Short Communication

Specific amplification of iron receptor genes in *Xylella fastidiosa* strains from different hosts

Flávia Teresa Hansen Pacheco[1], Maria Estela Silva-Stenico[1], Augusto Etchegaray[1],
José Elias Gomes[1], Emanuel Carrilho[2] and Siu Mui Tsai[1]

[1]*Universidade de São Paulo, Centro de Energia Nuclear na Agricultura, Laboratório de Biologia Celular e Molecular, Piracicaba, SP, Brazil.*
[2]*Universidade de São Paulo, Instituto de Química de São Carlos, Laboratório de Cromatografia, São Carlos, SP, Brazil.*

Abstract

Bacterial production of siderophores may involve specific genes related to nonribosomal peptide and polyketide biosynthesis, which have not been fully identified in the genome of *Xylella fastidiosa* strain 9a5c. However, a search for siderophore-related genes in strain 9a5c indicated five membrane receptors, including siderophore, ferrichrome-iron and hemin receptors. All these biomolecules are thought to be associated with iron transport and utilization. Eighty isolates obtained from citrus orchards containing trees that developed citrus variegated chlorosis (CVC) were screened for siderophore production. The results demonstrated that only 10 of the isolates did not produce siderophores. Additional strains obtained from coffee, almond, mulberry, elm, ragweed, periwinkle and grape also infected by *X. fastidiosa* were also shown by the chromeazurol bioassay to produce siderophores. In order to correlate siderophore production with the presence of siderophore-related genes, a polymerase chain reaction (PCR) was developed using specific primers for the catechol-type ferric enterobactin receptor (*pfe*A) and the hydroxamate-type ferrisiderophore receptor (*fiuA*) genes of strain 9a5c. The PCR results confirmed our hypothesis by demonstrating that amplification products were detected in all strains except for those isolates that did not produce siderophores.

The Gram-negative, xylem-limited bacterium *Xylella fastidiosa* causes a range of economically important plant diseases, including citrus variegated chlorosis (CVC), Pierce's disease (PD) of grapevine, alfalfa dwarf, leaf scorch of almond, coffee, elm, sycamore, oak, plum, mulberry and maple, and wilt in periwinkle and ragweed (Chang *et al.*, 1993) but the molecular mechanisms through which *X. fastidiosa* establishes the infection process have not yet been fully determined.

The genome of *X. fastidiosa* 9a5c contains 67 genes encoding proteins involved in iron metabolism and has been reported to contain five membrane receptors, including siderophore, ferrichrome-iron and hemin receptors, all of which are thought to be associated with iron transport and utilization (Simpson *et al.*, 2000).

In microbes, the demand for iron often induces excretion of siderophores, low molar mass metabolites with high affinity for ferric iron (Ankenbauer *et al.*, 1994). Most bacterial siderophores are nonribosomal bioactive peptides synthesized by the multifunctional nonribosomal peptide synthetases (NRPSs) (Kleinkauf and von Doehren, 1996; Etchegaray *et al.*, 2004). Nonribosomal peptide synthetase homologues have been shown to be involved in the biogenesis of siderophore molecules such as yersiniabactin (or yersianiophore), an iron-chelating virulence factor of the human pathogen *Yersinia pestis* (Gehring *et al.*, 1998a) expressed during iron starvation. There is considerable homology between yersiniabactin synthetase and NRPSs, especially enterobactin synthetase (Gehring *et al.*, 1998b).

Preliminary evidence supporting the putative production of siderophores by *X. fastidiosa* developed by our group (Silva-Stenico *et al.*, 2005) shows that the presence of *X. fastidiosa* siderophores is related to iron stress.

Send correspondence to Flávia Teresa Hansen Pacheco. Universidade de São Paulo, Centro de Energia Nuclear na Agricultura, Laboratório de Biologia Celular e Molecular, Av. Centenário 303, 13400-970 Piracicaba, SP, Brazil. E-mail: flapache@hotmail.com.

Specific analysis of the genome of *X. fastidiosa* strain 9a5c for the presence of nonribosomal peptide biosynthetic genes has shown putative NRPS and polyketide synthase (PKS) homologues. Since NRPS and PKS genes can be involved in both siderophore biosynthesis and pathogenicity, we have also investigated these types of genes employing specific primers based on the sequences of the NRPS, PKS and siderophore-receptor genes identified in the genome of strain 9a5c. These results demonstrate a correlation between siderophore production and the presence of genes involved in its transportation and reception by *X. fastidiosa*.

