FEMS Microbiology Ecology, 96, 2020, fiaa138

doi: 10.1093/femsec/fiaa138
Advance Access Publication Date: 0 2020
Research Article

RESEARCH ARTICLE

Participation of two general stress response proteins from *Xanthomonas citri* subsp. *citri* in environmental stress adaptation and virulence

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One sentence summary: Two general stress response proteins from a plant pathogen involved in environmental stress adaptation and virulence

Editor: Kornelia Smalla

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ABSTRACT

Xanthomonas citri subsp. *citri* (Xcc) is the bacteria responsible for citrus canker. During its life cycle Xcc is found on leaves as epiphyte, where desiccation conditions may occur. In this work, two Xcc genes, XAC0100 and XAC4007, predicted *in silico* to be involved in general stress response, were studied under salt, osmotic, desiccation, oxidative and freezing stress, and during plant-pathogen interaction. Expression of XAC0100 and XAC4007 genes was induced under these stress conditions. Disruption of both genes in Xcc caused decreased bacterial culturability under desiccation, freezing, osmotic and oxidative stress. Importantly, the lack of these genes impaired Xcc epiphytic fitness. Both Xac0100 and Xac4007 recombinant proteins showed protective effects on *Xanthomonas* cells subjected to drought stress. Also, *Escherichia coli* overexpressing Xac4007 showed a better performance under standard culture, saline and osmotic stress and were more tolerant to freezing and oxidative stress than wild type *E. coli*. Moreover, both Xac0100 and Xac4007 recombinant proteins were able to prevent the freeze-thaw-induced inactivation of L-Lactate dehydrogenase. In conclusion, Xac0100 and Xac4007 have a relevant role as bacteria and protein protectors; and these proteins are crucial to bacterial pathogens that must face environmental stressful conditions that compromise the accomplishment of the complete virulence process.

Keywords: desiccation; plant-pathogen interaction; epiphytic fitness; hydrophilins

INTRODUCTION

Water is essential to maintain the structure and function of biological molecules and water availability can influence cell physiology of both prokaryotes and eukaryotes at some stage of their life cycles. Therefore, some organisms, among them: bacteria, higher and lower plants, insects, yeasts, fungi and crustacean, have evolved strategies to overcome conditions of water deficit. Air drying can induce water stress in bacterial cells, and desiccation tolerance in these microorganisms comprises structural, physiological and molecular changes to survive under this stress condition (Potts 1994). Bacteria exert important influences on ecological processes, and therefore it is crucial to study specific traits of these organisms and how they cope with changes in

Received: 4 October 2019; **Accepted:** 6 July 2020

the environment. This knowledge will provide insights into the functioning of microbial systems and thus, contribute to deepen in microbial functional ecology (Escalas *et al.* 2019). The ecological niche of a microorganism is a complex picture of its performance in a particular environment. Most pathogenic bacteria subsist as epiphytes on healthy plants preceding infection, and low water availability is considered one of the most important barriers encountered by microbes on leaf surfaces (Freeman *et al.* 2013). At this stage, bacteria are subjected to constant modifications in water availability, temperature, light intensity and radiation. And specifically, fluctuations in water accessibility challenge epiphytic bacteria to withstand these stringent environmental conditions (Lindow, Andersen and Beattie 1993; Yu *et al.* 2013; Beattie and Lindow 1994).

