ABSTRACT

PAGANI, MARIA CRISTINA. An ABC transporter protein and molecular diagnoses of *Xanthomonas arboricola* pv. *pruni* causing bacterial spot of stone fruits (under the direction of Dr. David F. Ritchie).

The purpose of the research was to develop a detection and identification system for *Xanthomonas arboricola* pv. *pruni* using polymerase chain reaction (PCR) analysis and Southern hybridization with a DNA probe. Random amplified polymorphic DNA (RAPD) analysis was used to identify a specific DNA sequence strictly associated and conserved among all *X. arboricola* pv. *pruni* strains tested and obtained from various locations and hosts. PCR primers Y17CoF and Y17CoR specific to this DNA fragment were synthesized and evaluated as a diagnostic tool. Primers amplified a 943-bp DNA fragment in all strains previously identified as *X. arboricola* pv. *pruni* on the basis of biochemical and physiological tests, and failed to amplify DNA from other xanthomonads and non-xanthomonads including saprophytes and epiphytes associated with *Prunus* spp. The PCR assay detected between 25 and 50 cells. A digoxigenin-labeled DNA probe, XPRUNI14, was developed and used to detect an extensive collection of this bacterium through dot-blot and Southern analysis. Results indicated that *X. arboricola* pv. *pruni* could be accurately detected and identified by PCR analysis and probe hybridizations on symptomatic and asymptomatic plant materials avoiding the need for prior isolation of this phytopathogen. Sequence analysis of this 943-bp DNA fragment revealed the presence of an open reading frame (ORF) predicted to encode a putative protein of 243 amino acids with a calculated molecular mass of 26.8 kDa. *In silico* analysis predicted this protein to share similarity to the ABC transporter family, with a FtsX conserved domain possibly involved in cell division. In the present study we overexpressed the putative protein and results were confirmed with western and immunoblot analysis. Gene disruption with a streptomycin cassette resulted in small

colony phenotype, with no evidence of bacterial mass increase suggestive of impaired division that led them to cell death.

AN ABC TRANSPORTER PROTEIN AND MOLECULAR DIAGNOSES OF *XANTHOMONAS ARBORICOLA* PV*. PRUNI* CAUSING BACTERIAL SPOT OF STONE FRUITS

by

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BIOGRAPHY

Maria Cristina Pagani was born and raised in Montevideo, Uruguay and received a BS in Agronomy from the University of Uruguay. After graduating, she worked for the National Institute of Agricultural Research (INIA) as a researcher in Bacteriology. In 1994 she received her Master's Degree from the Department of Plant Pathology at North Carolina State University working on genetic variability of *Xanthomonas arboricola* pv. *pruni* under the direction of Dr. David F. Ritchie. After completing her Master's Degree in 1994, she returned to Uruguay and continued working at INIA "Las Brujas" Experimental Station on basic and applied research providing strategies to improve integrated pest management practices for farmers growing temperate fruits and vegetables. She participated and served as liaison in several international collaborative projects, particularly with Japan and Spain. In 2001 she began pursuing a doctoral degree in the department of Plant Pathology at North Carolina State University working on the development of a molecular detection system, involving PCR, a DNA-probe and the expression and characterization of a putative protein of *Xanthomonas arboricola* pv. *pruni* under the guidance of the same advisor.

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TABLE OF CONTENTS

CHAPTER 3

In vitro expression of a putative protein and insertional mutagenesis of a predicted ORF in *Xanthomonas arboricola* pv. *pruni* with similarity to the ABC (ATP-binding cassette) system.

LIST OF TABLES

Chapter 1

Chapter 2

LIST OF FIGURES

Chapter 1

Chapter 2

INTRODUCTION

Xanthomonas arboricola pv. *pruni* (Vauterin *et al*., 1995, syn. *X. campestris* pv. *pruni* [Smith]) is a gamma, gram-negative plant pathogenic bacterium that causes bacterial spot disease on *Prunus* spp. such as peach, nectarine, apricot, plum and cherry (Ritchie, 1995). This is a serious economic disease described for the first time by Erwin Smith in 1903 on Japanese plums from Michigan (Smith, 1903). Since then, bacterial spot has been reported to occur in almost all major stone fruit producing areas of the world (Appendix 1). This disease is a serious threat for susceptible cultivars grown in warm, humid areas of the eastern United States and other countries. In the European Union *X. arboricola* pv*. pruni* is subject to phytosanitary legislation through the EEC Directive no.92/103 (S.I. Nº 219, 2003) because of its establishment and recent appearance in several countries of that region (OEPP/EPPO, 2003).

Symptoms occur on leaves, fruits and twigs, ranging from necrotic angular lesions on leaves and sunken lesions on fruits to cankers on twigs. *X. arboricola* pv*. pruni* can be very damaging in years when severe infections occur on fruits of highly susceptible cultivars (Ritchie, 1999). Management of bacterial spot consists of the use of resistant cultivars if available and a limited number of chemicals, primarily copper and oxytetracycline, applied in a protective spray program or when symptoms appear, with often poor results (Ritchie, 1995).

X. arboricola pv*. pruni* can persist and be transmitted in budwood, thus buds used in plant propagation can disperse the pathogen to areas or countries previously free of this disease (Goodman and Hattingh, 1986; Zaccardelli *et al.*, 1998). Traditional diagnosis of bacterial spot is based on techniques that usually lack the sensitivity required to detect latent infections (Lelliott and Stead, 1987; Schaad, 2001). The availability of a highly sensitive, accurate and specific assay for detecting *X. arboricola* pv*. pruni* had been lacking. Nucleic acid hybridization and polymerase chain reaction (PCR) offer a specific and highly sensitive approach for identification of *X. arboricola* pv*. pruni.* Both technologies could provide useful tools to clarify epidemiological aspects of bacterial spot necessary for disease management in

integrated pest management (IPM) systems. They could also be used by regulatory agencies charged with the responsibility of detecting *X. arboricola* pv*. pruni* in interstate or international commerce reducing the risk of disseminating the pathogen into disease free regions. Thus, the first objective of this work was to identify a genetic marker specific to *X. arboricola* pv*. pruni* and to develop and apply a molecular detection system involving PCR primers and a DNA probe.

In recent years, several bacterial genomes have been sequenced and informatic programs have been used to predict structural and functional properties of genes and the proteins they encode (Linton and Higgins, 1998; Marchal *et al*., 2004). Consequently, a vast number of predicted genes are initially known only by their putative amino acid sequence through comparative studies (Meidanis *et al*., 2002; Tekaia *et al*., 1999). Several computer programs perform genome-wide screening using all the completely sequenced genome species available (Altschul *et al*., 1997; Tusnády, 2001). Furthermore some of these programs focus specifically on the analysis of certain protein families (Ren *et al*., 2004). An example of one the most studied protein families, subjected to detailed characterization through bioinformatic tools is the ABC (ATP-binding cassette) transporter family (Holland *et al*., 2003). This is one of the most important protein families found in all phyla and it performs functions affecting processes as diverse as import or export of a wide range of solutes, cell division and regulation of metabolic processes among others (Bisbal *et al*., 2000; de Leeuw *et al*., 1999; Higgins, 1992). The majority of the ABC transporter proteins currently identified have been *in silico* characterized through sequence similarity (Zhao, 2001). Only some of them have been functionally analyzed, mainly in bacteria and mammals, and to some degree in yeast and other fungi (Bauer, 1999; de Leeuw *et al*., 1999; Silva *et al*., 2001; Tomii and Kanehisa, 1998). Even though considerable progress has been made in the past two decades in understanding structure and function of some ABC transporters, especially the ones that are involved in human diseases and multidrug resistance, little is known about the function of many members of this family (Allikmets *et al*., 1997; Klein, 1999).

With the complete sequence of several plant bacterial genomes, including two *Xanthomonas* species, the identification and characterization of numerous ABC transporters represent an essential contribution to the progress in this area (Meidanis *et al*., 2002). Although the genomes of various bacterial plant pathogens are now available, (da Silva *et al*., 2002; Meidanis *et al*., 2002) little is known about the genome of *X. arboricola* pv*. pruni*, and to date, the whole genome of this bacterium is not available. However, we had previously identified a 943-bp DNA fragment, specific to *X. arboricola* pv*. pruni,* which contained a predicted open reading frame (ORF). As an initial step towards the elucidation of its possible function, the second aim of this study was to characterize the predicted *X. arboricola* pv*. pruni* ORF through bioinformatic programs in order to glean initial information about its structure and function. *In silico* analysis of the 943-bp DNA fragment identified an ORF predicted to encode a putative protein with similarity to the ABC (ATP-binding cassette) family, with a conserved domain possibly implicated in bacterial cell division. The conserved domain predicted in the *X. arboricola* pv*. pruni* putative protein was found to share similarity to the FtsX membrane protein in *Escherichia coli.*

One of the essential roles of ABC proteins is their involvement in cell division (de Leeuw *et al*., 1999; Schmidt *et al*., 2004). Cell division and DNA replication are processes central to life. Most rod-shaped bacteria divide symmetrically during normal growth, constricting at the middle by building a central septum across the middle of the cell (Bramhill, 1997). Cell division in *E. coli* involves the coordinated action of many proteins encoded by different clusters of genes (Gill *et al*., 1986). One of these clusters is the *fts*YEX operon, associated with the inner membrane, which encodes three proteins known to be required for cell division, FtsY, FtsE and FtsX. It is known that FtsX and FtsE interact and are co-transcribed, however their function in cell division it is not completely known. They may use the energy of ATP hydrolysis to complete cell separation but also they may act indirectly, translocating proteins involved in potassium transport and probably other proteins involved in cell division into the cytoplasmic membrane (Kempf and McBride, 2000). During the past

10 years, FtsE and/or FtsX mutants have been reported in some organisms exhibiting morphological defects suggestive of impaired division (Kempf and McBride, 2000; Merino *et al*, 2001).

Several experiments demonstrated restoration of viability of *fts*E and *fts*X mutants obtained by addition of high KCl concentrations (Ukai *et al*., 1998), or NaCl in the culture medium (Schmidt *et al*, 2004). The ability of salt to rescue division in FtsEX null mutants could be explained by its effect on folding, assembly and/or function of one or more of the downstream division proteins FtsK through FtsN.

The last goal of the present study was to analyze an ORF in *X. arboricola* pv. *pruni* predicted to encode a putative protein with similarity to some members of the ABC (ATP-binding cassette) family and to a conserved domain of the cell division FtsX protein in *E. coli*. The expression of the *X. arboricola* pv. *pruni* putative protein was achieved and mutations in the corresponding gene were obtained by homologous recombination in order to understand how this previously undescribed ORF in *X. arboricola* pv. *pruni* contributes to the normal function of the cell.

LITERATURE CITED

Allikmets, R., Shroyer, N. F., Singh, N., Seddon, J. M., Lewis, R. A., Bernstein, P. S., *et al*. 1997. Mutation of the Stargardt disease gene (ABCR) in age-related macular degeneration. Science 277: 1805-1807.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zheng Zhang, J. Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acid Res. 25: 3389-3402.

Bauer, B. E., Wolfger, H., and Kuchler, K. 1999. Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. Biochim. Biophys. Acta 1461: 217-236.

Bisbal, C., Silhol, M., Laubenthal, H., Kaluza, T., Carnac, G., Milligan, L., Le Roy, F., and Salehzada, T. 2000. The 2'-5' oligoadenylate/ RNase L/RNase L inhibitor pathway regulates both MyoD mRNA stability and muscle cell differentiation. Mol. Cell. Biol. 20: 4959-4969.

Bramhill, D. 1997. Bacterial cell division. Annu. Rev. Cell Dev. Biol. 13: 395-424.

da Silva, A., Ferro, J., Reinach, F., *et al*. 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. Nature 417: 459-463.

de Leeuw, E., Graham, B., Phillips, G., tenHagen-Jongman, C., Oudega, B., and Luirink, J. 1999. Molecular characterization of *Escherichia coli* FtsE and FtsX. Mol. Microbiol. 31: 1-13.

Gill, D. R., Hatfull, G. F., and Salmond, G. P. 1986. A new cell division operon in *Escherichia coli*. Mol. Gen. Genet. 205: 134-145.

Goodman, C. A., and Hattingh, M. J. 1986. Transmission of *Xanthomonas campestris* pv. *pruni* in plum and apricot nursery trees by budding. HortScience 21: 995-996.

Higgins, C. F. 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. 8: 67-113.

Holland, I. B., Cole, S. P., Kuchler, K., and Higgins, C. F. 2003. ABC proteins. from bacteria to man. Acad. Press.

Kempf, M., and McBride, M. 2000. Transposon insertions in the *Flavobacterium johnsoniae ftsX* gene disrupt gliding motility and cell division. J. Bacteriol. 182: 1671-1679.

Klein, I., Sarkadi, B., and Varadi, A. 1999. An inventory of the human ABC proteins. Biochim. Biophys. Acta 1461: 237-62.

Lelliott, R. A., and Stead, D. E. 1987. Methods for the Diagnosis of Bacterial Diseases of Plants. Blackwell Scientific Publications, Oxford, UK.

Linton, K., and Higgins, C. 1998. The *Escherichia coli* ATP-binding cassette (ABC) proteins. Mol. Microbiol. 28: 1-5.

Marchal, K., De Keersmaecker, S., Monsieurs, P., van Boxel, N., Lemmens, K., Thijs, G., Vanderleyden, J., and De Moor, B. 2004. *In silico* identification and experimental validation of PmrAB targets in *Salmonella typhimurium* by regulatory motif detection. Genome Biol. 5: R9. http://genomebiology.com/2004/5/2/R9.

Meidanis, J., Braga, M., and Verjovski-Almeida, S. 2002. Whole-genome analysis of transporters in the plant pathogen *Xylella fastidiosa*. Microbiol. Mol. Biol. Rev. 66: 272-299.

Merino, S., Altarriba, M., Gavin, R., Izquierdo, L., and Tomas, J.M. 2001. The cell division genes (*ftsE and X*) of *Aeromonas hydrophyla* and their relationship with opsonophagocytosis. FEMS Microbiol. Letters 198: 183-188.

OEPP/EPPO, 2003. Data sheets on quarantine organisms. *Xanthomonas arboricola* pv. *pruni*. www.eppo.org/QUARANTINE/BACTERIA/Xanthomonas_pruni/XANTPR_ds.pdf

Ren, Q., Kang, K. H., and Paulsen, I. T. 2004. TransportDB: a relational database of cellular membrane transport systems. Nucleic Acids Res. 32 Database issue D284-D288.

Ritchie, D.F. 1995. Bacterial Spot. Pages 50-52 in: Compendium of Stone Fruit Diseases, Ogawa, J. M., Zehr, E. I., Bird, G. W., Ritchie, D. F., Uriu, K., and Uyemoto, J. K., eds. APS Press, St. Paul, MN.

Ritchie, D. F. 1999. Sprays for control of bacterial spot of peach cultivars having different levels of disease susceptibility, 1998. Fung. Nematic.Tests 54: 63.

Schaad, N. W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. 3nd. Ed. APS Press, St. Paul, USA.

Schmidt, K., Peterson, N., Kustusch, R., Wissel, M., Graham, B., Phillips, G., and Weiss, D. 2004. A predicted ABC transporter, FtsEX, is needed for cell division in *Escherichia coli*. J. Bacteriol. 186: 785-793.

S.I. Nº 219, 2003. European Communities. Introduction of Organisms Harmful to Plants or Plant Products. Prohibition. Amendment Regulations 2003.

http://www.agriculture.gov.ie/legislation/SI2003/S219-03F.pdf

Silva, P., Bigi, F., Santangelo, M., Romano, M., Martin, C., Cataldi, A., and Ainsa, J. 2001. Characterization of P55, a multidrug efflux pump in *Mycobacterium bovis* and *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 3: 800-804.

Smith, E. 1903. Observations on a hitherto unreported bacterial disease, the cause of which enters the plant through ordinary stomata. Science 17: 456-457.

Tekaia, F., Gordon, S.V., Garnier, T., Brosch, R., Barrell, B.G., and Cole, S.T. 1999. Analysis of the proteome of *Mycobacterium tuberculosis in silico*. Tubercle Lung Dis. 79: 329-342.

Tomii, K., and Kanehisa, M. 1998. A comparative analysis of ABC transporters in complete microbial genomes. Genome Res. 8: 1048-1059.

Tusnády, G. E., and Simon, I. 2001. The HMMTOP transmembrane topology prediction server. Bioinformatics 17: 849-850.

Ukai, H., Matsuzawa, H., Ito, K., Yamada, M., and Nishimura, A. 1998. ftsE (Ts) affects translocation of K-pump proteins into the cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. 180: 3663-3670.

Vauterin, L., Hoste, B., Kersters, K., and Swings, J. 1995. Reclassification of *Xanthomonas*. Int. J. Syst. Bacteriol. 45: 472-489.

Zaccardelli, M., Malaguti, S., and Bazzi, C. 1998. Biological and epidemiological aspects of *Xanthomonas arboricola* pv. *pruni* on peach in Italy. J. Plant Pathol. 80: 125-132.

Zhao, Z. 2001. ABC transporters: one of the largest protein families involved in various functions both in prokaryotes and eukaryotes ww.sfu.ca/”zyzhao/abc/review.html.

Development and Evaluation of a Molecular Detection System Involving PCR Primers and a DNA Probe for Detection of Bacteria Causing Bacterial Spot of Stone Fruits

ABSTRACT

Xanthomonas arboricola pv. *pruni* is the causal agent of bacterial spot on stone fruits. Specific detection and identification of this bacterial pathogen was possible using polymerase chain reaction (PCR) analysis and Southern hybridization with a DNA probe. Random amplified polymorphic DNA (RAPD) analysis was initially used to identify a specific DNA sequence strictly associated with *X. arboricola* pv. *pruni*. Among 61 arbitrarily chosen primers, primer Y17 revealed a unique DNA fragment conserved among 50 *X. arboricola* pv. *pruni* strains obtained from various locations and hosts including peaches, plums, nectarines and apricots. This DNA fragment was cloned, sequenced and specific PCR primers were designed for the detection of the pathogen. Primers Y17CoF and Y17CoR amplified a 943-bp DNA fragment in all strains previously identified as *X. arboricola* pv. *pruni* on the basis of biochemical and physiological tests, and failed to amplify DNA from other xanthomonads and non-xanthomonads including saprophytes and epiphytes associated with *Prunus* spp. The PCR assay detected between 25 and 50 cells of *X. arboricola* pv. *pruni*. A digoxigenin-labeled DNA probe, XPRUNI14, was developed and used to assay a collection of 167 non-*X. arboricola* pv. *pruni* strains and 138 strains of *X. arboricola* pv. *pruni* through dot-blot and Southern analysis. Results indicated that *X. arboricola* pv. *pruni* could be accurately detected and identified by PCR analysis and Southern hybridizations on symptomatic and asymptomatic plant materials avoiding the need for prior isolation of this phytopathogen.

INTRODUCTION

Bacterial spot on stone fruits caused by *Xanthomonas arboricola* pv. *pruni* (Vauterin *et al*., 1995, syn. *X. campestris* pv. *pruni* [Smith]) has become an increasingly important problem in almost all-major stone fruit producing areas of the world. In years when bacterial spot is severe, losses on highly susceptible cultivars can approach 100% of the fruit (Ritchie, 1999). Current controls consist of the use of resistant cultivars if available and the application of a limited number of chemicals which are often ineffective and costly. The absence of resistant cultivars when environmental conditions are highly conducive for the disease, and the desire by consumers for some susceptible cultivars make the management of this disease very difficult once the bacterium is established in an orchard (Ritchie, 1995; Werner and Ritchie, 1986).

X. arboricola pv*. pruni* can persist on stems and buds of peach and plum trees through the entire year (Ritchie, 1995; Shepard and Zehr, 1994). Contaminated buds used in plant propagation can disperse the pathogen to areas or countries free of this disease (Goodman and Hattingh, 1986; Zaccardelli *et al.*, 1998). Therefore, exclusion of the pathogen from a region by use of disease or pathogen-free plant material is important (Ritchie, 1995). In addition, early detection on fruits, buds and leaves before symptoms develop early in the growing season would be advantageous to speed regulatory actions in areas free of the disease and to time the application of bactericides in regions where *X. arboricola* pv*. pruni* is already present.

This implies the need for a rapid, specific and highly sensitive assay to detect *X. arboricola* pv*. pruni* on colonized plant material*.* Traditional diagnosis of bacterial spot is based on attempts to isolate the pathogen on conventional (Schaad, 2001), semi-selective (Dhanvantari *et al*., 1978), or selective media (Civerolo *et al.,* 1982), and following purification, to characterize it by biochemical, physiological and pathogenicity tests (Lelliott and Stead, 1987; Randhawa and Civerolo, 1985; Vauterin *et al*., 1990). This process is laborious and time-consuming. Furthermore,

bacterial populations can be present in numbers well below the detection levels of these techniques. Although amplified fragment length polymorphism (AFLP) fingerprints have been applied to distinguish *X. arboricola* pv*. pruni* strains from those of *X. arboricola* pv*. juglandis* and other *Xanthomonas* spp., its reliability has not been assessed when compared to a wider collection of pathovars and species in addition to saprophytic and epiphytic strains associated with stone fruits (Zaccardelli *et al*. 1999). While a commercial serological test is currently available for *X. arboricola* pv*. pruni* it does not ensure the detection of this pathogen when the population is low.

