XYLELLA FASTIDIOSA BACTERIAL POLYSACCHARIDES WITH A POTENTIAL ROLE IN PIERCE'S DISEASE OF GRAPES

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INTRODUCTION

Pierce's disease (PD) causes symptoms of leaf scorch and fruit cluster wilt on wine, table and raisin grapes, and is caused by the bacterial pathogen *Xylella fastidiosa*. These fastidious, gram-negative bacteria occur only in the xylem of infected plants and are transmitted by xylem-feeding insects; leafhoppers and sharpshooters. The close association of plants with bacteria, either pathogenic or symbiotic, is often mediated by cell surface polysaccharides that may become modified during the infection process (Price, 1999). *Xylella* has 9 genes homologous to the *gum* genes of *Xanthomonas campestris* that direct the synthesis of a highly viscous exopolysaccharide gum (EPS). The biosynthesis and secretion of EPS is often tightly regulated either by the availability of nutrients or of specific small-molecule inducers in plant exudates. In addition, *Xylella* undergo developmental changes from rippled to smooth cell walls during the infection process (Huang et al., 1986), implicating a possible involvement of cell surface lipopolysaccharides (LPS). LPS consists of membrane-anchored lipid A, a core oligosaccharide, and a carbohydrate O-antigen repeat that typically contains phosphorylated, acetylated or methylated sugars that can profoundly affect its physical properties. Since it forms the outermost bacterial surface, the *Xylella* LPS may be an important factor in mediating interactions such as recognition and adhesion between the bacteria and host plant, or the bacteria and the insect vector.

OBJECTIVES

The stated hypothesis of our research is that bacterial polysaccharides produced by *X. fastidiosa*, particularly cell wall lipopolysaccharides and an exopolysaccharide analogous to xanthan gum, may be causative agents of Pierce's disease on grapes. The prioritized research objectives were stated as:

1. Characterize structurally the bacterial exopolysaccharide(s) from the xylem sap of PD-infected and non-infected vines (using two grape varieties, Chardonnay and Carbernet), and to ascertain its potential involvement in Pierce's disease.
2. Characterize structurally the lipopolysaccharide O-antigen from *Xylella fastidiosa* grape pathovar. isolated from the xylem of PD-infected and non-infected vines (two varieties, Chardonnay and Cabernet).
3. Characterize structurally the exopolysaccharides and O-antigen from *Xylella fastidiosa* grown in culture, and to assess potential changes in response to changing growth conditions and/or cultures additives.

RESULTS AND CONCLUSIONS

Carbohydrate and Genomic Analysis

Sequence data from the *Xylella fastidiosa* clone 9a5c (Simpson, 2000), a causative agent of citrus variegated chlorosis, indicated that this strain has 9 genes with close homology to the xanthan gum biosynthetic cluster of *Xanthomonas campestris*, i.e. *gumBCDEFHJKM*, but lacked *gumGIL*. In *Xanthomonas* the *gum* genes direct the synthesis of a highly viscous exopolysaccharide gum (xanthan) that is widely used in the food industry as a thickening agent. The production of a comparable gum by *Xylella* in the xylem of infected vines would likely block the plants' water uptake system and produce Pierce-type pathogenic symptoms. For the citrus pathovar (CVC clone 9a5c) these *gum* genes are clustered on a single operon. Examination of the preliminary genomic data from *X. fastidiosa* grape, oleander and almond pathovars. (at the time of writing these data are not yet published) identified similar components of *gum* genes, suggesting the exopolysaccharide (EPS) production is common to all four pathovar. strains. Hence the EPS may be symptomatic of *Xylella*-induced plant diseases, but is unlikely to be a determinant of host-pathovar. specificity.