In the study described in the present paper most of the *X. fastidiosa* strains were isolated by us using BCYE (buffered charcoal yeast extract) medium (Wells *et al.*, 1987) from four citrus orchards located in, or near, the towns of Gavião Peixoto, Neves Paulista, Paraíso and Santa Rita do Passa Quatro in the Brazilian state of São Paulo, although some strains were purchased from culture collections or received as a gift from laboratories (Table 1). A total of 80 *X. fastidiosa* strains were used in our study, all strains being maintained on PW (periwinkle wilt) medium (Davis *et al.*, 1981) irrespective of their origin.

To screen for siderophore production by the 80 *X. fastidiosa* strains we developed an agar siderophore production screening test based on the method of Schwyn and Neilands (1987) using PW-CAS (Chromeazurol S) agar prepared as follows: 60.5 mg CAS was dissolved in 50 mL of ultra-pure water and 10 mL of iron (III) solution (1 mmol L^{-1} $FeCl_3.6H_2O$ in 10 mmol L^{-1} HCl) added, the mixture being stirred and slowly mixed with 72.9 mg of hexadecyl-trimethylammonium bromide (HDTMA) dissolved in 40 mL of water, the resultant dark blue solution being autoclaved and then mixed with PW medium. The PW-CAS medium was poured into Petri plates which were inoculated with bacteria and incubated at 28 °C for 15 days. Positive results were indicated by formation of a colorless halo around the colonies. Each assay was performed in triplicate.

Extraction of DNA was performed according to Duningan (1997) and the concentration of DNA estimated by electrophoreses on 0.8% (m/v) agarose gels against known molecular markers, absorbance at 260 and 280 nm being used to confirm the quality of the DNA. The primers designed for specific sequences are listed in Table 2, appropriate PCR protocols being developed as follows. For the XF599F-599R and XF2134F-2134R primer set the mixture contained 0.1 μM of each primer, 0.1 μg of extracted *X. fastidiosa* DNA in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 μM of each deoxynucleoside triphosphate and 2.5 units of *Taq* DNA polymerase in a final volume of 43 μL. The negative control contained no *X. fastidiosa* DNA. The PCR was initiated by a 4 min denaturation step at 94 °C followed by 25 cycles at a denaturation temperature of 94 °C for 1 min, primer annealing at 47 °C for 1 min and extension at 72 °C for 1 min with a final 5 min

extension. Aliquots (5 μL) of the PCR products were visualized on ethidium bromide-stained 1% agarose gel.

For the XF2135F-2135R and XF2276F-2276R primer set the mixture contained 0.1 μM of each primer, 0.1 μg of extracted *X. fastidiosa* DNA in 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 20 mM $MgCl_2$, 20 μM of each deoxynucleoside triphosphate and 2.5 units of *Taq* DNA polymerase in a final volume of 23 μL. The negative control contained no *X. fastidiosa* DNA. The PCR started with a 5 min denaturation step at 96 °C followed by 5 cycles at a denaturation temperature of 95 °C for 1 min, primer annealing at 45 °C for 1 min and extension at 72 °C for 1 min followed by another 3 cycles at a denaturation temperature of 95 °C for 1 min, primer annealing at 45 °C for 1 min and extension at 72 °C for 2.5 min with a final 5 min extension. Aliquots (5 μL) of the PCR products were visualized on ethidium bromide-stained 1% agarose gel.

The PCR products were sequenced on an ABI Prism 3100 automatic sequencer with BigDye chemistry (Applied Biosystems, Foster City, California) using about 100 mg of DNA, sample preparation being performed according to the manufacturer's instructions. Sequences were compared to the public database and edited using the Blast 2.0, Align and Clustal W software.

Screening for siderophore production by *X. fastidiosa* can be made by growth on specific media at different iron concentrations. Results demonstrate the effect on the production of new biomolecules in the medium, induced by iron limitation. During this phase, the chemical composition of the medium can be changed and optimized in order to define the most adequate component concentration for siderophore-related gene expression and production.

In our experiments, siderophore production was determined by the formation of an orange/yellow halo around the bacterial colony. Time required for the formation of halos varied amongst the different *X. fastidiosa* isolates tested, isolates from grape presenting halos within 5 to 6 days of inoculation onto PW-CAS agar, while isolates from citrus required about 10 to 12 days before any halo could be detected. We found that the time needed for halos to appear (due to iron uptake) for the different *X. fastidiosa* isolates grown on PW-CAS agar was apparently the same when compared to the period required for the appearance of colonies on PW medium. Nevertheless, a comparative analysis between the *X. fastidiosa* isolates showed that on both PW or PW-CAS agar strains isolated from grape vines showed the fastest and citrus isolates the slowest growth rates.