Here we report the development of a DNA probe and a set of PCR primers that allow the rapid, sensitive and specific detection and identification of *X. arboricola* pv*. pruni* in culture and in colonized plant tissue.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *X. arboricola* pv*. pruni* strains and genomic DNA, together with other non-*X. arboricola* pv*. pruni* strains (names according to Vauterin *et al*., 1995) used in this study, are listed in Tables 1 and 2 respectively. All strains except *Escherichia coli* were routinely cultured on sucrose-peptone-agar medium (SPA) containing 20 g of sucrose, 5 g of peptone, 0.5 g of K_2HPO_4, 0.25 g $MgSO_4$ and 15 g of agar per liter. Strains were grown at 28°C and stored at -80°C in 20% glycerol. *E. coli* was cultured on Luria-Bertani medium (Sambrook *et al*., 1989) at 37°C. In order to isolate naturally occurring saprophytic and epiphytic bacteria associated with *Prunus* plants, leaves, fruits, buds and twigs from peaches and plums were cultured and shaken for 2 h in sterile distilled water, the supernatant centrifuged, and the pellet resuspended in 0.5 ml of sterile water. Samples (50 µl) were spread using an L-shaped glass rod over the surface of SPA agar dishes and incubated for 2 to 3 days at 28°C. Individual colonies were isolated, tested for hypersensitivity reaction in tobacco leaves, labeled and stored at -80°C for further studies.

DNA extraction and sample preparation. RAPD and PCR amplifications were performed using extracted genomic DNA from pure cultures and naturally infected plant tissues, and intact bacterial cells as direct templates, collected from agar media or from infected plant material. Total genomic DNA from pure bacterial cultures was extracted by the method of Ausubel *et al*. 1994. In order to extract total genomic DNA from naturally infected samples, small portions of infected leaves, buds, twigs and fruits were collected in microfuge tubes, immersed in liquid nitrogen to freeze them and ground to a fine powder. A simple procedure for extracting nucleic acids from woody hosts was used based on the cetyltrimethylammonium bromide (CTAB) method (Zhang *et al.* 1998). For direct detection of *X. arboricola* pv*. pruni* from plant parts, small pieces of infected tissues were shaken in distilled water for 2 h, supernatants were centrifuged, pellets resuspended in 100 µl of sterile distilled water, and 5 µl of the resultant suspensions were added to PCR mixtures. For direct identification of *X. arboricola* pv*. pruni* cells from pure cultures, a loopful of

bacteria from single colonies was resuspended in 100 µl of sterile distilled water and 5 µl added to the PCR mixture without further treatment.

RAPD analysis and separation of bands. For RAPD analysis, a set of 61 10-base oligonucleotide arbitrary primers was screened against strains Xap14 (from Georgia), Xap40 (from North Carolina) and Xap70 (from Brazil). This technique used direct assay of whole bacteria cells as described above, collected from 24 to 48 h-old cultures grown on solid media and used as direct templates. Amplifications were carried out in a total volume of 25 µl containing 25-50 ng of genomic DNA, 2.5 mM $MgCl_2$, 100 µg of bovine serum albumin per ml, 0.2 µM primer, 1.2 U of *Taq* DNA polymerase (Boehringer-Mannheim), 0.2 mM (each) dNTP's (Boehringer-Mannheim) in 10 mM Tris-HCl (pH 8.3) and 50 mM KCl. A DNA thermal cycler (Perkin-Elmer Cetus) was used, programmed for 40 cycles of 2 min at 95°C, 1 min at 92 °C, 1 min at 35 °C, 2 min at 72°C and a final extension period of 5 min at 72°C. Aliquots (10 µl) of PCR products were analyzed by electrophoresis in 1.2% agarose gels in 1 x TBE buffer (8.9 mM Tris, 0.25 mM Na_2EDTA, 0.89 mM boric acid [pH8.3]) at 75 V for 2 h, stained with ethidium bromide. Fingerprint profiles generated with all primers were visually assessed on the basis of migration patterns of the amplified products. Primer Y17 (5'-GACGTGGTGA) was selected due to its ability to amplify a distinctive 943-bp DNA fragment specific to *X. arboricola* pv*. pruni*, subsequently confirmed in further studies.

DNA cloning, sequence and primer design. The specific DNA fragment generated by RAPD analysis (band 3 Fig. 1A and 2) was gel extracted with a QIAquick Gel extraction kit (QIAGEN, Valencia, CA) and cloned into a pCR4-TOPO vector using a TOPO TA Cloning kit (Invitrogen, Carlsbac, CA) according to the manufacturer's instructions. The nucleotide sequence of the fragment was determined with a Cycle Sequencing Ready Reaction kit on an ABI PRISM® 377 DNA automatic sequencer (Applied Biosystems, Foster City, CA). Two oligonucleotides, Y17CoF (5'-GACGTGGTGATCAGCGAGTCATTC-3') and Y17CoR (5'-GACGTGGTGATGATGATCTGC-3'), located near the 3' and 5' end of the Y17 fragment were designed and synthesized (Invitrogen, USA) for specific amplification

of *X. arboricola* pv*. pruni* by PCR analysis. The nucleotide sequence generated with primers Y17CoF and Y17CoR is shown in Chapter 2, Fig.1.

PCR assays of microbial populations. Polymerase chain reactions were conducted using total DNA of the bacterial strains listed in Tables 1 and 2. DNA templates (approximately 50 ng) were included in a 25 µl reaction volume containing 0.2 mM (each) dNTP's (Boehringer-Mannheim), 1.2 U of Fast start Taq polymerase (Roche Diagnostics GmbH), 1 µM primers, 2 mM $MgCl_2$, 4% dimethyl sulfoxide, 5% glycerol in 10 mM Tris-HCl (pH 8.3) and 50 mM KCl. Amplifications were performed with a DNA thermal cycler (Perkin-Elmer Cetus) programmed for one cycle of 4 min at 95°C to ensure complete denaturation of the template DNA, and 30 cycles of 1 min at 92°C, 1 min at 53°C, 2 min at 72°C and a final extension period of 5 min at 72°C. After PCR amplification, DNA samples were electrophoresed in 1% agarose gels in 1x TBE buffer (8.9 mM Tris, 0.25 mM Na_2EDTA, 0.89 mM boric acid [pH8.3]) stained with ethidium bromide, and photographed.

DIG-Probe construction and hybridization analysis. A DNA probe, XPRUNI14, was constructed with the 943-bp PCR-amplified fragment specific for *X. arboricola* pv*. pruni*. The fragment was recovered and purified from agarose gel by using the Geneclean kit (Bio 102, Inc.San Diego, Calif.), and randomly labeled with digoxigenin-11-dUTP (DIG-dUTP) according to the manufacturer's instructions (Boehringer-Mannheim). Prehybridization, hybridization and washings for Southern hybridization were performed at 65°C. Resolution was done by colorimetric detection with NBT and BCIP following the manufacturer's recommendations (Boehringer-Mannheim). Amplified RAPD products were transferred from agarose gels to nitrocellulose membranes (Micron Separations Inc., MA) by Southern blot as previously described (Sambrook *et al*., 1989). Dot blots were prepared with plant lesion extracts and with dilutions of bacterial cultures adjusted to an optical density of 0.3 absorbance at 600 nm approximately 4 $x10^8$ CFU/ml. A sample of 1µl of each dilution was spotted on nitrocellulose membranes and allowed to air-dry. Cell dots were lysed by placing the membranes on 1.5 M NaCl and 0.5 M NaOH for 15 min and neutralized for 15 min in 1.0 M Tris-HCl and 1.5 M NaCl. After neutralization,

membranes were air-dried and UV-cross-linked (Cross-linker model XL-1500 UV, Spectronics Corp.).

Restriction fragment length polymorphism (RFLP) analysis. DNA fragments were generated by restriction enzyme digestion with *EcoR*I, according to the conditions defined by the manufacturer, using 5 U of enzyme per µg of DNA. *EcoR*I digested genomic DNA (2.5 µg per lane) from *X. arboricola* pv*. pruni* and non-*X. arboricola* pv*. pruni* strains were immobilized on nitrocellulose membranes (Micron Separations Inc., MA) by Southern analysis probed as above.

Estimation of limits of detection. To determine the minimum number of bacterial cells detected by PCR and DNA hybridization, serial dilutions of *X. arboricola* pv*. pruni*, strain Xap14 were made in sterile distilled water and in plant extract fluids and assayed by PCR and dot blot analysis. Tests were repeated at least three times. Concentration of *X. arboricola* pv*. pruni* was determined by a standard plate count procedure on SPA medium to enumerate the number of CFU per reaction. A dilution series of total genomic DNA extracted from strain Xap14 was also prepared and assayed as described above to estimate the limit of detection for purified homologous DNA.

RESULTS

RAPD amplification. To identify primers that generate RAPD patterns distinctive to *X. arboricola* pv*. pruni*, 61 different 10-base arbitrary primers were tested with three strains of the pathogen. The DNA pattern generated with primer Y17 yielded three major bands of approximately 3.0, 1.5 and 0.98 Kb, designated bands 1, 2, and 3 respectively (Fig. 1A). Band 1 was only present in the *Erwinia* strain, while band 2 was present in all the xanthomonad and pseudomonad strains tested. Band 3 however, was unique to the *X. arboricola* pv*. pruni* strains. Primer Y17 consistently generated 2 DNA fragments (bands 2 and 3) (Fig. 2) for all the 138 *X. arboricola* pv*. pruni* strains tested (Table 1). This revealed pattern was clearly distinguishable from those produced by the 167 other non- *X. arboricola* pv*. pruni* strains tested (Table 2).

PCR Amplification. The DNA band unique to *X*. *arboricola* pv. *pruni* (band 3) was cloned and sequenced. Primers Y17CoF and Y17CoR were designed for the amplification of this 943-bp region. The applicability of this region in a specific detection system for *X. arboricola* pv*. pruni* was first analyzed by screening the 138 *X. arboricola* pv*. pruni* strains for the predicted size fragment through PCR analysis. All *X. arboricola* pv*. pruni* amplifications resulted in a single DNA fragment of 943 bp (Fig. 3).

Specificity of the 943-bp DNA fragment. In order to determine whether the DNA in band 3 was specific to *X. arboricola* pv*. pruni*, a panel of 167 non- *X. arboricola* pv*. pruni* strains was screened by PCR analysis. No amplification products were observed when non-*X. arboricola* pv*. pruni* strains were screened (Fig. 3). The specificity of the reaction products was confirmed in all cases by the absence of a product in the negative controls, by the predicted product size, and by Southern blotting. To test the specificity of this DNA sequence, a DIG-labeled probe XPRUNI14 was constructed and hybridized to total *X. arboricola* pv*. pruni* DNA digested with *EcoR*I (data not shown) and to RAPD amplification products (Fig. 1B and 4B) of the panel of 138 *X. arboricola* pv*. pruni* representing different hosts and

geographical locations and to 167 non-*X. arboricola* pv*. pruni* strains. All *X. arboricola* pv*. pruni* strains listed in Table 1 gave a strong hybridization signal in Southern and dot blot hybridizations with the probe (Fig. 4A). The probe did not hybridize to any of the strains from other genera, species or pathovars.

Sensitivity of PCR and dot blot assays. A dilution series of whole *X. arboricola* pv*. pruni* cells, strain Xap14, were directly assayed using Y17CoF and Y17CoR primers. The PCR amplification of the dilution series yielded a limit of detection between 25 and 50 CFU per reaction (Fig. 5A). Dilution series of genomic DNA of strain Xap14 were also prepared, and aliquots used as templates for PCR amplification. Aliquots that contained 15 ng of DNA were successfully detected after PCR analysis. An additional test of the sensitivity of the probe was determined by directly spotting aliquots of a dilution series of strain Xap14 cells onto hybridization membranes and probing with XPRUNI14 labeled DNA. It was possible to detect populations as low as 4×10^2 CFU (Fig. 5B.).

Detection of *Xap* in infected leaves, twigs, buds and fruits. The PCR assay was evaluated for detection of *X. arboricola* pv*. pruni* in infected plant tissues as would be required for routine diagnosis. DNA was extracted directly from woody tissue using the CTAB protocol of Zhang *et al*. (Zhang *et al.*, 1998). Specific amplification of target DNA was obtained when DNA preparations from plant homogenates containing 2×10^8 to 2×10^1 CFU per milliliter of strain Xap14 were tested. Aliquots that contained between 50 and 75 CFU per reaction were successfully detected with PCR analysis using Y17CoF and Y17CoR primers. Amplification products were not observed when extracts from healthy non-inoculated plant homogenates were assayed as controls. Naturally infected plant tissues were also used to determine the ability of the probe to detect *X. arboricola* pv*. pruni* cells and results were confirmed with traditional techniques (Fig. 3). Results showed that the presence of *X. arboricola* pv*. pruni* could be detected directly on *Prunus* tissues (leaves, buds, fruits and twigs) at a minimum number of 4×10^2 CFU when using membrane hybridization and up to 50 and 75 CFU when using PCR.

TABLE 1. List of *Xanthomonas arboricola* pv. *pruni* strains or DNA used in this study.

Geographical Source	Host plant	Number of isolates
USA		
North Carolina	*Prunus persica*	95
	Prunus persica [nectarine group]	4
	Prunus salicina	3
	Prunus armeniaca	2
	Prunus domestica	1
South Carolina	*Prunus persica*	2
Virginia	*Prunus persica*	3
Georgia	*Prunus persica*	2
	Prunus persica [nectarine group]	1
BRAZIL	*Prunus persica* [x]	3
	Prunus persica [nectarine group] [x]	1
	Prunus salicina [x]	3
URUGUAY	*Prunus persica* [y]	2
AUSTRALIA	*Prunus persica* [z]	5
	Prunus persica [nectarine group] [z]	2
	Prunus salicina [z]	9

[x], obtained from EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária), Brazil; [y],obtained from INIA (Instituto Nacional de Investigacion Agropecuaria) Las Brujas, Uruguay;
[z], obtained as DNA samples.

TABLE 2. List of non-*Xanthomonas arboricola* pv. *pruni* strains used in this study.

Bacteria	**Host plant**	**Number of isolates**
Agrobacterium tumefaciens	*Prunus* sp.	2
Clavibacter michiganensis subsp. *michiganensis*	*Lycopersicon* sp.	2
Escherichia coli	-	2
Erwinia amylovora	*Pyrus* sp.	3
carotovora subsp atroseptica	*Solanum* sp.	2
carotovora subsp *carotovora*	*Solanum* sp.	7
chrysantemi	*Solanum* sp.	3
Pseudomonas cichorii	*Lactuca* sp.	3
corrugata	*Lycopersicon* sp.	1
syringae pv.*phaseolicola*	*Phaseolus* sp.	3
syringae pv.*syringae*	*Prunus* sp.	6
syringae pv.*tabaci*	*Nicotiana* sp.	3
syringae pv.*tomato*	*Lycopersicon* sp.	2
Xanthomonas axonopodis pv.*begoniae*	*Begonia* sp.	4
axonopodis pv.*dieffenbachiae*	*Dieffenbachia* sp.	4
axonopodis pv.*vesicatoria*	*Capsicum* sp.	19
campestris pv.*campestris*	*Brassica* sp.	7
campestris pv.*zinniae*	*Zinnia* sp.	8
fragariae	*Fragaria* sp.	6
hortorum pv.*pelargonii*	*Geranium* sp.	9
oryzae	*Oryza* sp.	3
Saprophytic and epiphytic strains associated with *Prunus* plants	*Prunus* sp.	68

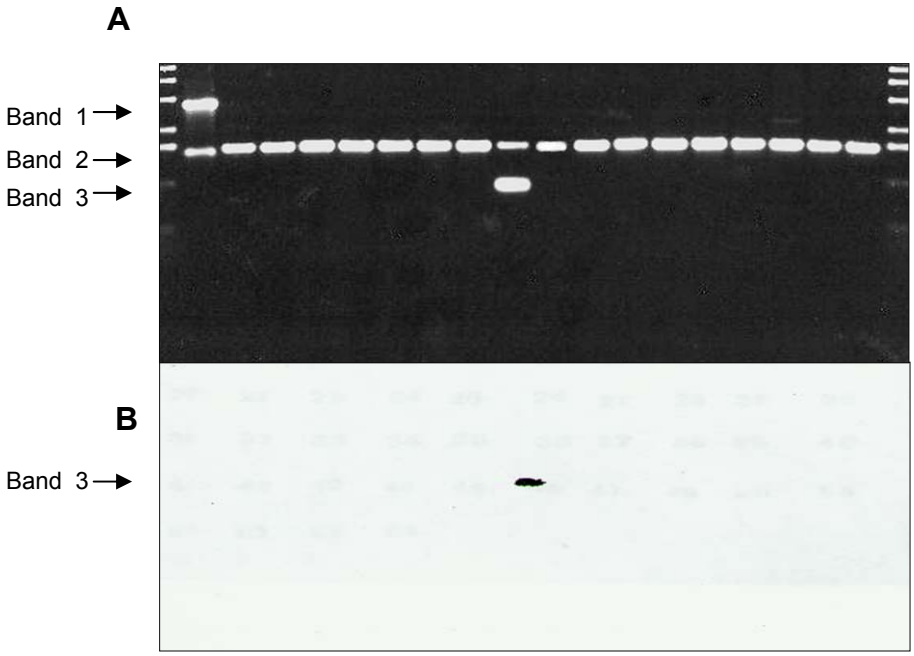


Fig. 1. A, Ethidium bromide-stained agarose gel of RAPD-DNA products amplified with primer Y17. Lanes: 1 and 20, 1Kb DNA Ladder; 2, *Erwinia carotovora* pv. *carotovora*; 3, *Xanthomonas campestris* pv. *begoniae*; 4, *X. campestris* pv. *begoniae*; 5, *X. campestris* pv. *begoniae*; 6, *X. fragariae*; 7, *X. fragariae*; 8, *X. fragariae*; 9, *Pseudomonas cichorii*; 10, *X. arboricola* pv. *pruni*; 11, *X. campestris* pv.*oryzae*; 12, *X. campestris* pv. *campestris*; 13, *P. corrugata*; 14, *X. campestris* pv. *campestris*; 15, *X. campestris* pv. *campestris*; 16, *X. campestris* pv. *campestris*; 17, *X. campestris* pv. *zinniae*; 18, *X. campestris* pv. *zinniae*; 19, *X. campestris* pv. *zinniae*.
B, Southern blot of the gel in panel A, hybridized with the digoxigenin-11-dUTP XPRUNI14 probe (943 bp).The Band 3 show the specific 943-bp product and the hybridized band.

Fig. 2. Ethidium bromide-stained agarose gel of RAPD-DNA products amplified with primer Y17 from different *Xanthomonas arboricola* pv. *pruni* isolates. Lanes: 1 and 20, 1Kb DNA Ladder; lane 2,XapL1;lane 3,XapL2;lane 4,XapCLE2;lane 5,XapCLE5; lane 6,XapCLE7;lane 7,XapW1;lane 8,XapW3;lane 9,XapGJ1;lane 10,XapGJ2;lane 11,SenC2-1;lane 12,Xap19-1F-5;lane 13,Xap19-1F-7;lane 14,XapSH5;lane 15,XapSH7;lane 16,XapPlumB31;lane 17,XapOH-4-C2;lane 18,XapOH-7 and lane 19,XapR2-1.

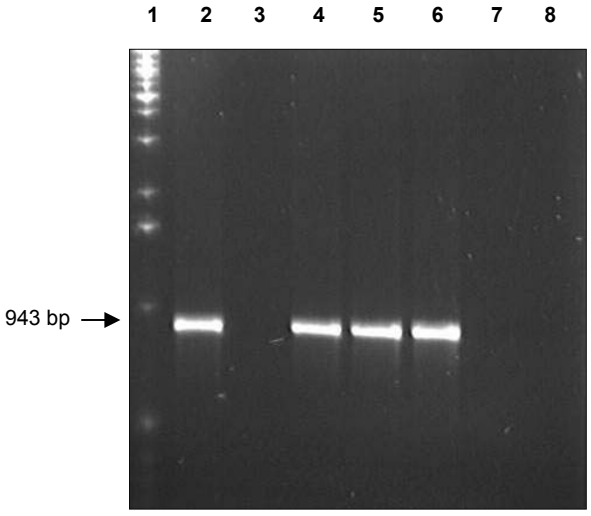


Fig. 3. Detection of *Xanthomonas arboricola* pv. *pruni* by PCR with primers Y17CoF and Y17CoR from infected peach cankers. Lane1, 1Kb DNA Ladder; lane 2, positive control (Xap14); lane 3, negative control (*X. axonopodis* pv. *vesicatoria*); lanes 4-6: DNA extracted from peach canker samples; lane 7,DNA extracted from a non-infected peach-twig sample; Lane 8, negative control (distilled H_2O);

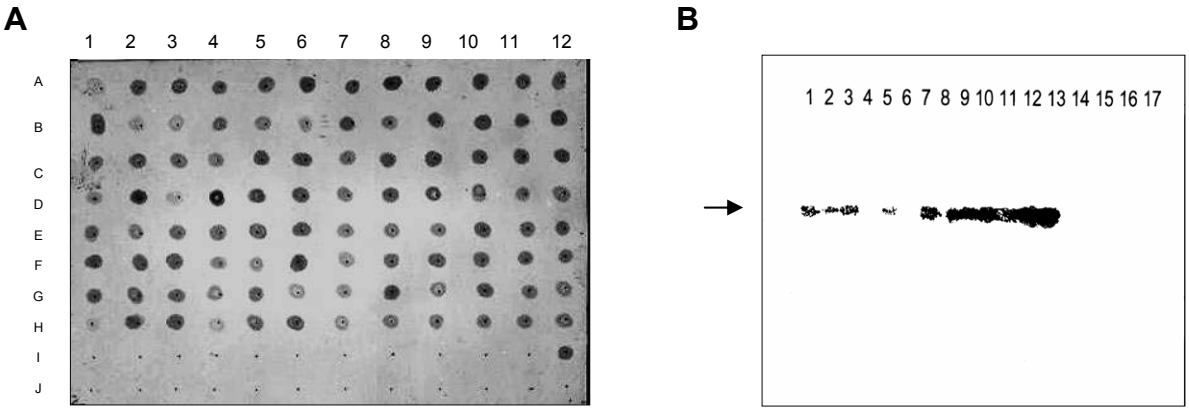


Fig. 4. **A,** Dot blot hybridization with the DIG-labeled probe XPRUNI14
Dots A1-12; B1-12; C1-12; D1-12;E1-12;F1-12;G1-12;H1-12;I12, represent different *Xanthomonas arboricola* pv*. pruni* strains. Dots I1-11 and J1-12 represent non-*X. arboricola* pv*. pruni* strains. Approximately, 1 x10^5 cells were used for each dot.
B, Southern hybridization with the DIG-labeled probe XPRUNI14. Lane 1,Xap54; lane 2,Xap56; lane 3,Xap61; lane 4,Xco1; lane 5,Xap63; lane 6,Xf49; lane 7,Xap70; lane 8,Xap72; lane 9,Xap73; lane 10,Xap74; lane 11, Xap75; lane 12,Xap77; lane 13,Xap78; lane 14, Xf50; lane 15,Xcb3; lane 16, Xf22 and lane 17 Xcc1.