Biochemical studies have shown that in *Xanthomonas* the first nine *gum* genes encode enzymes involved in the biosynthesis of the xanthan gum tetrasaccharide repeat unit, D-GlcA-(beta-1,2)-6-O-acetyl-D-Man-(alpha-1,3)-D-Glc-(beta-1,4)-D-Glc, while *gumGIL* encode for a terminal mannosyl transferase (GumI) and for two enzymes involved in decorating the mannosyl residue, the acetyltransferase GumG and the pyruvate ligase GumL. Hence the overall structure of xanthan gum is a cellulosic backbone that is branched at the 3-position on every other Glc residue with a 4,6-pyruvyl-O-acetyl-Man-GlcA-6-O-acetyl-Man side chain. The lack of *gumGIL* in the *Xylella* genome suggested that its exopolysaccharide should be closely analogous to xanthan gum, but lacking the terminal 4,6-puyruvyl-O-acetylmannose residue. Our initial aim was to determine whether xanthan lacking this outermost sugar residue would still form a viscous polymerized gum likely to clog xylem vessels, or whether the intrinsic viscosity would be lost.

Xanthan gum forms a very stable gel in aqueous solution that retains its gel-state even after centrifugation. The gel assume a double stranded conformation that is stabilized by high concentration of metal ions, whereas denatured, single-stranded forms exist at low ion strength or elevated temperature. Hence, the presence of metal ions such as Mg2+ or Ca2+ tend to stabilize the ordered, double-stranded conformation of xanthan, and thereby stabilize the gel-like properties. Acid treatment of xanthan to selectively cleave the outermost mannose residue, leaves a polysaccharide directly analogous to the proposed *Xylella* gum. We report that the gel viscosity was apparently unaffected by this treatment. After dialysis, the diasylate contained a single monosaccharide identified by thin layer chromatography (TLC) after methanolic-H_2SO_4 charring as D-mannose. The lyophilized residual gel was rehydrated to 0.5% w/v and gave a stable gel indistinguishable to the hydrated xanthan. These data suggest that the outermost β-Man is not necessary in maintaining the ordered conformation of the predicted *Xylella* gum which is therefore likely to have physical properties very similar to xanthan. If produced in the xylem of vine plants, even at relatively low concentration (0.5% w/v), it would therefore likely occlude them, blocking the plants' water uptake system and leading to the water-stress damage symptomatic of Pierce's disease.

Hydrolysis/Methanolysis

Polysaccharide analysis generally requires hydrolysis (or methanolysis) to the component monosaccharides (methylglycosides) prior to compositional analysis. Controlled acid hydrolysis of the predicted *Xylella* gum would produce D-glucose and D-mannose plus a characteristic disaccharide, D-GlcA-(beta-1,2)-D-Man arising from the extra stability of the glucuronic acid (GlcA) glycosidic bond. Xanthan gel was still viscous after acid hydrolysis in TFA or 1% aq. sulfuric and gave a streak at the origin on TLC plates. In 10% aqueous sulfuric the gel-form was denatured, and a single spot was observed on the TLC plates probably corresponding to co-eluting component monosaccharides. Methanolysis at concentrations less than 1 M HCl were not viscous (in methanol) but the majority of the polysaccharide was undissolved. In 1 M methanolic HCl 80-90% of the xanthan dissolved to a non-viscous clear solution containing the predicted D-Man, D-Glc, and GlcA-β1,2-Man, plus free GlcA as a minor component. The GlcA-β1,2-Man disaccharide should therefore be characteristic of this type of polysaccharide gum and might be useful as a "fingerprint" diagnostic of the *Xylella* EPS in xylem exudates or vine cuttings.