The genus *Xanthomonas* are Gram-negative bacteria from the γ-proteobacteria subdivision. Members of this genus have a wide environmental distribution and can cause disease in a great number of plant species (Brunings and Gabriel 2003). Nevertheless, individual *Xanthomonas* strains usually cause disease on only a few plant species and are greatly adapted to their hosts and environment (Jacques *et al.* 2016). Hence, the importance of studying proteins involved in stress response in this microorganism. *Xanthomonas citri* subsp. *citri* (Xcc) strains are responsible for citrus canker which affects rutaceous plants mainly *Citrus* spp., *Fortunella* spp. and *Poncirus* spp. (Brunings and Gabriel 2003). Xcc is able to survive as epiphyte, until entering the plant tissue mainly via stomata and wounds (Gottig *et al.* 2009). One of the strategies used by Xcc to resist environmental stress conditions to guarantee full bacterial virulence on host tissues is the synthesis of the non-reducing disaccharide trehalose, which functions as a compatible solute with osmoprotection capacity (Piazza *et al.* 2015). Other osmoprotectants well characterized in bacteria include glycine betaine, proline (Weber, Kogl and Jung 2006; Wood 2011; Freeman *et al.* 2013) and ectoine (Peter, Burkovski and Kramer 1998). Accordingly, a proteomic analysis of Xcc subjected to sodium chloride stress revealed an increased amount of proteins related to the biosynthesis of these compounds (Barcarolo *et al.* 2019).

A family of proteins implicated in stress response was first described in plant species in early 80´s and was named late embryogenesis abundant (LEA) proteins. Because seeds are able to withstand severe dehydration, LEA proteins have been associated with desiccation tolerance (Tunnacliffe and Wise 2007). These proteins comprise a group of small and hydrophilic polypeptides predominantly composed of repeated hydrophilic amino acids arrangements forming a highly hydrophilic and heat stable structure (Battaglia *et al.* 2008). Roles for LEA proteins in water depletion processes include: protection of proteins, membranes and cellular structures by retention of water, sequestration of ions, and renaturation of misfolded proteins (Wise 2003). Although LEA proteins were primarily found in plants, a number of putative LEA genes have been found in non-plant species, including the bacteria *Haemophilus influenzae, Bacillus subtilis* (Stacy and Aalen 1998), *Deinococcus radiodurans* (Battista, Park and McLemore 2001), *Azotobacter vinelandii* (Rodriguez-Salazar, Moreno and Espin 2017) and the nematodes *Caenorhabditis elegans* and *Aphelenchus avenae* (Browne, Tunnacliffe and Burnell 2002). Some LEA proteins were named hydrophilins based on their high hydrophilicity (>1.0) and high glycine contents (>6%) (Garay-Arroyo *et al.* 2000), including the *E. coli* YjbJ protein. This small 69-amino acid protein is highly abundant during early stationary phase (Link, Phillips and Church 1997), is induced under osmotic stress imposed by sodium chloride and acts as a general stress response protein (Garay-Arroyo *et al.* 2000). Moreover, YjbJ resembles the general stress response CsbD protein from *B. subtilis* (Weber, Kogl and Jung 2006).

Here, the role of two Xcc bacterial genes, which encode proteins with sequence similarity to YjbJ protein, during stressful conditions, was analyzed. Xcc mutant strains lacking XAC0100 and XAC4007 displayed decreased bacterial culturability under desiccation, freezing, osmotic and oxidative stress. Also, bacterial fitness was impaired when these mutants were evaluated as epiphytes on leaves, affecting their capability to develop citrus canker disease under natural infection conditions. Moreover, Xac4007 overexpression in *E. coli* conferred advantages to this bacterium on salt, osmotic, oxidative and freezing stress.

MATERIALS AND METHODS

Bacterial strains, culture conditions and media composition

Escherichia coli DH5α and BL21-CodonPlus-RIL cells were grown at 37°C in Luria Bertani (LB) medium (Sambrook 2001). *Xanthomonas citri* subsp. *citri* wild-type strain Xcc99–1330 (Ficarra *et al.* 2015) and mutant strains were cultured at 28°C in Silva Buddenhagen (SB) medium (5 g L^{-1} sucrose, 5 g L^{-1} yeast extract, 5 g L^{-1} peptone, and 1 g L^{-1} glutamic acid, pH 7.0) (Dunger *et al.* 2007) or XVM2 medium (20 mM NaCl, 10 mM $(NH_4)_2SO_4$, 1 mM $CaCl_2$, 0.01 mM $FeSO_4$, 5 mM $MgSO_4$, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4, 10 mM fructose, 10 mM sucrose and 0.03% (w/v) casein acid hydrolysate (casaminoacid), pH 6.7) (Wengelnik *et al.* 1996). Antibiotics were used with the following final concentrations: ampicillin (Ap), 100 μg mL^{-1} for *E. coli* and 25 μg mL^{-1} for Xcc, gentamycin (Gm), 20 μg mL^{-1} both for *E. coli* and for Xcc, kanamycin (Km) 40 μg mL^{-1} for *E. coli* and 25 μg mL^{-1} for Xcc, chloramphenicol (Cm) 30 μg mL^{-1} for *E. coli*. LB medium was supplemented with sucrose 10% (w/v) for mutant selection.

