Interactions of Xylella fastidiosa and Endophytic Bacteria in Citrus: A Review

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ABSTRACT

Citrus variegated chlorosis (CVC) is a disease of sweet orange [Citrus sinensis (L.)] caused by Xylella fastidiosa subsp. pauca, a phytopathogenic bacterium that has been shown to infect all sweet orange cultivars. Xylella fastidiosa is a fastidious Gram-negative, xylem-limited bacterium which was rapidly disseminated by infected nursery trees and by several xylem-feeding sharpshooter insect vectors. In Brazil, CVC is the most economically important of several plant diseases caused by X. fastidiosa. One factor that may confer apparent resistance to CVC is the endophytic microbial community colonizing individual C. sinensis plants. Endophytes are microorganisms that do not visibly harm the host plant, but can be isolated from the internal tissues of surface-disinfected plants. Furthermore, as they colonize an ecological niche similar to that of certain plant pathogens, they are likely candidates for biocontrol agents. There is evidence that X. fastidiosa interacts with endophytic bacteria present in the xylem of sweet orange, and that these interactions, particularly with Methylobacterium mesophilicum and Curtobacterium flaccumfaciens, may affect disease progress. Studies of endophytic bacterial populations in sweet orange suggest that symptoms of CVC in sweet orange could be influenced by the relative populations of Methylobacterium spp., C. flaccumfaciens and X. fastidiosa subsp. pauca. Symbiotic control is a new strategy that uses symbiotic endophytes as biological control agents to antagonize or displace pathogens. Candidate endophytes for use in symbiotic control of CVC must occupy the xylem of host plants and attach to the precibarium of sharpshooter insects in order to have access to the pathogen. In the present review, we focus on interactions between endophytic bacteria from sweet orange plants and X. fastidiosa subsp. pauca, especially those that could result in some strategy for symbiotic control of CVC.

Keywords: endophytes, Citrus sinensis, Curtobacterium flaccumfaciens, Methylobacterium mesophilicum, symbiotic control, Xylella fastidiosa subsp. pauca

Abbreviations: Axd, Alcaligenes denitrificans var. xylosoxidans; CVC, Citrus variegated chlorosis; DGGE, Denaturing gradient gel electrophoresis; DsRed, red fluorescent protein; GFP, green fluorescent protein; GWSS, glassy-winged sharpshooter; PBS, phosphate buffered saline; PD, Pierce’s disease; PPFM, pink-pigmented, facultatively methylotrophic; SC, symbiotic control; S1, antibody fragment

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THE PATHOGEN XYLELLA FASTIDIOSA

The first report of symptoms caused by what we now call Xylella fastidiosa was in 1884 in the grape-growing region of southern California (US). A disease syndrome, known today as Pierce’s disease (PD), was later described in detail (Pierce 1892). Subsequently, similar diseases were reported on many fruit tree and ornamental species, especially in North and South America (Hopkins 1989). Xylella fastidi-os is a fastidious Gram-negative xylem-limited bacterium, rod-shaped with distinctive rippled cell walls. It is non-flagellate, does not form spores and measures 0.1-0.5 × 1-5 μm (Nylund et al. 1973; Bradbury 1991). This Gram-negative bacterium was formally named only in 1987 (Wells et al. 1987), and is characterized by being extremely slow-growing in culture. These traits have made the pathogen difficult to study, and have contributed to its previous obscurity. The taxonomic position of X. fastidiosa (Wells et al. 1987) is: Bacteria, Gracilicutes, aerobic rods, Category I, Group 4, Subgroup 4 A (Holt 1994). Natural transmission occurs via insects feeding suctorially on xylem sap. Transmission efficiency varies widely among vector species. The bacterium overwinters in the xylem of the host plant as well as in weeds.
The bacterium *X. fastidiosa* (Wells et al. 1987), as mentioned above, resides in the xylem vessels of a broad range of perennial plants in the New World, and has been shown to cause important diseases in a variety of fruit trees and vines. These include PD in grapevines (Davis et al. 1981; Hopkins and Purcell 2002), leaf scorch of pecan (Sanderlin and Heyderich-Alger 2000; Sanderlin and Melanson 2006), pear (Leu and Su 1993), plum (Raju et al. 1983), almonds (Minc and Heyderich-Alger 2000; Minc and Melanson 2001), mulberry (Kostka et al. 1978), hop (Hearon 1979), phony peach disease (Goheen et al. 1981), periwinkle wilt (McCoy et al. 1978), and citrus variegated chlorosis (Chang et al. 1993; Hartung et al. 1994). Strains of *X. fastidiosa* have a wide host range in the native flora, where they exist without inducing symptoms of disease, and they are transmitted by common sharpshooter insects (Freytag 1951; Freytag and Frazier 1954). These attributes contribute to the current lack of adequate disease control measures.

