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Research Article

In silico identification of potential chaperone genes that belong to type III and type IV secretion systems in *Xanthomonas axonopodis* pv *citri*

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Abstract

The secretion of bacterial virulence factors and flagellar components requires the assistance of specific type III and flagellar chaperones. Standard computational annotation of the genome of *Xanthomonas axonopodis* pv *citri*, a plant pathogen that causes citrus canker, initially did not identify any genes belonging to these chaperone categories since the primary sequence homology between them was very low. However, in a search for hypothetical proteins with characteristics similar to these chaperones, we have now identified 30 chromosomal and 10 plasmidial potential genes encoding chaperones belonging to types III/IV, and flagellar secretion systems in this organism. The significance of these findings is discussed.

Key words: chaperone, protein secretion, type III and type IV secretion systems, *Xanthomonas.*

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Introduction

Bacterial pathogens use the type III and type IV secretion systems to transfer virulence proteins to animal or plant cells (Wattiau *et al.*, 1996; Winans *et al.*, 1996; Hueck, 1998). Hence, these secretion systems are critical for bacterial pathogenicity and the initiation of disease (Gálan and Collmer, 1999; Cornelis and Van Gijsegem, 2000). The type III and type IV secretion systems require cell-to-cell contact and molecular chaperones for secretion (Deng *et al.*, 1999), and are involved in the assembly of extracelullar filaments or pili (Christie, 1997). The flagellum-specific secretion pathway is considered to be the archetype of the type III secretion system in bacteria that interact with plants and animals, and is thus part of the type III superfamily. Thus, the proteins that form the type III apparatus are homologous to proteins that are essential for the assembly of surface flagella required for bacterial motility (Aizawa, 1996; Stephens and Shapiro, 1996; Hueck, 1998; Minamino and MacNab, 1999; MacNab, 2003). The type IV system resembles bacterial conjugation systems and is involved in host cell contact-mediated protein translocation (Covacci *et al.*, 1999).

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Although the details of the signaling pathway involved in delivery to the target are still uncertain (see, for instance, Anderson and Schneewind, 1997; Aldridge and Hughes, 2001; Lloyd *et al.*, 2001), it is generally accepted that chaperone binding prevents non-productive pre-secretory associations with its substrates (Hueck, 1998; Wattiau and Cornelis, 1993). The main role suggested for type III chaperones is to maintain the bound proteins in a partially folded conformation, or secretion-competent state, that is able to engage the secretory machinery and travel through the secretory pathway (Woestyn *et al.*, 1996; Bennett and Hughes, 2000; Stebbins and Gálan, 2001).

In general, chaperones of the effector class are responsible for only one secreted protein, the effector, whereas chaperones of the translocator class bind two related proteins (Cornelis and Van Gijsegem, 2000). To date, three classes of type III chaperones have been distinguished based on their target category (Page and Parsot, 2002). The SycE-like family (alpha-beta class) associates with only one effector protein, the SycD/LcrH family associates with

two translocator proteins, and members of the Spa 15 associates with several effector proteins (Page *et al.*, 2002; Pallen *et al.*, 2003 and references therein). The SycD/LcrH family is distinguished by a tetratricopeptide repeat (TPR) motif (Pallen *et al.*, 2003), which is an imperfect 34 amino acid repeat often arranged in tandem arrays (Goebl and Yanagida, 1991).

