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Variations in the molecular masses of the capsular exopolysaccharides amylovoran, pyrifolan and stewartan

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ABSTRACT

Erwinia amylovora, causing fire blight of apple, pear and some ornamentals, *Erwinia pyrifoliae*, causing Asian pear blight, and *Pantoea stewartii*, causing Stewart's wilt of sweet maize, synthesize capsular extracellular polysaccharides (EPSs) with a high molecular mass. The EPSs are virulence factors and form viscous aggregates, which participate in clogging vessels of infected plants and causing wilting. The sizes of EPSs produced under different environmental growth conditions were determined by analysis with large pore HPLC columns. Their molecular mass of ca. 5 MDa, when isolated from agar plates, decreases to ca. 1 MDa for *E. amylovora* amylovoran from freeze-dried supernatants from liquid cultures and to 2 MDa for freeze-dried preparations of *P. stewartii* stewartan. Size changes were also found following growth in various other media and for different strains. Stewartan, amylovoran and *E. pyrifoliae* pyrifolan were also shown to be completely degraded by a bacteriophage EPS depolymerase.

1. Introduction

The capsular EPS of *Erwinia amylovora*, *Erwinia pyrifoliae* and *Pantoea stewartii* subsp. *stewartii* surrounds the bacterial cells in order to protect them against recognition by plant defense mechanisms or desiccation and it also sequesters ions and nutrients in the vicinity of the bacteria [1]. The exopolysaccharides are important virulence factors for these pathogens affecting apple, pear or corn [2–4]. Bacterial biofilm formation, especially visible in flow cell experiments, has also been associated with amylovoran and stewartan [5,6]. Synthesis of stewartan is down-regulated by acyl-homoserine lactone (AHL) [7], whereas *E. amylovora* does not produce AHL [8]. On the other hand, amylovoran synthesis is affected by autoinducer 2 (Wensing et al., unpublished). The repeating units of the EPSs are highly polymerized. Amylovoran consists of five different sugar residues and a glucose residue in a second side chain [9]. Pyrifolan, produced by *E. pyrifoliae*, and stewartan are related to amylovoran, but the glucose of the second side chain is absent in pyrifolan and a galactose residue in the backbone of stewartan is substituted by glucose [10]. Also the main side chain of stewartan terminates in glucose rather than pyruvate. Their biosynthesis depends on large gene clusters. The *ams* gene cluster, for the synthesis of amylovora, contains 12 genes that are transcribed as a polycistronic mRNA [2], by the cluster may also include additional internal transcriptional starts. Similar *cps*/*wce* gene clusters have been described for the synthesis of stewartan [11] and pyrifolan [1,12]. The genes for EPS synthesis of *P. stewartii* and *E. pyrifoliae* apparently have analogous functions of the *ams* genes of *E. amylovora*. These biosynthetic steps may need additional genes located in other chromosomal regions [13]

The sequential involvement of *ams*-encoded UDP-glycosyl transferases in the biosynthesis of the pentasaccharide has partially been dissected using an in vitro system consisting of EDTA-treated cells of *E. amylovora ams*-mutants [11]. A similar approach was used to dissect the subcellular synthesis of succinoglycan of *Rhizobium meliloti* [14]. Assigned gene functions were supported by interspecific genetic complementations and protein sequence analysis.

The molecular masses of amylovoran and stewartan were previously determined to be in the range of 1–2 MDa [15]. Those preparations were processed by dialysis and freeze-drying, which may cause EPS breakage. Commercially important EPSs have a low molecular weight such as inulin (5 kDa) [16] or are large as xanthan (10 MDa) [17]. The size of an EPS may change during processing and depend on various environmental conditions for cell growth.

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Table 1
Bacterial strains used in the experiments.

Strain	Source	Additional information
E. amylovora		
CFBP1430	Hawthorn, France	[34]
Ea1/79	Apple, Germany	[35]
Ea1/79-D26	Ea1/79 with Tn5 in *amsF*	[2]
Ea11/88	Apple, Germany	[35]
EaRb7	Raspberry, Canada	[34]
Ejp546	Nashi pear, Japan	[36]
Ejp556	Nashi pear, Japan	[36]
Ep1/96	Nashi pear, Korea	[37]
Ep16/96	Nashi pear, Korea	[37]
P. stewartii		
DC283	Corn, Illinois, USA Nal	[10]
DM2237	*cpsH*::*aphT* mutant of DC283, Km	This study
DM2211	*cpsJ*::*aphT* mutant of DC283, Km	This study
Plasmids		
pDM449	pES2144 with Tn5 in *cpsF*	[4]

We have determined the molecular mass of amylovoran, pyrifolan and stewartan with size-exclusion chromatography (SEC) applying large pore columns for comparison of various EPS preparations.

2. Materials and methods

2.1. Bacteria

Bacterial strains used in the experiments are listed in Table 1. The non-polar *cpsH* and *cpsJ* mutants of *P. stewartii* were constructed in the following manner. The *cpsH* and *cspJ* ORFs were subcloned into pBluescript. Using unique restriction sites, an *aphT* (km) cartridge was ligated into the gene (the *Nar*I site of *cpsH* and the *Nco*I site of *cpsJ*). The mutant ORFs were recloned into a suicide plasmid pKNG101 [18] and then recombined into the chromosome of wild-type strain DC283 using sucrose to enrich for double recombinations and loss of the suicide plasmid. The marker exchanges were verified by Southern blotting and the phenotypes were confirmed by complementation with the corresponding wild-type subclone.