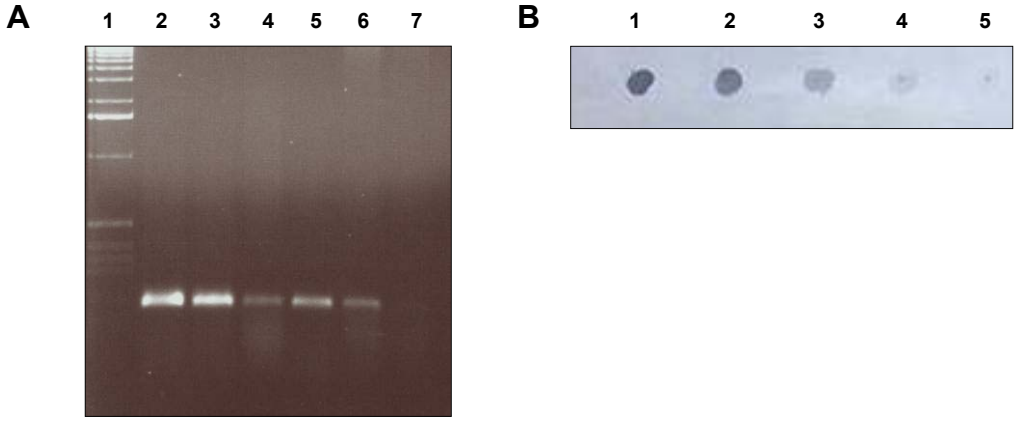


Fig. 5. A, Detection limit of the *Xanthomonas arboricola* pv. *pruni*-specific DNA primers and PCR. Tenfold serial dilutions of *X. arboricola* pv*. pruni* strain Xap14 were used; 5 µl of each dilution was added directly to the PCR mixture. Lane1, 1Kb DNA Ladder; lane 2, 5 x10^7 ; lane 3, 5 x10^6 ; Lane 4, 5 x10^5 ; lane 5, 5 x10^4 ; lane 6, 5 x10^3 ; lane 7, 5 x10^2 CFU of *X. arboricola* pv*. pruni* per ml.
B, Dot blot hybridizations of 10-fold serial dilutions of *X. arboricola* pv*. pruni* strain Xap14 cells probed with the DIG labeled probeXPRUNI14; 1 µl of each dilution was spotted directly to the membrane. Dots 1-5 contained respectively, 4 x10^8 ; 4 x10^7 ; 4 x10^6 ; 4 x10^5 ; 4 x10^4 CFU of *X. arboricola* pv*. pruni* per ml.

DISCUSSION

A specific 943-bp DNA fragment unique and conserved in all *X. arboricola* pv. *pruni* strains tested was identified using the RAPID method. This fragment was used as a DIG-probe and its DNA sequence provided the basis for the synthesis of specific oligonucleotide primers Y17CoR and Y17CoF useful as sensitive detection tools for *X. arboricola* pv. *pruni.* This is the first report describing a DNA probe and PCR primers specific for the detection and identification of *X. arboricola* pv. *pruni*. The use of these primers and hybridization with the XPRUNI14 probe should provide a powerful tool for phytosanitary agencies and epidemiological studies.

X. arboricola pv. *pruni* is considered of quarantine significance for the Inter-African Phytosanitary Council (IAPSC) as well as for many countries where this bacterium is absent or not yet established (OEPP/EPPO, 2003). The pathogen is listed as an A2 quarantine pest by the European and Mediterranean Plant Protection Organization (OEPP/EPPO, 1978) implying that although present in some areas, is not widely distributed and is subject to official control (OEPP/EPPO, 2003).

Colonized buds have been suggested as one of the main vehicles for the spread of bacterial spot (Zaccardelli *et al*., 1998). For conducting effective quarantine measures as well as providing reasonable disease control, it is important to utilize a specific, rapid and sensitive detection system for *X. arboricola* pv. *pruni* in order to detect latent infections in propagation materials and to uncover possible new sources of inoculum of this bacterium. Many questions regarding the epidemiology of bacterial spot have remained unanswered partly because of the lack of sensitive and precise identification methods. Although dispersal of *X. arboricola* pv. *pruni* by rain and wind is usually limited to neighboring areas, it can be carried long distances in international trade, such as in contaminated budwood (Zaccardelli *et al*., 1998). Therefore, exclusion of this pathogen from a region by use of disease or pathogen-free plant material is one of the most important phytosanitary measures (OEPP/EPPO, 2003).

DNA-based methods provide powerful tools to identify and detect microorganisms with high sensitivity and specificity. *X. arboricola* pv. *pruni* can be detected by traditional techniques involving a series of biochemical tests of purified colonies (Gitaitis *et al*., 1988; Schaad, 2001) and by selective media (Civerolo *et al*., 1982). In spite of allowing for high plating efficiency, selective media often fail to recover bacteria that enter in a non-culturable but viable state, not detectable with traditional techniques. Current serological techniques for diagnosis of *X. arboricola* pv. *pruni* (ADGEN, Co), although considered reliable and reasonably rapid, may not be sensitive enough to detect low numbers of the pathogen particularly in buds or as latent infections. PCR and DNA probes on the other hand, are techniques that have been proven to rapidly and specifically detect very low numbers of bacteria in a sample without the need for prior cultivation or enrichment of the pathogen. The suitability of the use of DNA based techniques for *X. arboricola* pv. *pruni* is also validated by the fact that, despite studies reporting some differential virulence to *Prunus* spp. (Du Plessis, 1988), no major genetic diversity has been reported worldwide in strains of *X. arboricola* pv. *pruni*.

The PCR primers designed in this study were shown to be specific to *X. arboricola* pv. *pruni* and able to detect small numbers of this bacterium in field samples and pure cultures. We detected as few as 25 to 50 cells in suspensions of pure culture, with 30 rounds of amplification. A slightly decreased sensitivity (between 50 to 75 cells) was observed when PCR was performed on inoculated plant homogenates possibly attributed to PCR-interfering compounds present in *Prunus* plants. Results exhibited clear differences between positive and negative samples (Fig. 3). Identical results were obtained when PCR was performed on naturally infected plant tissues. PCR applied to purified DNA or cells from other species or to extracts from healthy plants failed to generate products of any size. Primers Y17CoF and Y17CoR allowed the detection of *X. arboricola* pv. *pruni* in asymptomatic colonized buds located at different distances from a bacterial spot canker as a source of inoculum and revealed the presence of this bacterium in 40% of the buds situated at the closest proximity with the canker (data not shown).

Although the sensitivity obtained does not represent the detection of a single cell which is theoretically possible with the PCR technique, an enrichment step with a selective medium added before amplification or a second round of PCR could allow an even higher sensitivity possibly to one bacterial cell. A drawback associated with DNA tests applied on quarantine assays is that they do not provide information about the viability of the pathogen. This disadvantage could be circumvented with the amplification of mRNA template by reverse transcription PCR assuming that mRNA endures in live cells but quickly degrades after cell death.

The specific 943-bp fragment labeled with DIG-probe was a sensitive detection tool for *X. arboricola* pv. *pruni*. Development of specific DNA probes for identification and detection of several bacterial pathogens has been previously reported (Kuflu *et al*, 1997; Schaad, 2001; Ward and De Boer, 1994). This is an easy technique for assaying large numbers of samples in a relatively short time providing an effective means for identifying field *X. arboricola* pv. *pruni* samples. In our studies, XPRUNI14 specifically hybridized to *X. arboricola* pv. *pruni* in dot blot assays containing as few as 4 x 10^2 bacteria. Although this sensitivity should be satisfactory for the detection of the pathogen in latently infected plants, it is not optimal for regulatory purposes. In our assays, dot blot hybridizations were detected using a colorimetric method. Although this method is quicker, less expensive and more suitable to routine assays, it is generally less sensitive than a chemiluminescent detection method. In a survey of field samples with bacterial spot-like symptoms (data not shown), dot blot assays identified 100% of presumptive bacterial spot lesions, which were later confirmed with traditional techniques. The signals obtained for these dot blots exhibited clear differences between positive and negative results.

The lack of cross-reactions between *X. arboricola* pv. *pruni* and non-*X. arboricola* pv. *pruni* strains and the ability to identify all *X. arboricola* pv. *pruni* strains tested indicates that this fragment is a useful diagnostic tool for detecting this pathogen. Both methods of detection (PCR and labeled DNA probe) have the potential to circumvent many of the problems currently associated with a conclusive

detection and identification of this bacterium. It was possible to target and amplify a DNA sequence specific to *X. arboricola* pv. *pruni* even in the presence of plant extracts and without prior isolation of the pathogen from plant tissues. In addition to its sensitivity, this detection system ensures selectivity against non-target microorganisms that are usually associated with *Prunus* plants and plant parts.

LITERATURE CITED

ADGEN, Co. http://www.adgen.Co.UK; http://www.shigematsu- bio.com/J/products/ research/infect/topic/adgen/identkit/bacteria.html.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1992. Current protocols in molecular biology, vol.I. Greene Publishing Associates and Wiley-Intersceince, NY.

Civerolo, E. L., Sasser, M., Helkie, C., and Burbage, D. 1982. Selective medium for *Xanthomonas campestris* pv. *pruni*. Plant Dis. 66: 39-43.
Dhanvantari, B. N., Dirks, V. A., and Brown, R. J. 1978. Effectiveness of antibiotics for control of bacterial spot of peach in southwestern Ontario. Can. J. Plant Sci. 58: 953-959.

du Plessis, H. J. 1988. Differential virulence of *Xanthomonas campestris* pv. *pruni* to peach, plum and apricot cultivars. Phytopathology 78: 1312-1315.

Gitaitis, R. D., Hamm, J. D., and Bertrand, P. F. 1988. Differentiation of *Xanthomonas campestris* pv. *pruni* from other yellow-pigmented bacteria by the refractive quality of bacterial colonies on an agar medium. Plant Dis. 72: 416-417.

Goodman, C. A., and Hattingh, M. J. 1986. Transmission of *Xanthomonas campestris* pv. *pruni* in plum and apricot nursery trees by budding. HortScience 21: 995-996.

Kuflu, K. M., and Cuppels, D. A. 1997. Development of a diagnostic DNA probe for Xanthomonads causing bacterial spot of peppers and tomatoes. Appl. Environ. Microbiol. 63: 4462-4470.

Lelliott, R. A., and Stead, D. E. 1987. Methods for the diagnosis of bacterial diseases of plants. Blackwell Scientific Publications, Oxford, UK.

OEPP/EPPO, 1978. Data sheets on quarantine organisms No.62, *Xanthomonas pruni*. Bulletin OEPP/EPPO Bulletin 8.

OEPP/EPPO, 2003. Data sheets on quarantine organisms. *Xanthomonas arboricola* pv. *pruni*. www.eppo.org/QUARANTINE/BACTERIA/Xanthomonas_pruni/XANTPR_ds.pdf.

Randhawa, P. S., and Civerolo, E. 1985. A detached-leaf bioassay for *Xanthomonas campestris* pv. *pruni*. Phytopathology 75: 1050-1063.

Ritchie, D.F. 1995. Bacterial Spot. Pages 50-52 in: Compendium of Stone Fruit Diseases, Ogawa, J. M., Zehr, E. I., Bird, G. W., Ritchie, D. F., Uriu, K., and Uyemoto, J. K., eds. APS Press, St. Paul, MN.

Ritchie, D. F. 1999. Sprays for control of bacterial spot of peach cultivars having different levels of disease susceptibility, 1998. Fung.Nematic.Tests 54: 63.

Sambrook, J., Frisch, E. F., and Maniatis, T. 1989. Molecular cloning laboratory Manual. 2nd. Ed. Cold Spring Harbor Laboratory, Cold Harbor, NY.

Schaad, N. W. 2001. Laboratory guide for identification of plant pathogenic bacteria. 3nd. Ed. APS Press, St. Paul, USA.

Shepard, D. P., and Zehr, E. I. 1994. Epiphytic persistence *of Xanthomonas campestris* pv. *pruni* on peach and plum. Plant Dis. 78: 627-629.

Vauterin, L., Swings, J., Kersters, K., Gillis, M., Mew, T. W., Schroth, M. N., Palleroni, N. J, Hildebrand, D. C., Stead, D. E., Civerolo, E. L., Hayward, A. A. C., Maraite, H., Stall, E. E., Vidaver, A. K., and Bradbury, J. F. 1990. Towards an improved taxonomy of *Xanthomonas*. Int. J. Syst Bacteriol. 40: 312-316.

Vauterin, L., Hoste, B., Kersters, K., and Swings, J. 1995. Reclassification of *Xanthomonas*. Int. J. Syst. Bacteriol. 45: 472-489.

Ward, L. J., and De Boer, S. H. 1994. Specific detection of *Erwinia carotovora* subsp.*atroseptica* with a digoxigenin-labeled DNA probe. Phytopathology 84: 180-186.

Werner, D. J., and Ritchie, D. F. 1986. Susceptibility of peaches and nectarines, plant introductions, and other *Prunus* species to bacterial spot. HortScience 21: 127-130.

Zaccardelli, M., Ceroni, P., and Mazzucchi, U. 1999. Amplified fragment length polymorphism fingerprinting of *Xanthomonas arboricola* pv. *pruni*. J. Plant Pathol. 81: 173 -179.

Zaccardelli, M., Malaguti, S., and Bazzi, C. 1998. Biological and epidemiological aspects of *Xanthomonas arboricola* pv. *pruni* on peach in Italy. J. Plant Pathol. 80: 125-132.

Zhang, Y., Uyemoto, J. K., and Kirkpaatrick, B. C. 1998. A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. J. Virol. Methods 71: 45-50.

In silico identification and characterization of a putative protein in *Xanthomonas arboricola* pv. *pruni* with similarity to the ABC (ATP-binding cassette) system

ABSTRACT

Xanthomonas arboricola pv. *pruni* (Vauterin *et al*., 1995, syn. *X. campestris* pv. *pruni* [Smith]) is a gamma, gram-negative plant pathogenic bacterium that causes bacterial spot disease on *Prunus* spp. Previously a 943-bp DNA fragment from *X. arboricola* pv. *pruni* was identified, cloned and sequenced and has proven to be a useful tool for detection and identification of this bacterium. Sequence analysis of the 943-bp DNA fragment revealed the presence of an open reading frame (ORF) encoding a putative protein of 243 amino acids with a calculated molecular mass of 26.8 kDa. BLAST searches revealed significant amino acid sequence similarity between the *X. arboricola* pv. *pruni* putative protein and other known proteins of the ABC transporter family. The highest similarity at the amino acid level was found with two proteins of the related phytopathogenic bacteria X. *axonopodis* pv. *citri* (39% identity) and *X. campestris* pv. *campestris* (32% identity). Bioinformatic programs for subcellular localization predicted the location of this protein to be at the cytoplasmic membrane. Additional programs suggested the presence of three transmembrane helices located at the carboxyl terminal region and the absence of signal peptide sequences. Database searches predicted a conserved domain in the *X. arboricola* pv. *pruni* putative protein, 25% identical and 67% similar to the FtsX permease in *Escherichia coli*. FtsX is a membrane protein and a member of a cell division operon (FtsE/FtsX) in *E. coli* considered essential for cell division. Results of the *in silico* analysis suggested the presence of an ORF in the *X. arboricola* pv. *pruni* DNA fragment encoding a putative protein with similarity to members of the ABC transporter family and possibly involved in cell division. The identification and potential characterization of this putative protein using bioinformatic tools represents only the beginning of the understanding of its function. In order to gain a better

knowledge of its possible biological function, further research was conducted in our laboratory with results that support the initial predictions.

INTRODUCTION

Several bacterial genomes have been sequenced recently and bioinformatic programs used to predict structural and functional properties of many genes and the proteins they encode (Mulder and Apweiler, 2001). Little is known however, about the genetics of *Xanthomonas arboricola* pv. *pruni* (Vauterin *et al*., 1995, syn. *X. campestris* pv. *pruni* [Smith]), the causal bacterium of bacterial spot of peach and other *Prunus* spp.

Previously a 943-bp DNA fragment from *X. arboricola* pv*. pruni* was identified, cloned and sequenced and proven useful as a tool for detection and identification of this bacterium (Pagani and Ritchie, 2002). Sequence analysis revealed the presence of an open reading frame (ORF) with the potential to encode a protein of 243 amino acids. This predicted ORF was compared with protein sequences from several databases (Altschul *et al*., 1997; Gasteigen *et al.,* 2003). *In silico* analysis suggested the presence of a putative protein with significant similarity to the ABC (ATP-binding cassette) transporter system.

ABC transporters represent one of the largest protein families, widely spread among prokaryotes and eukaryotes (Higgins, 1992). They use the hydrolysis of ATP to energize several physiological processes usually involved in the export and import of a wide variety of compounds, ranging from inorganic ions to large polypeptides (Higgins, 1992). These proteins are involved in preserving the dynamic stability of cells, and maintaining efficient communications with their environment (Holland *et al*., 2003). ABC transporter proteins are considered central to many biological functions, including the uptake of nutrients, export of antigenic products that are involved in infection of human cells, secretion of signaling molecules and multidrug resistance phenomenon (Abele and Tampe, 1999; Allignet *et al*., 1992; Gaudet and Wiley, 2001; Hyde *et al*., 1990). Furthermore, some human inheritable diseases, like

cystic fibrosis, sitosterolemia, adrenoleukodystrophy and Stargardt's disease are also caused by defective ABC transport systems (Abele and Tampe, 1999; Allikmets *et al*., 1997; Klein *et al*., 1999; Quinton and Bijman, 1983; Salen *et al*., 1992).

Records of the complete number of putative ABC transporters present in most sequenced genomes are now available (Holland *et al.,* 2003). *Saccharomyces cerevisiae* was the first eukaryotic organism to have its complete genome sequenced, revealing 30 genes encoding ABC proteins (Decottignies and Goffeau,1997). Some of these ABC proteins participate in cell functions such as development of drug resistance, in peroxisome biogenesis processes, mitochondrial functions and pheromone secretion (Bauer *et al*., 1999; Taglicht and Michaelis, 1998).

In plant pathogenic fungi and bacteria, ABC transporters play an important role in several defense mechanisms. Exogenous toxic compounds that enter the pathogen can be pumped out of the cell by these membrane proteins in order to reduce the toxin concentration inside the cell. Numerous studies report the role of ABC transporters protecting pathogens against fungicides such as fenpiclonil and fludioxonil (Schoonbeek *et al*., 2001; Vermeulen *et al*., 2001), against the antibiotic phenazine-1-carboxamide (PCN) produced by *Pseudomonas* strains (Schoonbeek *et al*., 2002), or plant defense compounds (Fleissner *et al*., 2002).

In bacteria, ABC transporters constitute the largest family of proteins. They include 5% of all proteins encoded by the *Escherichia coli* and *Bacillus subtilis* genomes (Blattner *et al*., 1997; Kunst, 1997). Recently two multidrug efflux pumps (AcrB and NorM) were identified in the plant pathogenic bacteria *Erwinia amylovora*. They were reported to play a role in the resistance of this bacterium to the antibiotic tetracycline, to an antibiotic produced by the biocontrol bacterium *Pantoea agglomerans,* and to phytoalexins produced by apple trees (Burse *et al*., 2004,a,b). Another ABC transporter in *Erwinia chrysanthemi*, *ybiT* was found to be essential for competitive fitness against endophytic bacteria during plant infection (Llama-Palacios *et al*., 2002).

Completion of the *E. coli* genome sequence revealed a total of 79 ABC transporter proteins (Linton and Higgins, 1998). They perform functions affecting diverse processes, involving the regulation of translation of polypeptides, control of cell volume in response to osmotic shock, cell surface integrity, export and import of solutes, osmoprotection, and cell division (Higgins, 1995; Holland *et al*., 2003). The number of ABC transporters present in different organisms varies according to species. Bacterial species requiring a greater level of adaptation to diverse environments like *E. coli*, possess considerably more ABC transporter proteins compared to other species that occupy more restricted niches (Holland *et al*., 2003). This is clearly evidenced by the number of major transport proteins per megabase of DNA present in different organisms. *E. coli*, *B. subtilis* and *Haemophilus influenzae* are reported to have as many as twice the number of transport proteins compared to *Xylella fastidiosa* and other bacterial plant pathogens (Paulsen *et al*., 2000).