The importance of secretion chaperones to bacteria is enormous since intact chaperones are required for substrate secretion (Wattiau *et al.*, 1994; Iriarte and Cornelis, 1998) and may act as regulators of virulence gene expression (Lloyd *et al.*, 2001). Thus, to understand the secretion systems of bacteria, the chaperones involved in each pathway need to be identified. *Xanthomonas axonopodis* pv *citri* is the causal agent of citrus canker, a serious but still poorly understood disease that affects most commercial citrus cultivars in more than 30 countries (for a recent review of *Xanthomonas,* see Brunings and Gabriel, 2003). This bacterium has the type III cluster present in its chromosome, and two type IV secretion systems, one present in the chromosome and the other in the plasmid pXAC64 (da Silva *et al.*, 2002). Bonas and coworkers (Fenselau *et al.*, 1992; Buttner and Bonas, 2002 and references therein) showed that certain *Xanthomonas* species use their type III system to inject virulence effector proteins into eukaryotic cells. The genome of *Xanthomonas axonopodis* pv *citri* has been fully sequenced (da Silva *et al.*, 2002) and its initial annotation, based on DNA sequence homology, showed no type III chaperone genes. We have reanalyzed the genome of this bacterium using bioinformatic tools to search for hypothetical proteins with predominant type III and flagellar chaperone characteristics (see Bennett and Hughes, 2000 and others for discussion of these characteristics). The results of this analysis indicate the presence of secretion system chaperone genes in the genome of *Xanthomonas axonopodis* pv *citri.*

Material and Methods

Prevalent structural characteristics of type III chaperones used to analyse the genome of *Xanthomonas axonopodis* pv *citri*

The genes encoding particular type III chaperones are often located adjacent to the gene encoding their particular effector in the genome. Thus, the co-expression of these genes increases the performance of the system. Although primary sequence identity is low or not evident among type III and flagellar chaperones, these proteins share several features that can be used to classify them in their respective classes. Such features include a low molecular mass (< 20 kDa), a generally low isoelectric point, and a secondary structure predicted to be predominantly helical with a putative amphipathic α-helix located at the N- or C-terminus (for a discussion of these features see Wattiau *et al.*, 1996; Gygi *et al.*, 1997; Fraser *et al.*, 1999; Bennett and Hughes, 2000). The presence of such features in a hypothetical gene sequence provides the first indication of the function of the protein until more data become available.

Homology search strategy

To find potential type III and flagellar chaperone genes in *Xanthomonas axonopodis* pv *citri,* we used two independent strategies to search the genome of this pathogen (Figure 1). In the first round, a database containing sequences belonging to type III and flagellar chaperones from several species of bacteria was generated using the Entrez databank (http://www.ncbi.nlm.nih.gov/Entrez/). These sequences were aligned using the DIALIGN 2 (Morgenstern, 1999) and CLUSTAL W (Thompson *et al.*, 1994) programs. This alignment allowed redundant sequences in the type III chaperones database to be discarded and decreased their number. BlastP software (Altschul *et al.*, 1990) was then used to compare the generated database with the *Xanthomonas axonopodis* pv *citri* genome in order to find potential type III and flagellar chaperone genes. The default parameters used in this analysis included BLOSUM62 matrix, an e-value for inclusion in subsequent iterations of 0.005, a word size of 3, gap costs of existence 10 and extension 1, and no filter. The ANTHEPROT (ANalyse THE PROTeins; Deleage *et al.*, 1988, 2001) and Compute pI/Mw (Bjellqvist *et al.*, 1993, 1994) software packages,