**CITRUS VARIEGATED CHLOROSIS (CVC)**

Citrus variegated chlorosis (CVC) is a disease of the sweet orange (*C. sinensis* (L.)), which is caused by *Xylella fastidiosa* subsp. *pauca* (Chang et al. 1993; Hartung et al. 1994; Schaad et al. 2004) a phytopathogenic bacterium that has been shown to infect all sweet orange cultivars (Li et al. 1994; Schaad et al. 2004) a phytopathogenic bacterium that has been shown to infect all sweet orange cultivars (Li et al. 1994; Schaad et al. 2004). CVC was first reported in Brazil in 1987 and has been shown to infect all sweet orange cultivars (Li et al. 1994; Schaad et al. 2004) and citrus variegated chlorosis (Chang et al. 1993; Hartung et al. 1994). Strains of *X. fastidiosa* have a wide host range in the native flora, where they exist without inducing symptoms of disease, and they are transmitted by common sharpshooter insects (Freytag 1951; Freytag and Frazier 1954). These attributes contribute to the current lack of adequate disease control measures.

Endophytes can be isolated from surface-disinfected plant tissue or the inner parts of plants and are defined as bacteria that live within a plant for at least a part of its life cycle, but which do not cause apparent harm to the plant. Petrim et al. (1989; Hallmann et al. 1997; Azevedo et al. 2000). The most comprehensive definition was proposed by Azevedo and Araújo (2007), who described endophytes as all microorganisms that may or may not be successfully cultured, that either internally colonize the host plant and do not cause apparent damage and/or visible external structures. Endophytes were reported to contribute to host plant protection and ultimately survival (Sturz and Matheson 1996; Hallmann et al. 1998; Azevedo et al. 2000; Newman and Reynolds 2005). Since endophytes colonize an ecological niche similar to that of phytopathogens, they are possible biocontrol agents (Hallmann et al. 1997). The potential for practical applications of endophytes has led to studies addressing the bacterium’s ability to control both disease and insect infestations, as well as promoting plant growth (Azevedo et al. 2000; Koždějová et al. 2004; Kavino et al. 2007).

Indeed, previous work suggested that endophytic microorganisms have the potential to control pathogens (Sturz and Matheson 1996; Duijf et al. 1997; Sharma and Nowak 1998; Sturz et al. 1998; Lacava et al. 2004, 2007a), insects (Petrim et al. 1989; Azevedo et al. 2000), and nematodes (Hallmann et al. 1997). In some cases, endophytes can also accelerate seed emergence, help plant establishment under adverse conditions (Chanway 1997), and increase plant growth and development (Bent and Chanway 1997; Lazarovits and Nowak 1997; Pillay and Nowak 1997; Nassar et al. 2005).

Endophytic bacteria may play a significant role in protection against plant pathogens and in the overall productivity of an agricultural ecosystem (Hallman et al. 1997; Sturz et al. 2000). The mode of action of the endophytic bacterial community may be through induction of disease resistance mediated by the synthesis of structural compounds, such as siderophores and extracellular enzymes (Benhamou and Nicole 1999), and the induction and expression of general molecular-based plant immunity (Benhamou and Nicole 1999). Alternatively, they may act by niche competition, preventing pathogens from becoming established in a host (Sturz et al. 2000).

In mature citrus trees, the endophytic environment becomes more stable over time. This may result from selection of particular genotypes within each local microbial population (Araújo et al. 2002). Consequently, bacteria living in an endophytic environment may show a tendency to adapt themselves to this more stable environment, resulting in intense interactions among them (Lacava et al. 2004).

Recent results highlighted the relationships among bacterial populations and suggest that CVC symptoms in citrus plants could be influenced by the population balance among...

