RESEARCH LETTER

In vivo proteome analysis of *Xanthomonas campestris* pv. *campestris* in the interaction with the host plant *Brassica oleracea*

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Abstract

The genus *Xanthomonas* is composed of several species that cause severe crop losses around the world. In Latin America, one of the most relevant species is *Xanthomonas campestris* pv. *campestris*, which is responsible for black rot in cruciferous plants. This pathogen causes yield losses in several cultures, including cabbage, cauliflower and broccoli. Although the complete structural genome of *X. campestris* pv. *campestris* has been elucidated, little is known about the protein expression of this pathogen in close interaction with the host plant. Recently, a method for *in vivo* analysis of *Xanthomonas axonopodis* pv. *citri* was developed. In the present study, this technique was employed for the characterization of the protein expression of *X. campestris* pv. *campestris* in close interaction with the host plant *Brassica oleracea*. The bacterium was infiltrated into leaves of the susceptible cultivar and later recovered for proteome analysis. Recovered cells were used for protein extraction and separated by two-dimensional electrophoresis. Proteins were analysed by peptide mass fingerprinting or *de novo* sequencing and identified by searches in public databases. The approach used in this study may be extremely useful in further analyses in order to develop novel strategies to control this important plant pathogen.

Introduction

World agriculture is severely affected by numerous diseases caused by species belonging to the *Xanthomonas* genus. One common example is black rot disease, which is caused by *Xanthomonas campestris* pv. *campestris*. This phytopathogen causes one of the most destructive diseases in cruciferous plants, which include a variety of economically important plants cultivated for food and spices and as ornamentals. Although black rot has been partially controlled by the use of resistant cultivars, *X. campestris* pv. *campestris* still represents a threat to agriculture, and therefore it is necessary to develop more efficient methods to control it. Although the structural genome sequence of *X. campestris* pv. *campestris* has been revealed (da Silva *et al.*, 2002), information regarding the protein expression of this phytopathogen during interactions with the host plant is remarkably limited.

Interest in proteome analysis has recently increased, especially for organisms that have a known genome, as a high number of sequences are available for direct comparisons. The analysis of the proteome from an organism can be carried out by exploring the high resolution of two-dimensional electrophoresis (2-DE) (Jungblut & Wittmann-Liebold, 1995) coupled to MS. These data, when complemented by *de novo* sequencing, allow the unequivocal identification of proteins involved in various pathways, including pathogenic processes, cell growth and others. The proteomic technique has been considered an important method in gaining an understanding and identifying the functions of proteins that are differentially expressed (Joo & Kim, 2005). This method has been used for the study of several microorganisms, such as *Helicobacter pylori* and *Thiobacillus ferrooxidans* among others (Bumann *et al.*, 2002; He *et al.*, 2005), and several proteins with crucial functions have been identified.

Regarding phytopathogenic bacteria, this approach has only rarely been used. The expression of proteins in *Xylella fastidiosa* grown in the medium BCYE was reported, and 111 proteins were identified by MS (Smolka *et al.*, 2003). More recently, the extracellular proteome of *X. campestris* grown in culture media was determined (Watt *et al.*, 2005). However, little is known about the protein/gene expression in species of *Xanthomonas* during the interaction with the host plant. Some genes and proteins that are differentially expressed in response to leaf extract have been identified (Mehta & Rosato, 2001; Tahara *et al.*, 2003). Although genes of *Xanthomonas* involved in pathogenicity have been isolated, the mechanisms involved in this process are not well established. The involvement of polysaccharides and extracellular enzymes that degrade the cell wall in host plants in the infection process is well known (da Silva *et al.*, 2002), but no holistic studies have been carried out to analyse *in vivo* conditions.

The present study sheds some light on the protein expression of *X. campestris* pv. *campestris* in the interaction with the host plant using an *in vivo* system. Recently, a method for *Xanthomonas axonopodis* pv. *citri* cell recovery from infiltrated leaves of *Citrus sinensis* was developed (Mehta & Rosato, 2003). This method is used here in an attempt to characterize the *in vivo* protein expression of *X. campestris* pv. *campestris* during host-plant infection.

Materials and methods

Bacterial strain and culture conditions

Xanthomonas campestris pv. *campestris* 11078 (ATCC 33913), obtained from the culture collection of phytopathogenic bacteria of the Instituto Agronômico do Paraná (IAPAR), was used in this analysis. This strain, used in the genome sequencing project, was cultured in the complex medium Nutrient Yeast Glycerol (NYG) (Daniels *et al.*, 1984) at 28 °C.