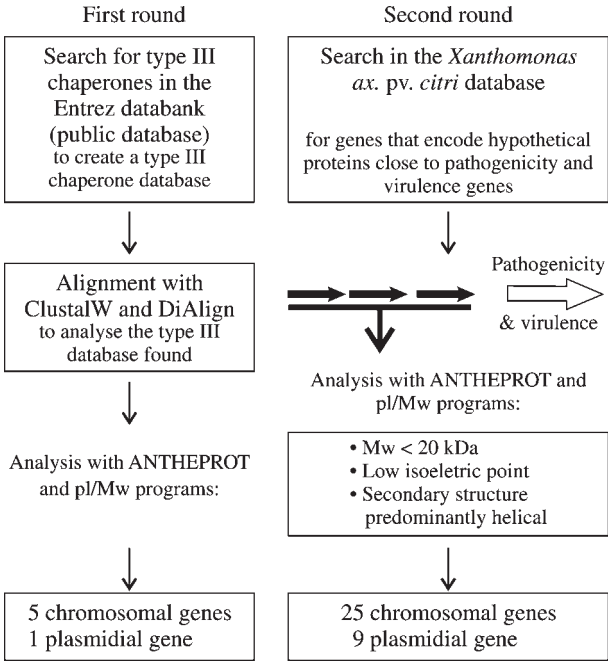

Figure 1 - Schematic representation of the two strategies used to search the *Xanthomonas axonopodis* pv *citri* genome for potential type III chaperone genes. The figure schematically represents the two strategies used to search the *Xanthomonas axonopodis* pv *citri* genome (da Silva *et al.*, 2002) for potential type III chaperone genes. See Material and Methods for details and software references.

which perform large scale protein sequence analyses to predict protein secondary structure and physico-chemical parameters, were used to determine whether the potential *Xanthomonas axonopodis* pv *citri* type III and flagellar chaperones genes found had the global type III chaperone characteristics described above. In the second round of analysis, the genes in the *Xanthomonas axonopodis* pv *citri* genome (da Silva *et al.*, 2002) located within 1.5 kb of pathogenic and virulence clusters, were analysed individually with the ANTHEPROT and Compute pI/Mw packages to identify potential physico-chemical characteristics similar to type III chaperones.

PSI-BLAST search for conserved domains in secretion chaperones.

A PSI-BLAST search (Altschul *et al.*, 1997) was performed on the NCBI site (http://www.ncbi.nlm.nih.gov/BLAST/) using the established default parameters (see above) in order to verify whether the genes selected have characteristic secretion chaperone domains, such as tetratricopeptide (TPR)-like repeats (Pallen *et al.*, 2003), Spa15 family characteristic residues (Page *et al.*, 2002), or similarity to the SycE family (Woestyn *et al.*, 1996).

Results

The analysis for redundancy in the type III chaperone database generated from the Entrez databank reduced the number of sequences belonging to type III and flagellar system chaperones to 120. The *Xanthomonas axonopodis* pv *citri* genome was searched for genes homologous to each of these 120 sequences. The other hypothetical genes close to pathogenic and virulence clusters in the *Xanthomonas axonopodis* pv *citri* genome were analyzed individually for potential physico-chemical characteristics similar to those of type III chaperones using the programs described in the Material and Methods section. The sequences selected by these two approaches were then analyzed for their cellular location in order to eliminate sequences not predicted to be in the periplasm. Five of the putative secretion chaperone genes found in the first round were located on the chromosome and one in the plasmid pXAC33, and had type III and flagellar chaperone characteristics, as described in Material and Methods. This approach allowed the identification of proteins with ≥ 45% similarity to type III and flagellar chaperones of other bacteria (Table 1). The second approach selected 25 chromosomal genes and 9 plasmidial genes with secretion chaperones characteristics (Table 2). A total of 40 potential type III/IV or flagellar chaperone genes were identified in the *Xanthomonas axonopodis* pv *citri* genome, with 30 located on the chromosome and 10 located in the two plasmids, pXAC33 and pXAC64 (da Silva *et al.*, 2002).

As an example of this approach, the analysis for two potential type III chaperones in *Xanthomonas axonopodis* pv *citri* is shown in Figure 2. This figure illustrates the amphipathic α-helix wheel predicted from the primary amino acid sequences of the potential chaperones NP_640774 (Figure 2a) and NP_640641 (Figure 2b). The amphipathic α-helix has predominantly nonpolar amino acid residues along one side and polar residues along the other. The presence of an amphipathic α-helix is one of the characteristics of secretion chaperones.

Discussion

Secretory systems are of fundamental importance for several species of bacteria that use the systems to assemble the flagellum and to secrete important, mainly pathogenic factors, into the environment (Hueck, 1998; Gálan and Collmer, 1999; Cornelis and Van Gijsegem, 2000; MacNab, 2003). The acquisition of a type III secretory system allows many gram-negative bacteria to become pathogens since mutants with a disabled type III system are nonpathogenic (Hueck, 1998; Neyt and Cornelis, 1999).

