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Absence of Classical Heat Shock Response in the Citrus Pathogen *Xylella fastidiosa*

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Abstract. The fastidious bacterium *Xylella fastidiosa* is associated with important crop diseases worldwide. We have recently shown that *X. fastidiosa* is a peculiar organism having unusually low values of gene codon bias throughout its genome and, unexpectedly, in the group of the most abundant proteins. Here, we hypothesized that the lack of codon usage optimization in *X. fastidiosa* would incapacitate this organism to undergo quick and massive changes in protein expression as occurs in a classical stress response. Proteomic analysis of the response to heat stress in *X. fastidiosa* revealed that no changes in protein expression can be detected. Moreover, stress-inducible proteins identified in the closely related citrus pathogen *Xanthomonas axonopodis* pv *citri* were found to be constitutively expressed in *X. fastidiosa*. These proteins have extremely high codon bias values in the *X. citri* and other well-studied organisms, but low values in *X. fastidiosa*. Because biased codon usage is well known to correlate to the rate of protein synthesis, we speculate that the peculiar codon bias distribution in *X. fastidiosa* is related to the absence of a classical stress response, and, probably, alternative strategies for survival of *X. fastidiosa* under stressfull conditions.

The citrus industry is one of the most important economical activities in Brazil. However, plant diseases such as citrus variegated chlorosis (CVC) [1–3] and citrus canker (CC) [2] cause severe economic losses and are considered the important limiting factors for citrus production. The Gram-negative, plant-pathogenic bacteria *Xylella fastidiosa* and *Xanthomonas axonopodis* pv. citri are the causal agents of CVC and CC, respectively. *X. fastidiosa* infects the plant xylem and can be vectored in the foregut of sharpshooter leafhoppers, which feed on the sap of the plant xylem. Basically, the insect vector delivers the bacteria directly into the xylem system of host plants, where they multiply in a biofilm obstructing the vessels, causing symptoms of chlorosis in the leaves and obstructing the sap flow [4–6]. Fastidiousness is a marked characteristic of *X. fastidiosa*, which shows a doubling time of over 14 hours when cultured in liquid media. In contrast, *X. citri* is a fast growing bacteria having a doubling time of 3 hours in liquid media. It does not need a specific vector since it can be spread to other plants by agents like rain, wind, and infested tools. The most common symptoms of *X. citri* infection are canker on fruits, leaves, and twigs. These structures may suffer abscission and can be the cause of plant death [2, 7].

We have recently shown that *X. fastidiosa* is a peculiar organism having unusual low values of gene codon bias throughout its genome and, most unexpectedly, in the group of most abundant proteins, including ribosomal and housekeeping proteins [8]. Speculation then arises as to the ability of *X. fastidiosa* to rapidly and massively synthesize proteins, as occurs in a classical stress response [9, 10]. In bacteria, Heat Shock Proteins (HSPs) such as DNAK, HspA, and the system GroeL/

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GroeS play important roles in the response to several stresses, mainly functioning as chaperones to correct protein folding [9]. It is well known that genes encoding HSPs have high CBI (Codon Bias Index) values, which is probably important for these proteins to be abundantly synthesized during a stress response [11, 12]. In a classical heat shock response, the abundance of several HSPs is dramatically affected, and such changes in abundance are easily detected using standard techniques of protein analysis, such as Two-Dimensional Electrophoresis (2D) [13].

To determine whether *X. fastidiosa* is able to respond to stress conditions through a quick induction in the expression of HSPs, we subjected *X. fastidiosa* to heat stress (42°C) and monitored protein expression using 2D electrophoresis. *X. citri*, which also infects citrus plants, was used as a control of a classical fast growing bacterium possessing the major codon bias distribution and classical stress response.

Materials and Methods

Bacterial Strains and Culture Conditions

Xylella fastidiosa. The pathogenic strain, 9a5c, of *X. fastidiosa*, originally isolated from Brazilian sweet orange, was used in the current study. The complete genome sequence of this isolate is available at the *Xylella* Genome Project Web site (http://aeg.lbi.ic.unicamp.br/xf) [4]. Bacteria were cultured on liquid PW medium (peptone 0.5%, Hemin chloride 0.1%, Phenol red 0.2%, K_2HPO_4 0.12%, KH_2PO_4 0.1%, $MgSO_4$.7H2O 0.05%) at 28°C for 4 days. Cultures of *X. fastidiosa* were centrifuged at 2000*g* for 5 minutes and cells were collected and immediately stored in liquid nitrogen for later processing. Heat-shocked cells had the same treatment.