A typical ABC transporter contains conserved domains known as nucleotide binding domains (NBDs) and several α-helical transmembrane domains (TMDs). The amino acid sequence of the NBDs usually has three major conserved motifs, the Walker A and Walker B motifs commonly found in ATPases together with a specific signature motif. The minimal structural requirement for an active ABC transporter seems to be two TMDs and two NBDs. ABC transporters in multicellular organisms are commonly duplicated and arranged as tandem paralogue clusters fused to yield a single polypeptide chain, whereas bacterial ABC transporters are built from individual subunits (Holland *et al*., 2003).

Phylogenetic analyses of prokaryotic and eukaryotic genomes confirm the presence of conserved NBDs in ABC proteins suggesting a common evolutionary origin even in the likelihood of an independent evolutionary process (Holland *et al.*, 2003). Computer-assisted methods have been applied (Fath and Kolter, 1993; Higgins *et al*., 1986; Saurin *et al*., 1999) in an attempt to classify ABC systems. Phylogenetic studies place ABC proteins into three classes. Class 1 includes systems with fused NBDs and TMDs, which are involved in export; class 2 systems lack known TMDs and are involved with non-transport cellular processes and

antibiotic resistance, and class 3 are systems with unfused TMDs and ABC domains carried by independent polypeptide chains which involve substrate binding proteins and other systems not known to be importers (Dassa and Bouige, 2001; Holland *et al*. 2003).

While ABC transporters can be easily identified by conserved motifs in the NBD eg, Walker A, and B, signature motif, their physiological function and the identity of the substrates they transport often are unknown (Schneider and Hunke, 1998). Most ABC transporters are associated with the transport of substrates across biological membranes, although there are several exceptions (Doolittle *et al*., 1986). Some proteins, like UvrA from *E. coli* and the elongation factor EF-3 of yeast, have been recognized as typical ABC proteins but they clearly serve functions other than transport (Chakraburtty, 1999; Goosen and Moolenaar, 2001).

The objective of this research was to analyze and characterize a predicted *X. arboricola* pv*. pruni* ORF using an *in silico* approach with several bioinformatic programs as an initial step towards the elucidation of a possible biological function.

MATERIALS AND METHODS

ORF prediction. The 943-bp nucleotide sequence (Fig. 1) generated with primers Y17CoR and Y17CoF was translated and analyzed with the Translator tool program (ExPASy, Export Protein Analysis System) Proteomics Server, of the Swiss Institute of Bioinformatics (Gasteiger *et al.,* 2003) and with the ORF Finder program (NCBI) (Altschul *et al*., 1997) to predict possible ORFs.

BLAST, domain searches and sequence alignment. Comparisons were done using the *X. arboricola* pv. *pruni* predicted ORF as a query sequence against the National Center for Biotechnology Information (NCBI) database (GenBank). Sequence homologies to the predicted ORF were searched by using BLASTP which compares an amino acid query against a protein sequence database, and BLASTX program, which compares a nucleotide query sequence translated in all reading frames, against a protein sequence database with the best probability scores recorded (Altschul *et al*., 1997). The *X. arboricola* pv*. pruni* ORF sequence also was BLAST to a Membrane Transport System database, Transport DB, a specific database for cellular membrane transporter proteins (Ren *et al*., 2004). Among the bioinformatic programs offering multiple sequence alignment, the ClustalW multiple alignment program (Thompson *et al.,* 1994) was chosen to compare and align sequences with the highest similarity to the *X. arboricola* pv*. pruni* ORF. To identify conserved domains a search was performed with the BLASTP (NCBI) (Altschul *et al*., 1997) and the SMART (Simple Modular Architecture Research Tool) (Letunic *et al*., 2004) programs followed by an alignment with the Pfam: Swiss/PROT program (Bairoch and Apweiler, 1999).

Phylogenetic analysis. A phylogenetic tree was created by amino acid sequence comparisons using scores derived from the BLASTP algorithm through the ClustalW program (Thompson *et al.,* 1994). The PHYLIP (PHYLogeny Inference Package) program (Felsenstein, 1993) was used to illustrate phylogenic distances between the *X. arboricola* pv*. pruni* putative protein and other ABC transporter proteins with homology to the queried protein.

Identification of ABC transporter motifs, signal sequences and protein localization. ABC transporter motifs were analyzed through the program PATTINPROT/IBCP (Combet *et al*., 2000). The SignalP 3.0 program (Center for Biological Sequence Analysis) (Bendtsen *et al*., 2004) was used to identify potential signal peptides in the predicted protein. This program predicts the location of signal peptides based on neural networks (NN) and hidden Markov models (HMM) trained on separate sets of prokaryotic and eukaryotic sequences. Neural networks and hidden Markov models trained on gram-negative bacteria were used in this study. In order to assess the likelihood of the predicted *X. arboricola pv. pruni* putative protein being located at a specific site in the cell, the PSORT-B v.2.0 program (Gardy *et al.,* 2003) was chosen for the prediction of localization of proteins in gram-negative bacteria.

Location of transmembrane helices. The location of transmembrane helices can be predicted by analyzing the distribution of hydrophobic amino acids. The TMHMM Server v.2.0 program, Prediction of Transmembrane Helices in proteins allows for prediction of the location of transmembrane alpha helices and intervening loop regions (Tusnády and Simon, 2001). This program also will predict which loops are on the inside or outside of the cell or organelle. Prediction of secondary structure and transmembrane helices was also performed by the SOSUI program (Hirokawa *et al.,* 1998). This program has an accuracy of 99% for the classification of proteins and 97% for the corresponding value for the transmembrane helix prediction (Hirokawa *et al.,* 1998). Our results were also confirmed with the SMART program.

Protein characteristics prediction. Various characteristics of the *X. arboricola pv. pruni* putative protein were estimated with program ProtParam (Expasy) (Gasteiger *et al.,* 2003). Some of the above prediction programs were also used to analyze and compare the *X. axonopodis* pv. *citri* AE012014 protein which shares the highest sequence similarity with the *X. arboricola* pv. *pruni* putative protein.

RESULTS

ORF prediction. Both prediction programs, the Translator tool (ExPASy) and the ORF Finder (NCBI), identified an ORF of 732 nucleotides in length with a start codon at nucleotide 121 and a stop codon at nucleotide 852 within the 943-bp DNA fragment (Fig. 2).

BLAST, domain searches and sequence alignment. BLASP and BLASTX (NCBI) programs revealed significant similarity between the identified *X. arboricola* pv*. pruni* ORF and three ABC transporter ATP-binding proteins (E values: 1e -32, 40% identity; 2e-19, 31% identity and 3e -11, 27% identity) from the bacteria: *Xanthomonas axonopodis* pv. *citri* strain 306; *X. campestris* pv. *campestris* strain ATCC 33913 and *Lysobacter lactamgenus* (Fig. 3). Alignments with the two proteins that presented the highest similarity scores are shown in Fig. 4. Searches also revealed the presence of a putative conserved domain (FtsX, pfam 02687) in the queried protein sequence (Fig. 5). Proteins that contain this domain often are predicted permeases and transmembrane proteins. Additional information provided by these programs described the presence of the FtsX, pfam 02687 domain in several ABC transporters involved in cell division (IPR004513) and chromosome partitioning (COG2177) (Altschul *et al*., 1997). BLAST to a Membrane Transporter System database (Transport DB) exhibited similar identity scores to the same proteins (Identities: 91/232; 39% to *X. axonopodis* pv. *citri* protein (AE012014) and 84/280; 30% to *X. campestris* pv. *campestris* protein *(AE012154)*. BLASTP (NCBI) showed the presence of a predicted permease domain (FtsX) located between amino acids 118 and 178 of the queried protein (Fig. 5). When the total FtsX protein sequence was compared to the *X. arboricola* pv*. pruni* amino acid sequence with the Pfam:Swiss/PROT program, the alignment revealed similarities to a more extended portion of the *X. arboricola* pv*. pruni* sequence (between amino acids 84 and 235), with 25% identity and an E value of 1.9 e-10 (Fig. 6). When the *X. axonopodis* pv. *citri* AE012014 protein was subjected to BLASTP searches, a conserved domain

was predicted (SalY). This domain is described as involved in an ABC-type antimicrobial peptide transport system (Fig. 7).

Phylogenetic analysis. A phylogenic analysis of the ABC transporter proteins with the highest sequence similarities to the *X. arboricola* pv. *pruni* putative protein was performed. An evolutionary tree of aligned sequences calculated by ClustalW and PHYLIP showed molecular relationships between the putative *X. arboricola* pv. *pruni* protein and other ABC transporters (Fig. 8). The *X. arboricola* pv. *pruni* putative protein grouped into the same cluster with ABC proteins of one saprophytic bacterial soil and several plant pathogenic bacterial proteins. The tree-fitting value was 0.30. According to normal standards (>1 =poor fit; <1= good fit) this is considered a good fitting value.

Identification of ABC transporter motifs, signal sequences and protein localization. ABC motif prediction programs identified the presence of a Walker A motif located between amino acids 75 and 85, with a similarity of 50%; a Walker B motif was identified between amino acids 92 and 96, with a 53% similarity and between amino acids 231 and 235, with a similarity of 54%. A signature motif was identified between amino acids 69 and 74, with a 52% similarity. Results of the SignalP program indicated that the putative *X. arboricola pv. pruni* protein is a non-secretory protein. There was no evidence for a signal peptide since the signal peptide probability was zero (Fig. 9). The absence of signal peptides also was confirmed by PSORT B v.2.0 program (Fig. 10). The PSORT B. v.2.0 program predicted the localization of the *X. arboricola* pv.*pruni* putative protein at the cytoplasmic membrane with a score of 9.46 (Fig. 10). The analysis of the *X. axonopodis* pv. *citri* AE012014 protein with program SignalP suggested a signal peptide with 0.993 probability (Fig. 11).

Location of transmembrane helices. The TMHMM program indicated the presence of three transmembrane helices located in the C-terminal domain, in the amino acid positions: 119-141; 168-190 and 205-227 (Fig. 12). Similar results were obtained with the SOSUI program locating three transmembranes helices at amino acid positions: 117-139; 158-180 and 208-230 (Fig. 13). The SOSUI program

predicted the putative *X. arboricola* pv*. pruni* protein to be a membrane protein (Fig. 13). A graphical representation of the secondary structures of the *X. arboricola pv. pruni* putative protein is also shown in Fig. 13.

Predicted protein characteristics. The ProtParam program estimated the molecular weight of the *X. arboricola* pv. *pruni* putative protein as 26,816.8 ; the theoretical isoelectric point 5.98; the total number of negatively charged residues (Asp + Glu): 27, and the total number of positively charged residues (Arg + Lys): 24. The instability index (II) was computed to be 56.13. This classifies the protein as unstable (Table 1). The analysis of the *X. axonopodis* pv. *citri* AE012014 protein with the same program predicted a 47,835 molecular weight, an isolelectric point of 9.01; the total number of negatively charged residues (Asp + Glu): 39 and the total number of positively charged residues (Arg + Lys): 45. The instability index (II) was computed to be 35.59, classifying this protein as stable (Table 2).

TABLE 1. **Predicted protein characteristics - ProtParam tool -** *Xanthomonas arboricola* pv. *pruni* putative protein.

Number of amino acids: 243
Molecular weight: 26816.8
Theoretical pI: 5.98
Amino acid composition:

Ala (A)	28	11.5%
Arg (R)	18	7.4%
Asn (N)	2	0.8%
Asp (D)	15	6.2%
Cys (C)	5	2.1%
Gln (Q)	14	5.8%
Glu (E)	12	4.9%
Gly (G)	18	7.4%
His (H)	5	2.1%
Ile (I)	11	4.5%
Leu (L)	29	11.9%
Lys (K)	6	2.5%
Met (M)	8	3.3%
Phe (F)	11	4.5%
Pro (P)	7	2.9%
Ser (S)	16	6.6%
Thr (T)	11	4.5%
Trp (W)	3	1.2%
Tyr (Y)	6	2.5%
Val (V)	18	7.4%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 27
Total number of positively charged residues (Arg + Lys): 24

Atomic composition:

Carbon	C	1190
Hydrogen	H	1890
Nitrogen	N	332
Oxygen	O	347
Sulfur	S	13

Formula: $C_{1190}H_{1890}N_{332}O_{347}S_{13}$
Total number of atoms: 3772

Extinction coefficients:
Conditions: 6.0 M guanidium hydrochloride
0.02 M phosphate buffer
pH 6.5

Extinction coefficients are in units of M^{-1} cm^{-1} .
The first table lists values computed assuming ALL Cys residues appear as half cystines, whereas the second table assumes that NONE do.

276 nm	278 nm	279 nm	280 nm	282 nm

Ext. coefficient	25190	25454	25290	24990	24240
Abs 0.1% (=1 g/l)	0.939	0.949	0.943	0.932	0.904

	276 nm	278 nm	279 nm	280 nm	282 nm
Ext. coefficient	24900	25200	25050	24750	24000
Abs 0.1% (=1 g/l)	0.929	0.940	0.934	0.923	0.895

Estimated half-life:
The N-terminal of the sequence considered is M (Met).
The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index:
The instability index (II) is computed to be 56.13
This classifies the protein as unstable.

Aliphatic index: 97.20
Grand average of hydropathicity (GRAVY): 0.098

TABLE 2. **Predicted protein characteristics - ProtParam tool -** *Xanthomonas axonopodis* pv. citri AE012014 protein.

Number of amino acids: 433
Molecular weight: 47835.0
Theoretical pI: 9.01

Amino acid composition:

Ala (A)	46	10.6%
Arg (R)	29	6.7%
Asn (N)	16	3.7%
Asp (D)	23	5.3%
Cys (C)	4	0.9%
Gln (Q)	19	4.4%
Glu (E)	16	3.7%
Gly (G)	36	8.3%
His (H)	4	0.9%
Ile (I)	17	3.9%
Leu (L)	53	12.2%
Lys (K)	16	3.7%
Met (M)	11	2.5%
Phe (F)	20	4.6%
Pro (P)	16	3.7%
Ser (S)	28	6.5%
Thr (T)	20	4.6%
Trp (W)	9	2.1%
Tyr (Y)	16	3.7%
Val (V)	34	7.9%
Asx (B)	0	0.0%

Glx (Z) 0 0.0%
Xaa (X) 0 0.0%

Total number of negatively charged residues (Asp + Glu): 39
Total number of positively charged residues (Arg + Lys): 45

Atomic composition:

Carbon	C	2159
Hydrogen	H	3384
Nitrogen	N	588
Oxygen	O	611
Sulfur	S	15

Formula: $C_{2159}H_{3384}N_{588}O_{611}S_{15}$
Total number of atoms: 6757

Extinction coefficients:

Conditions: 6.0 M guanidium hydrochloride
0.02 M phosphate buffer
pH 6.5

Extinction coefficients are in units of M^{-1} cm^{-1} .

The first table lists values computed assuming ALL Cys residues appear as half cystines, whereas the second table assumes that NONE do.

	276 nm	278 nm	279 nm	280 nm	282 nm
Ext. coefficient	72090	73054	72700	71930	69840
Abs 0.1% (=1 g/l)	1.507	1.527	1.520	1.504	1.460

	276 nm	278 nm	279 nm	280 nm	282 nm
Ext. coefficient	71800	72800	72460	71690	69600
Abs 0.1% (=1 g/l)	1.501	1.522	1.515	1.499	1.455

Estimated half-life:
The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index:
The instability index (II) is computed to be 35.59
This classifies the protein as stable.
Aliphatic index: 96.44

Fig . 1. Nucleotide sequence generated with primers Y17CoF and Y17CoR. Primer locations are shown on yellow.

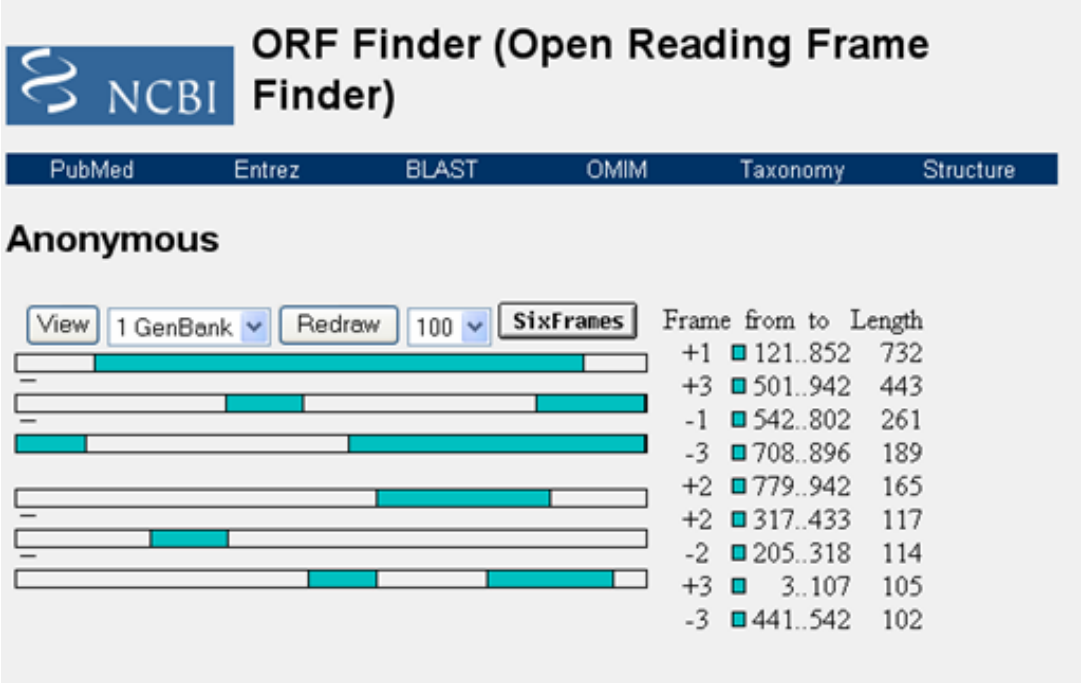
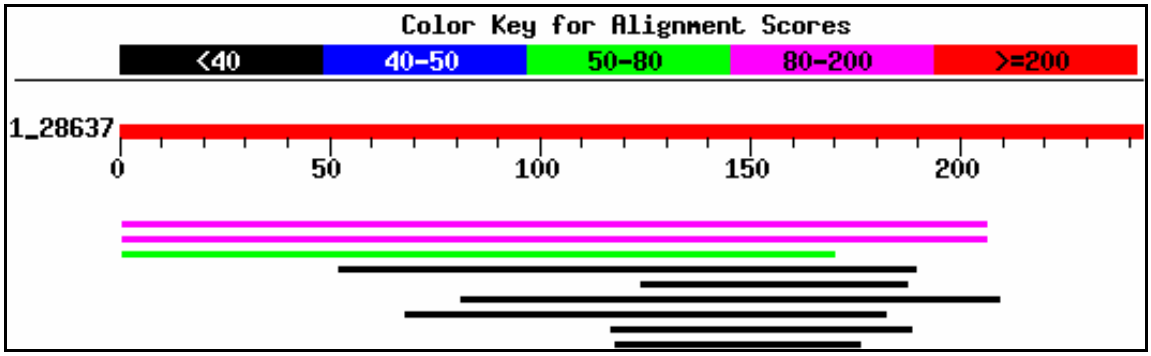


Fig . 2. Prediction of ORFs with ORF Finder (Open Reading Frame Finder) NCBI.



Sequences producing significant alignments:		Score (bits)	E Value
qi|21110022|qb|AAM38483.1|	ABC transporter ATP-binding prot...	141	1e-32
qi|21111562|qb|AAM39882.1|	ABC transporter ATP-binding prot...	98	2e-19
qi|13272378|qb|AAK17128.1|	putative membrane transporter pr...	71	3e-11

Fig . 3. BLASTP search (NCBI) with *Xanthomonas arboricola* pv. *pruni* ORF sequence.