Table 1 - Potential type III chaperone genes identified by sequence homology and predicted protein physico-chemical characteristics. The *Xanthomonas axonopodis* pv *citri* genome was searched for homology to type III chaperones. Five of the identified genes are localized in the chromosome and one in the plasmid pXAC33 (da Silva *et al.*, 2002). All 6 were confirmed for type III chaperone characteristics as described in Material and Methods. This method allowed the identification of genes with 45% (or higher) homology to type III and flagellar chaperones.

Putative secretion chaperone						
Xanthomonas axonopodis pv *citri* Gene ID	Genome localization	Predicted physico-chemical parameters			Protein homologue / gene number	S (%)
		MW (kDa)	pI	Residues involved in the Amphipathic domain		
NP_642798.1	C	17.8	5.8	98-111	FliS / gi2276420	49
NP_641681.1	C	10.9	5.6	88-100	FliJ / gi1518878	51
NP_642046.1	C	12.7	6.8	100-117	YscB / gi2635354	63
NP_643331.1	C	20.2	4.5	158-175	FliJ / gi1518878	45
NP_641682.1	C	17.1	5.5	92-102	FliJ / gi1518878	48
NP_644711.1	P	21.3	6.0	152-162	Spa15/gi13449095	56

C, chromosomal gene; P, plasmidial gene; S, similarity.

Table 2 - Potential type III chaperone genes determined by analysis of sequences localized near pathogenic and virulence clusters. Hypothetical genes in the *Xanthomonas axonopodis* pv *citri* genome (da Silva *et al.*, 2002) localized near (< 1.5 kb) pathogenic and virulence clusters, were individually analyzed with the programs described in the Material and Methods section for potential physico-chemical characteristics similar to type III chaperones. This method allowed the identification of 34 potential secretion system chaperone genes, 25 localized at the chromosome and 9 localized at the two plasmids, pXAC33 and pXAC64.

Putative secretion chaperone				
Xanthomonas axonopodis pv *citri* Gene ID	Genome localization	Predicted physico-chemical parameters		
		MW (kDa)	pI	Residues involved in the Amphipathic domain
Near avirulence genes (avr)				
NP_640641.1	C	18.6	7.0	69-97
NP_643529.1	C	14.7	9.1	69-84
NP_641699.1	C	16.3	5.5	72-83
NP_643565.1	C	12.9	6.9	54-66
NP_641577.1	C	18.1	6.9	61-76
Near hypersensitivity response and pathogenicity genes (Type III cluster)				
NP_641599.1	C	14.3	8.5	54-79
NP_640774.1	C	10.7	7.0	67-91
NP_642316.1	C	12.1	6.7	22-38
Near host cell wall degradation genes				
NP_640498.1	C	11.3	10.3	1-18
NP_640495.1	C	17.1	6.2	16-30
NP_640494.1	C	18.6	7.7	147-165
NP_640523.1	C	14.0	11.3	36-52
NP_643871.1	C	16.6	10.8	80-96
NP_643873.1	C	12.3	7.7	100-114
NP_640383.1	C	15.7	5.62	21-36
NP_643815.1	C	13.3	11.3	1-14
NP_642683.1	C	10.5	4.5	61-77
In the type IV secretion system cluster				
NP_641701.1	C	22.6	6.2	148-165
NP_642723.1	C	17.8	5.2	42-58
NP_642935.1	C	14.7	6.0	55-71
NP_640924.1	C	15.4	4.7	42-60
NP_642924.1	C	17.1	8.1	71-84
NP_642919.1	C	17.2	8.4	53-65
NP_644761.1	P	8.4	5.6	01-13
NP_644763.1	P	15.8	7.9	110-122
NP_644770.1	P	14.2	9.8	123-129
NP_644771.1	P	9.4	9.1	28-41
NP_644776.1	P	13.9	8.6	87-97
NP_644777.1	P	13.6	8.5	71-82
Nearby virB6 protein				
NP_642918.1	C	16.6	5.5	126-142
NP_642914.1	C	16.4	10.2	95-108
Near pathogenicity, virulence and adaptation genes				
NP_644726.1	P	16.1	10.9	96-110
NP_644791.1	P	17.2	12.1	5-21
NP_644792.1	P	16.2	11.7	110-126