**ENDOPHYTIC BACTERIA FROM CITRUS PLANTS AND INTERACTIONS WITH XYLELLA FASTIDIOSA**

Araújo et al. (2001) isolated several endophytic bacteria from citrus trees. The genus *Methylobacterium* was the most frequently isolated endophytic bacterium from CVC-symptomatic citrus plants (*C. sinensis*) (Araújo et al. 2002; Lacava et al. 2004, 2006a, 2006b). Also, Araújo et al. (2002) and Lacava et al. (2004) provided data to suggest an interaction between *Methylobacterium* species and *X. fastidiosa* subsp. *pauca* because the genus *Methylobacterium* is frequently isolated from the citrus plants with symptoms of CVC and *M. mesophilicum* could reduce the growth of *X. fastidiosa*, while *M. extorquens* could stimulate the growth of *X. fastidiosa in vitro*. Lacava et al. (2004) suggested that CVC symptoms in citrus plants could be influenced by the population balance among the endophytic bacteria *Methylobacterium* spp., *C. flaccumfaciens* and *X. fastidiosa* subsp. *pauca*.

We have focused on the interaction between members of endophytic bacterial community, such as *Methylobacterium* spp. and *C. flaccumfaciens*, which occupy the same ecological niche as *X. fastidiosa* subsp. *pauca* in the xylem vessels of citrus plants (Araújo et al. 2002). The genus *Methylobacterium* is classified in the α2 subgroup of the *Proteobacteria* and includes a group of strictly aerobic, Gram-negative, pink-pigmented, facultatively methylotrophic (PPFM) bacteria characterized by their ability to utilize single-carbon compounds like methanol and formaldehyde via the serine pathway, as well as a wide range of multi-carbon growth substrates (Green 1992 Urakami et al. 1993; Wood et al. 1998; Doronina et al. 2000; McDonald et al. 2001; Doronina et al. 2002; Aken et al. 2004; Anesti et al. 2004; Van Jourand et al. 2004; Gallego et al. 2005a, 2005b, 2005c, 2006). The genus *Curtobacterium* had been defined by Yamada and Komagata (1972) as Gram positive and aerobic bacteria and for some so-called motile brevibacteria. *Curtobacterium* strains had been isolated from rice and other plants and *C. flaccumfaciens*, in particular, is a well-established plant pathogen (Collins and Jones 1983). However, *Curtobacterium* have been isolated as endophytes from many crops, including red clover (Sturz et al. 1998), rice (Sturz et al. 2000), potato (Sturz and Matheson 1996), yam (Tor et al. 1992), prairie plants (Zimmer et al. 2002), and citrus (Araújo et al. 2001). Several reports have indicated that *C. flaccumfaciens* can function as a biological control agent against many pathogens, and may function either by the triggering of induced systemic resistance (Raupach and Kloepper 1998) or by antibiotic (Sturz and Matheson 1996).

The bacterium *M. mesophilicum* seems to reduce the growth of *X. fastidiosa* subsp. *pauca*, while *M. extorquens* could stimulate the growth of *X. fastidiosa* subsp. *pauca* in vitro (Lacava et al. 2004). The conclusion is that the symptoms of CVC could be affected by the populations of *Methylobacterium* spp. and also *C. flaccumfaciens* (Araújo et al. 2002; Lacava et al. 2004).

Lacava et al. (2006a) evaluated the co-inoculation of *M. mesophilicum* and *X. fastidiosa* subsp. *pauca* into *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle), an excellent experimental host plant for *X. fastidiosa* subsp. *pauca* and endophytic bacteria (Monereo et al. 2001; Andreote et al. 2006). These authors verified that the population of endophytic *Methylobacterium* was lower when inoculated with *X. fastidiosa* subsp. *pauca* than when inoculated alone. Also, the population of *X. fastidiosa* subsp. *pauca* was in turn reduced by *M. mesophilicum*. These initial results are from a single time point only, but are consistent with other data that suggest interactions between *M. mesophilicum* and *X. fastidiosa* subsp. *pauca* in vitro (Lacava et al. 2004, 2006a).

The bacterium, *C. flaccumfaciens*, was more frequently isolated from CVC-asymptomatic than from CVC-symptomatic orange (*C. sinensis*) and tangerine (*Citrus reticulata*) plants (Araújo et al. 2002; Lacava et al. 2004), and it was also suggested, based on in vitro interaction experiments, that the growth of *X. fastidiosa* subsp. *pauca* could be inhibited by endophytic *C. flaccumfaciens*. It was also demonstrated that symptoms of *X. fastidiosa* subsp. *pauca* infection in *C. roseus*, such as shortened internodes, reduced flowering and stunting and chlorosis of leaves with occasional scorch symptoms, were reduced or prevented entirely by co-inoculation with *C. flaccumfaciens* (Lacava et al. 2007a).