The tests performed on PW-CAS agar confirmed that 10 of our *X. fastidiosa* isolates do not produced siderophore, suggesting that siderophore producers contain genes involved in siderophore production and transportation or which produce ferri-siderophores and/or proteins probably involved with *X. fastidiosa* pathogenicity. The sizes of the

Table 1 - Strains of *Xylella fastidiosa* used in this study.

Strain	Biological origin	Geographic origin	Collection[a]
Brazil			
9a5c*	*Citrus sinensis* (citrus)	São Paulo	INRA
J1a12	*Citrus sinensis*	São Paulo	CCT
CM1*	*Coffea arabica* (coffee)	São Paulo	CCT
CSR1	*Coffea arabica*	São Paulo	CCT
CSR2	*Coffea arabica*	São Paulo	CCT
P3*	*Coffea arabica*	São Paulo	CCT
6755	*Coffea arabica*	São Paulo	CCT
USA			
MUL-1*	*Morus nigra* (mulberry)	Massachusetts	ATCC 35868
MUL-3*	*Morus nigra*	Massachusetts	ATCC 35869
ALS-BC*	*Prunus amygdalus* (almond)	California	ATCC 35870
ELM-1*	*Ulmus americana* (elm)	Washington	ATCC 35873
RGW-R	*Ambrosia artimeisiifolia* (ragweed)	Florida	ATCC 35876
PWT-22*	*Vinca minor* (periwinkle)	Florida	ATCC 35878
PCE-GG	*Vitis vinifera* (grapevine)	California	ATCC 35877
PCE-RR*	*Vitis vinifera*	Florida	ATCC 35879
PCE-FG	*Vitis vinifera*	Florida	ATCC 35881
Temecula*	*Vitis vinifera*	California	ATCC 700964
Fetzer	*Vitis vinifera*	California	BC
Trever	*Vitis vinifera*	California	BC

[a]INRA = Institut National de la Recherche Agronomique et Université Victor Ségale, Bordeaux, France; CCT = Coleção de Culturas Tropical, Fundação André Tosello, Campinas, Brazil; ATCC = American Type Culture Collection, Manassas, VA, USA; BC = B.C. Kirkpatrick, Department of Plant Pathology, University of California, Davis, California, USA. *Used for sequence analysis.

halos also varied among the *X. fastidiosa* isolates tested with isolates from grapes presented large intensely-colored halos as compared to other strains, possibly due to the fact that these grape isolates grew faster than the citrus isolates. Due to the difficulty in establishing nutritional conditions for microorganisms that induce the production of different siderophore-types or limit their synthesis little is known about the consequences of siderophore production by phytopathogens in plants.

To confirm the presence of the genes involved in iron metabolism or virulence and to assess their potential correlation with pathogenicity we performed PCR analyses of iron metabolism and virulence genes (enterobactin, pyoverdine, NRPS and PKS genes) of *X. fastidiosa* strain 9a5c and the other isolates. The sequences were analyzed using the Sequence Navigator program and were identified by consulting the nucleotide database at the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov).

Siderophores belonging to enterobactin-type are produced by a great number of bacteria and are always involved in their pathogenicity. This class of siderophore is found in microorganisms with the capacity to obtain iron from their hosts and has been found in many enteric bacteria, including *E. coli* and also in other highly pathogenic species such as *Yersinia* species (*enterocolitica, pestis, pseudotuberculosis*) and *Vibrio cholerae*. Another example is the enterobacterium *Erwinia chrysanthemi* that infects *Saintpaulia ionantha* and produces the catechol-type siderophore chrysobactin which causes iron deficiency in the leaves of *Saintpaulia* (Neema *et al.*, 1993).

The amplification of all *X. fastidiosa* isolates presented a single fragment of about 1,600 bp using the primer sets shown in Table 2. The amplification products showed that DNA from citrus, grape, mulberry, almond, periwinkle, ragweed, elm and coffee *X. fastidiosa* isolates have genes potentially involved in the biogenesis of enterobactin-type siderophores.

A single PCR fragment of about 1,000 bp was obtained by amplification using DNA from 70 of our *X. fastidiosa* isolates plus additional strains from coffee, almond, grape, mulberry, ragweed, periwinkle and elm. This fragment indicates that these *X. fastidiosa* isolates have genes responsible for the synthesis of pyoverdine-type siderophores which are known to be associated with the increase of virulence in the host (Cox and Adams, 1985).