C. chromosomal gene; P. plasmidial gene.

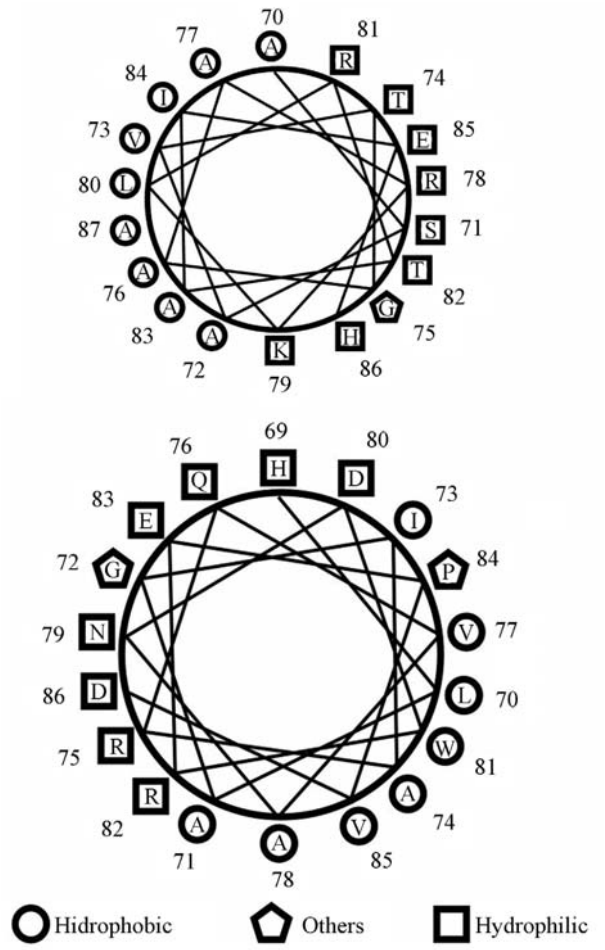

Figure 2 - Predicted amphiphatic α-helical wheel of potential secretion chaperone. The genes NP_640774 (a) and NP_640641 (b) were selected as potential secretion chaperones because they were located near pathogenic and virulence clusters and because they had predicted protein physico-chemical characteristics similar to those of type III chaperones (see Table 2). Analysis with the ANTHEPROT software package (ANalyse THE PROTeins; Deleage *et al.*, 1988; Deleage *et al.*, 2001) allowed the prediction of a potential amphipathic α-helical wheel at the protein C-terminus. Amphipatic α-helices have predominantly nonpolar amino acid residues along one side and polar residues along the other side.

Type III secretory systems have been found in animal and plant pathogens, and show varying degrees of homology, from highly conserved (some proteins of the secretory apparatus) to quite divergent (chaperones and translocators) or very diverse (effectors) (Bennett and Hughes, 2000; Subtil *et al.*, 2000). The secreted proteins must be targeted to the secretory apparatus in order to be secreted. One of the most important mechanisms for labeling and delivering the proteins to be secreted involves chaperones that recognize the target proteins and direct them to the secretory apparatus (Wattiau and Cornelis, 1993; Hueck, 1998; Bennett and Hughes, 2000; Stebbeins and Gálan, 2001). Curiously, no type III chaperone genes were annotated in the fully sequenced genome of *Xanthomonas axonopodis* pv *citri* (da Silva *et al.*, 2002).