Xanthomonas axonopodis pv. Citri. The pathogenic strain Xac306 of *X. axonopodis* pv. citri, originally isolated from citrus, was used in the current study. The complete genome data are available at the ONSA Fapesp Brazil *Xanthomonas* Genome Project Web site (http://cancer.lbi.ic.unicamp.br/xanthomonas) [7]. Bacteria were cultured on liquid NB (Nutrient Broth) medium (peptone 0.5%, meat extract 0.3%, NaCl 0.1%) at 28°C for 15 hours. Cultures of *X. axonopodis pv. citri* were centrifuged at 5000 rpm for 5 minutes and cells were collected and immediately stored in liquid nitrogen for later processing. Heat-shocked cells had the same treatment.

Stress conditions and sample preparation. Both bacteria, grown at log phase containing almost 10^9 cells/mL as determined using the dilution plating method, were exposed to 42°C for 15, 30, 45, 180, and 360 minutes in a constant temperature shaker Innova 4000 (New Brunswick Scientific). Both bacteria were exposed to 28°C during the same time used for the heat-shocked cells.

For the preparation of whole cell heat-shocked and control protein extracts, approximately 100 mg (wet weight) of bacteria were washed three times in 1 mL of washing buffer containing 10 mM Tris (pH 8.8), 3 mM KCl, 50 mM NaCl, 5 mM EDTA, and 1 mM PMSF and centrifuged for 2 minutes at 1600*g*. The pelleted cells were then lysed with 200 μL of the following solution: 10 mM Tris (pH 8.8), 0.5 % (w/v) SDS, 5 mM EDTA, 1 mM PMSF. After adding 100 mM DTT, the sample was stored at −70°C. Bacterial concentrations were determined using the dilution plating method to verify and quantify the viability of cells after each step of stress.

Two-dimensional gel electrophoresis and peptide mass fingerprinting. Protocols used are described in Smolka et al. [8].

Calculation of Codon Bias. The codon bias value of the gene sequences was based on the Codon Bias Index (CBI), as defined by Bennetzen and Hall [14]. It was calculated using the CodonW program, accessible at URL: www.molbiol.ox.ac.uk/cu. Based on all the gene sequences present in the *X. fastidiosa* and *X. citri* genome, the program computes the relative frequency of each codon and attributes a value for each gene that varies between −1 and +1. The more the most used codons are present in a given gene sequence, the greater is the codon bias value.

Results and Discussion

We hypothesized that the random codon distribution in *X. fastidiosa* could compromise rapid protein synthesis, as required in a classical stress response, leading to slow or no response under stress situations. So, we subjected both bacteria to heat shock conditions and monitored protein expression.

As shown in Figure 1A and B, 45 minutes after exposure of *X. citri* to heat stress, there is an increase in the expression of the heat shock proteins HspA and GroeS, in addition to a previously uncharacterized protein. These proteins were identified by MALDI-TOF mass spectrometry and were found to have high CBI values (see Fig. 3). HspA and GroeS are well known to participate in bacterial stress responses [15, 16].

Conversely, when *X. fastidiosa* was exposed to heat shock, no changes in protein expression were observed (Fig. 1C and D), even after 6 hours of elevated temperature. To discard the possibility that the heat shock condition was not sufficient to evoke a classical stress response in *X. fastidiosa*, we exposed the bacteria to higher temperatures (45°C and 47°C) and longer periods of time (until 12 hours). Total protein profiles observed in 2D gels did not exhibit any significant difference when compared to 2D profiles from bacteria that were not exposed to the treatment (data not shown). The heat stress conditions used were shown to be lethal for *X. fastidiosa* since no colonies were recovered when aliquots of the culture were plated on solid medium (data not shown). Comparison of the total protein profiles of *X. fastidiosa* and *X. citri* by SDS-PAGE confirmed the data visualized on 2D maps (Fig. 2A and B). While the appearance of new bands was clear in *X. citri* starting 45 minutes after heat stress, no obvious changes in the *X. fastidiosa* SDS-PAGE profile could be visualized.