**BIOLOGICAL CONTROL OF CVC**

Lacava et al. (2004) reported that the growth of *X. fastidiosa* subsp. *pauca* was inhibited by endophytic *C. flaccumfaciens* in vitro and Lacava et al. (2007a) demonstrated that *C. flaccumfaciens* reduced the severity of disease symptoms when co-inoculated with *X. fastidiosa* subsp. *pauca* in periwinkle (*C. roseus*) plants (Fig. 1).

The use of isolation and denaturing gradient gel electrophoresis (DGGE) techniques revealed several genera of bacteria as colonizers of glassy-winged sharpshooter (GWSS) heads and as identified by 16S sequencing, these included *M. extorquens* and *C. flaccumfaciens*. The GWSS, *Homalodisca vitripennis* Germar (Hemiptera: Cicadellidae) (formerly *H. coagulata* (Takiya et al. 2006)) is the most widespread sharpshooter insect vector of *X. fastidiosa* in the United States. In addition, Kirkpatrick and Wilhelm (2007) have also isolated strains of *C. flaccumfaciens* as part of the endophytic bacterial community of grapevine in California. In Brazil, *C. flaccumfaciens* is consistently isolated as an endophytic bacterium from citrus plants (Araújo et al. 2002; Lacava et al. 2004).

It is likely that endophytic bacteria are introduced into sweet orange trees by sharpshooter insects in the same manner as *X. fastidiosa* subsp. *pauca*. In the study of Gai (2006), *Curtobacterium* sp. was the most important bacterium colonizing the heads of the insect vectors of *X. fastidiosa* subsp. *pauca* in Brazil. To isolate the bacterial community associated to head of insect vectors of *X. fastidiosa* subsp. *pauca*, Gai (2006) started with surface sterilization which involved placing sharpshooter body in 75% ethanol for two minutes, transferring them to a container with sodium hypochlorite solution (2% available Cl-) for two minutes, and rinsing them twice in sterile double-distilled water. After the surface sterilization step, each sharpshooter was transferred into a sterile Petri dish and its head and eyes were removed using a sterile scalpel. The head, which contained the foregut, was removed, the eyes excised and the remainder of the head was used in the isolation, then placed into a sterile 1.5 ml microcentrifuge tube containing 150 μl of sterile phosphate buffered saline (PBS). The mixture was mace-
oliquet of 100 μl of this solution was plated on 5% TSB media (Tryptone Soy Broth, DIFCO). Plates were incubated at 28°C for 5 days and colonies were counted, classified according to morphological groups and the number of colony forming units per insect head (CFU/insect head) was determined.

Curtobacterium flaccumfaciens was implicated in playing an important role in the prevention of CVC symptoms in California (Lacava et al. 2002; Durvasula et al. 2004, 2007a). The citrus endophyte, Curtobacterium sp., colonizing vector insect heads could explain why the transmission efficiency of X. fastidiosa subsp. pauca by vectors is low (5 to 10%), when compared to the transmission of X. fastidiosa subsp. fastidiosa by GWSS, which transmit PD (45%) (Krugnet et al. 2000; Redak et al. 2004). Endophytic bacteria could influence disease development by reducing the efficiency of transmission by insects due to competition with pathogens in host plants and also in insect foreguts (Gai 2006). In addition, the bacterial communities in the foregut of insect vectors of X. fastidiosa subsp. pauca changed with time, environmental conditions and in different insect species. However, since members of the genus Curtobacterium were consistently detected in the insect vectors of X. fastidiosa subsp. pauca (Gai 2006), they may be candidates for biological control of X. fastidiosa subsp. pauca, which requires endophytic bacteria (Lacava et al. 2007a) that can colonize both the insect vectors of CVC and citrus plants.

SYMBIOTIC CONTROL (SC)

The technique of paratransgenesis was developed as a novel method to create conditions that render insect vectors incompetent. The strategy of symbiotic control (SC) employs both paratransgenic (defined below) and non-recombinant methods to control disease or health problems. In some cases, these solutions may result in competitive displacement of the pathogen with a more benign microbe.

