CONTROL OF PIERCE'S DISEASE THROUGH DEGRADATION OF XANTHAN GUM

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ABSTRACT
Acinetobacter johnsonii GX123, a *Xylella* gum-degrading endophyte was co-inoculated with *Xylella fastidiosa* strain Texas in oleander plants to determine its efficacy as a biocontrol agent in preliminary experiments. Symptoms appeared in both plants inoculated with *X. fastidiosa* alone and plants co-inoculated with the endophyte. However, symptoms were more severe and appeared earlier in plants inoculated with *X. fastidiosa* than in those co-inoculated with the endophyte. *A. johnsonii* GX123 seems to be a promising candidate to control *X. fastidiosa*. Experiments using a sequential strategy of inoculating the *Xylella* gum-degrader endophyte prior to *X. fastidiosa* are ongoing and its effects on symptom expression are still under investigation.

INTRODUCTION
Pierce's disease (PD) of grapevine and other leaf scorch diseases caused by *Xylella fastidiosa* (*Xf*) are associated with aggregation of bacteria in xylem vessels, formation of a gummy matrix, and subsequent blockage of water uptake. In the closely-related pathogen, *Xanthomonas campestris* (Xc), xanthan gum is known to be an important virulence factor (Katzen et al, 1998), probably contributing to bacterial adhesion, aggregation, and plugging of xylem. The published genome sequence of *Xf* (Simpson et al, 2000; Bhattacharyya et al, 2002; Van Sluys et al, 2003) revealed that this pathogen also has genes for producing an exopolysaccharide with a very similar structure to that of xanthan gum. In PD, this *Xylella* gum is likely to contribute to plugging of the grapevine xylem (Keen et al, 2000) and possibly to the aggregation of the bacterium in the mouthparts of the glassy-winged sharpshooter. Because of its importance as an industrial thickener and emulsifier, xanthan gum synthesis and degradation have been extensively studied (Becker et al, 1998). Bacteria that produce xanthan-degrading enzymes have been isolated from soils using enrichment techniques with xanthan gum as the sole carbon source (Sutherland 1987; Ruijssenaars et al, 2000).

The purpose of this project is to identify bacteria that produce xanthan-degrading enzymes to target this specific virulence factor of *Xf*. This approach has the potential to significantly reduce the damage caused by PD in grapes and potentially in other hosts of *Xf* such as almond and oleander. If the gum is important in the aggregation of the pathogen in the insect vector, then our approach may also reduce the efficiency of transmission of PD. Our first approach will be to develop endophytic bacteria that produce these enzymes in the xylem of grapevines, but another approach is to engineer grape plants to produce these enzymes. Through the cloning and characterization of genes encoding xanthanases and xanthan lyases we will facilitate possible efforts to transform grapevines to produce these enzymes.

Previously, we used modified xanthan gum that mimics *Xylella* gum from a Xc mutant as the sole carbon source for enrichment culture from infected grapevines and oleanders. The *Xylella* gum biosynthetic operon in the *Xf* genome is different than the one in Xc from which the commercial xanthan gum is obtained. Since it is not feasible to produce *Xylella* gum for our studies from the slow-growing *Xf*, we genetically modified a strain of Xc to produce a modified xanthan gum that is predicted to have the same chemical structure as that from *Xf*. This was accomplished by deleting the *gumI* gene from the biosynthetic operon. Over 100 bacterial strains were initially recovered from enrichment experiments, and 11 were subsequently confirmed to effectively degrade *Xylella* gum. These strains were then tested for cellulase activity. Degradation of the cellulosic backbone of the gum polymer would be desirable, but we do not want enzymes that recognize and degrade plant cellulose. One particular strain (GX123) with high gum-degrading activity but no cellulase activity isolated from oleander was identified as *Acinetobacter johnsonii* (Aj), and characterized in more detail. In vitro, growth and biofilm production by GX123 were enhanced by *Xylella* gum as a substrate and by cells of *Xf* added to a minimal medium. The gum was degraded rapidly during log-phase growth of this endophyte, and viscosity was reduced almost to non-detectable levels. GX123 colonized stems and leaves of oleander systemically (10^4-10^5 cfu/g of plant tissue 20 days after inoculation), and systemic colonization was enhanced by co-inoculation with *Xf*. The effect of using GX123 as an endophyte to reduce the ability of *Xf* to produce disease symptoms in oleander was studied.