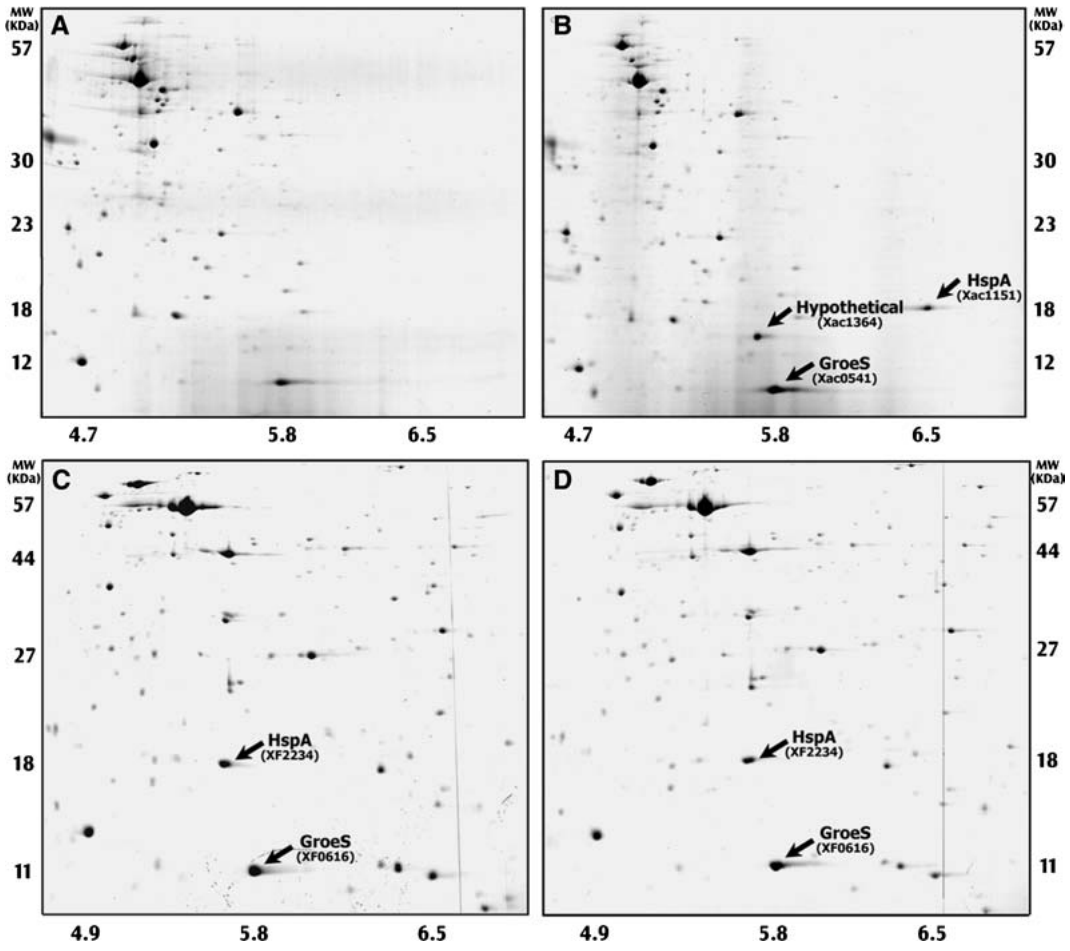

Fig. 1. Effect of heat-shock on the 2D pattern of *X. citri* (A: Control, 28°C; B: Heat Shocked, 42°C) and *X. fastidiosa* (C: Control, 28°C; D: Heat Shocked, 42°C). Heat induced proteins in *X. citri* are marked. Homologs of HspA and GroeS in *X. fastidiosa* are also indicated in C and D. In *X. fastidiosa*, these genes are constitutively expressed and not induced after heat shock. The gels are Comassie blue stained and the experiments were carried out in triplicates and results were found to be consistent. Representative gels are shown.

Figure 3 shows the CBI values of the heat-inducible proteins in *X. citri* and of their homologues in *X. fastidiosa*. It is clear that the CBI values of these proteins in *X. fastidiosa* are not as high as their homologues in *X. citri*. Stress-inducible HSPs, such as HspA and GroeS, generally exhibit high CBI values and may be rapidly expressed under stress conditions [11, 12]. Interestingly, as shown by 2D gel analysis in Figure 1C, *X. fastidiosa* constitutively expresses, at relatively high levels, proteins that in *X. citri* are mainly stress inducible, such as HspA and GroeS. Based on a viability assay, although there was a reduction in the viable population of *X. citri* over the first 15 minutes, it rapidly recovered, and 45 minutes after the treatment was initiated, *X. citri* exhibited population levels similar to those of untreated cultures (Fig. 4). In the case of *X. fastidiosa*, the number of viable cells under stress conditions never reached the same number as the untreated sample, and total death of the bacteria population occurred after 180 minutes at 42°C (Fig. 4).