The strategy, paratransgenesis, was developed in order to prevent the transmission of pathogens by insect vectors to humans (Beard et al. 1998, 2001, 2002; Rio et al. 2004). The key concept in paratransgenesis is the genetic alteration of symbiotic microbes that are carried by insects (therefore, they are paratransgenic insects). The genetic alterations of the symbiotic microbes are designed to increase their competitiveness within the insect vector at the expense of the pathogen. This overall strategy of disease prevention is an example of SC and is a variation on the theme of symbiotic therapy (Ahmed 2003). Genetic manipulation has fitness costs that must be factored in to the application (Durvasula et al. 1997; Miller 2007).

The key to SC, and therefore paratransgenesis, is to find a local candidate microbe having an existing association with the pathosystem that includes the problem or condition at hand. The local candidate microbe should occupy the same niche as, or have access to, the target pathogen or condition (Durvasula et al. 1997). The local origin of the biocontrol microbe in SC differs from classical biological control, where microbes, herbivores, parasites or predators are sought from outside of the local ecosystem for establishment in the local ecosystem to control a pest, such as a plant or invertebrate (Miller 2007). In SC, all elements originate at the local site and are already co-evolved with and established in the pathosystem; foreign exploration is not only unnecessary, but also most likely counter-productive. Because of these requirements, a suitable symbiotic candidate may not always be found or may not be amenable to practical manipulation (Miller 2007).

Microbes chosen for symbiotic control must be able to pass subsequent regulatory scrutiny (Miller 2007). Once a candidate symbiont is identified as a control agent for paratransgenesis, all genetic or other manipulations can be local. Indeed, a symbiotic control or paratransgenic solution developed for a specific location may not be suitable for another site or condition elsewhere (Durvasula et al. 1999, 2003; Miller 2007).

Once a microbe is identified as having potential for symbiotic or paratransgenic control, it is studied to define requirements for culture and reintroduction into the pathosystem, and suitability for genetic alteration, if necessary. The methods selected have to be adaptable to ordinary practices in the target area. In the case of paratransgenic control, a gene or genes to be introduced into the endosymbiont to influence its interaction with the pathogen must be identified. Beard et al. (2001) have isolated and characterised symbiont bacteria from various triatomine species, vector of Chagas disease, and developed a method for genetically transforming them. These authors have reintroduced them into triatomine species, thereby producing stable paratransgenic insects that are able to express heterologous gene products.

SYMBIOTIC CONTROL OF PIERCE’S DISEASE AS A MODEL FOR CVC CONTROL

PD was first detected in Southern California in 1884, where it destroyed approximately 40,000 acres of grapes inAnaheim, CA, during a 5-year outbreak (Pierce 1892; Goodwin and Purcell 1992). After this devastating experience, PD has continued to be a serious pest in West Coast viticulture for decades until the mid-1990s, when the GWSS became established in California. The GWSS is a major concern for horticultural industries beyond viticulture due to its ability to transmit X. fastidiosa strains causing scorch diseases in a number of host plants, including X. fastidiosa subsp. fastidiosa that causes PD in grapevines (Purcell 2005). As with other sharpshooter insects, H. vitripennis is a xylophagous insect that feeds on hundreds of plant species (Purcell and Hopkins 1996; Purcell and Saunders 1999); citrus is one of its preferred hosts (Blua et al. 2001). Perring et al. (2001) demonstrated a relationship between PD incidence in grapes and the proximity of vineyards to citrus orchards. This leafhopper, which can serve as a vector of X. fastidiosa, has the capacity to feed on more than 70 different plant species and can survive winter temperatures as low as -6°C (Park et al. 2006). Moreover, compared with other X. fastidiosa-carrying insects associated with PD and native in California, GWSS has a longer flight range (up to a quarter mile). These traits make the GWSS a very serious threat to the wine industry of Southern and Central California (Castle et al. 2005). Indeed, since the first identification of GWSS in the California vineyards, programs aimed at controlling the dissemination of this insect as a strategy to prevent PD outbreaks have involved more than US $16 million of direct investment in US $300 million of indirect investment (USDA phpps/pdcp/). Control of any of the GWSS transmitted diseases of horticultural crops in California by a SC or paratransgenic approach would be of immediate interest to other industries as well. The objective or rationale for developing a method of SC for PD is to disrupt vector transmission with the least effect on other crops. SC would be available to local vineyards for local control instead of area-wide treatments of alternative host plants such as is done now. Treatment of citrus with systemic insecticides for GWSS to reduce the chance of acquiring and spreading pathogens in adjacent vineyards cannot be seen as a long-term solution. SC would be more selective and have less side-effects on other biological control practices. The SC organisms inhabit the xylem fluid of the target plants yet do not contaminate the berries of the grapevines. It remains to be seen if one treatment would be effective for an entire season (Miller 2007).