Infiltration and recovery of the bacterium from the host plant

The infiltration and recovery of bacterial cells from the susceptible host plant *Brassica oleracea* (cv. coração de boi) was performed as described by Mehta & Rosato (2003). Briefly, young leaves of cabbage were infiltrated with *X. campestris* pv. *campestris* ($A_{600\,nm} = 0.6$) and recovered 6 days after inoculation, when the bacteria reached the stationary phase. The infiltrated leaves were cut and submersed in *c.* 20 mL of distilled water in Petri dishes for 45 min. The bacteria were recovered from the suspension by centrifugation and used for protein extraction. Water-infiltrated leaves were used as the control condition. The same number of leaves as used for bacterial protein recovery was collected for protein extraction. The protein profile of the bacterium *in vivo* was compared with that of the bacterium cultured in the complex medium NYG. The bacteria were grown for 12 h in NYG, until the stationary phase was reached ($A_{600\,nm} = 1.2$), and used for protein extraction. Population dynamics was performed by sampling 6-mm-diameter discs of infiltrated leaves at 0, 1, 2, 4 and 6 days after inoculation (DAI). The discs were macerated in 1 mL of sterile distilled water and diluted to appropriate concentrations. A total of 100 μL was plated onto NYG medium and incubated for a period of 2–3 days.

Protein extraction

Total proteins were extracted from bacterial cells and water-infiltrated leaves according to de Mot & Vanderleyden (1989) for at least three different experiments for each of the three conditions. Extraction buffer (0.7 M sucrose, 0.5 M Tris-HCl, 30 mM HCl, 50 mM EDTA, 0.1 M KCl and 40 mM dithiothreitol) and phenol (100%) were used in the same volume (750 μL). Proteins were precipitated with ammonium acetate 0.1 M in methanol, washed with acetone 80%, dried and resuspended in 30 μL of lysis buffer (9.8 M urea, 0.2% (v/v) Nonidet P-40 (Sigma), 100 mM dithiothreitol and 2% (v/v) of a mixture of ampholytes pH 5–8 and 3–10 (BioRad) in the ratio 5 : 1). Protein samples were stored at − 20 °C, and protein quantification was performed according to Bradford (1976).

2-DE

Isoelectric focusing was performed according to de Mot & Vanderleyden (1989), using 11-cm polyacrylamide gels containing 3.6% acrylamide, 0.21% bis-acrylamide, 7.2% ampholyte pH 5–7 and 3–10 in the proportion of 5 : 1 (v : v), 2% Nonidet P-40 and 55% urea. Approximately 150 μg of proteins was loaded onto the gel after a prerun. Electrophoresis was performed at 400 V for 18 h, using NaOH 20 mM in the upper compartment of the chamber and H_3PO_4 10 mM in the lower. Molecular mass separation was performed according to Laemmli (1970) using 16 cm × 16 cm polyacrylamide gels 10%. At least three repetitions were performed for each condition. Silver staining was carried out according to Blum *et al.* (1987).

Image analysis

The 2-D gel images were analysed using the BIONUMERICS software v. 4.5 (Applied Maths NV, Belgium). First, a calibration with a grey scale was performed to transform grey levels into OD values for each pixel (px) of the gel image. The gel pictures were analysed as tiff files, and the wizard detection method proposed by the software was used to detect the spots with the following parameters: 30 px for

estimated spot size, 3 px for minimum spot size, and a spot contrast enhancement of 75%. Automatically detected spots were manually checked and some of them were manually added or removed. Following the detection procedure, a normalization step was carried out to attribute a common spot identity for the same spots derived from different images utilizing the reference gel construct and automatically matching options of the BIONUMERICS software. The overlapped images were based on landmark spots showing same pI and Mw.