Genomic Analyis of Xylella Lipopolysaccharide (LPS) Biosynthesis

An initiative of the American Vineyard Foundation and California Dept. Food and Agriculture to sequence the grape pathovar. genome is presently in progress, and *X. fastidiosa* pathovars. of citrus, almond and oleander are completed. These genomic data are invaluable to understanding comparative polysaccharide biosynthesis by *Xylella*, particularly for predictions of diverse or pathovar-specific carbohydrate structures, such as LPS O-antigen. Our sequence analysis of the published genome of *Xylella* clone 9a5c (CVC strain) (Simpson , 2000) indicates that the gram-negative *Xylella* has the full genetic compliment required for lipid A biosynthesis (*lpxABCDK*), but is unique in having multiple copies of several of these genes (Table 1). Four copies of the N-acyltransferase gene (*lpxD*) are present instead of the usual single copy, and there are two copies of the O-acyltransferase gene (*lpxA*). One *lpxA/lpxD* pair is linked (XF1043 and XF1045) and is also associated with the disaccharide synthase gene (*lpxB*, XF1042). However, the 4'-kinase (*lpxK*, XF1082) and deacetylase (*lpxC*, XF0803) genes are positioned elsewhere on the genome, as are the repeat copies of *lpxA* and *lpxD*. The multiple copies of the acyltransferases may indicate the *Xylella* has the ability to hyper-acylate its lipid A under certain conditions, thereby increasing its lipophilic character. Alternatively, there may be specific LpxA and LpxD proteins expressed to transfer specialized lipid motifs, such as the C28:27-OH fatty acid found on *Rhizobium* lipid A (Simpson, 2000).

Table 1: Genes implicated in Xylella LPS and Surface Antigen Biosynthesis.

XF1289	44 %	2-dehydro-3-deoxyphosphooctonate aldolase (KDSA) {*Escherichia coli*}
XF0105	44 %	3-deoxy-D-manno-octulosonic acid transferase (KDTA) {*Escherichia coli*}
XF2299	50 %	3-deoxy-manno-octulosonate cytidylyltransferase (KDSB) {*E. coli*}
XF1419	26 %	acetyltransferase (LPXD OR FIRA OR OMSA) {*Escherichia coli*}
XF0918	32 %	acyl-[ACP]-UDP-N-acetylglucosamine (LPXA OR AQ_604) {*Aquifex aeolicus*}
XF1994	29 %	beta 1,4 glucosyltransferase (HI0653) {*Haemophilus influenzae*}
XF0612	24 %	dolichol-phosphate mannosyltransferase (dmt) {*Aquifex aeolicus*}
XF1638	34 %	dolichyl-phosphate mannose synthase related protein {*Pyrococcus abyssi*}
XF0257	75 %	dTDP-4-dehydrorhamnose 3,5-epimerase (rfbD) {*Xanthomonas campestris*}
XF0258	54 %	dTDP-4-keto-L-rhamnose reductase (rfbC) {*Xanthomonas campestris*}
XF0255	76 %	dTDP-glucose 4,6-dehydratase (RFBB) {*Xanthomonas campestris*}
XF0611	63 %	dTDP-glucose 4-6-dehydratase (rfbB) {*Synechocystis sp.*}
XF1637	29 %	glycosyl transferase (spsQ) {*Sphingomonas sp. S88*}
XF1082	38 %	lipid A 4'-kinase (LPXK OR HI0059) {*Haemophilus influenzae*}
XF0104	40 %	lipid A lauroyl acyltransferase (HTRB OR WAAM) {*Escherichia coli*}
XF1348	26 %	lipid A lauroyl acyltransferase (HTRB OR WAAM) {*Escherichia coli*}
XF1042	45 %	lipid A disaccharide synthase (LPXB OR PGSB) {*Escherichia coli*}
XF0879	26 %	lipopolysaccharide biosynthesis protein (rfbU) {*Escherichia coli*}
XF2434	37 %	lipopolysaccharide core biosynthesis(rfb303) {*Pseudomonas aeruginosa*}
XF0980	56 %	lipopolysaccharide synthesis enzyme (kdtB) {*Serratia marcescens*}
XF0778	26 %	O-antigen acetylase (oafA) {*Salmonella typhimurium*}
XF1413	45 %	polysialic acid capsule expression protein (kpsF) {*Aquifex aeolicus*}
XF2154	73 %	saccharide regulatory protein (opsX) {*Xanthomonas campestris*}
XF0176	43 %	sugar transferase (SC4A2.10c) {*Streptomyces coelicolor A3(2)*}
XF1045	41 %	UDP-3-O-(R-3-hydroxymyristoyl)-GlcN-acyltransferase (LPXD) {*Salmonella}*
XF1646	28 %	UDP-3-O-(R-3-hydroxymyristoyl)-GlcN-acyltransferase (LPXD){*Rickettsia}*
XF0486	31 %	UDP-3-O-[3-hydroxymyristoyl] GlcN-acyltransferase (lpxD) {*Chlamydia}*
XF0803	59 %	UDP-3-O-[3-acyl] GlcNAc deacetylase (lpxC) {*Pseudomonas aeruginosa*}
XF1043	44 %	UDP-N-acetylglucosamine acyltransferase (LPXA) {*Escherichia coli*}