Three potential bacterial candidates, Alcaligenes sp., Chryseomonas sp., andRalstonia sp., for SC of PD were collected from GWSS in southern California (Bextine et al. 2004). All were endophytes transmitted to different host plants by GWSS in a manner analogous to the pathogen; thus, the candidates had access to the pathogen in host plants or in the insect vectors, providing the needed access property, Alcaligenes denitrificans var. xylosoxidans (Axli)
was selected for further development because the endophytic bacterium should have most of the requirements for a successful paratransgenesis strategy such as: a) a population of microbes that is amenable to culture and genetic manipulation in vitro must exist within a disease-transmitting vector; b) facile methods for isolating and transforming the endophytic bacteria must be present; c) transformation of the symbiotic/endophytic bacteria must result in stable mutation; d) genetic manipulation of the bacteria should not affect their symbiotic functions in the host vector; e) genetic manipulation of symbiotic bacteria should not render them virulent, either to the target vector or other organisms in the environment. Furthermore, bacteria chosen as gene-delivery vehicles must not be pathogens themselves.

Successful delivery to and colonization of Axd in the foregut regions of GWSS suggest that a paratransgenic approach to manage, prevent, and/or control Pierce’s disease is possible (Bextine et al. 2004).

Lacava et al. (2007b) used isolation and denaturing gradient gel electrophoresis (DGGE) techniques to identify several genera of bacteria as colonizers of heads of GWSS collected in orange groves. As identified by 16S rRNA sequencing, these included Bacillus, Cryocoryx, Microbacterium, Micrococcus, and Pedobacter. In addition, Methylobacterium extorquens, Curtobacterium flaccumfaciens, Bae
mannia cicadellinicoa and various Pseudomonas and Wolbachia species were found. Of these genera, Bacillus, Pseudomonas, Methylobacterium and Curtobacterium were previously described as endophytes that are able to colonize citrus plants. The work of Araújo et al. (2002) strongly indicated interactions among Methylobacterium spp., C. flaccumfaciens and X. fastidiosa subsp. pauca. These results reinforced the idea that all of these bacteria could interact in the vector insect as well as in the host plant.

Furthermore, Lacava et al. (2004) suggested CVC symptoms in citrus plants could be influenced by the interactions among these three species. In a study of the diversity of bacterial communities associated with GWSS foreguts, they used culture-dependent methods as well as procedures based on sequence polymorphisms (DGGE) of the 16S rRNA gene present in total DNA extracted from GWSS foreguts. Lacava et al. (2007b) suggested that the diversity profile obtained with culture dependent (isolation in culture) techniques indicated a low bacterial diversity. However, the same authors described higher bacterial diversity when using PCR-DGGE, a culture-independent method. These initial results from Lacava et al. (2007b) showed that PCR-DGGE is suitable for the analysis of bacterial diversity in GWSS heads. In the future, species such as C. flaccumfaciens, Methylobacterium spp., Bae
mannia cicadellinicoa, Pedobacter spp., and various Pseudomonas and Wolbachia species were found. Of these genera, Bacillus, Pseudomonas, Methylobacterium and Curtobacterium were previously described as endophytes that are able to colonize citrus plants. The work of Araújo et al. (2002) strongly indicated interactions among Methylobacterium spp., C. flaccumfaciens, and X. fastidiosa subsp. pauca. These results reinforced the idea that all of these bacteria could interact in the vector insect as well as in the host plant.