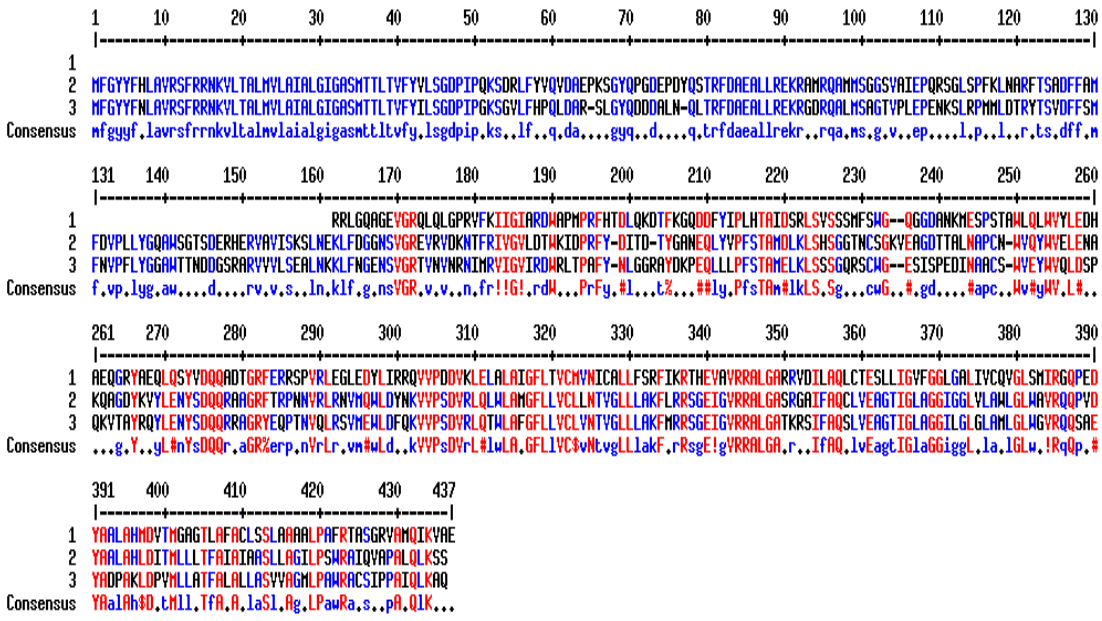
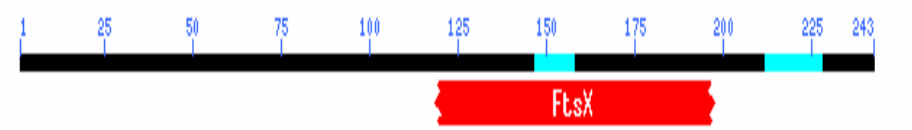


Fig . 4. Alignment of ABC transporter proteins (Clustal W).
1= ORF sequence of *Xanthomonas arboricola* pv. *pruni*; 2= *Xanthomonas axonopodis* pv. *citri*, ABC protein (AE012014) and 3= *Xanthomonas campestris* pv. *campestris,* ABC protein *(AE012154)*.Red color shows perfect identity and blue color shows similarity.

A



B

gnl|CDD|23940, pfam02687, FtsX, Predicted permease. This is a family of predicted permeases and hypothetical transmembrane proteins. One member has been shown to transport lipids targeted to the outer membrane across the inner membrane. Two members have been shown to require ATP.

CD-Length = 172 residues, only 47.1% aligned
Score = 38.8 bits (90), Expect = 5e-04

```
Query:  118  LALAIGFLTVCMVNICALLFSRFIKRTHEVAVRRALGARKVDILAQLCTESLLIGVFGGL  177
Sbjct:  52   ILIAVLILLVAALGIGNTLLLSVAERRREIGILKALGASRRQILRIFLLEGLLLGLLGGL  111

Query:  178  GALIVCQVGLSMIRGQPEDYA  198
Sbjct:  112  LGLLLGYLLAYLLLVALSYF  132
```

Fig . 5. A, Conserved domain FtsX pfam 02687 identified in the *Xanthomonas arboricola pv.pruni* putative protein sequence (BLASTP, NCBI).
B, Alignment of FtsX pfam 02687 domain with the *Xanthomonas arboricola* pv.*pruni* putative protein sequence.

Pfam HMM search results. Pfam 15.0.

	Model	Seq-from	Seq-to	HMM-from	HMM-to	Score	E-value	Description
!!	FtsX	84	235	1	167	45.2	**1.9e-10**	Predicted permease

Alignments of top-scoring domains:

```
FtsX:  1 of 1, from 84 to 235: score 45.2, E = 1.9e-10
                  *->vtaivvkakdpanvdalakklekleilelfsalssiktllgliamvi
                     +++ + +  +p++++ l + l +       +++     l +++a +
       PRUNI    84    ADTGRFERRSPVRLEGLEDYLIRR------QVVPDDVKLELALA-IG

                     sllvaalgigntllmsvteRtrEIgiLrAlGAsrrqIlriFllEglllgl
                      l v+ ++i  +l   ++ Rt+E++++rAlGA+r +Il+++ +E+ll+g+
       PRUNI   124 FLTVCMVNICALLFSRFIKRTHEVAVRRALGARRVDILAQLCTESLLIGV

                     lGgllGlllGllanltliflayllllalsyflstlplslsp.avllalll
                     +Ggl +l++  +       +  ++   +    ++   +++ +  +++l++
       PRUNI   174 FGGLGALIVCQV-------GLSMI--RGQPEDYAALAHMDVtMGAGTLAF

                     alligllagllParrAarldP<-*
                     a l +l a++lPa r a   +
       PRUNI   215 ACLSSLAAAALPAFRTASGRV    235
```

Fig. 6. Alignment of the FtsX amino acid sequence with the *Xanthomonas arboricola* pv.*pruni* putative protein (Pfam:Swiss/PROT).

Query = (433 letters)

Putative conserved domains have been detected

Fig . 7 . Conserved domain SalY identified in the *Xanthomonas axonopodis* pv.*citri* AE012014 protein (BLASTP, NCBI).

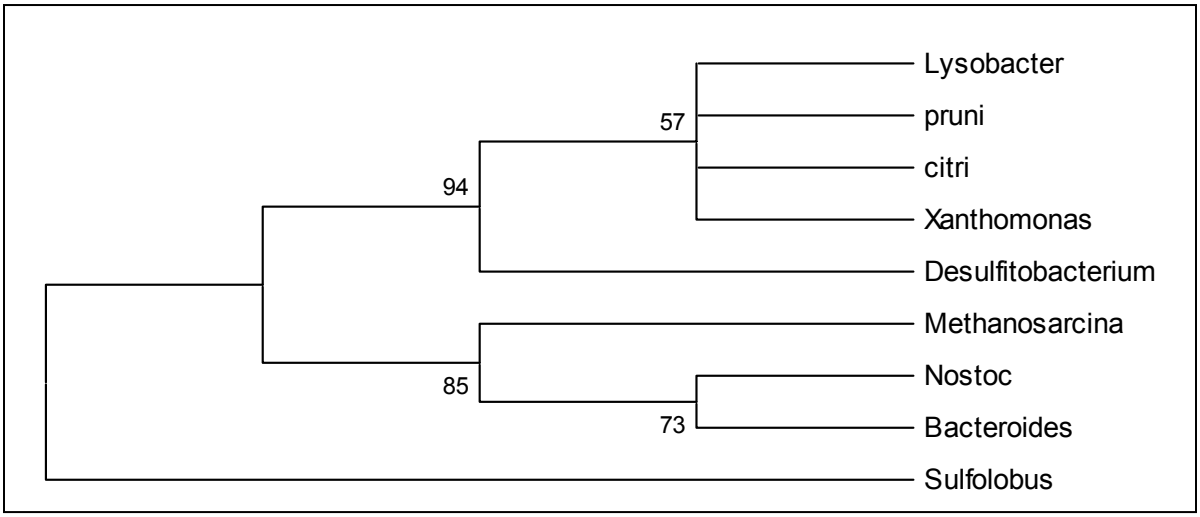


Fig . 8 . Phylogenetic Tree, of several ABC transporter proteins with similarity to the identified *Xanthomonas arboricola pv. pruni* ORF (PHYLIP).
Lysobacter*: Lysobacter lactamgenus* (AF315510); pruni: *X. arboricola* pv. *pruni* (putative protein); citri: *X. axonopodis* pv. *citri* (AE012014); Xanthomonas: *X. campestris* pv. *campestris* (AE012154); Desulfitobacterium: *Desulfitobacterium hafniense* (gi23120215); Methanosarcina: *Methanosarcina acetivorans* (AE010705); Nostoc: *Nostoc punctiforme* (gi 23128707); Bacteroides: *Bacteroides thetaiotaomicron* (29346625); Sulfolobus: *Sulfolobus solfataricus* (AE006695)

SignalP 3.0 Server

SignalP-NN result

# Measure	Position	Value	Cutoff	signal peptide?
max. C	26	0.046	0.52	NO
max. Y	42	0.068	0.33	NO
max. S	1	0.457	0.92	NO
mean S	1 – 41	0.129	0.49	NO
mean D	1 – 41	0.099	0.44	NO

SignalP-HMM result:

Prediction: Non-secretory protein
Signal peptide probability: 0.000
Max cleavage site probability: 0.000 between pos. 41 and 42.

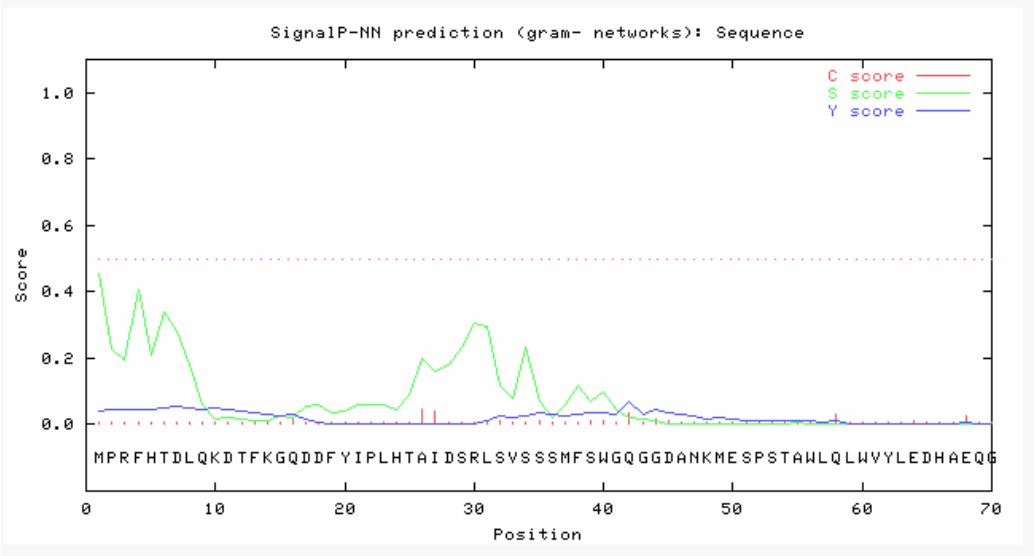


Fig . 9 . Predictions of signal sequences (SignalP Server 3.0) in the *Xanthomonas arboricola* pv. *pruni* ORF.

PSORT-B Results

```
SeqID: MPRFHTDLQK
  Analysis Report:
    Signal-           Unknown             [No signal peptide detected]
  Localization Scores:
    Cytoplasmic             0.04
    Cytoplasmic Membrane     9.46
    Periplasmic             0.07
    OuterMembrane           0.18
    Extracellular           0.25
  Final Prediction:
    Cytoplasmic Membrane     9.46
```

Fig . 10 . Prediction of the *Xanthomonas arboricola* pv.*pruni* putative protein localization (PSORT-B v.2.0).

SignalP 3.0 Server

SignalP-NN result:

>Sequence	#	Measure	Position	Value	Cutoff	signal peptide?
max. C	27		0.045	0.52		NO
max. Y	29		0.130	0.33		NO
max. S	26		0.988	0.92		YES
mean S	1-28		0.725	0.49		YES
mean D	1-28		0.428	0.44		NO

Most likely cleavage site between pos. 28 and 29: AIA-LG

SignalP-HMM result:

>Sequence
Prediction: Signal peptide
Signal peptide probability: 0.993
Max cleavage site probability: 0.686 between pos. 45 and 46

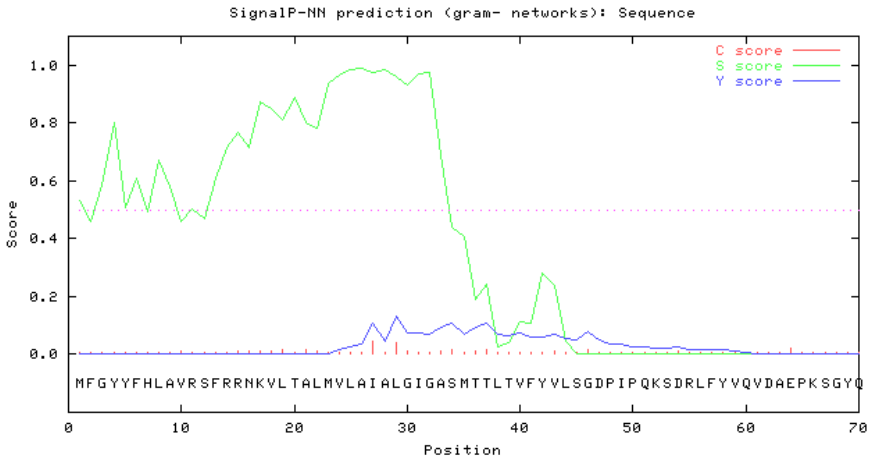


Fig . 11. Predictions of signal sequences (SignalP Server 3.0) in the *Xanthomonas axonopodis* pv. *citri* AE012014 protein.

TMHMM result

Sequence Length: 243

# Sequence Number of predicted		TMHs: 3
# Sequence Exp number of AAs in		TMHs: 68.09591
# Sequence Exp number, first 60 AAs:		0.08692
# Sequence Total prob of N-in:		0.00713
Sequence TMHMM2.0	outside	1 118
Sequence TMHMM2.0	TMhelix	119 141
Sequence TMHMM2.0	inside	142 167
Sequence TMHMM2.0	TMhelix	168 190
Sequence TMHMM2.0	outside	191 204
Sequence TMHMM2.0	TMhelix	205 227
Sequence TMHMM2.0	inside	228 243

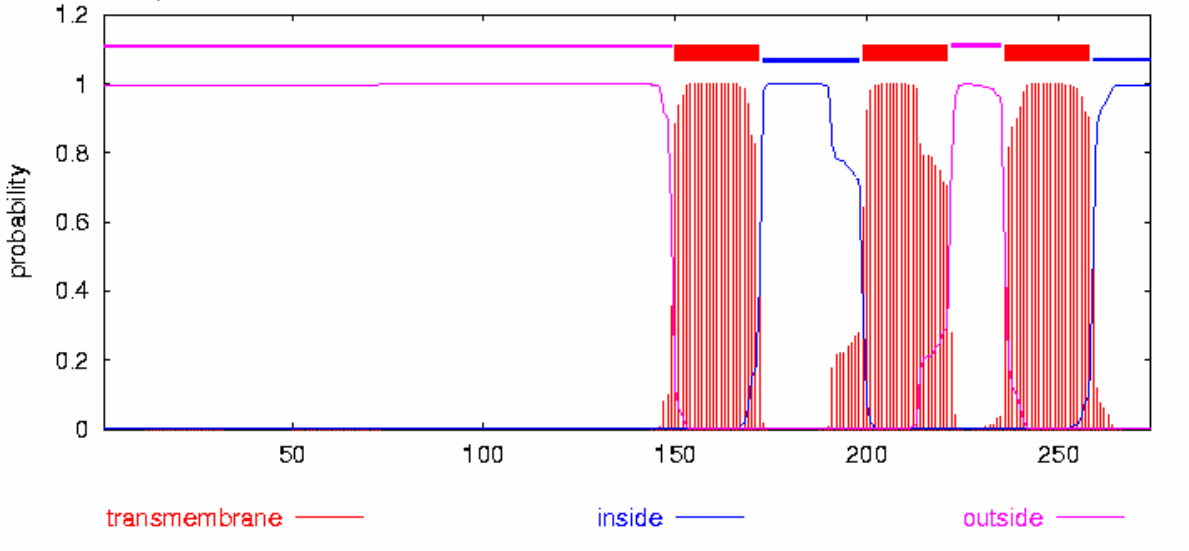


Fig . 12 . Prediction of transmembrane helices (TMHMM Server v.2.0) in the *Xanthomonas arboricola* pv.*pruni* ORF.

SOSUI Result

Total length : 243 A. A.

Average of hydrophobicity : 0.098354

This amino acid sequence is of a MEMBRANE PROTEIN which have 3 transmembrane helices.

No.	N terminal	transmembrane region	C terminal	type	length
1	117	ELALAIGFLTVCMVNICALLFSR	139	PRIMARY	23
2	158	VDILAQLCTESLLIGVFGGLGAL	180	SECONDARY	23
3	208	GAGTLAFACLSSLAAAALPAFRT	230	SECONDARY	23

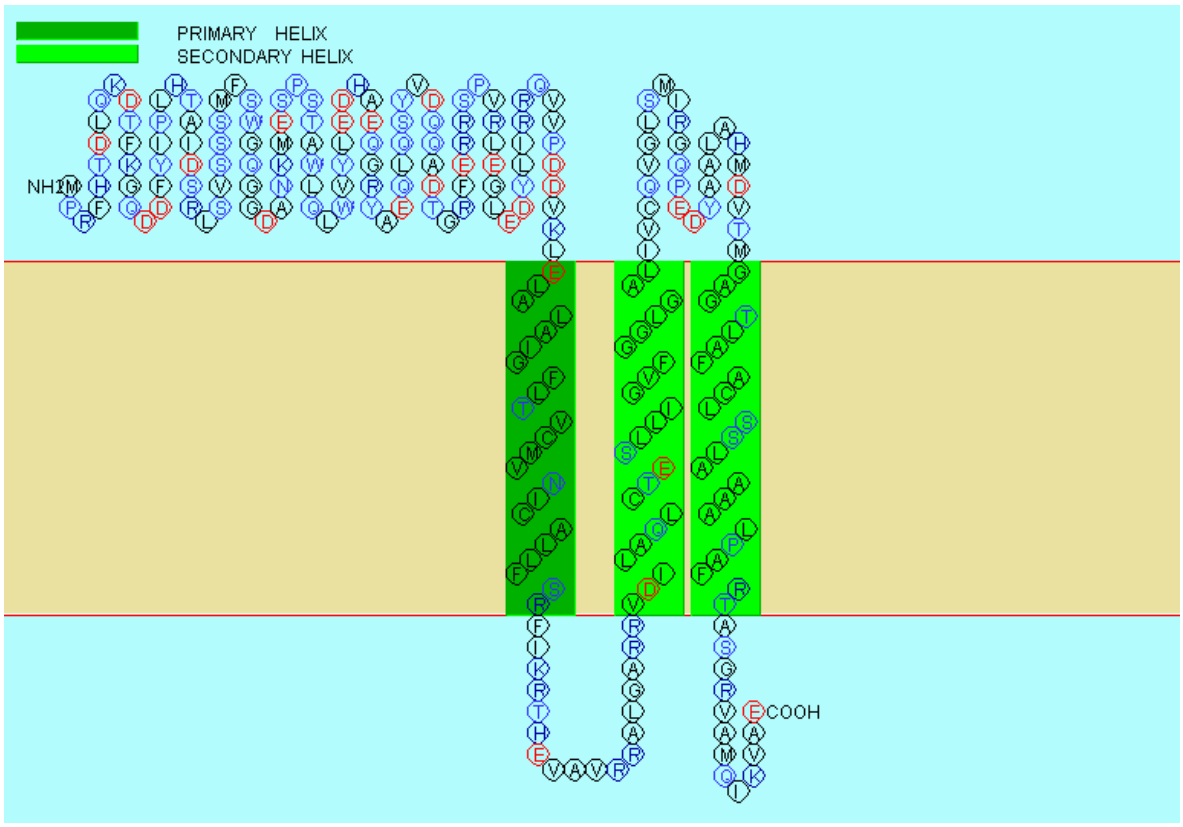


Fig. 13. Prediction of secondary structures in the *Xanthomonas arboricola* pv. *pruni* putative protein with SOSUI program.

DISCUSSION

Recently, bioinformatic programs have provided the tools to explore protein structures and to predict putative functions (Altschul *et al*., 1997; Ren *et al*., 2004). They provide the features to examine in detail specific domains and motifs and to make comparisons with other proteins. Predictive methods also allow an understanding of the possible structure and function of proteins whose existence is only hypothesized based on genomic sequence data (Bairoch and Apweiler, 1999).

The aim of this work was to analyze a DNA sequence identified in *X. arboricola* pv*. pruni* through the use of bioinformatic programs to glean information about its possible function. In this study we *in silico* identified and characterized an ORF found in a 943-bp DNA fragment of the genome of this bacterium. The characterization of this ORF was an initial step towards understanding its biological function. Results of the *in silico* analysis suggested the presence of an ORF in the *X. arboricola* pv. *pruni* DNA fragment with the potential to encode a protein with similarity to members of the ABC transporter family and possibly involved in cell division.

So far, several bacterial transport systems have been found to contain integral membrane proteins with similar sequences (Reizer *et al*, 1992). The ABC transporter system is one of the active transport systems in the cells, capable of using the hydrolysis of ATP to energize diverse functions. ABC transporter proteins of bacterial origin appear to perform functions affecting processes as diverse as the export and import of a wide variety of solutes, cell division and regulation of translation of peptides. Initially, members of this family were solely considered ATPases with a distinct role in the export or import of solutes (Holland *et al*. 2003). Since 1989, several new members of this family have been discovered, increasing the knowledge of the diversity of the functions they perform. There are now well documented examples of ABC transporters involved in a varied range of functions such as osmoprotection, cell division, control of cell volume in response to osmotic shock, (Fe/S) cluster assembly, among some (Booth, I., 1992; Garland *et al*., 1999;

Schmidt *et al*., 2004). However, there are still examples of numerous ABC proteins where their functions remain unknown (Holland *et al.,* 2003).

The comparisons with amino acid sequences available at the time of the search (August, 2004), indicated that the putative *X. arboricola* pv. *pruni* protein shares similarity (39% and 32% amino acid identity) with two ABC transporter ATP binding proteins from the closely related plant pathogens *X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris,* respectively. These two ABC transporters were described as membrane components with an ATP-binding function. They are putative proteins predicted to be components of the ABC-type antimicrobial peptide transport system (NCBI, CDART). The functional prediction of these putative proteins remains to be confirmed since it has been inferred from informatics and was not subjected to functional experimentation.

The role of bioinformatics and computational biology in proteomics research is frequently challenged with the presence of functional diversity in closely related proteins (Wu *et al*., 2004). Conversely, often the reaction chemistry that determines the functional role of a sequence remains conserved even when sequences diverge almost beyond recognition. This fact emphasis the need for an integrated approach for the functional assignment of predicted ORFs considering simultaneously the whole protein sequence and the domain information (Wu *et al.,* 2004).