Rfa-type genes involved in core assembly are also present, as are *kds*/*kdt* genes involved in CMP-KDO biosynthesis. We have identified both ADP-heptose synthetase (*rfaD*) and two heptosyltransferase (*rfaC, rfaF*) genes suggesting that *Xylella* has a conserved inner core structure α Hep-1,3-α Hep-1,5-α KDO-2,6-lipidA, similar to *E. coli* or *Salmonella*. Rfb-type genes that are likely to encode for synthesis of the outermost carbohydrate portion (the O-antigen) are also present on the genome of the *Xylella*. Structurally O-antigen tends to be very diverse, perhaps reflecting adaptation to particular environments and in this respect it is noticeable that several of the *Xylella rfb* genes are also conserved in *Xanthomonas campestris*. For example, the *rfbBCD* cluster (XF0255, XF0258 and XF0257) are probably involved in the synthesis of dTDP-L-rhamnose, a sugar known to be part of *Xanthomonas* LPS. As stated earlier, *Xanthomonas* LPS has been implicated in adhesion to its plant host.

SDS-PAGE Analysis of *Xylella* Lipopolysaccharide (LPS)

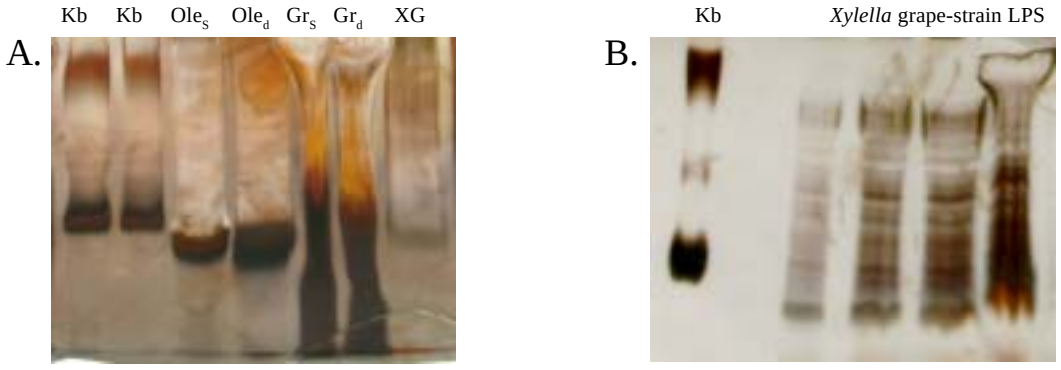

Figure 1. SDS-PAGE analysis of Silver-Alcian Blue stained LPS from *Xylella fastidiosa* grape (Gr) and oleander (Ole) pathovars. *Klebsiella* LPS (Kb) is included as a standard, and XG is xanthan gum. A. 18% continuous; B. 5%, 18% discontinuous.

X. fastidiosa undergoes developmental changes from rippled to intermediate to smooth walled during the course of Pierce's disease indicating a possible involvement of LPS, although *Xylella* LPS has never been identified previously. High molecular weight LPS typically consists of repeating units which are resolved into ladders by gel electrophoresis. SDS-treated LPS extracts of *Xylella* grape strain and oleander strain behaved very differently on 18% SDS-PAGE gels, indicating gross structural differences. On continuous gels LPS from the oleander strain (Ole_s and Ole_d) ran as a single low M.wt. band indicative of "rough" LPS lacking significant O-antigen (Figure 1A). In contrast, grape-strain *Xylella* LPS (Gr_s and Gr_d) comprised a ladder series of highly acidic (Alcian blue binding) O-antigen forms most readily resolved on discontinuous gels (Figure 1B). These data represent the first evidence of LPS production by *Xylella* species, and the first indication that diverse LPS structures may be host-pathovar. specific.