2.2. Preparation of EPS

Cells of overnight cultures were streaked on a cellophane disk placed on CPG or MM2C agar [10,19]. After 3 d incubation at 28 °C, the cell cultures were suspended in 2 ml water and gently vortexed. The cells were pelleted in an Eppendorf centrifuge, the supernatant dialyzed in a Pierce Slide-a-lyzer mini dialysis unit (exclusion size 10 kDa) and stored frozen at −80 °C (prep A). For other preparations, the cells were grown on P2 [20] or MM1 [19] agar. After growth in MM2 medium the supernatant was purified by ultracentrifugation at 100,000 × *g*, dialysis against water and freeze-dried (prep B). These preparations were stored at room temperature.

2.3. EPS depolymerase and degradation

The gene for synthesis of EPS depolymerase was cloned from phage ϕEa1h (protein ID: CBX44510) and the enzyme was expressed in *Escherichia coli* from a His-tag fusion and purified on Ni-columns [21]. The EPS depolymerase was added to stewartan and the mixture incubated in 50 mM sodium acetate buffer (pH 4.7) for various times.

2.4. EPS analysis

The EPS preparations were analyzed on the linked Tosohaas large pore columns PWXL 6000, PWXL 5000 and PWXL 4000 in the Beckman HPLC system Gold with an RI detector. The flow rate was 0.4 ml/min and the elution buffer was 10 mM acetic acid or 10 mM trichloro acetic acid. Degraded EPS was separated on a Tosohaas Toyopearl HW-40S column (90 cm × 1 cm; carrier particles 30 μm) in 10 mM trifluoro acetic acid and eluted compounds were analyzed with a light scattering detector (LSD Sedex 75). For freeze-dried material, absolute molar mass distribution X was determined by means of SEC-mass/LS experimental setup with a set of TSK PW columns (pre + PWM + PW6000 + PW5000 + PW4000 + PW3000, Toyo Soda) and 0.05 M aqueous NaCl as an eluent. Molar mass sensitive light scattering was monitored by KMX-6 LLAS instrument (Thermo Separation) at a scattering angle of 5° and wavelength of 632 nm; corresponding weight fractions were measured by an interferometric refractometer (Wyatt Optilab 903) at 630 nm [22].

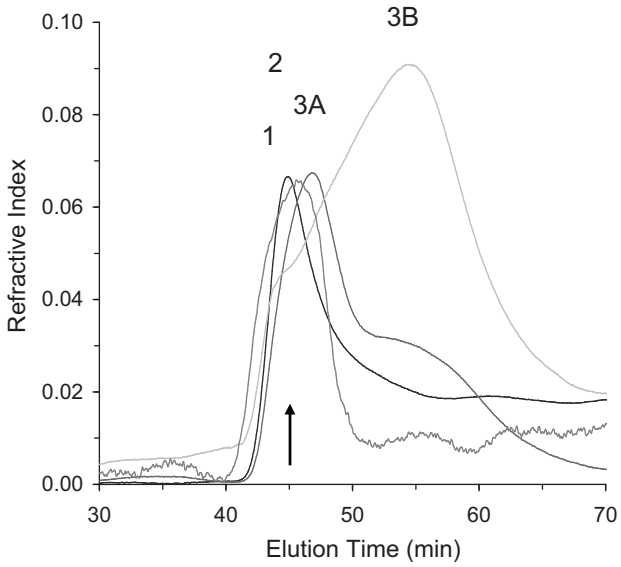

Fig. 1. Separation of EPS preparations using large pore size HPLC columns. The cells were grown on MM2C agar for 3 d at 28 °C. Peak 1. levan; peak 2. pyrifolan from *E. pyrifoliae* Ep16/96; peak 3A. amylovoran from *E. amylovora* Ea1/79, grown on plates and extracted immediately after harvesting the cells; peak 3B. amylovoran from *E. amylovora* Ea1/79, grown in liquid culture and freeze-dried; arrow: 6 MDa size marker (levan, analyzed with SEC-MALS). An elution time of 46 min indicated a molecular mass of 5 MDa, 56 min a size of 1 MDa (Fig. 2).

3. Results

3.1. Molecular mass

Amylovoran from *E. amylovora* or pyrifolan from *E. pyrifoliae* produce a single peak of 5 MDa on large pore columns when freshly isolated from agar plates (Fig. 1). The molecular mass distribution of amylovoran from liquid cultures that had been freeze-dried was much more disperse than amylovoran from agar plate cultures that had been extracted and analyzed immediately. The former peaked at 1 MDa (Fig. 1, 3A) and the latter had a more homogeneous peak at 5 MDa (Fig. 1, 3B). This demonstrates that the size of amylovoran from *E. amylovora* can be heterogeneous depending on the growth the bacteria and steps of isolation. Interestingly, the size of levan, synthesized by the enzyme levansucrase secreted from *E. amylovora*, was as large as 6 MDa after isolation from culture supernatants, which is in agreement with previous data [16].