Together, our data show that *X. citri* undergoes some changes in protein expression in response to heat stress, as expected in a classical stress response. Contrastingly, no change in protein expression could be detected for *X. fastidiosa* using our techniques. The results support the hypothesis that *X. fastidiosa* is unable to respond to stress conditions through the fast regulation of gene expression, as occurs in classical stress responses.

We propose that this phenomenon is a consequence of the absence of a preferred codon usage in its genome. Constitutive expression of stress-related proteins may partially alleviate *X. fastidiosa* to cope with sudden environmental changes/stresses. Understanding how

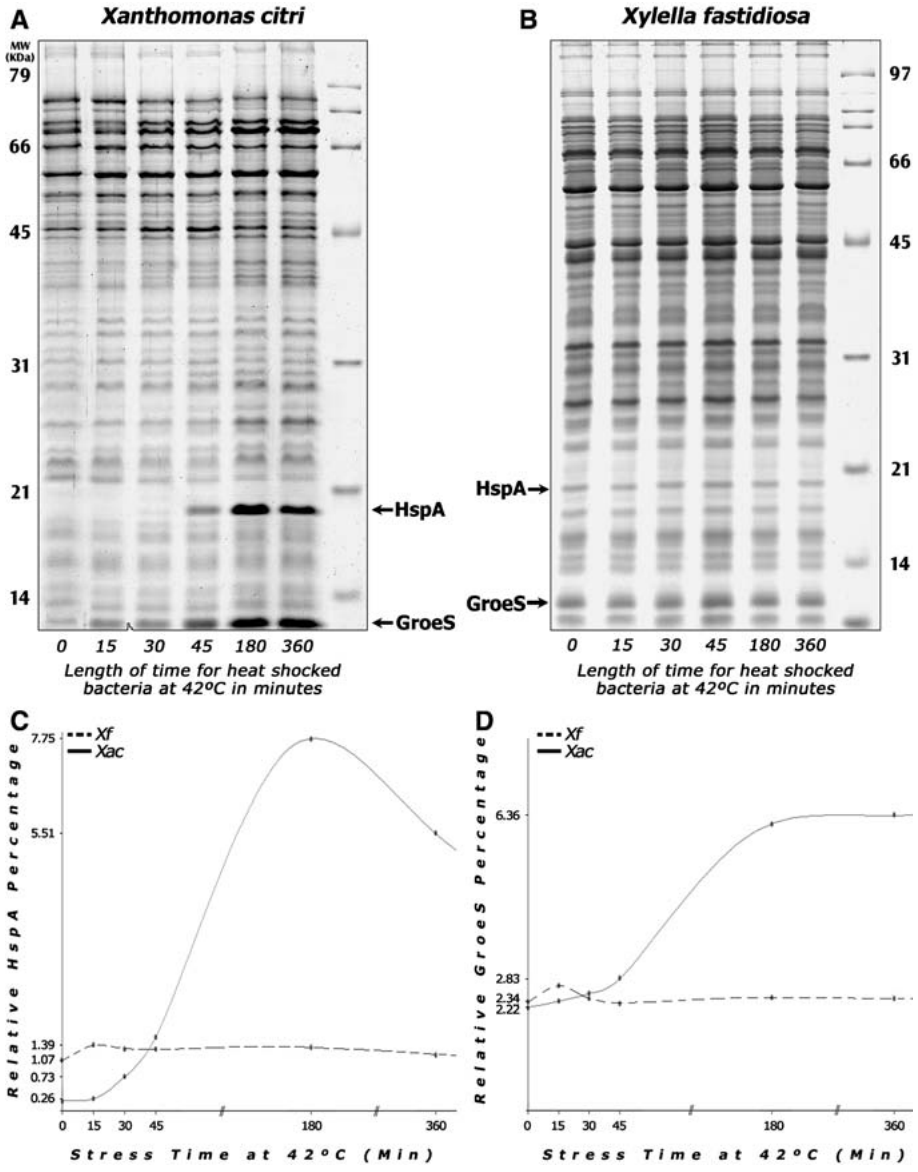

Fig. 2. Kinetics of expression of HspA and GroeS in (A) *X. citri* and (B) *X. fastidiosa* after heat stress (42°C). Densitometric analysis of the bands corresponding to (C) HspA and (D) GroeS in *X. fastidiosa* (dashed line) and *X. citri* (solid line). The percentage is relative protein amount compared to total protein in cell extract. The gels are Comassie blue stained.