The *Xylella* LPS may be important in mediating interaction such as recognition and adhesion between the bacteria and host plant, or the bacteria and the insect vector, while EPS "xanthan" gum production is likely common to all strains. Our findings indicate that the EPS gum is not produced constituitively by *Xylella* but may be up-regulated during the development of the disease. In addition, SDS-PAGE analysis of cell wall extracts demonstrates the presence of *Xylella* LPS for the first time, and highlights considerable differences between LPS from oleander- and grape-specific Xylella strains. These structural differences may be potential determinants of the *Xylella* host-pathogen specificity and may provide a fuller understanding of Pierce's disease at the molecular level. Our present aims are therefore: 1. To investigate the *in planta* production of *Xylella* EPS gum during the later stages of the vine growing season; and 2. to chemically characterize *Xylella* LPS, and to compare cultured *Xylella* LPS with that from *Xylella* grown on grape xylem exudate or *in planta*.

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PRUNING FOR CONTROL OF PIERCE'S DISEASE

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INTRODUCTION

Because there are no practical therapeutic measures for grapevines with Pierce's disease (PD), we tested the effects of drastic pruning as a method of rapidly regenerating healthy vines from vines with PD symptoms. If such a practice were to be successful, it could speed the replacement of affected vines by preserving an established root system to support vigorous regrowth. Our studies are designed to provide useful information for growers even if our pruning experiments do not produce methods that eliminate the disease from infected vines. Some growers have experimented with pruning practices against PD and claim worthwhile successes but have not compared their results to negative controls. These efforts may be a waste of money for labor and lost time in vine replacement. Our goals were to determine either that pruning would reduce the time required to restore a diseased vine to productivity or that such practices are not worthwhile. Finally, the results of our proposed experiments would provide new data on the distribution and overwinter survival of *Xylella fastidiosa* relative to PD symptoms.

Preliminary pruning trials in 1977 discouraged further testing of pruning to eradicate PD from individual vines. The incidence of PD symptoms was mapped in a plot within a commercial vineyard of 'Ruby Cabernet' in Fresno County. Vines that had only a single cordon with PD symptoms were noted and the diseased cordon was sawed off of every other such vine during October. In the fall of 1978, about 35% of the marked vines had no PD symptoms, but pruning had no influence on the likelihood of recovery. The same percentages of vines recovered regardless of severe pruning.

In another pilot experiment begun in late October 1996, we mapped the occurrence of PD in about 1000 Cabernet Sauvignon vines in Napa Valley. We marked, photographed, and took samples of symptomatic leaves from vines that had very early symptoms of PD only on one or several canes in these quadrilateral-trained vines (4 cordons). The samples were stored frozen at -70 C to process with PCR at a later date to confirm our diagnosis of PD. We then immediately removed all canes with symptoms from half of the vines. The following fall, 12 of 19 of the early-pruned vines showed no symptoms of PD. However there were no disease symptoms in 16 of the 18 unpruned control vines. We concluded that removing canes with light symptoms was not a promising method to eliminate PD from infected vines.

Some growers in Napa and Sonoma county have claimed to have eliminated PD from about 60% of the vines by cutting the trunk of the vine near the ground and retraining a new shoot to form a new trunk. None of these growers left similarly diseased vines as unpruned controls, so the effectiveness of their drastic pruning to control PD could not be assessed. If this pruning practice were to be adopted on a widespread basis without further critical evaluation, it is possible that a lot of expense may be incurred with no real economic return.