OBJECTIVES

1. Characterize xanthan-degrading enzymes from endophytic bacteria isolated from grape
2. Explore applications of naturally-occurring endophytic bacteria that produce xanthan-degrading enzymes for reduction of Pierce's disease and insect transmission
3. Clone and characterize genes encoding xanthan-degrading enzymes for enzyme overproduction and construction of transgenic endophytes and plants

RESULTS

Co-inoculation of the Xylella Gum-degrader Endophyte and X. fastidiosa in Oleander Plants

GX123 was co-inoculated with *Xf* strain Texas in 3 different cultivars of oleander in the green house: White, Single Red and Betty. At the same time, controls were inoculated with GX123 alone, *Xf* alone or PBS buffer. Four plants were used per inoculation condition and per cultivar, totaling 48 plants obtained commercially. The appearance of symptoms was checked at approximately monthly intervals. Chlorotic mottling along the edges of leaves (Purcell et al, 1999) started to appear approximately in the eighth month after the inoculations, slowly developing into generalized chlorotic mottling and dried tissue (Table 1). The oleander cultivars White and Single Red were the first ones to show symptoms, while the cultivar Betty started to show symptoms 12 months after the inoculations. For all the cultivars, symptoms appeared in both plants inoculated with *Xf* and plants co-inoculated with the endophyte. However, the severity of the symptoms was less for the plants co-inoculated with the endophyte than for the plants not co-inoculated (Figures 1-3). Symptoms were more severe and appeared earlier in plants inoculated with *Xf* than in those co-inoculated with GX123 (Table 1 and 2). One year after being inoculated with *Xf* alone all the plants infected by *Xf* (positive result in ELISA test) showed symptoms, while one year after co-inoculations only 75% of the plants infected by *Xf* showed symptoms (Table 3). On the other hand, one year after inoculations *Xf* was detected in infected plants (10^5-10^6 ufc/g of plant tissue), while GX123 was not detected, showing a probable need for re-inoculation of the endophyte for a long term survival or a different strategy of introducing the biocontrol endophyte.

Table 1. Severity of the symptoms in oleander plants, regardless of the cultivar, inoculated with *X. fastidiosa* strain Texas alone or co-inoculated with GX123; 12 plants total per inoculation condition per month sampling.

	X. fastidiosa strain Texas				*X. fastidiosa* strain Texas/GX123			
Months	8	10	12	14	8	10	12	14
(+)	2	0	2	3	3	1	2	3
+	3	1	0	0	2	2	3	0
++	2	3	4	1	0	4	4	2
+++	0	3	3	4	0	0	0	5
AD	0	0	0	1	0	0	0	0
D	0	0	0	2	0	0	0	0

(+) chlorotic mottling along the edges of a few leaves; + chlorotic mottling along the edges of many leaves evolving into a uniform chlorotic mottling; ++ chlorotic mottling of many leaves, starting to wrinkle and dry; +++ chlorotic mottling of many leaves and zones of dead tissue (dried, straw color), smaller leaves; AD many dried leaves, plant almost dead; D plant dead.

Table 2. Number of symptomatic plants after inoculation with *X. fastidiosa* strain Texas alone, co-inoculated with GX123, GX123 alone or PBS buffer; 12 plants total per inoculation condition per month sampling.

Months	*X. fastidiosa* strain Texas	*X. fastidiosa* strain Texas/GX123	GX123	PBS
8	7	5	0	0
10	7	7	0	0
12	9	9	0	1
14	11	10	0	1

Table 3. Symptomatic plants and ELISA results after 1 year of inoculation; 12 plants total per inoculation condition.

Inoculations	*X. fastidiosa* strain Texas	*X. fastidiosa* strain Texas/GX123
Symptomatic plants	9	9
Positive ELISA for *X. fastidiosa*	9	12

Figure 1. Oleander 'White' after 1 year of inoculation with *X. fastidiosa* strain Texas.

Figure 2. Oleander 'White' after 1 year of co-inoculation with *X. fastidiosa* strain Texas and GX123.

Figure 3. Oleander 'White' after 1 year of inoculation with GX123.

Sequential Inoculation of the Xylella Gum-degrader Endophyte and X. fastidiosa in Oleander Plants

To examine the effect of different strategies to introduce the *Xylella* gum-degrader endophyte to control *Xf* in plants, GX123 was inoculated in oleander plants (cultivar white) prior to *Xf*. Sequential inoculation of *Xf* was done 20 days after GX123 was inoculated in the same point when the titers of GX123 were already around 10^4-10^5 cfu/g of plant tissue. This experiment is still ongoing and symptoms have not developed yet, consequently the effect on disease expression is still unknown.

CONCLUSIONS

The *Xylella* gum-degrader endophyte *Acinetobacter johnsonii* GX123 colonized plants and delayed symptoms of infected oleander plants in preliminary experiments. It is a potential candidate as a biocontrol agent for *Xylella fastidiosa,* and therefore a promising tool to fight Pierce's disease.

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