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

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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 (*pfeA*) and the hydroxamate-type ferrisiderophore receptor (*fhuA*) 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.

Key words: citrus variegated chlorosis, plant pathogen, iron-transportation, pyoverdine, enterobactin, polyketide synthase, peptide synthetase.

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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 yersianiphore), 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.
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 (periwinlkie 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 FeCl₃·6H₂O in 10 mmol L⁻¹ HCl) added, the mixture being stirred and slowly mixed with 72.9 mg of hexadecyltrimethylammonium 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 Duninang (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 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 MgCl₂, 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 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 MgCl₂, 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 ng 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
Due to the difficulty in establishing nutritional conditions that these grape isolates grew faster than the citrus isolates. Halos as compared to other strains, possibly due to the fact with isolates from grapes presented large intensely-colored 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 chrysobatin 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.

<table>
<thead>
<tr>
<th>Primers</th>
<th>5'-3' Oligonucleotide sequence</th>
<th>GenBank accession numbers</th>
<th>Function (gene or abbreviation)</th>
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<tr>
<td>599F</td>
<td>GTC TCA AGA AGG CCT GCA AC</td>
<td>XF0599</td>
<td>Ferri-pyoverdine TonB receptor (fpvA)</td>
</tr>
<tr>
<td>599R</td>
<td>GTA GTG AAC CCT GCC GAC AT</td>
<td>XF0599</td>
<td>Ferri-pyoverdine TonB receptor (fpvA)</td>
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<tr>
<td>2134F</td>
<td>GAC ATC AGC CAA CAA GAG CA</td>
<td>XF2134</td>
<td>Ferri-enterobactin receptor (fpvA)</td>
</tr>
<tr>
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<td>Ferri-enterobactin receptor (fpvA)</td>
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<tr>
<td>2135F</td>
<td>CAA GAA GTC CTT TTG GAG CG</td>
<td>XF2135</td>
<td>Polyketide synthase (PKS)</td>
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<tr>
<td>2135R</td>
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<td>XF2135</td>
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</tr>
<tr>
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<td>ACA ATC GTA CAG CAG CAA CG</td>
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<td>Non-ribosomal peptide synthetase (NRPS)</td>
</tr>
<tr>
<td>2276R</td>
<td>ACC TGC TCG GTA TAC AAC GG</td>
<td>XF2276</td>
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</table>
Nonribosomal peptide synthetases (NRPSs) have also been 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 for the additional strains isolated from citrus, grape, coffee, almond, mulberry, 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

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References


<table>
<thead>
<tr>
<th>Orchards</th>
<th>Number of siderophore producers</th>
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<th>Pyoverdine</th>
<th>NRPS</th>
<th>PKS</th>
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</thead>
<tbody>
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<td>Negative</td>
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<td>Negative</td>
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<td>15</td>
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</table>

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)).

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