Table 2 - Primers based on the specific sequences of *Xylella fastidiosa* strain 9a5c that were used in this study.

Primers	5'-3' Oligonucleotide sequence	GenBank accession numbers	Function (*gene* or abbreviation)
599F	GTC TCA AGA AGG CCT GCA AC	XF0599	Ferri-pyoverdine *TonB* receptor (*fpvA*)
599R	GTA GTG AAC CCT GCC GAC AT	XF0599	Ferri-pyoverdine *TonB* receptor (*fpvA*)
2134F	GAC ATC AGC CAA CAA GAG CA	XF2134	Ferri-enterobactin receptor (*pfeA*)
2134R	GTG ATT GCG TGA GCG ATA GA	XF2134	Ferri-enterobactin receptor (*pfeA*)
2135F	CAA GAA GTC CTT TTG GAG CG	XF2135	Polyketide synthase (PKS)
2135R	TTC AAC TGA GCC AGT GCA TC	XF2135	Polyketide synthase (PKS)
2276F	ACA ATC GTA CAG CAG CAA CG	XF2276	Non-ribosomal peptide synthetase (NRPS)
2276R	ACC TGC TCG GTA TAC AAC GG	XF2276	Non-ribosomal peptide synthetase (NRPS)

Table 3 - Screening of *Xylella fastidiosa* isolates for siderophore and genes associated with iron-uptake (enterobactin, pyoverdine-receptor, polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS)).

Orchards	Number of isolates									
	Number of sidero-phore producers		Enterobactin		Pyoverdine		NRPS		PKS	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
S.R. do Passa Quatro	17	3	17	3	18	2	17	3	17	3
Neves Paulista	18	2	9	11	5	15	18	2	18	2
Paraíso	17	3	14	6	17	3	17	3	17	3
Gavião Peixoto	18	2	16	4	15	5	18	2	18	2

Nonribosomal peptide synthetases (NRPSs) have also been shown to be involved in siderophore production (Gehring *et al.*, 1998a), because of which we selected a set of primers designed to amplify a peptide synthetase based on the related sequence of *X. fastidiosa* strain 9a5c. Homologous non-ribosomal peptide synthetases have been studied for their involvement in the biogenesis of siderophore molecules as an iron chelating virulence factor expressed during iron deficiency. We were able to obtain 560 bp PCR amplification products for 70 of our *X. fastidiosa* isolates. The region amplified is common in genes coding for peptide synthetases and other enzymes such as acetyl-coenzyme A synthetase that hydrolyze ATP to activate substrates such as amino acids, carboxylic acids and hydroxy acids. Such enzymatic activities are found in the open reading frames of *X. fastidiosa* strain 9a5c, *i.e.* the XF287, XF2255 and XF2276 ligases. We calculated the specificity of these ligases and specific amino acids, and our results indicated that, hypothetically, the XF287 ligase is specific for amino acids (Etchegaray *et al.*, 2004). Even so, these results need to be confirmed by the expression of these ligases in *E. coli* and their full biochemical characterization. It is also important to study the expression of adjacent ORFs, because most of the biosynthesis of non-ribosomal peptides usually requires the support of correlated enzymes (Keinkauf and Von Döhren 1996). These regions will be our targets for future site-directed mutagenesis studies involving the estimation of siderophore production using PW-CAS agar.

A single fragment of about 300 bp was also obtained during the PCR amplification of 70 of our *X. fastidiosa* isolates and for the additional strains isolated from citrus, grape, coffee, almond, mulberry, periwinkle, elm and ragweed. This indicates that all of these isolates contained genome regions encoding sequences homologous to the PKS gene of *X. fastidiosa* strain 9a5c.

Our PCR study was performed in order to screen some *X. fastidiosa* isolates recovered from trees infected with citrus variegated chlorosis for specific genes that may be involved in pathogenicity. In this study we have shown that *X. fastidiosa* isolates from citrus, coffee, grape, almond, elm, mulberry, ragweed and periwinkle produce enterobactin pyoverdine type siderophores and also carry genes related to nonribosomal peptide synthetases and polyketide synthase (Table 3). We believe that the ability of *X. fastidiosa* isolates to produce siderophores and absorb iron from its hosts could be involved in pathogenicity.

Acknowledgments

References

Ankenbauer R, Skiyosachati S and Cox CD (1994) Effects of siderophores on the *Pseudomonas aeruginosa* in human serum and transferrin. Infect Immun 49:132-140.