OBJECTIVE

1. Determine if severe pruning can eliminate Pierce's disease from grapevines with symptoms of the disease.

RESULTS AND CONCLUSIONS

Severe pruning (just above the graft union) in the winter of 1998-99 successfully regenerated healthy grapevines from trellised vines in Napa Valley that had severe symptoms of PD during fall, 1998. Grape varieties used were Cabernet sauvignon, Merlot, Pinot noir, Chardonnay, and Cabernet franc. Vine ages were from 2 to over 8 years. Recovery rates ranged from 87 to 100% for vines with the least severe symptoms; from 71 to 95% for vines in the "moderate" severity category; and from 38 to 85% for the most severe category. For the least severe disease category, rates of recovery for pruned vines were not substantially or significantly greater than normal dormant pruning in some plots. Visual ratings of PD agreed with results from using a sensitive molecular diagnostic test (Polymerase Chain Reaction, PCR) for PD for 79% of the least severe category, 80% of the moderate category, and 97% of the severe category. These results appeared to demonstrate that it is feasible to regenerate healthy vines from vines with PD more quickly by severe pruning than by pulling and replanting the vines, but the results for the following year drastically changed this conclusion.

To test pruning as a PD control tool, we established as many plots in commercial Napa Valley vineyards as possible during fall, 1998. We defined three disease severity categories, based on the severity (extent) of PD symptoms from (1) light PD symptoms on one to a few leaves, (2) moderate PD symptoms on only one side of the vine, or (3) moderate to severe symptoms on both sides of the vine and with some fruit rasining. We photographed each vine in the experiment and pruned half of the vines in each category, leaving the remaining vines as controls for normal dormant pruning. In the fall of 1999 we evaluated the PD status of all vines in our plots. Two growers in three of our plots pruned most of our designated control vines, so we did not re-map PD in one of these and got limited information from two other plots.

The results for the first year were very promising (Weber et al. 2000), with recovery rates of 87% to 100% for light PD symptoms (category 1), 71-95% (category 2), and 38-85% (category 3) (Table 1). However, the second year (2000), most of these same vines in categories 2 and 3 had PD symptoms. It is possible that severe pruning in summer months may be a more effective approach, but this has yet to be tested. The success of severe pruning in eliminating PD probably is influenced heavily by grape variety. More susceptible varieties support faster movement of the causal bacterium (Purcell 1981). Pruning may also be more successful in climates in which the bacteria moves more slowly and has lower overwinter survival rates.

Based on our results, we conclude that severe pruning will not eliminate PD from the remaining vine at rates that are more profitable than removing and replanting. Severe pruning of category one vines (light symptoms) eliminates production for 2 years from all the severely pruned vines, over half of which will recover with normal dormant pruning. Severe pruning is not reliable or effective enough for programs that aim to reduce the amount of inoculum for vine-to vine spread of PD. Removing suspect vines as soon as possible is currently the only method to reduce PD inoculum levels in grape.

Table 1. First and Second Year Results for Regenerated Vines After Severe Pruning (Pr.) and for Controls (Check) of 1998 Symptoms in Five Vineyards. Pruning was in the winter of 1998-99.

Treat-ment	Cultivar	Number (No.) mapped in 1998 and percentage recovery (%) in three disease categories at the end of two growing seasons.								
		Category One			Category Two			Category Three		
		No. in 1998	% in 1999	% in 2000	No. in 1998	% in 1999	% in 2000	No. in 1998	% in 1999	% in 2000
Pruned	Cabernet Sauvignon	32	97	47	32	84	31	32	38	0
Check		32	81	78	32	31	25	28	0	0
Pruned	Cabernet Franc	30	87	70	10	80	30	9	55	0
Check		30	63	53	10	0	0	9	0	0
Pruned	Pinot Noir	30	97	47	14	71	42	62	85	0
Check		30	90	63	14	29	29	38	0	0
Pruned	Cabernet Sauvignon	14	100	64	20	95	60	17	70	13
Check		20	70	55	26	19	12	17	1	0
Pruned	Chardonnay	6	100	83	25	84	8	13	85	17
Check		6	50	50	19	16	16	13	0	0

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