Using methods perfected in previous studies (Lampe et al. 1999, 2000), Axd was genetically altered to contain a DsRed fluorescent marker gene in the chromosome (Bextine et al. 2004) to demonstrate the ability of DsAx to colonize the bacterial region of the GWSS foregut for up to 5 weeks post-exposure. The conclusion was that Axd occupies the same region in the foregut as the pathogen, X. fastidiosa. Using methods perfected in previous studies (Lampe et al. 1999, 2000), Axd was genetically altered to contain a DsRed fluorescent marker gene in the chromosome (Bextine et al. 2004) to demonstrate the ability of DsAx to colonize the bacterial region of the GWSS foregut for up to 5 weeks post-exposure. The conclusion was that Axd occupies the same region in the foregut as the pathogen, X. fastidiosa. (Bextine et al. 2004). DsRed Axd was found to be transmitted by GWSS and to colonize various plants (Bextine et al. 2004, 2005). DsRed Axd could be introduced into grapevines by misting the leaves or by soil drenching or by direct injection of the stem of the grapevine. Interestingly, Axd appeared to be better adapted to citrus than to grapevine (Bextine et al. 2005). Indeed, the original samples of GWSS from southern California were obtained from citrus groves in the Agricultural Operations plots at the University of California, Riverside, so it is likely that the endophytes in the GWSS sampled originally came from citrus. Bextine et al. (2004) describe the successful delivery of Axd to, and colonization of, the foregut of GWSS by Axd. These results suggest that a paratransgenic approach to manage, prevent, and/or control PD by SC may be possible. We proposed (Fig. 2) a sequence of steps needed to develop a strategy of SC, using endophytic bacteria, to disrupt the disease cycle where the causal agent is X. fastidiosa (adapted from Bextine et al. 2004, 2005).

A number of candidate antimicrobial peptides were screened against X. fastidiosa (Kuzina et al. 2006). In this study the authors show that antibiotics and antimicrobial peptides have some activity against the pathogen, X. fastidiosa and may have application in protecting plants from developing PD. The potential use of these antimicrobial peptides in the protection of grapevines will depend on the development of a delivery system, such as SC (Kuzina et al. 2006). Also, Lampe et al. (1999, 2000) further screened single chain antibodies from a phage antibody library for ability to bind the coat protein of the pathogen, X. fastidiosa. These authors selected an antibody fragment, designated S1, that was specific for the strain of X. fastidiosa causing PD and which did not recognize closely related X. fastidiosa strains.

**STRATEGY OF SYMBIOTIC CONTROL FOR CVC**

The key to symbiotic control is finding a candidate microbe having an existing association with the ecosystem that includes the problem or condition at hand and that occupies the same niche as or has access to the target pathogen (Miller 2007). Bacteria of the genus Methylobacterium are known to occupy the same niche as X. fastidiosa subsp. pauca inside citrus plants (Araújo et al. 2002; Lacava et al. 2004). During feeding, insects could acquire not only the plant system and the transmission of this endophyte by Bucephalogonia xanthophis, a sharpshooter insect vector of X. fastidiosa subsp. pauca.
Methylobacterium mesophilicum, originally isolated as an endophytic bacterium from citrus plants (Araújo et al. 2002), was genetically transformed to express GFP (Green Fluorescent Protein) (Gai et al. 2007). The GFP-labeled strain of M. mesophilicum was inoculated into C. roseus (model plant) seedlings and was observed colonizing its xylem vessels. The transmission of M. mesophilicum by B. xanthophis was verified with insects feeding on fluids containing the GFP-labeled bacterium. After inoculation, the plants exhibited endophytic colonization by M. mesophilicum, confirming this bacterium as a nonpathogenic, xylem-associated endophyte (Gai 2006). These data demonstrate that M. mesophilicum not only occupies the same niche as X. fastidiosa subsp. pauca inside plants, but also that it may be transmitted by B. xanthophis. The transmission, colonization, and genetic manipulation of M. mesophilicum is a prerequisite to examine the potential use of paratransgenic-SC to interrupt transmission of X. fastidiosa subsp. pauca, the bacterial pathogen causing CVC, by insect vectors. We propose M. mesophilicum as a candidate for a paratransgenic-SC strategy to reduce the spread of X. fastidiosa subsp. pauca. It is known that X. fastidiosa subsp. pauca produces a fastidian gum (da Silva et al. 2001) which may be responsible for the obstruction of xylem in affected plants (Lambais et al. 2000), so the production of endothe-cause by genetically modified endophytic bacteria may transform the endophytes into symbiotic control agents for CVC. Azevedo and Araújo (2003) have used the replicative vector pEGLA160 to produce genetically modified Methylo- bacterium expressing antibiotic resistance and endogluca-nase genes. Furthermore, other strategies can be evaluated such as the production of genetically modified Methylobac-terium to secrete soluble anti-Xylella protein effecting citrus, such as Lampe et al. (2006) suggested in the Escherichia coli u-hemolysis system for use in Axd to secrete soluble anti-Xylella protein effectors in grapevine and GWSS. Also, Lampe et al. (2007) suggested the evaluation of proteins secreted from the grapevine bacterial symbiont Pantoaea agglomerans for use as secretion partners of anti-Xylella protein effectors. One strategy that can be adopted as the next step for SC control of CVC is producing a genetically modified endophytic bacteria, like Methylobacterium, to secrete anti-Xylella protein effectors.