Trypsin digestion and MS analysis

Proteins were excised from the 2-D gels and enzymatically digested into peptide fragments using Trypsin Gold (Promega) according to Shevchenko *et al.* (1996). Aliquots of each hydrolyzed sample were mixed with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid, spotted onto MALDI target plates, and allowed to air dry. Monoisotopic masses of the molecular components ranging from *m/z* 600 to 6000 were determined by MS using an UltraFlex II MALDI-TOF/TOF (Bruker Daltonics, Bilerica, MA) and a 4700 MALDI-TOF/TOF (Applied Biosystems, Framingham, MA) controlled by the manufacturer's software. All spectra were obtained in positive reflector mode, using an accelerating voltage of about 20 kV for the MS mode. Molecular ions displaying sufficient signal were submitted to MS/MS analyses, carried out in the positive mode precursor ion fragmentation at a laser frequency of 50 Hz. The MS/MS spectra were acquired in the reflector-positive mode after collision-induced dissociation (CID) or LIFT™ (Bruker Daltonics) fragmentation with external calibration. The resulting data were analysed using FLEXANALYSIS v. 2.4 or DATAEXPLORER v. 4.5, and MS/MS spectra were further interpreted automatically and manually (*de novo* sequencing) using PEPSEQ software running under MASSLYNX v. 4.0 (Micromass, Manchester, UK). Peptide masses obtained were used for identification with the MASCOT program (Matrix Science, London, UK) using the MSDB database, and only matches to *Xanthomonas*, *Brassica* or *Arabidopsis* species were considered as positive identifications. Peptide sequences obtained by *de novo* sequencing were analysed using the BLAST program.

Results and discussion

In the present study, the *in vivo* expression method previously developed for *X. axonopodis* pv. *citri* (Mehta & Rosato, 2003) was used for the protein analysis of *X. campestris* pv. *campestris* in connection with the interaction with the host plant. Initially, a population dynamics study of the bacterium *in vivo* was performed. The analysis of bacterial growth at various sampling times revealed that, 1 day after inoculation (DAI), there was a clear decrease in the population (from 5.4×10^6 to 1.6×10^5 CFU cm^{-2}). The bacterial growth was regained at 2 DAI (1.4×10^7 CFU cm^{-2}) and maintained at 4 DAI (1.8×10^7 CFU cm^{-2}) and at 6 DAI (3.8×10^7 CFU cm^{-2}), with a slight increase in the population at 4 DAI. The initial symptoms of black rot could be observed 3 DAI, and the beginning of plant tissue necrosis was visualized 6 DAI. After this period, severe necrosis was observed as well as leaf abscission, and therefore the recovery of *X. campestris* pv. *campestris* for protein extraction was performed at 6 DAI, when the bacterium attained maximum growth. One of the limitations of the recovery method is the small numbers of bacterial cells obtained. The sampling point of 6 DAI was chosen in order to maximize the number of bacteria recovered from infiltrated leaves. Attempts were made to perform an analysis at other sampling points by increasing the number of infiltrated leaves. However, the protein analysis revealed the detection of a high number of plant proteins. Moreover, the precipitated proteins obtained were not completely solubilized, probably as a result of higher amounts of interfering plant compounds in the samples.

The protein map of water-infiltrated leaves (Fig. 1), used as the control condition, was compared with the profile obtained from bacterial cells recovered at 6 DAI *in vivo*. The protein maps of both conditions were overlapped using the molecular marker as well as some protein spots present in both profiles (Fig. 2). This procedure allowed the identification of 10 overlapped spots (Fig. 2) and 204 differentially expressed proteins, including 181 proteins exclusive to the *in vivo* experiment (Fig. 3). The overlapped proteins observed indicated that these spots represented plant proteins. Indeed, some of these proteins, shown in Fig. 3 with numbers preceded by a 'p', were identified by peptide mass fingerprinting as ATP synthase subunits from *Arabidopsis thaliana* and *Brassica napus* or as hypothetical proteins from *A. thaliana* (Table 1). Although plant proteins were identified in the *X. campestris* pv. *campestris in vivo* profile, most proteins identified were of bacterial origin, showing that the *in vivo* method is valid to characterize protein expression associated with direct interaction with the host plant.

Bacterial cells recovered at 6 DAI in the *in vivo* condition were also compared with cells cultured in NYG medium at same growth stage. To date, most studies showing the characterization of *Xanthomonas* proteomes have been performed using culture media (Mehta & Rosato, 2001; Watt *et al.*, 2005; Chung *et al.*, 2007). In this study, these two growth conditions were overlapped (data not shown), and proteins commonly found in the *X. campestris* pv. *campestris* profile were observed. The comparison of these two growth conditions allowed the identification of 23 overlapped spots. A total of 118 and 210 proteins were exclusive to the *in vivo* and NYG growth conditions, respectively, revealing a high diversity in the protein expression profiles. A higher number of proteins was observed in the map of the bacterium grown in NYG (Fig. 4). Similar