X. fastidiosa adapts and survives in hostile environments, such as the plant xylem, is central to the study of its pathogenesis and the development of novel approaches for disease control.

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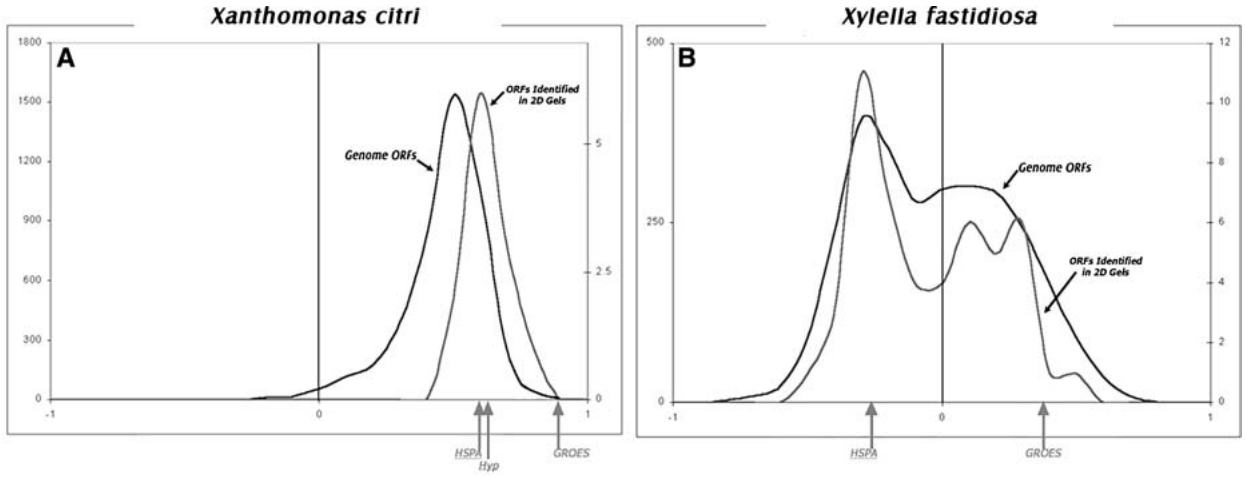

Fig. 3. CBI value distribution of all genome ORFs in *X. citri* and *X. fastidiosa*. The gray line shows the CBI value distribution of the gene products identified in 2D gels (''Identified ORFs''), representatives of highly expressed genes. In *X. citri*, highly expressed genes have higher CBI values, a tendency that is not observed in *X. fastidiosa*. All identified heat-induced proteins in *X. citri* (HspA, GroeS, and Hypothetical) also have relatively high CBI values. In *X. fastidiosa*, HspA has a relatively low CBI.

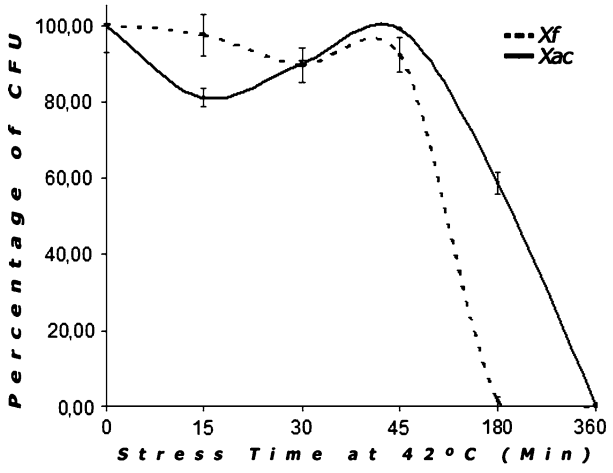

Fig. 4. Survival curves for *X. citri* (solid line) and *X. fastidiosa* (dashed line) under heat stress. Bacterial survival was measured by percentage of Colonies Formation Units (CFU) after indicated times at 42°C. *X. citri* shows that there was an initial reduction in the population, but recovered rapidly when maintaining heat stress for 45 minutes. A great reduction of cell viability was observed after 360 minutes, although viable cells were still present. *X. fastidiosa* shows a gradual reduction in the number of cells. All the cells died before 180 minutes at 42°C. The experiments were made in triplicate.

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