In our studies, the *X. arboricola* pv. *pruni* putative protein based on sequence alignment analysis was found to share the highest similarity with the *X. axonopodis* pv. *citri* AE012014 protein of the ABC transporter family, even though, further characterization and comparisons between both proteins showed potential divergence in their functions.

A fundamental distinction between secretory and non-secretory proteins is the presence of a cleavable N-terminal sorting signal in secretory peptides predicted by the SignalP program. Signal peptides are peptide sequences that direct the proteins from the cytosol to different destinations. They typically serve as signals for entering the secretory pathway. Because of the high probability of protein AE012014 of presenting a signal peptide (Fig. 11), it is likely to be considered a secretory protein

or in plasmalemma. In the case of the *X. arboricola* pv. *pruni* putative protein this probability is predicted as zero (Fig. 9), suggesting a functional difference between both.

The BLASTP and Pfam programs predicted the presence of the putative conserved domain FtsX, pfam 02687 in the *X. arboricola* pv. *pruni* protein sequence. FtsX is an ABC transporter, transmembrane protein only found in eubacteria and belonging to the class 3 system (ABC proteins which apparently are not involved in import) and to the CDI familiy involved in cell division (Holland *et al*., 2003; Schmidt *et al*, 2004). This class of ABC proteins appears to be involved in cellular processes like DNA repair or cell division, rather than in transport. The FtsX protein, homodimerizes with another protein (FtsE) in order to form an essential operon (FtsE/FtsX) directly involved according to numerous studies in the processes of cell division and in the assembly and stability of a septum at the middle of the cell (Schmidt *et al*., 2004). In contrast, protein AE012014 presented a conserved domain SalY involved in the transport of antimicrobial peptides (Fig. 7) essential in competitive fitness against other bacteria.

By protein sequence comparison using scores derived from the BLAST algorithm, we constructed a phylogenetic tree with amino acid sequences from organisms that exhibit the highest similarity scores with the *X. arboricola pv. pruni* putative protein. Recent comparative analysis of ABC transporters in microbial genomes (Tommi and Kanehisa, 1998) suggest that ancestral ABC transporter operons may have arisen early in evolution before the speciation of bacteria and archae. This is not perceived in our analysis, where the putative *X. arboricola* pv*. pruni* protein is clustered in the same clade with other related gram-negative plant pathogenic bacterium (*X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris*) and one gram-negative soil bacteria (*Lysobacter lactamgenus*) (Fig. 8). Correspondingly, members of archae (*Sulfolobus solfataricus*, and *Methanosarcina acetivorans*) and cyanobacteria (blue green algae) (*Nostoc punctiforme*) all share a different clade.

Additional information was inferred through the alignment of the *X. arboricola* pv*. pruni* putative protein with conserved consensus domains of the ABC protein

family. Even though the conserved motifs Walker A, Walker B and signature motif, which characterize this family were identified in this putative protein, they did not present significant values. Our results are in agreement with functional predictions made by Tommi and Kanehisa, 1998, which show that although most of the membrane proteins in the class 3 possess conserved regions, their similarity is somewhat weak. According to these authors, the class 3 cellular division (CDI) family contains the largest number of ATP-binding proteins having unknown functions.

Studies conducted by Conti and others, 1998 (Conti *et al*., 1998) demonstrated that some proteins have intrinsic signals that govern their transport and localization in the cell. One of the crucial properties of a protein is its subcellular location and prediction of protein sorting is an important question in bioinformatics. Analysis of the *X. arboricola* pv*. pruni* putative protein localization with the PSORT B program revealed with high confidence that this putative protein is located at the cytoplasmic membrane (Fig. 10).

A remarkable similarity on the content of leucine, alanine and valine as the three most abundant amino acids was found between the putative *X. arboricola* pv*. pruni* protein and FtsX in *E. coli*. The content of these three amino acids represent 30.8% and 31% of the total protein, respectively (Blattner *et al*, 1997).

Prediction of transmembrane helices with TMHMM and SOSUI programs clearly show the location of three transmembrane helices mapping in the C-terminal hydrophobic domain (Fig. 13). Interestingly, protein FtsX in *E. coli* and in *M. tuberculosis*, however containing four transmembrane helices, three of them are located in the C-terminal hydrophobic domain. These proteins were also found in the same cellular location as the *X. arboricola* pv*. pruni* putative protein (Tekaia *et al*., 1999 and Tyagi *et al.*,1996)

Secondary structure predictions for most ABC transporters typically estimate six transmembrane α-helices per monomer (Holland *et al*., 2003). However, several exceptions have been found in the literature that provide evidence that exceptions to this rule in ABC transporters might be more common than previously thought.

Recent studies show that ABC proteins that belong to the cell division family have usually four transmembrane segments rather than six (Tomii and Kanehisa, 1998). Molecular analysis on the xylose ABC transporter protein XylH in *Thermoanaerobacter ethanolicus* show the presence of 11 and 12 transmembrane segments, although XylH protein in *E. coli* presents 10 and 11 (Erbeznik *et al*. 2004). Differences in the number of transmembrane segments are also present between transporters with broad and narrow solute-specificity. General amino acid permeases usually have eight or nine transmembrane segments, whereas integral membrane proteins with narrow-solute specificity are typically predicted to have five. (Walshaw *et al*.,1997). One potential explanation of the divergence between the prevalent numbers of transmembrane segments found in the vast majority of ABC proteins and our results is the possibility that the *X. arboricola* pv. *pruni* putative protein sequence does not comprise in fact the entire protein, and the stop codon predicted could be the result of an error in the sequenced fragment. The fact that this is a new family and there is not yet much understanding about its organization and structure, leaves an open question about this point. Even though it is possible to have a prediction of the structure and function of this protein from its sequence analysis, the adaptable nature of ABC transporters ensures that exceptions will certainly challenge our expectations.

The data gathered here from a number of prediction programs agree on several points. All provide persuasive evidence to postulate that the predicted *X. arboricola* pv. *pruni* putative protein is an integral membrane protein with similarity to the ABC (ATP-Binding cassette) system and with a domain related to the cell division family. It remains to be determined whether this protein is indeed functional under normal growth conditions and whether it does in fact participate in cellular division processes.

LITERATURE CITED

Abele, R., and Tampe, R. 1999. Function of the transport complex ATP in cellular immune recognition. Biochim. Biophys. Acta 1461: 405-419.

Allignet, J., Loncle, V., and El Solh, N. 1992. Sequence of a staphylococcal plasmid gene, *vga*, encoding a putative ATP-binding protein involved in resistance to virginiamycin A-like antibiotics. Gene 117: 45-51.

Allikmets, R., Shroyer, N. F., Singh, N., Seddon, J. M., Lewis, R. A., Bernstein, P. S., *et al*. 1997. Mutation of the Stargardt disease gene (ABCR) in age-related macular degeneration. Science 277: 1805-1807.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zheng Zhang, J. Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acid Res. 25: 3389-3402.

Bairoch, A., and Apweiler, R. 1999. The SWISS-PROT protein sequence data bank and its supplement TrEMBL. Nucleic Acid Res. 27: 49-54.

Bauer, B. E., Wolfger, H., and Kuchler, K. 1999. Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. Biochim. Biophys. Acta 1461: 217-236.

Bendtsen, J.D., Nielsen, H., von Heijne, G., and Brunak, S. 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340: 783-795.

Blattner, F. R., Plunkett, G. I., Bloch, C. A. Perna, N. T., Burland, V., Riley, M. *et al.* 1997. The complete genome sequence of *Escherichia coli* K12. Science 28: 1453-1462.

Booth, I.R. 1992. Regulation of gene expression during osmoregulation: the role of potassium glutamate as a secondary signal of osmotic stress. Pages: 205-224 *in*: Alkali Cation Transport Systems in Prokayotes (ed.Bakker). CRC Press.

Burse, A., Weingart, H., and Ullrich, M. 2004 a. NorM, an *Erwinia amylovora* multidrug efflux pump involved in in vitro competition with other epiphytic bacteria. Appl. Environ. Microbiol. 70: 693–703

Burse, A., Weingart, H., and Ullrich, M.S. 2004 b. The phytoalexin-inducible multidrug efflux pump AcrAB contributes to virulence in the fire blight pathogen, *Erwinia amylovora*. Mol. Plant-Microbe Interact. 17: 43-54.

Chakraburtty, K. 1999. Functional interaction of yeast elongation factor 3 with yeast ribosomes. Int. J. Biochem. Cell Biol. 31: 163-173.

Combet, C., Blanchet, C., Geourjon, C., and Deleage, G. 2000. NPS@: Network Protein Sequence Analysis TIBS 25. 291: 147-150.

Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. 1998. Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha. Cell 94: 193-204.

Dassa, E., and Bouige, P. 2001. The ABC of ABCs: a phylogenetic and functional classification of ABC systems in living organisms. Res. Microbiol. 152: 211-229.

Decottignies, A., and Goffeau, A. 1997. Complete inventory of the yeast ABC proteins. Nature Genetics. 15:137-145.

Doolittle, R. F., Johnson, M. S., Husain, I., Van, H. B., Thomas, D. C.,and Sancar, A. 1986. Domainal evolution of a prokaryotic DNA repair protein and its relationship to active-transport proteins. Nature 28: 451-453.

Erbeznik, M., Hudson, S., Herrman, A., and Strobel, H. 2004. Molecular analysis of the xylFGH Operon, coding for xylose ABC transport in *Thermoanaerobacter ehtanolicus*. Curr. Microbiol. 48: 295-299.

Fath, M. J., and Kolter, R. 1993. ABC transporters - bacterial exporters. Microbiol. Rev. 57: 995-1017.

Felsenstein, J. 1993. PHYLIP Version 3.c. Dept. of Genetics. University of Washington, Seattle.

Fleissner, A., Sopalla, C., and Weltring, K. 2002. An ATP-binding cassette multidrug-resistance transporter is necessary for tolerance of *Gibberella pulicaris* to phytoalexins and virulence on potato tubers. Mol. Plant-Microbe Interact. 15: 102-108.

Gardy, J., Spencer, C., Wang, K., Ester, M., Tusnády, G. E., Simon, I. *et al*. 2003. PSORT-B: improving protein sub cellular localization prediction for Gram-negative bacteria. Nucleic Acid Res. 31: 3613-3617.

Garland, S.A., Hoff, K., Vickery, L.E., and Culotta, V.C. 1999. *Saccharomyces cerevisiae* ISUI and ISU2: members of a well conserved gene family for iron-sulfur cluster assembly. J. Mol. Biol. 294: 897-907.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., and Bairoch, A. 2003. *ExPASy: the proteomics server for in-depth protein knowledge and analysis*. Nucleic Acid Res. 31: 3784-3788.

Gaudet, R., and Wiley, D.C. 2001. Structure of the ABC ATPase domain of human TAP1, the transporter associated with antigen processing. EMBO J. 20: 4964-4972.

Goosen, N., and Moolenaar, G. F. 2001. Role of ATP hydrolysis by UvrA and UvrB during nucleotide excision repair. Res. Microbiol. 152: 401-409.

Higgins, C. F., Hiles, I. D., Salmond, G. P., Gill, D. R., Downie, J. A., Evans, .I J., Holland, I. B., Gray, L., Buckel, S. D., Bell, A. W., *et al*. 1986. A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. Nature 323: 448-450.

Higgins, C. F. 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. 8: 67-113.

Higgins, C.F. 1995. P-glycoprotein and cell volume-activated chloride channels. J. Bioenerg. Biomembr. 27: 63-70.

Hirokawa, T., Boon-Chieng, S., and Mitaku, S. 1998. SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics. 14(4): 378-379.

Holland, I. B., Cole, S. P., Kuchler, K., and Higgins, C. F. 2003. ABC proteins. From Bacteria to Man. Acad.Press.

Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R. *et al*. 1990. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature 28: 362-365.

Klein, I., Sarkadi, B., and Varadi, A. 1999. An inventory of the human ABC proteins. Biochim. Biophys. Acta. 1461: 237-262.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignel,I S.C., Bron, S., Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.K., Codani, J.J., Connerton, I.F., Danchin, A., *et al*. 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature. 390: 237-238.

Letunic,I., Copley, R., Schmidt, S., Ciccarelli,F. D., Doerks, T., Schultz, J., Ponting, C. P., and Bork, P. 2004. SMART 4.0: towards genomic data integration. Nucleic Acid Res. Vol. 32 Database issue D142-D144.

Linton, K. J., and Higgins, C. F. 1998. The *Escherichia coli* ATP-binding cassette (ABC) proteins. Mol. Microbiol. 28: 5-13.

Llama-Palacios, A., Lopez-Solanilla, E., and Rodriguez-Palenzuela, P. 2002. The *ybiT* gene of *Erwinia chrysanthemi* codes for a putative ABC transporter and is involved in competitiveness against endophytic bacteria during infection. Appl. Environ. Microbiol. 68: 1624-1630.

Mulder, N.J., and Apweiler, R. 2001. Tools and resources for identifying protein families, domains and motifs. Genome Biol. 3(1) www.pubmedcentral.nih.gov/articlerender.

Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineering. 10: 1-6.

Pagani, M.C., and Ritchie, D.F. 2002. Detection of *Xanthomonas arboricola* pv. *pruni* with a PCR primer set and a digoxigenin-labeled DNA probe. (Abst.) Phytopathology 92:S62.

Paulsen, I.T., Nguyen, L., Sliwinski, M. K., Rabus, R., and Saier, M. H. 2000. Microbial genome analyses: comparative transport capabilities in eighteen prokaryotes. J. Mol. Biol. 301: 75-100.

Quinton, P. M., and Bijman, J., 1983. Higher bioelectric potentials due to decreased chloride absorption in the sweat glands of patients with cystic fibrosis. N. Engl. J. Med. 308: 1185-1189.

Reizer, J., Reizer, A., and Saier, M. 1992. A new subfamily of bacterial ABC-type transport systems catalyzing export of drugs and carbohydrates. Protein Sci. 1: 1326-1332.

Ren, Q., Kang, K. H., and Paulsen, I. T. 2004. TransportDB: a relational database of cellular membrane transport systems. Nucleic Acid Res. Vol. 32, Database issue D284-D288.

Salen, G., Shefer, S., Nguyen, L. Ness, G. C., Tint, G. S., and Shore, V. 1992. Sitosterolemia. J. Lipid Res. 33: 945-955.

Saurin, W., Hofnung, M., and Dassa, E. 1999. Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. J. Mol. Evol. 48: 22-41.

Schmidt, K., Peterson, N., Kustusch, R., Wissel, M., Graham, B., Phillips, G., and Weiss, D. 2004. A predicted ABC transporter, FtsEX, is needed for cell division in *Escherichia coli*. J. Bacteriol. 186: 785-793.

Schneider, E., and Hunke, S. 1998. ATP-binding-cassette (ABC) transport systems: Functional and structural aspects of the ATP-hydrolyzing subunits/domains. FEMS Microbiol. Rev. 22: 1-20.

Schoonbeek, H., Del Sorbo, G., and De Waard, M. 2001. The ABC transporter *BcathB* affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. Mol. Plant-Microbe Interact. 14: 562-571.

Schoonbeek, H., Raaijmakers, J., and De Waard, M. 2002. Fungal ABC transporters and microbial interactions in natural environments. Mol. Plant-Microbe Interact. 15: 1165-1172.

Taglicht, D. and Michaelis, S. 1998. *Saccharomyces cerevisiae* ABC proteins and their relevance to human health and disease. Methods Enzymol. 292: 130-162.

Tekaia, F., Gordon, S. V., Garnier, T., Brosh, R., Barrell, B. G., and Cole, S. T. 1999. Analysis of the proteome of *Mycobacterium tuberculosis in silico*. Tubercle Lung Dis. 79: 329-342.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acid Res. 22: 4673-4680.

Tomii, K., and Kanehisa, M. 1998. A comparative analysis of ABC transporters in complete microbial genomes. Gen. Res. 8: 1048-1059.

Tusnády, G. E., and Simon, I. 2001. The HMMTOP transmembrane topology prediction server. Bioinformatics 17: 849-850.

Tyagi, J. S., Das, T., and Kinger, A. 1996. An *M. tuberculosis* DNA fragment contains genes encoding cell division proteins ftsX and ftsE, a basic protein and homologues of PemK and Small protein B. Gene 177: 59-67.

Vauterin, L., Hoste, B., Kersters, K., and Swings, J. 1995. Reclassification of *Xanthomonas*. Int. J. Syst. Bacteriol. 45: 472-489.

Vermeulen, T., Schoonbeek, H., and De Waard, M. 2001. The ABC transporter *BcatrB* from *Botrytis cinerea* is a determinant of the activity of the phenylpyrrole fungicide fludioxonil. Pest Manage. Sci. 57: 393-402.

Walshaw, D.L., Lowthorpe, S., East, A., and Poole, P.S. 1997. Distribution of a sub-class of bacterial ABC polar amino acid transporter and identification of an N-terminal region involved in solute specificity. *FEBS* Lett. 414: 397-401.

Wu, C., Nikolskaya, Huang, H., Yeh,L., Natale, D.A., Vinayaka,C.R., Hu, Z., Mazumder, R., Kumar, S., Kourtesis, P., Ledley, R., Suzek, B., Arminski, L., Chen, Y., Zhang,J., Cardenas,J.L., Chung, S., Castro-Alvear, J., Dinkov, G., and Barker,

W. 2004. PIRSF: family classification system at the Protein Information Resource. Nucleic Acid Res. 32 (Database-Issue): 112-114.

In vitro expression of a putative protein and insertional mutagenesis of a predicted ORF in *Xanthomonas arboricola* pv*. pruni* with similarity to the ABC (ATP-binding cassette) system.

ABSTRACT

An open reading frame (ORF) was earlier identified in a DNA fragment of the plant pathogenic bacterium *Xanthomonas arboricola* pv. *pruni*. This 732 bp-ORF was predicted to encode a protein of 243 amino acids with sequence similarity to two proteins related to the ABC transporter family. The greatest similarity at the amino acid level was to *X. axonopodis* pv*. citri* (39% identity) and *X. campestris* pv. *campestris* (32% identity). *In silico* analysis previously characterized this *X. arboricola* pv. *pruni* putative protein as an integral membrane protein with similarity to the ABC (ie, ATP-binding cassette) system. A database search identified a conserved domain in the *X. arboricola* pv. *pruni* protein, 25% identical and 67% similar to the FtsX Pfam02687, a predicted permease transmembrane protein in *Escherichia coli,* which is part of the operon FtsE/FtsX involved in cell division. In the present study we examined the possible function of the predicted *X. arboricola* pv. *pruni* ORF by gene disruption through homologous recombination with a streptomycin cassette. ORF-disruptants were isolated and their phenotypes recorded. We cloned and analyzed the *X. arboricola* pv. *pruni* ORF designated *XapftsX* and expressed its gene product designated as *Xap*FtsX. The *Xap*FtsX protein was overexpressed in a pET system with *E. coli* cells. SDS-polyacrylamide gel electrophoresis revealed the presence of a protein with a molecular size of 26 kDa . Western and immunoblot analysis confirmed these results. The ORF disruption in *X. arboricola* pv. *pruni* cells resulted in attenuation of cell division and eventually cell death soon after its initial growth, possibly resulting from the depletion of salts in the sucrose-peptone-agar medium. Similar to homologous *fts*X in other organisms, our results suggest the involvement of *XapftsX* in cell division.

INTRODUCTION

ABC transporters comprise one of the largest protein families present in a wide variety of organisms from bacteria to humans (Higgins, 1992). They are membrane proteins capable of using the hydrolysis of ATP to energize several functions in the cell (Holland, 2003). ABC transporters have many roles in plant pathogens. They play an essential role in multidrug resistance (MDR), well described in filamentous fungi (Del Sorbo, 1997; Nakaune *et al*., 1998) protecting pathogens against a variety of compounds (Vermeulen *et al*., 2001). ABC transporters can also act as virulence factors; they mediate secretion of virulence factors or provide protection against plant defense compounds during pathogenesis (Zwiers, 2002).

Cell division and DNA replication are processes central to life. Most rod-shaped bacteria divide symmetrically during normal growth, constricting at the middle, by building a central septum across the middle of the cell. (Bramhill, 1997). One of the essential roles of ABC proteins is their involvement in cell division (de Leeuw *et al*., 1999; Schmidt *et al*., 2004). One of the first studies in cell division involving ABC transporter proteins was done by Hirota and others (1968). They identified genes in *Escherichia coli* involved in cell division by isolating conditional lethal mutants that formed long, non-septate filaments at a restrictive temperature (Hirota *et al*. 1968). Collectively, these mutants have been designated *fts* (filament-forming temperature sensitive) due to their ability to continue elongation in the absence of division. The absence of checkpoint controls to ensure the successful conclusion of cytokinesis before continuing with the next round of DNA synthesis allows the filaments to grow even when division is blocked by mutation (Bramhill, 1997).

ftsX is one of many (*fts)* filament-forming temperature sensitive genes. *fts* mutants of *E. coli* grow normally at 30º C but stop dividing and rapidly lose viability when shifted to the non-permissive temperature of 41ºC. In the case of the gram-negative bacterium *Flavobacterium johnsoniae*, mutants of the *F. johnsoniae ftsX*

gene (similar to *E. coli ftsX*) appear to be blocked at a late stage of cell division, since the filamentous mutants produce cross walls but cells fail to separate (Kempf and McBride, 2000).