Chang CJ, Garnier M, Zreik L, Rosseti V and Bové JM (1993) Culture and serological detection of *Xylella fastidiosa*, the xylem-limited bacterium associated with citrus variegated chlorosis disease. Current Microb 27:137-142.

Cox CD and Adams P (1985) Siderophore activity of pyoverdine for *Pseudomonas aeruginosa*. Infect Immun 48:130-138.

Davis MJ, French WJ and Schaad NW (1981) Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Current Microb 6:309-310.

Dunigan DD (1997) Preparations of genomic DNA from bacteria. Current Prot Molec Biol 241-242.

Etchegaray A, Silva-Stenico ME, Moon DH and Tsai SM (2004) *In silico* analysis of the nonribosomal peptide synthetases of *Xanthomonas axonopodis* pv. *citri*: Identification of putative siderophore and lipopeptide biosynthetic genes. Microbiological Research 159:425-437.

Gehring AM, DeMoll G, Fetherston JD, Mori I, Mayhew GF, Blattner FR, Walsh CT and Perry RD (1998a) Iron acquisition in plague: Modular logic in enzymatic biogenesis of yersiniabactin by *Yersinia pestis*. Chemistry Biology 5:573-586.

Gehring AM, Mori I, Perry RD and Walsh CT (1998b) The nonribosomal peptide synthetase HMWP2 forms a thiazoline ring during biogenesis of yersiniabactin, an iron-chelating virulence factor of *Yersinia pestis*. Biochemistry 37:11637-11650.

Kleinkauf H and von Dohren H (1996) A nonribosomal system of peptide biosynthesis. Europ J Biochem 236:334-351.

Neema C, Laulhére J-P and Expert D (1993) Iron deficiency induced by chrysobactin in *Saintpaulia ionantha* leaves inoculated with *Erwinia chrysanthemi*. Plant Physiol 102:967-973.

Schwyn B and Neilands JB (1987) Universal chemical-assay for the detection and determination of siderophores. Anal Biochem 160:47-56.

Silva-Stenico ME, Pacheco FTH, Rodrigues JLM and Tsai SM (2005) Growth and siderophore production of *Xylella fastidiosa* under iron-limited condition. Microb Res 160:429-436.

Simpson AJ, Reinach FC, Arruda P, Abreu FA, Acencio M, Alvarenga R, Alves LM, Araya JE, Baia GS, Baptista CS, Barros MH, Bonaccorsi ED, Bordin S, Bove JM, Briones MR, Bueno MR, Camargo AA, Camargo LE, Carraro DM, Carrer H, Colauto NB, Colombo C, Costa FF, Costa MC, Costa-Neto CM, Coutinho LL, Cristofani M, Dias-Neto E, Docena C, El-Dorry H, Facincani AP, Ferreira AJ, Ferreira VC, Ferro JA, Fraga JS, Franca SC, Franco MC, Frohme M, Furlan LR, Garnier M, Goldman GH, Goldman MH, Gomes SL, Gruber A, Ho PL, Hoheisel JD, Junqueira ML, Kemper EL, Kitajima JP, Krieger JE, Kuramae EE, Laigret F, Lambais MR, Leite LC, Lemos EG, Lemos MV, Lopes SA, Lopes CR, Machado JA, Machado MA, Madeira AM, Madeira HM, Marino CL, Marques MV, Martins EA, Martins EM, Matsukuma AY, Menck CF, Miracca EC, Miyaki CY, Monteriro-Vitorello CB, Moon DH, Nagai MA, Nascimento AL, Netto LE, Nhani A Jr, Nobrega FG, Nunes LR, Oliveira MA, de Oliveira MC, de Oliveira RC, Palmieri DA, Paris A, Peixoto BR, Pereira GA, Pereira HA Jr, Pesquero JB, Quaggio RB, Roberto PG, Rodrigues V, de M Rosa AJ, de Rosa VE Jr, de Sa RG, Santelli RV, Sawasaki HE, da Silva AC, da Silva AM, da Silva FR, da Silva WA Jr, da Silveira JF, Silvestri ML, Siqueira WJ, de Souza AA, de Souza AP, Terenzi MF, Truffi D, Tsai SM, Tsuhako MH, Vallada H, Van Sluys MA, Verjovski-Almeida S, Vettore AL, Zago MA, Zatz M, Meidanis J, Setubal JC (2000) The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406:151-157.

Wells JM, Raju BC, Hung HH, Weisburg WG, Mandelco-Paul L and Brennere DJ (1987) *Xylella fastidiosa* ge. nov., sp. nov.: Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. Int J Syst Bacteriol 37:136-143.

Associate Editor: Darcy Fontoura de Almeida