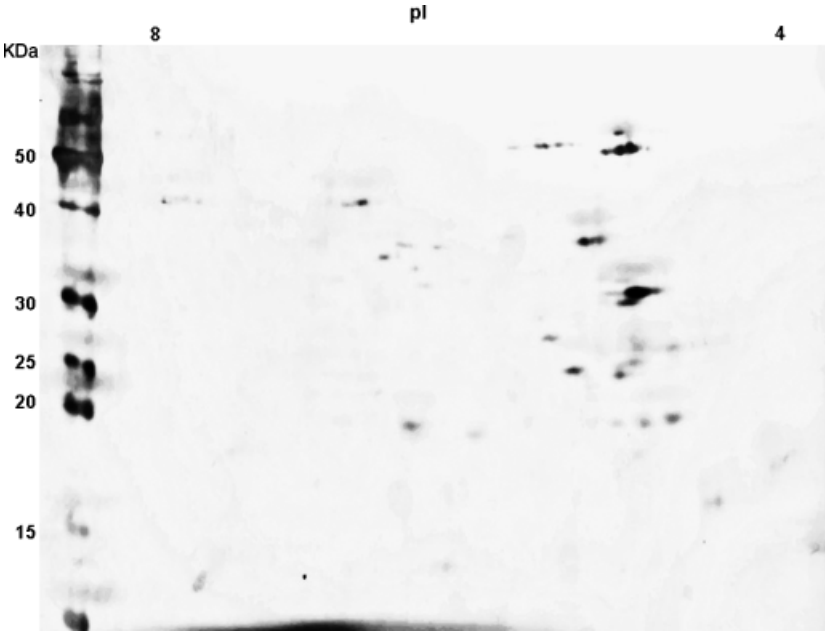

Fig. 1. 2-DE protein profile of water-infiltrated leaves.

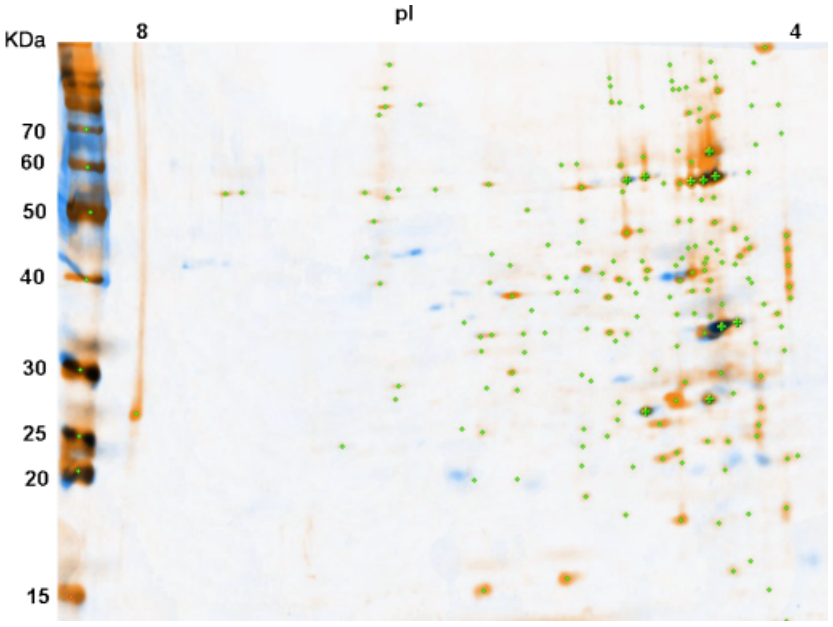

Fig. 2. Overlapped image of 2-D gels of *Xanthomonas campestris* pv. *campestris* for *in vivo* (orange) and water-infiltrated leaves (blue) conditions using the BIONUMERICS program. Crosses indicate proteins used for overlapping.

results were previously reported by Mehta & Rosato (2001) in a comparison analysis of *X. axonopodis* pv. *citri* grown in the presence and absence of leaf extracts and NYG medium.