Cell division in *E. coli* involves the coordinated action of many proteins encoded by several different clusters of genes (Gill *et al*., 1986). The *fts*YEX operon in *E. coli* is associated with the inner membrane and encodes three proteins known to be required for cell division, FtsY, FtsE and FtsX . These three proteins are organized in one operon and transcribed in the same direction (Gill and Salmon, 1990). FtsY belongs to the family of signal recognition particle (SRP)-type GTPases, involved in protein targeting (de Leeuw *et al.,* 1999). FtsE appears to be a hydrophilic ATP-binding component of an ABC transporter, and FtsX is supposed to be anchored to the cytoplasmic membrane and interact with FtsE at the cytoplasmic face of the membrane to form the ABC transporter complex (Kempf and McBride, 2000). However it is known that FtsX and FtsE interact and are co-transcribed, their function in cell division is not completely known. They may use the energy of ATP hydrolysis to complete cell separation but also they may act indirectly, translocating proteins involved in potassium transport and probably proteins involved in cell division into the cytoplasmic membrane (Kempf and McBride, 2000). Recently, studies demonstrated that FtsE and FtsX localize to the septal ring, implying that the FtsEX operon participates directly in the division process (Schmidt *et al.,* 2004). A model for the order of assembly of proteins into the septal ring in *E.coli* indicated that FtsEX localizes after FtsZ, FtsA and ZipA, and is important for recruitment of FtsK and other proteins (Schmidt *et al.,* 2004). During the past 10 years, several FtsE and/or FtsX viable mutants have been reported in several organisms exhibiting morphological defects suggestive of impaired division (Merino et al, 2001; Kempf and McBride, 2000).

There are divergent opinions about the classification of *ftsX* as an essential gene in *E.coli*. One of the sources used to identify essential genes in *E. coli* is described by Gerdes et al. (Gerdes *et al*., 2003). In their work, essentiality is based on the results of the analyses from three sources, the Profiling of *E. coli*

chromosome database (PEC database, 2003), the Blattner lab systematic Tn5 mutagenesis project (Blattner *et al*., 1997) and their own work (Gerdes *et al*., 2003). According to Gerdes and others, *ftsX* is classified as a non-essential, D2 gene in *E.coli* due to the fact that some of its mutants are salt remedial. In contrast, the PEC database considers this gene as an essential gene with no viable gene knockouts (PEC database, 2003). The major factor in the essentiality of *ftsX* and other members of the cell division operon in *E.coli* appears to be their dependance on the availability of salts. *fts*E and *fts*X do not appear to be essential genes when *E. coli* cells are grown in medium containing high salt concentration (de Leeuw, 1999). Mutant cells that lack FtsE protein show filamentous growth and require a high salt concentration in the medium, suggesting that FtsE/X complex is directly or indirectly involved in salt transport (de Leeuw, 1999). Several experiments demonstrated restoration of viability of *fts*E and *fts*X mutants obtained by addition of high KCl concentrations (Ukai *et al*., 1998), or NaCl to the culture medium (Schmidt *et al*, 2004). The ability of salts to rescue division in an FtsEX null mutant is explained by its effect on folding, assembly and/or function of one or more of the downstream division proteins FtsK through FtsN. The septal ring that forms at the middle of the cell in the division process fails to assemble or function properly if both salt and FtsEX are lacking, but although poorly, salt can improve the function of the ring in the absence of FtsEX (Schmidt *et al*, 2004).

The aim of the present study was to analyze a predicted ORF in *X. arboricola* pv. *pruni* with similarity to the cell division gene *fts*X in *E. coli*. Disruptants were obtained through homologous recombination with a streptomycin cassette in order to understand how this previously undescribed ORF in *X. arboricola* pv. *pruni* relates to the normal function of the cell.

MATERIALS AND METHODS

Strains, media and bacterial growth. *X. arboricola pv. pruni* isolate Xap14 was used for this study. *E. coli* DH5α (Invitrogene) was used for routine cloning procedures. *E. coli* strains used in this work were grown aerobically at 37ºC in Luria-Bertani medium (LB) consisting of 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl, and for plates 15 g of agar per liter. Strain Xap14 was grown aerobically at 28ºC in sucrose-peptone-agar medium (SPA) containing 20 g of sucrose, 5 g of peptone, 0.5 g of dibasic potassium phosphate, 0.25 g magnesium sulfate and 15 g of agar per liter. *X. arboricola* pv. *pruni* -knockout mutants were grown on SPA medium supplemented with 100 µg of spectinomycin/ml. Induction of expression cells was done by adding Isopropyl-β-D-galactopyranoside (IPTG) was added at the concentration of 50 µM.

General methods. Recombinant DNA techniques were carried out following established protocols (Sambrook *et al*., 1989). Plasmids were extracted with Qiagen Plasmid Mini and Maxi Kits (Qiagen, Valencia, CA) according to the manufacturer's instructions**.** Primers Y17CoF (5'-GACGTGGTGATCAGCGAGTCATTC-3') and Y17CoR (5'-GACGTGGTGATGATGATCTGC-3') were used for the PCR amplifications of the 943-bp DNA containing the *X. arboricola* pv*. pruni* ORF. PCR was conducted in a 25-µl reaction volume containing 0.2 mM each of dNTP's (Boehringer-Mannheim), 1.2 U of Fast start Taq polymerase (Roche Diagnostics GmbH), 1 µM primers, 2 mM $MgCl_2$, 4% dimethyl sulfoxide, 5% glycerol in 10 mM Tris-HCl (pH 8.3) and 50 mM KCl. For colony PCR, a single colony was suspended in the reaction mix. Amplifications were performed with a DNA thermal cycler (M. J. Research) programmed for one cycle of 4 min at 95°C to ensure complete denaturation of the template DNA, and 30 cycles of 1 min at 92°C, 1 min at 53°C, 2 min at 72°C and a final extension period of 5 min at 72°C. After PCR amplification, DNA samples were electrophoresed in 1% agarose gels in 1x TBE buffer (8.9 mM Tris, 0.25 mM Na_2EDTA, 0.89 mM boric acid [pH8.3]) stained with ethidium bromide, and photographed. Isolation of DNA fragments from agarose gels was done with a

Qiagen gel extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Enzymes used to manipulate DNA were from New England Biolabs (Beverly, Mass). Oligonucleotides were from Integrated DNA technologies (Coralville, Iowa).

Restriction fragment length polymorphism (RFLP) analysis. *X. arboricola pv. pruni* genomic DNA was digested separately with *Eco*RI and *Hind* III according to the conditions defined by the supplier, using 5 U of enzyme per µg of DNA. The resulting DNA fragments were electrophoresed on a 1% agarose gel and immobilized on nitrocellulose membranes (Micron Separations Inc., Westborough, MA) by Southern analysis as described by Sambrook *et al*.1989.

Ligations. One µl of T4 DNA Ligase (New England BioLabs, USA) was added to a total volume of 10 µl. Ligations were performed at 16º C overnight. The molar ratio of vector and insert probed was 1:1 and 1:3.

Construction of *Xap* knockout mutants. The *Xap* 943 bp-DNA fragment containing the predicted *XapftsX* ORF was amplified by PCR from strain *Xap*14 with primers Y17CoF and Y17CoR. The PCR product was cleaned and ligated to a pCR®2.1-TOPO vector (Invitrogen, Life technologies, CA) with a TOPO TA cloning kit according to the manufacturer's instructions resulting in plasmid p*Xap*. One shot chemical transformation was performed with chemically competent *E. coli* cells and plated onto LB plates supplemented with X-Gal and 100 µg/ml ampicillin for selection. After 24 h, white colonies were recovered. Colonies were cultured overnight in LB broth containing 100 µg/ml ampicillin and plasmids extracted with Qiagen Plasmid Mini Kit (Qiagen, Valencia, CA). Analysis of plasmids was done by digestion with *Eco*RI and *Hind* III and the resulting fragments subjected to electrophoresis on 1% agarose gels. A unique *Sal* I restriction site was identified in the pXap sequence. This site is located at nucleotide 237 from the predicted *XapftsX* ORF initiation codon. Digestion with *Sal* I resulted in plasmid pXap/*Sal*I. After the fragment was blunt ended with the Klenow enzyme, pXap/*Sal*I/Kl was analyzed on a 1% agarose gel. The resulting 4,874 bp fragment was cleaned and ligated to a 2,082 bp omega (Ω) cassette (Prentki and Krisch, 1984) which encodes for resistance to

spectinomycin and streptomycin. Digestions with *EcoR*I and *Hind* III confirmed the presence of the insert. Transformation was performed with competent *E. coli* cells, and then plated on LB medium containing 100 µg/ml ampicillin and 100 µg/ml spectinomycin. PCR analysis was performed with primers Y17CoF and Y17CoR to confirm the presence of the insert. Transformants were grown overnight on LB broth containing 100 µg/ml ampicillin and 100 µg/ml spectinomycin. Plasmid pXa/*Sal*I/*Kl*/Ω was extracted with Qiagen Plasmid Maxi Kit (Qiagen, Valencia, CA) and electroporated with *X. arboricola pv. pruni* competent cells.

Electroporation of *X. arboricola* pv*. pruni* cells. Electrocompetent *X. arboricola pv. pruni* cells were prepared by the method of Dower *et al*, 1988 and stored in 10% glycerol stock tubes at -80ºC. Stocks were thawed on ice, and plasmid pXa/*Sal*I/*Kl*/Ω (50 ng) added. This mixture was kept on ice for 1 min and was then added to ice-cooled electroporation cuvettes. Electroporation was performed in Gene Pulser/*E.coli* cuvettes with a 0.2 cm electrode gap (Bio-Rad Laboratories Inc. Richmond, Calif.). Electroporator conditions were set to 25 µF, 400 Ω, and 2.5 KV pulse (9 msec delay) on a Gene Pulser (Bio-Rad Laboratories Inc. Richmond, Calif.). One ml of SPA medium was immediately added and mixed with the cell solution. Electroporated cells were pipetted into eppendorf tubes. After growing for 1 h with shaking at 28ºC, cells were plated in 100 µl aliquots on SPA agar plates containing 100 µg/ml spectinomycin and incubated at 28ºC. Transformants were subsequently screened by PCR to confirm a double crossover event.

Viability assay. Knockout mutants XapT13 and XapT15 were stored in 20% glycerol stock tubes at -80ºC. To study the effects of different concentrations of NaCl and different temperatures on the survival of mutants, strains XapT13 and XapT15 were cultured on SPA medium containing 100 µg/ml spectinomycin and 1% NaCl and on SPA standard medium, with plates incubated at 28ºC.

Expression of the *Xap* putative protein in a pET System (Novagen Inc.). *E. coli* BL21(DE3) and *E. coli* BL21(DE3)LysS were used for the expression of the *X. arboricola* pv. *pruni* putative protein cloned in the pET-28a expression vector (Studier *et al.,* 1990). *E. coli* BL21(DE3) is a general purpose expression host with

no antibiotic resistance. *E. coli* BL21(DE3)LysS is a high-stringency expression host with chloramphenicol resistance, which carries on its chromosome the T7 RNA polymerase gene under the control of the *lac* promoter. pET-28a is a 5,369 bp-expression vector with a T7lac promoter and encoding kanamycin resistance that carries a His-fusion tag. In order to clone the *X. arboricola pv. pruni* putative protein-coding region into a pET expression vector, two primer adaptors (pET*BamH*I and pET*Xho*I) were designed, synthesized, and used in a PCR reaction to amplify the entire *XapftsX*ORF. Primers pET*BamH*I (5'- CGGGATCCATGCCTCGTTTCCAC-3') and pET*Xho*I (5'-CGCTCGAGTCATTCTGCCACCTTG-3') amplified a 747 bp-DNA fragment from the genome of *X. arboricola pv. pruni* and created restriction sites *BamH*I and *XhoI* to allow a directional cloning. After preparation of the vector and the insert according to the manufacturer's instructions, cloning was performed. pET recombinants were analyzed by sequencing and by PCR with primers pETF (5'-ATGCCTCGTTTCCAC-3') and pETR (5'-TCATTCTGCCACCTTG-3') in order to confirm insert orientation and size.

Induction of protein expression. *E. coli* strains BL21(DE3)pLysS and BL21(DE3) were grown aerobically at 37ºC to an optical density at 660 nm of 0.5 and induced with IPTG (1 mM final concentration) for 5 h. Cells were harvested at various time points for analysis of proteins by SDS-PAGE.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Sambrook *et al*., 1989. Samples were solubilized by the addition of an equal volume of 2 X SDS-sample buffer, boiled for 5 min before loading and electrophoresed. Gels were stained with Coomassie blue.

Western blotting and immunostaining. The SDS-PAGE was blotted onto a polyvinylidene fluoride (PVDF) membrane presoaked in methanol according to Sambrook *et al*., 1989. The expression of the *Xanthomonas arboricola* pv*. pruni* putative protein was examined by immunoblot analysis with His-Tag monoclonal antibody (1:2000 diluted) (Novagen Inc.) and alkaline phosphatase-conjugated anti-mouse immunoglobulin G (whole molecule; Sigma Corporation, United Kingdom).

Expression of the *X. arboricola* pv. *pruni* putative protein with Expressway™ Plus Expression System (Invitrogen, Life Tech.). We utilized pX28 expression vector previously prepared for the expression of this putative protein in the pET system to generate the DNA template in an *in vitro* Expressway expression system. *In vitro* transcription and translation was performed according to the manufacturer's instructions (Invitrogen, Life Tech.). Samples were analyzed on a 12% SDS-PAGE stained with Coomasie blue.

RESULTS

Estimation of the *XapftsX*-like sequence copy number. Total DNA of strains Xap14 and Xap40 was completly digested with *HindIII* to determine the number of copies of the sequence to be disrupted (Fig.1). *HindIII*- digests were subjected to Southern transfer and hybridization with a XPRUNI14 digoxigenin-labeled DNA probe as previously described (Chapter 1). A single hybridizing band was detected suggesting the presence of only one copy of the *XapftsX*-like sequence (Fig.2).

Construction of a knockout mutant. A 943 bp-DNA fragment was obtained from the PCR amplification of *X. arboricola* pv. *pruni* total genomic DNA with primers Y17CoF and Y17CoR (Fig. 3). This fragment was ligated to a pCR®2.1-TOPO vector resulting in p*Xap* with an expected size of 4,874 bp. The analysis of the p*Xap* nucleotide sequence revealed a single restriction site for *Sal*I at nucleotide 358 from the initiation of the DNA-fragment. Digestion with *Sal*I resulted in plasmid p*Xap*/*Sal*I (Fig.4). After blunt ended with the Klenow enzyme, pXa/SalI/KI was ligated with an omega (Ω) cassette generating pXa/SalI/KI/Ω whose size was verified by digestion with *EcoRI* and *HindIII*. Digestion with *EcoRI*, resulted in two fragments of 2,948 and 3,984 bp and digestion with *HindIII* produced three fragments of 4,294, 2,014 and 625 bp. Fragment sizes corresponded with the expected values (Fig.5). pXa/SalI/KI/Ω was introduced into the wild-type *X.arboricola* pv. *pruni* strain Xap14 by electroporation and after incubation, streptomycin-resistant colonies were selected. Since pXa/SalI/KI/Ω is not able to replicate in *X.arboricola* pv. *pruni*, the streptomycin resistance gene is likely to have been integrated into the chromosome of the cells that were able to survive in the selective media. Several attempts were needed in order to recover transformants. In some cases colonies were never obtained, while in others we could recover colonies and confirm them by PCR, but they rapidly stop growing and died. Despite many efforts in transferring and maintaining these colonies, they resulted in an extremely small colony phenotype and in a rapid cell death. Of the several colonies tested by PCR analysis, three of

them amplified a fragment of approximately 3,000 bp (Fig.6). We were able to keep two transformants, labeled XapT13 and XapT15 in 15% glycerol stocks at -80ºC.

Viability assay. In order to analyze the effect of NaCl on the disrupted mutants, XapT13 and XapT15 were transferred and incubated on SPA medium containing spectinomycin and 1% NaCl at 28ºC. The presence of 1% NaCl in the medium did not restore growth in mutants although wild-type cells of X*. arboricola* pv*. pruni* exhibited no changes in their normal phenotype.

Expression of the *X. arboricola* pv. *pruni* putative protein in a pET System. The coding sequence for the putative *X. arboricola* pv*. pruni* protein (*Xap*FtsX) was amplified by PCR with adaptor primers pET*BamH*I and pET*Xho*I to introduce restriction sites at appropriate positions in the resulting product (Fig. 7). Digestion of the PCR product with the corresponding restriction enzymes *BamH*I and *Xho*I yielded a 731 bp-fragment (Fig.7),which was then cloned into the T7 expression vector pET-28a to produce pX28. *E. coli* strains BL21(DE3) and BL21 (DE3) LysS were transformed with plasmid pX28, which contains the entire coding region of the putative *X. arboricola* pv*. pruni* protein. pET recombinants were analyzed by PCR with primers pETF and pETR to confirm the presence of the insert and several recombinants were identified (Fig. 8). Furthermore, size and insert orientation was also verified by sequence analysis. Expression of the fusion protein was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). This induction resulted in a very weak expression generating a very weak band (Fig. 9 A.). Growth curves of induced BL21(DE3) and BL21(DE3)LysS *E. coli* cells expressing the putative *Xap*FtsX protein grew more slowly than the non-induced cells and in general after 30 min from the time of induction, the concentration of cells decreased (Fig. 10).

Expression of the *X.arboricola* pv*. pruni* putative protein with Expressway™ Plus Expression System. Expression with an *in vitro*, cell-free transcription and translation system successfully revealed the presence of a protein in *E. coli* extracts containing vector pX28. Cell lysates were analyzed using 12% SDS-polyacrylamide gel electrophoresis, and stained with Coomassie blue. A band of 26 kDa was visible only in samples containing vector pX28 (Fig.9 B).These

results were confirmed by Western blot analysis with an anti-His tag monoclonal antibody (Fig.11).

Fig. 1. Digestion of total DNA,strains Xap14 and Xap40.Lane 1, Undigested Xap14 total genomic DNA; 2, Undigested Xap40 total genomic DNA; 3, Total Xap14 genomic DNA digested with *HindIII* and 4, Total Xap40 genomic DNA digested with *HindIII*.

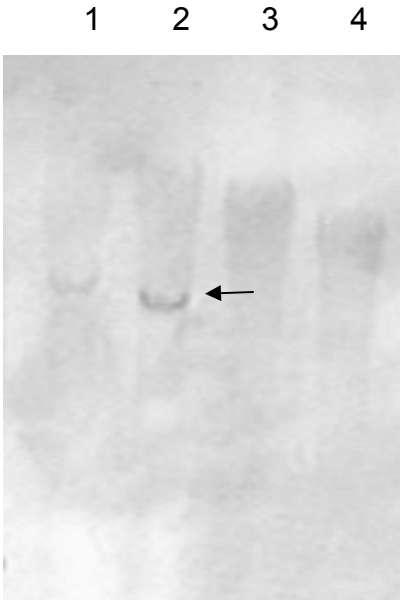


Fig. 2. Southern transfer and hybridization with digoxigenin-labeled DNA Probe XPRUNI14. Lane 1, Total Xap14 genomic DNA digested with *HindIII* ; 2, Total Xap40 genomic DNA digested with *HindIII;* 3,Undigested Xap14 total genomic DNA; 4, Undigested Xap40 total genomic DNA.

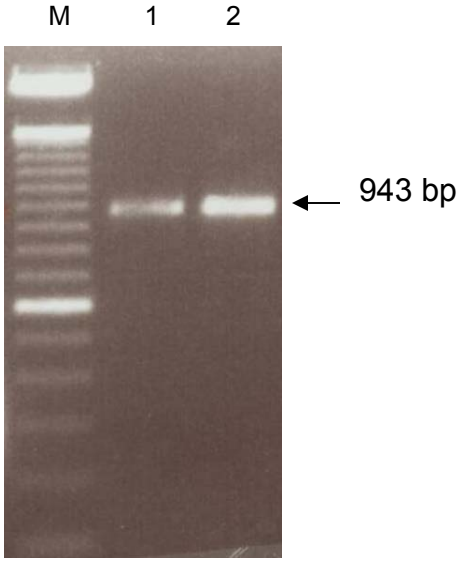


Fig. 3. Lanes 1 and 2, PCR amplification of strain Xap14 with primers Y17CoF and Y17CoR. Lane M, DNA molecular marker (100-bp ladder).

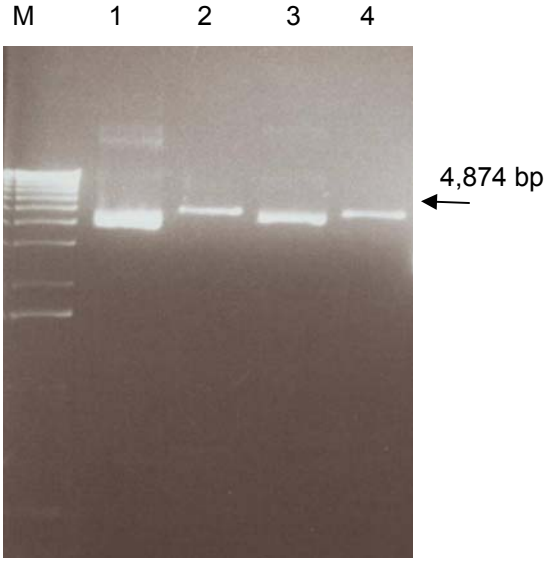


Fig. 4. *SalI* digestion of plasmid pXap. Lanes 1 and 3, undigested pXap; Lanes 2 and 4, pXa/SalI. Lane M, DNA molecular marker (1 Kb Ladder).

