kb fragment with <i>P. stewartii cpsF</i> and <i>cpsG</i> in vector pGEM-T	This study
pEScpsG	1.1 kb fragment with <i>P. stewartii cpsG</i> in vector pGEM-T	This study
Primers for creation of pEScpsFG and pEScpsG		
CPSFG	TGCTGCCGGAAGTCTCT	
CPSFGc	CATCTCCAGCCCGTTTA	
CPSG	CGATCGGCAGGATTGTC	

*, equivalent to Ea7/74-K15, described in [12].

Table 2

Effect of the *galE*-mutation and the transcriptional activator RcsA on incorporation of radioactively labeled UDP-galactose into cells of wild-type strain Ea1/79 and *ams* mutants after EDTA treatment. An aliquot of the organic phase from the extracted reaction mixture was dried and the radioactivity measured in a scintillation counter. Values in bold indicate the genetic background chosen for further analysis of the synthesized products. Plasmid pEA101 encodes for the activator RcsA.

Strain	Genotype	Incorporation (cpm)	Mutant/ plasmid	Incorporation (cpm)
Ea1/79	wild-type	378	<i>galE</i>	1794
-D41	<i>amsE</i>	300	<i>amsE</i>	1111
			<i>galE</i>	
-D12	<i>amsC</i>	172	<i>amsC</i>	1064
			<i>galE</i>	
Ea1/79- <i>galE</i>	wild-type	2022	+pEA101	14137
-G10- <i>galE</i>	<i>amsG</i>	24	+pEA101	75
-D12- <i>galE</i>	<i>amsC</i>	1064	+pEA101	4928
-D26- <i>galE</i>	<i>amsF</i>	689	+pEA101	59
-D41- <i>galE</i>	<i>amsE</i>	327	+pEA101	1432
-D44- <i>galE</i>	<i>amsB</i>	621	+pEA101	267
-D50- <i>galE</i>	<i>amsD</i>	33	+pEA101	342
-K14- <i>galE</i>	<i>amsK</i>	744	+pEA101	322

Legends to the figures

Fig. 1. The structures of capsular EPS from *E. amylovora* and *P. stewartii*. Glycosyl residues and their linkages in the repeating units of amylovoran and of stewartan are shown. The positions P1 to P6 and pyr in () and bold letters refer to peaks in the elution profiles from methylation analysis [3] (see also Fig. 4). The number 0.5 refers to the glucose residue added to half of the repeating units. Differences in the repeating units are indicated in bold letters.

Fig. 2. Colinearity of the *E. amylovora* *ams* region and the *P. stewartii* *cps* region. Genetic maps for the *ams* region of *E. amylovora* [12] and the *cps* region of *P. stewartii* are shown. The pointers link corresponding proteins encoded by both gene clusters. The dotted line indicates a pair of glycosyltransferases that have very low homology. The alternative w-- nomenclature used for polysaccharide synthesis genes in the Enterobacteriaceae is also shown [27]. The EMBL/Genbank Nucleotide Sequence Database accession number for the *ams* genes of *E. amylovora* is X77921 and for the *cps* genes of *P. stewartii* is AF077292. The ORFs for both gene clusters were calculated with Glimmer version 3.

Fig. 3. HPLC profile of the labeled intermediate of amylovoran after incorporation of galactose from UDP-[³H]galactose in EDTA-treated cells of *E. amylovora* wild type (A) and an *amsD* mutant (B). The arrows indicate the positions of four size markers (from left to right: BSA, cellopentaose, cellotetraose, and galactose). Elution profile B shows that label is only found in lipid carrier-linked monomeric galactose extracted from *E. amylovora* Ea1/79-D50. S1, position of monosaccharide; S2, disaccharide; S5, pentasaccharide; D, dimer of repeating unit; E, EPS exceeding dimers.

Fig. 4. Profiles from methylation analysis of amylovoran, stewartan and transgenic EPS from an *E. amylovora* *amsD* mutant complemented with the *P. stewartii* *cpsFG* genes. A: EPS from Ea1/79; B: EPS from DC283; C: EPS from Ea1/79-D50(pEScpsFG). The assignments P1 to P6 and pyr [26] refer to the positions indicated in Fig. 1.

Fig. 5. A proposed scheme for sequential attachment of UDP-sugars (arrow) by the *ams*-gene products of *E. amylovora* and by the *cps* gene products of *P. stewartii* to undecaprenylphosphate, a membrane-bound lipid carrier (Und). Similarities for corresponding proteins and the w-- nomenclature gene names [27] are indicated. *, glycosyl site for attachment of the repeating unit to the

existing EPS chain. Underlined: replacement of galactose/pyruvyl residue in amylovoran by glucose in stewartan. Bold indicates the residue attached in a new step. \$, from reference [34]. There is no known correlation for *E. amylovora* and *P. stewartii* in the attachment of steps 6 and 7.

Fig. 1

Amylovoran

Stewartan

Fig. 2

***E. amylovora* Ea1/79**

***P. stewartii* DC283**

identity of corresponding proteins:

➡ >58% ➡ 30% - 35% ➡ 15%

Fig. 3

A

B

Fig. 4

Fig. 5

	Stewartan		Amylovoran		
	Sequence of addition	Transfer by protein	Similarities of Cps and Ams proteins	Transfer by protein	Sequential attachment of UDP sugars to lipid carrier
↓	1. Gal	CpsA/WceG	86%	AmsG/WceG	Gal -P-P-Und
	2. Glc	CpsG/WceN	44%	AmsD/WceN	Gal -Gal-P-P-Und
	3. Gal	CpsF/WbdN	34%	AmsE/WceM	Gal -Gal-Gal-P-P-Und
	4. GlcA	CpsE/WceB	78%	AmsB/WceB	GlcA -Gal-Gal-Gal-P-P-Und
	5. Gal	CpsK/WceK	80%	AmsK/WcaL	Gal -GlcA-Gal-Gal-Gal-P-P-Und
	additional attachments:				
	6. <u>Glc</u>	WceO\$		AmsJ/WceJ	<u>pyr</u> Gal-GlcA-Gal-Gal-Gal-P-P-Und
				pyr Gal-GlcA-Gal*-Gal-Gal-P-P-Und	
	7. Glc	unknown protein			Glc