The intense protein spots observed in the gel from the *in vivo* condition (*c.* 40) were excised and analysed by MS or MS/MS, and positive identification was obtained for 21 proteins. Six of these spots represented plant proteins, as discussed above; therefore, a total of 15 spots could be identified as bacterial proteins expressed *in vivo* (Table 1). The other proteins were in insufficient amounts for the identification analysis or did not return reliable matches with the MASCOT program. This is probably because of a low quantity of proteins and/or the low ionization capacity of molecular components present in the samples analysed. By *de novo* peptide sequencing, two proteins (N2 and N11) expressed only in the culture medium condition (Fig. 4) as well as one protein from the *in vivo* condition (spot 8) were identified. The peptides searched in the NCBI database showed 100% identity to *X. campestris* pv. *campestris* protein sequences.

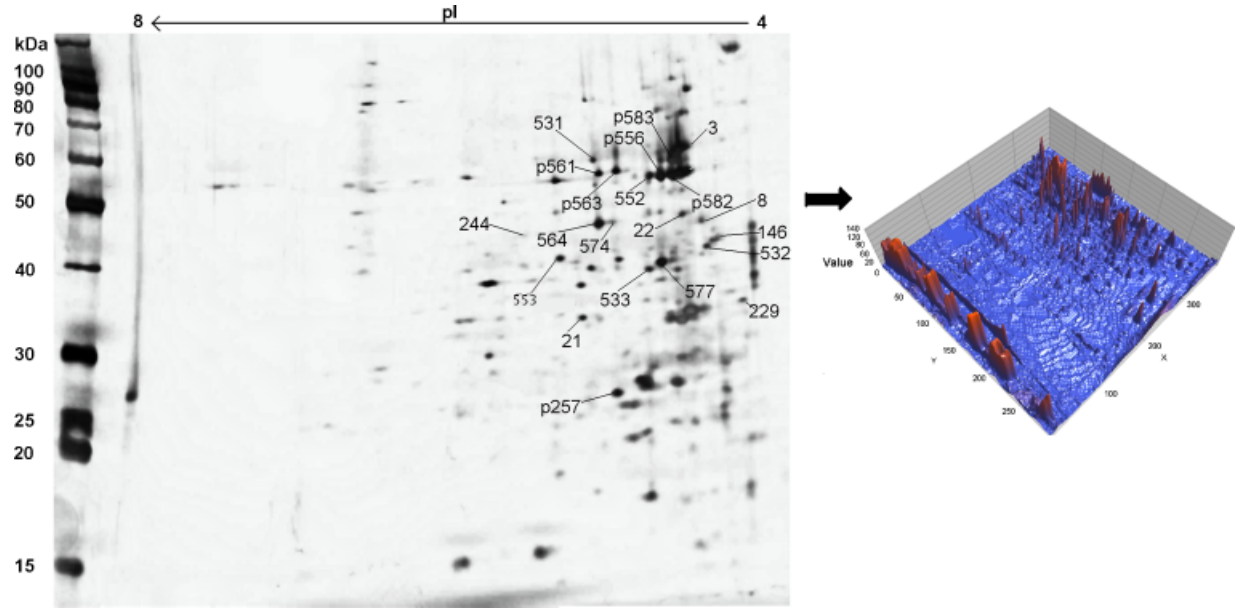

Fig. 3. 2-DE protein profile of *Xanthomonas campestris* pv. *campestris in vivo* and the corresponding 3-D image. Protein spots indicated by numbers were identified by MS. Spot numbers preceded by a 'p' indicate plant proteins identified by peptide mass fingerprinting.

Table 1. Proteins identified by peptide mass fingerprinting or *de novo* sequencing