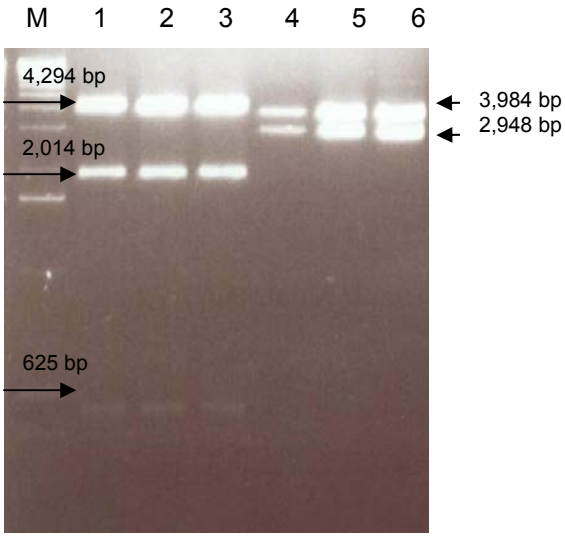


Fig. 5 *Hind II*l and *EcoR*I digestion of pXa/SalI/Kl/Ω. Lanes 1,2,3 *HindIII* digestion; Lanes 4,5 and 6, *EcoRI* digestion. M, DNA molecular marker (1Kb Ladder)

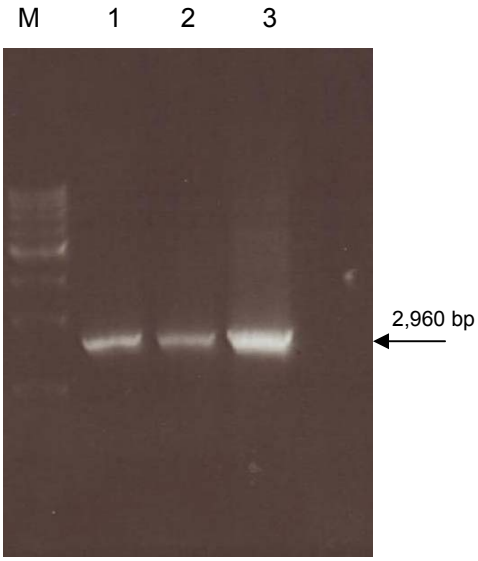


Fig. 6. PCR amplification of knockout mutants with primers Y17CoF and Y17CoR. Lanes 1, transformant XapT12; Lane 2, transformant XapT13 and Lane 3, transformant XapT15. M, DNA molecular marker (1Kb Step Ladder.)

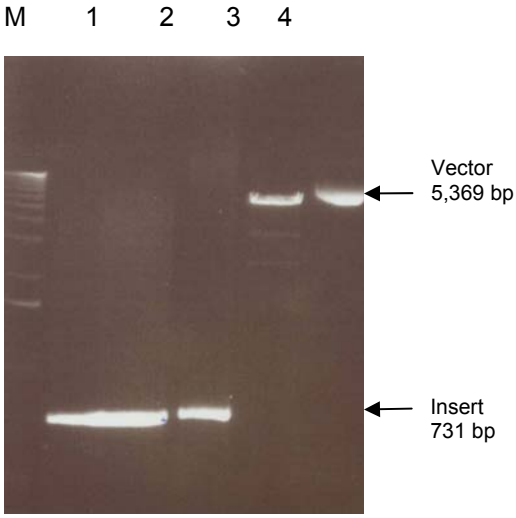


Fig. 7. PCR amplification of the predicted ORF in *X. arboricola* pv. *pruni*, with adaptor primers pETBamHI and pETXhoI. Lane 1, digested insert; 2,undigested insert; 3, digested vector pET28a and 4,undigested vector pET28a. M, DNA molecular marker.

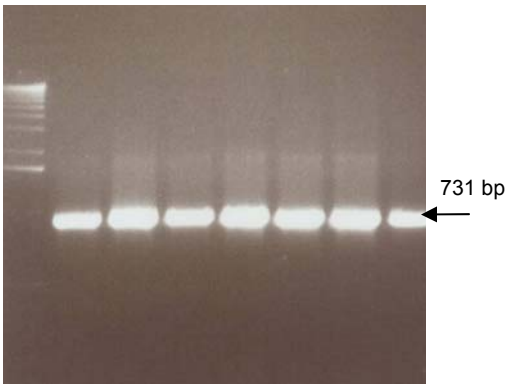


Fig. 8. Recombinants analized by PCR with primers pETF and pETR confirming presence of the insert. Lanes 1,2,3,4,5,6 and7, recombinants. M, DNA molecular marker (1Kb ladder).

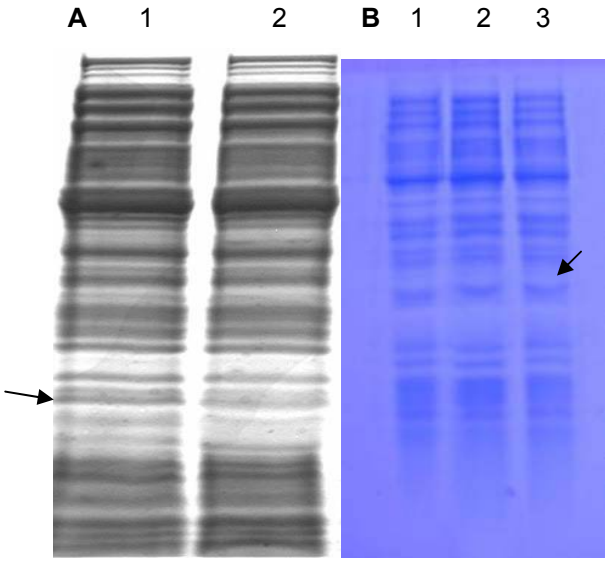


Fig. 9. Expression of the *X. arboricola* pv. *pruni* putative protein on 12% SDS-PAGE. **A,** Protein expression with a pET system. Lane 1, BL21(DE3) cell extracts induced with IPTG; Lane 2, BL21(DE3) cell extracts without IPTG. **B,** Protein expression in a cell-free system. Lane 1, *E.coli* extracts without vector pX28, lanes 2 and 3, *E.coli* extracts with vector pX28.

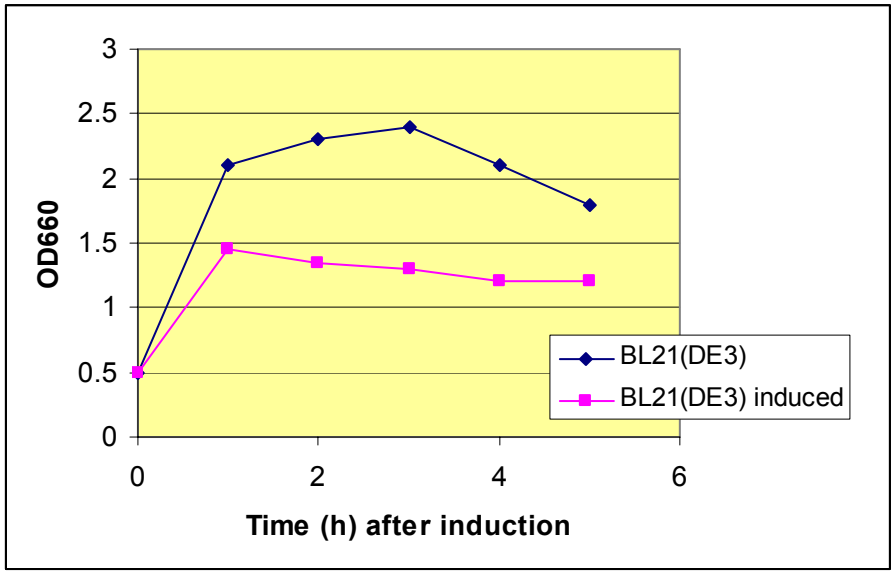


Fig. 10. Growth curves of BL21(DE3) not induced and induced with 1 mM IPTG.

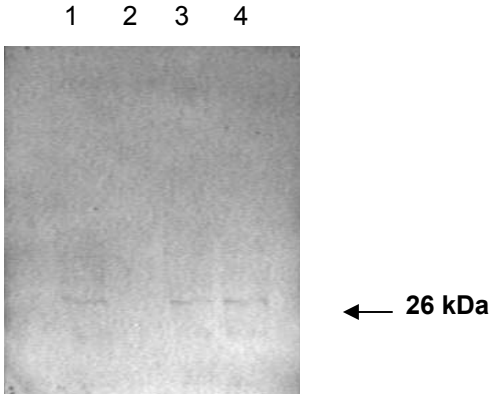


Fig. 11. Detection of the 26 kDa protein by Western blotting with anti-His tag antibody. Lanes 1,3 and 4, *E.coli* extracts overexpressed the putative protein. Lane 2, E.coli extracts with no DNA.

DISCUSSION

The aim of the present study was to analyze a predicted ORF found in a DNA fragment of *X. arboricola* pv*. pruni* . Previous *in silico* studies suggested that a putative integral membrane protein with similarity to the ABC (ATP-Binding cassette) system was predicted to be encoded by this ORF. Several bioinformatic programs also predicted a domain with high similarity to the FtsX protein in *E. coli* involved in cell division (Chapter 2).

As an initial step towards the elucidation of the potential role of this predicted ORF, knockout mutants were constructed in an attempt to understand its possible biological function. In order to initially evaluate the likelihood of this goal, we estimated the ORF sequence copy number through hybridization of the digested genome of two strains of *X. arboricola* pv. *pruni* with a probe created using the entire ORF nucleotide sequence. Although there are examples in the literature of duplicate copies of ABC transport genes in some organisms (Holland *et al*., 2003) (Erkeznik et al., 2003), in bacteria, gene duplication events of ABC transporters appears to be uncommon (Erkeznik et al., 2003). Based on our results, the predicted ORF is expected to occur once in the total genome of *X. arboricola* pv. *pruni*. Consequently, mutants were obtained through homologous recombination with a streptomycin/ Spectinomycin cassette. PCR analysis amplified a fragment of 3,028 bp (Fig. 6). This fragment size agrees with the assumption that a double crossover between the chromosomal predicted XapftsXORF and the XapORF sequences flanking the streptomycin resistance gene likely occurred.

Several *ftsX* mutants have been reported in various organisms, although some are described as viable but presenting morphological defects suggestive of impaired division (Kempf and McBride, 2000; Bernatches *et al*., 2000) others were unable to survive particularly in salt-free media (Schmidt *et al*., 2004). Three *XapftsX*-like mutants were identified in our experiments presenting an extremely small colony phenotype, with no evidence of growth or bacterial mass increase

suggestive of poor cell division ultimately leading to death. Transfering these mutants to medium amended with 1% NaCl did not result in colony development. Previous studies demonstrated the direct participation of FtsX along with FtsE in the division process of *E. coli* (Schmidt *et al*., 2004). It has been proposed that the capacity of salt to restore viability in *ftsEX* mutants is strain dependent and its effect is explained by the impact that ionic conditions have in folding, assembly and/or function of one or several downstream division proteins (Schmidt *et al*., 2004). Others propose that although the exact role of *E.coli* FtsX in cell division is not known, it is considered to be involved in protein translocation affecting the architecture of the cell (Ukai *et al*., 1998; Kempf and McBride, 2000) In our experiments the transfer of mutants XapT13 and XapT15 to a SPA medium amended with 1%NaCl did not revert the cell tendency for death. We believe that this could be the result of a belated transfer of the mutants to the amended medium, when the cells were already irreversibly damaged.

Expression of the *X. arboricola* pv. *pruni* putative protein in a pET expression system resulted in a strong inhibition of growth of BL21(DE3) and BL21(DE3)LysS cells (Fig. 9). In our studies, the accumulation of protein with this system was barely discernible. This is coincident with the molecular characterization of FtsX and FtsE in *E. coli* done by de Leeuw *et al*., 1999. They observed a clear growth inhibition and eventually cell death of the same expression host (BL21(DE3)) when FtsX was overexpressed (de Leeuw *et al*., 1999). This effect is supposed to be caused by the accumulation of a β-lactamase precursor that causes an interference with proteins mediated by signal recognition particles. A slight accumulation of β-lactamase precursor was found by the same authors in cells after overexpression of FtsE and/or FtsX.

Another possible explanation of this growth inhibition is related to the intrinsic properties of integral membrane proteins. High levels of expression of membrane proteins in *E.coli* are considered difficult due to toxic effects exerted by hydrophobic protein domains on the host cells. This explains the fact that bacterial expression of

membrane proteins has frequently been restricted to their soluble domains (Laage and Langosch, 2001).

We avoided this problem by using cell-free extracts for the *in vitro* expression of the protein (Wilkinson, 1999). An *in vitro* transcription and translation system was used by expressing the putative protein with an Expressway ™ Plus Expression System. This expression resulted in a moderate overexpression, but could be clearly distinguished upon SDS-PAGE and Western blotting at 26 kDa. (Fig.10).This is basically the same size predicted (26.8 kDa) with bioinformatic programs.

Previous *in silico* studies provided information to postulate the presence of an ORF in *X. arboricola* pv. *pruni* with the potential to encode an integral membrane protein with similarity to the FtsX involved in cell division in *E. coli.* Although our results are consistent with this hypothesis, additional work is still required to completely define and clarify the role that this predicted protein could have in the normal function of this bacterium.

Our results are the beginning of the understanding of a previously undescribed ORF in *X. arboricola* pv. *pruni*. The exact function of this FtsX protein remains under study, and is not clear in all bacterial species in which the corresponding gene has been identified (Lutkenhaus and Addinall, 1997; de Leeuw *et al*., 1999).

The elucidation of its function in *X. arboricola* pv. *pruni*, might contribute to the optimization of new drugs and/or the establishment of the basis of new drug development efforts, now based on a thorough understanding of the relationship between the drug and its target protein.

To date, no single gene has been associated with this bacterial plant pathogen. This is the first report of a putative gene in the genome of *X. arboricola* pv. *pruni*.

LITERATURE CITED

Bernatchez, S., Francis, F., Salimnia, H., Beveridge, T., Li, H., and Dillon, A. 2000. Genomic transcriptional and phenotypic analysis of *ftsE* and *ftsX* of *Neisseria gonorrhoeae*. DNA Res. 7: 75-81.

Blattner, F. R., Plunkett, G., Bloch, C. A., Perna, N. ., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B., and Shao, Y. 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277: 1453-1474.

Bramhill, D. 1997. Bacterial cell division. Annu. Rev. Cell Develop. Biol. 13: 395-424.

de Leeuw, E., Graham, B., Phillips, G. ten Hagen-Jongman, C, Oudega, B. and Luirink J. 1999. Molecular characterization of *Escherichia coli* FtsE and FtsX. Mol. Microbiol. 31: 983-996.

Del Sorbo, G., Andrade, A. C., Van Nistelroy, J. G. M., Van Kan, J. A. L., Balzi, E., and De Waard, M. A. 1997. Multidrug resistance in *Aspergillus nidulans* involves novel ATP-binding cassette transporters. Mol. Gen. Genet. 254: 417-426.

Dower, W. J., Miller, J. F. and Ragsdale, C. W. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acid Res. 16: 6127-6145.

Erkeznik, M., Hudson, S., Herrman, A and Strobel, H. 2003. Molecular analysis of the xylFGH operon, coding for xylose ABC transport, in *Thermoanaerobacter ethanolicus*. Curr. Microbiol. 48: 295-299.

Gerdes,S., Scholle,M. D.,D'Souza, M.,Bernal, A.,Baev, M.,Farrell, M.,Kurnasov, O., Daugherty, M., Mseeh,F., Polanuyer,B., Campbell,J., Anantha,S., Shatalin, K., Chowdhury, S., Fonstein, and Andrei, L. 2002. From genetic footprinting to antimicrobial drug targets: examples in cofactor biosynthetic pathways. J. Bacteriol. 184: 4555-4572.

Gill, D. R., Hatfull, G. F., and Salmond, G. P. 1986. A new cell division operon in *Escherichia coli*. Mol. Gen. Genet. 205: 134-145.

Gill, D. R., and Salmond, G.P.C. 1990. The identification of the *Escherichia coli ftsY* gene product: an unusual protein. Mol. Microbiol. 4: 575-583.

Higgins, C. F. 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. 8:67-113.

Hirota, Y., Ryter, A., and Jacob, F. 1968. Thermosensitive mutants of *E.coli* affected in the process of DNA synthesis and cell division. Cold Spring Harbor Symp. Quant. Biol. 33:677-694.

Holland, I. B., Cole, S. P., Kuchler, K., and Higgins, C. F. 2003. ABC proteins. From bacteria to man. Acad.Press.

Kempf, M., and McBride, M. 2000. Transposon insertions in the *Flavobacterium johnsoniae ftsX* gene disrupt gliding motility and cell division. J. Bacteriol. 182: 1671-1679.

Laage, R., and Langosch, D. 2001. Strategies for prokaryotic expression of eukaryotic membrane proteins. Traffic. 2: 99-106.

Lutkenhaus, J., and Addinall, S. 1997. Bacterial cell division and the Z ring. Annu. Rev. Biochem. 66: 93-116.

Merino, S., Altarriba, M., Gavin, R., Izquierdo, L., and Tomas, J.M. 2001. The cell division genes (*ftsE and X*) of *Aeromonas hydrophyla* and their relationship with opsonophagocytosis. FEMS Microbiol. Letters. 198: 183-188.

Nakaune, R., Adachi, K., Nawata, O., Tomiyama, M. Akutsu, K., and Hibi, T. 1998. A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. Appl.Envir.Microbiol. 64: 3983-3988.

PEC 2003 Profiling of *E.coli* chromosome database. National Institute of Genetics. http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp.

Prentki, P., and Krisch, H. 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene 29: 303-313.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Schmidt, K., Peterson, N., Kustusch, R., Wissel, M., Graham, B., Phillips, G., and Weiss, D. 2004. A predicted ABC transporter, FtsEX, is needed for cell division in *Escherichia coli*. J. Bacteriol. 186: 785-793.

Studier, F. W., Rosenberg, A. H. Dunn, J. J., and Dubendorff, J. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185: 60-89.

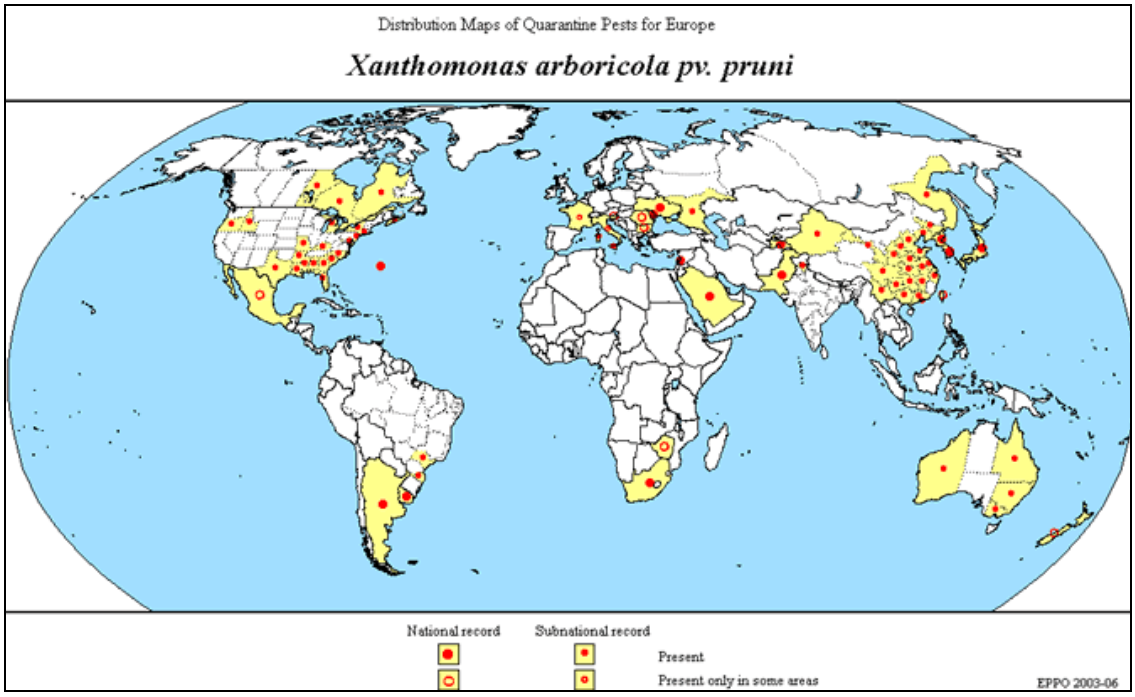
Ukai, H., Matsuzawa, H., Ito, K., Yamada, M., and Nishimura, A. 1998. ftsE (Ts) affects translocation of K-pump proteins into the cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. 180: 3663-3670.

Vermeulen, T., Schoonbeek, H., and De Waard, M. 2001. The ABC transporter *BcatrB* from *Botrytis cinerea* is a determinant of the activity of the phenylpyrrole fungicide fludioxonil. Pest Manage.Sci. 57: 393-402.

Wilkinson, D. 1999. Cell-Free and happy: In vitro translation and transcription/translation systems. The Scientist 13: 15.

Zwiers, L.H. 2002. ABC transporters of the wheat pathogen *Mycosphaerella graminicola.* Wageningen University dissertation no. 3189.

APPENDICES



Appendix A. Distribution map of *Xanthomonas arboricola* pv. *pruni*

Source: http://www.eppo.org/QUARANTINE/bacteria/Xanthomonas_pruni/XANTPR_map.pdf