CONCLUSION

Our strategy is similar to that developed by Bextine et al. (2004) for a paratransgenic strategy for SC of PD in grape- vine. Bextine et al. (2004) suggested that the genus Alcaligenes nonendophytic bacteria that can colonize the GWSS vector of X. fastidiosa subsp. fastidiosa, would be a candi-date for paratransgenic SC of PD in the USA. We believe that the endophytic bacterium M. mesophilicum from citrus plants is likewise a candidate for paratransgenic-SC of CVC. Our results indicate that this endophyte colonizes the same niche as X. fastidiosa subsp. pauca in citrus plants (Araújo et al. 2002; Lacava et al. 2004; Andreote et al. 2006; Lacava et al. 2004a). M. mesophilicum is also transmitted by an insect vector of X. fastidiosa subsp. pauca (Gai 2006).

Bacteria chosen as gene-delivery vehicles for paratrans-genesis-SC must not be pathogens themselves. M. mesophili-cum is not a pathogen and several requirements for suc-cessful paratransgenesis-SC strategy as described by Durva-sula et al. (2003) have been demonstrated a) M. mesophili-cum is amenable to culture and genetic manipulation in vitro; b) fastidiosa can be isolated, and transcript analysis of the endophytic bacteria have been developed; c) transformation of the symbiotic/endophytic bacteria has resulted in mutants that were stable in plants. Future genetic manipulation of M. mesophilicum to achieve paratransgenic-SC should not af-fect its symbiotic functions in the plant host and insect vec-tor, and of course genetic manipulation of symbiotic bac-teria should not render them virulent, either to the host plant or target.

C. flaccumfaciens is also a candidate for biological con-trol of CVC. Interaction and antagonism between C. flac-cumfaciens and X. fastidiosa subsp. pauca was strongly in-dicated on the basis of the frequency of isolation from sweet orange of C. flaccumfaciens (Araújo et al. 2002). In addition, in vitro interactions between X. fastidiosa and C. flaccumfaciens have been described, including the inhibi-tion of the growth of X. fastidiosa subsp. pauca by cell-free supernatants of nutrient medium in which C. flaccumfaciens was grown (Lacava et al. 2004a). Also, Lacava et al. (2007a) demonstrated that C. flaccumfaciens interacted with X. fastidiosa subsp. pauca in C. roseus, and reduced the severity of the disease symptoms induced by X. fastidiosa subsp. pauca (Fig. 1). The ability demonstrated by C. flaccumfaciens to colonize plant tissues in the presence of X. fastidiosa subsp. pauca and the reduction of disease symp-toms caused by X. fastidiosa subsp. pauca (Lacava et al. 2007a) are prerequisites for the use of this endophytic bac-terium as a biocontrol agent. Since members of the genus Curtobacterium were consistently detected in the insect vectors of X. fastidiosa subsp. pauca (Gai 2006), they fulfill another requirement of candidates for biological control of X. fastidiosa subsp. pauca (Lacava et al. 2007a), i.e. they can colonize both the insect vectors of X. fastidiosa subsp. pauca and citrus plants. In the case of biocontrol of X. fast-idiosa subsp. pauca and CVC disease, it would be desir-able if C. flaccumfaciens could be transmitted by budwood, but this has yet to be demonstrated. The reduction of disease symptoms caused by X. fastidiosa subsp. pauca in the presence of C. flaccumfaciens may be attributable to direct killing of X. fastidiosa subsp. pauca by C. flaccumfaciens. Consistent with this hypothesis, three bacteriocins showing activity against X. fastidiosa subsp. pauca have been re-cently described from C. flaccumfaciens (Cursino 2005).

We propose two complementary strategies for control of
CVC using endophytic bacteria from citrus plants. We sug-
gest the endophytic bacterium C. flaccumfaciens as a clas-
sical biological control agent and the endophytic bacterium
M. mesophilicum as a qualified candidate for a paratransge-
ic-SC strategy. The details of these strategies are sum-
marized in Fig. 3.

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