Spot *n*°	Peptide sequence	Protein identification	Accession #	Score	Mr (gel)	pI (gel)	Mr (cal)	pI (cal)
N2	NVAAHR	Asparaginase	NP_636247	–	16	4.5	17	5.6
N11	KLSASVPLHFINEAISPAG-KKQLIMHIDFQR	50S ribosomal protein L25	NP_636264	–	45	4.7	32	5.3
3	AAVEEGVVPGGGVALVR	Chaperonin GroEL	NP_635915	–	60	4.3	57	5.0
8	PVIGFQR	Elongation factor Ts	NP_636748	–	43	5.0	32	5.5
552		ATP Synthase β chain	Q5H4Y4	104	55	5.4	56	5.6
531		Phosphomannose isomerase	Q4UQM2	79	60	4.7	50	5.5
244		Citrate synthase	Q3BPS8	91	45	5.1	47	5.9
553		DNA-directed RNA polymerase α chain	P0A0Y2	103	42	4.9	36	5.5
21		UDP-glucose pyrophosphorylase	Q3BSP2	73	36	4.4	32	5.4
574		Elongation factor Tu	Q4URD7	125	47	4.6	43	5.4
564		Elongation factor Tu	Q4URD7	95	47	4.7	43	5.4
533		Elongation factor Tu	Q4USR1	76	40	4.4	30	5.2
22		Enolase	Q4UTP2	83	47	4.3	45	4.8
532		Elongation factor Tu	AAY47911	83	43	4.2	37	4.9
146		Phosphoglycerate kinase	Q4UY23	76	44	4.1	40	4.8
577		Aspartate semialdehyde dehydrogenase	Q4UWD8	91	40	5.9	36	5.1
229		Thioredoxin	Q4UWLO	103	35	4.0	31	4.6
P582/P556		ATP synthase subunit β	Q9MS49	153	55	5.6	53	5.2
P563		H^+-transporting ATP synthase β chain (mitochondrial) -like protein	CAC35873	74	57	5.3	59	6.0
P583		ATPase α subunit	BAA84370	105	60	5.7	55	5.1
P561		Hypothetical protein	Q8H135	86	55	5.2	48	5.4
P257		Mitochondrial F1 ATP synthase β subunit	CAC81058	80	27	5.3	63	6.5

The two differentially expressed protein spots N2 and N11, present only in the profile of the bacterium grown in NYG medium, were identified as an asparaginase and the 50S ribosomal protein L25, respectively. Asparaginases are tetrameric enzymes that catalyze the hydrolysis of asparagine to aspartic acid and ammonia. The up-regulation of this enzyme can be a result of intense bacterial growth in the complex medium. The 50S ribosomal proteins L25, L5 and L18 are 5S RNA binding proteins, and it has been reported that the presence of L25 is essential for the stimulation of L5

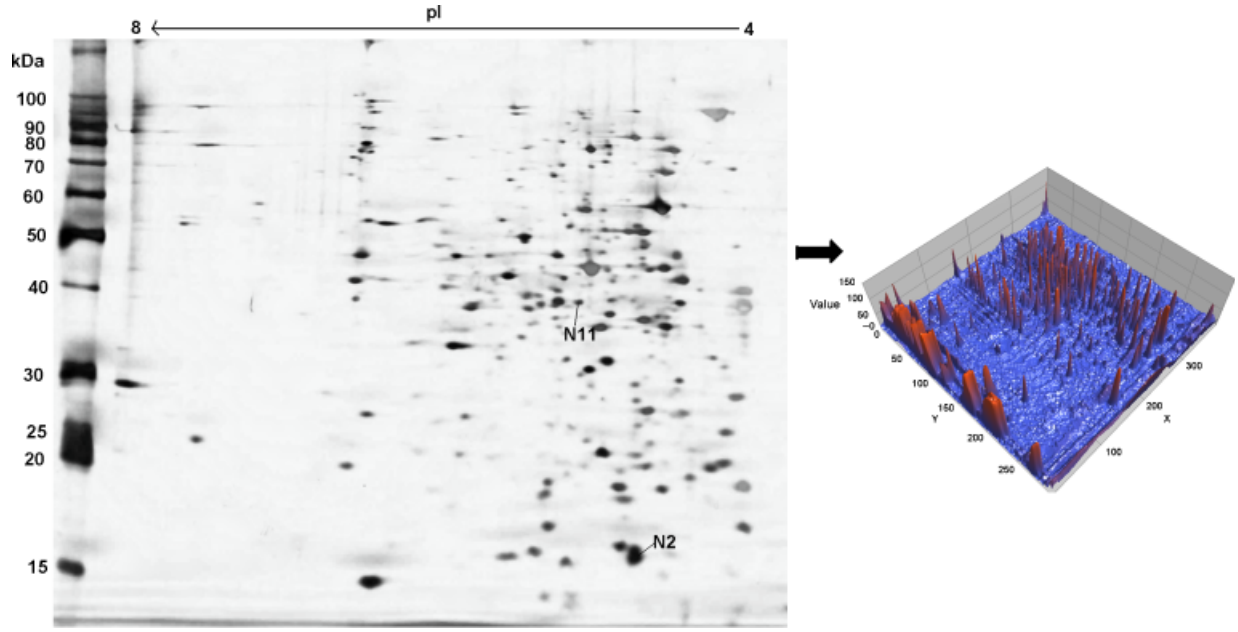

Fig. 4. 2-DE protein profile of *Xanthomonas campestris* pv. *campestris* cultured in NYG medium and the corresponding 3-D image. Protein spots indicated by numbers preceded by an 'N' were identified by MS.