Large-scale DNA sequencing efforts produce a huge amount of data that require detailed analysis to be well understood. The analysis of sequences and their annotation by homology is a suitable method to identify potential proteins in a sequenced genome. Since standard techniques did not annotate type III and type IV chaperones in the *Xanthomonas axonopodis* pv *citri* genome, we undertook a more specific analysis of this genome to search for these chaperones. For this, we used homology to search for specific sequences followed by a search for potential physico-chemical properties of the proteins. Several hypothetical genes in the *Xanthomonas axonopodis* pv *citri* genome were analyzed in order to identify those belonging to the secretion chaperone categories. These approaches have been used to show the presence of proteins in certain organisms (see for instance Lawson, 1999; Subtil *et al.*, 2000; Searls, 2000; and others). The comparison of multiple sequences can reveal gene functions that are not detected by simple sequence homology searches.

Secretion system chaperones vary in their amino acid sequences, but share a few common properties, including genome localization close to the gene encoding the target protein, a low isoelectric point, a low molecular weight, and a putative amphiphathic alpha-helix at the N- or C-terminus (Bennett and Hughes, 2000; Parsot *et al.*, 2003). Our analyses of the *Xanthomonas axonopodis* pv *citri* genome using the tools described here allowed the identification of 40 potential secretion system chaperone genes, 30 which were located on the chromosome and 10 in the two plasmids. The first round of the search for potential type III chaperones identified several hypothetical genes that shared similarity with type III chaperones from other bacteria. Two potential chaperones with similarity to the chaperone families SycE and Spa15 were found. The *Xanthomonas axonopodis* pv *citri* gene NP_642046.1 was similar to the secretion chaperone YscB that belongs to the SycE family and functions as a chaperone for the protein YopN in *Yersinia pestis* (Day and Plano 1998). *Xanthomonas axonopodis* pv *citri* gene NP_644711.1 was similar to the secretion chaperone Spa15 from *Shigella flexneri* and also to InvB from other bacteria that belong to a third class of chaperones in the type III secretion pathway (Page *et al.*, 2002). The potential chaperones identified here did not have the TPR domain characteristic of the SycD family (Pallen *et al.*, 2003). Genes belonging to the flagellum assembly apparatus were also identified in *Xanthomonas axonopodis* pv *citri*. Several of these sequences showed homology with FliJ from *Rhodobacter sphaeroides* and FliS from *Pseudomonas aeruginosa*. FliJ is a general flagellar chaperone related to the export process itself and FliS plays a role in protecting the target proteins before exportation (Minamino *et al.*, 2000).

Our results also indicated the presence of potential chaperone genes that may belong to the type IV secretion system because most of the genes identified in the second

round of searching were located close to the type IV system cluster. However, this characteristic may not be enough to support this conclusion and since some type IV chaperones share most of the type III chaperone characteristics (for instance VirE1; Deng *et al.*, 1999) we can only state that the annotated chaperones are related to type III/IV systems.

Type III secretion system occurs in other *Xanthomonas* species (Fenselau *et al.*, 1992; Buttner and Bonas, 2002) and our results indicated the presence of chaperones belonging to this system, and to the flagellar and type IV systems in *Xanthomonas axonopodis* pv *citri*. Together, the results presented here are important for understanding the pathogenicity of *Xanthomonas axonopodis* pv *citri* and may be useful in studies of the mechanism of disease caused by this pathogen in orange plantations. To date, few reports have described a requirement for chaperones in the type III secretion systems of plant pathogens (see for instance, Gaudriault *et al.*, 1997; van Dijk *et al.*, 2002). In conclusion, the study of *Xanthomonas axonopodis* will benefit from the identification of secretion system chaperone genes since uncovering the precise molecular events controlling the delivery of effector proteins should eventually allow the design of compounds that specifically interfere with these processes, hopefully without deleterious side effects to the host plant (Staskawicz, 2001).

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