binding (Newberry & Garrett, 1980). The differential expression of ribosomal proteins was reported by Mehta & Rosato (2005) when analysing the gene expression of *X. axonopodis* pv. *citri* in different culture conditions. In this study, the ribosomal protein L25 was also differentially expressed and is probably related to protein synthesis and again intense bacterial growth in the NYG medium. Although the bacterium was at the stationary phase at the sampling points for the *in vivo* and culture medium conditions, a more pronounced bacterial growth was observed in the NYG medium.

Several proteins expressed *in vivo* were also identified in this study and are related mainly to metabolism (Table 1). Spot 3 was identified as a chaperonin GroEL, which is expressed under stress conditions (Lindquist, 1986). The stress, probably caused by plant defence responses, may have induced the expression of this protein, which acts as a chaperone for the assembly of special complex and oligomeric proteins (Beckmann *et al.*, 1990) involved particularly in this condition. Protein spot 8 was identified as the elongation factor Ts, which is a key component of the translational machinery of bacteria (Seshadri *et al.*, 1999). Notably, other protein spots (564, 574, 533 and 532) observed in various positions in the gel were identified as the same elongation factor Tu (Table 1). Other proteins involved in metabolism were also identified, including ATP synthase (spot 552) and DNA-directed RNA polymerase (spot 553). Spot 577 showed a clear similarity to an aspartate semialdehyde dehydrogenase, which is involved in amino acid biosynthesis and has been considered essential for Gram-negative and Gram-positive bacteria (Cirillo *et al.*, 1994; Mei *et al.*, 1997; Becker *et al.*, 2006). Spot 22 was identified as an enolase, known to be involved in glycolysis-related machinery. Phosphoglycerate kinase was another key glycolytic enzyme to be identified (Spot 146): it catalyzes the reversible transfer of a phosphate from 1,3-bisphosphoglycerate to ADP to form 3-phosphoglycerate and ATP in the presence of magnesium (Lee *et al.*, 2006). This protein has also been associated with metal stress in *Rhodobacter capsulatus* (Mohamed Fahmy Gad El-Rab *et al.*, 2006). Although the primary functions of most genes involved in metabolism are well established regarding bacterial physiology, little is known about the relationship between *de novo* nutrient biosynthesis and pathogenicity (Smith, 1998). Other studies have also reported an up-regulation of several metabolic enzymes during pathogen infection and indicate that many metabolic genes are involved in bacterial pathogenesis (Qian *et al.*, 2005).

Two proteins involved in polysaccharide synthesis, which plays a crucial role in pathogenicity (Chatterjee & Vidaver, 1985), were also identified in the *in vivo* condition. Spot 531 was shown to be a phosphomannose isomerase, encoded by the *xanB* gene (Koplin *et al.*, 1992), which is important for the production of the extracellular polysaccharide produced by *X. campestris* known as xanthan gum. Another enzyme identified here, and reported to be involved in xanthan gum synthesis, was UDP-glucose pyrophosphorylase (spot 21). Wei *et al.* (1996) performed a complementation study of a nonmucoid isolate of *X. campestris* obtained by mutation, and determined that the gene responsible for the complementation was UDP-glucose pyrophosphorylase.

One protein spot (229) was identified as a thioredoxin and has been related to oxidative stress detoxification (Scholten *et al.*, 2007). Oxidative stress is a well-known plant defence response to microbial infection, and this protein was probably up-regulated in *Xanthomonas* as part of a protection system against plant reactive oxygen species.

Overall, the present study shows that the *in vivo* expression method developed for *X. axonopodis* pv. *citri* can be successfully employed for *X. campestris* pv. *campestris* for *in vivo* proteome studies and may be extended to other *Xanthomonas* species. The results obtained using this method provide an interesting approach for *in vivo* studies in plant pathogenic bacteria and can contribute to a better understanding of the mechanisms associated with the infection process of *Xanthomonas* species. Moreover, the up-regulated proteins identified here could be used as targets for further studies of the pathogenicity mechanisms of *X. campestris* pv. *campestris* as well as to determine the role of proteins associated with metabolism in pathogenicity. This knowledge could be utilized in the near future to develop biotechnological tools with activity against plant pathogens.

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