In silico analysis of proteins from *Xanthomonas*

BioCyC Database Collection (https://biocyc.org), Uniprot (http://www.uniprot.org) and NCBI (https://www.ncbi.nlm.nih.gov/) were used for sequence searching. The isoelectric point and molecular mass predictions were made by using Compute pI/Mw tool (http://expasy.org/tools/pi_tool.html). Sequence identities and similarities were determined using BLAST program with the GenBank database on the NCBI web-server (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Motif analysis was performed with Pfam tool (http://www.ebi.ac.uk/Tools/InterProScan/). Amino acid comparison and multiple alignments were analyzed with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). ProtParam (http://au.expasy.org/tools/protparam.html) was used to organize sequence information, the grand average of hydropathy (GRAVY) and instability index. RADAR (http://www.ebi.ac.uk/Tools/Radar/index.html) was used to detect sequence repeat motif and IUPred for prediction of intrinsically unstructured/disordered proteins (http://iupred.enzim.hu).

RNA preparation and quantitative real-time PCR (qRT-PCR)

Xcc were cultured either in SB medium for 6 h, as control, or in SB supplemented with 0.25 M NaCl and 0.5 M mannitol for 6 h until stationary growth phase; treated with 0.25 mM H_2O_2 for 20 min or grown in XVM2 medium overnight. Xcc maintained in desiccation conditions were recovered by soaking with 15 mM NaCl. In all the cases bacteria were harvested by centrifugation.

Bacterial recovery from sprayed leaves was done as previously described (Gottig *et al.* 2008). Briefly, 10 citrus leaves for each time point analysis were harvested and immediately sliced into thin pieces with a sterile razor blade and maintained for 1 h in sterile glass plates containing 15 mL of distilled water for bacterial exudation. Bacterial cells were recovered from the previously pipetted water by centrifugation. In all the cases, total bacterial RNA was isolated by using TriPure Isolation Reagent (Roche) according to the manufacturer's instructions. RNA quality was analyzed by electrophoresis in a 1% (w/v) agarose. cDNA was synthesized from 2 μg of total RNA using M-MLV Reverse Transcriptase (Promega) and the oligonucleotide dN6 (Invitrogen).

qPCRs were performed as previously described (Sgro *et al.* 2012). The oligonucleotide pairs used (Table S1, Supporting Information) were qPCRXAC0100, qPCRXAC4007 and qPCRrpoB, as an internal reference gene (Jacob *et al.* 2011). Values were normalized by the internal reference (Ct_r) according to the equation $\Delta Ct = Ct—Ct_r$, and quantified as $2^{-\Delta Ct}$. A second normalization by a control (Xcc cultured in early exponential phase of growth in SB medium or 1 day post-inoculation for plant-pathogen interaction) (Ct_c), $\Delta\Delta Ct = Ct—Ct_c$, produces a relative quantification: $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). The values are the mean of four independent experiments with n = 3. The results were analyzed using one-way analysis of variance (ANOVA), $P < 0.05$.

Generation of XAC0100 and XAC4007 deletion mutants

Mutant strains were performed by double crossover events. The flanking regions of XAC0100 and XAC4007 were amplified by PCR using two pairs of oligonucleotides for each one, rendering fragments of approximately 1000 bp. The pair used for the amplification of the region upstream XAC0100 was UpXAC0100F and UpXAC0100R. The pair used for the amplification of the region downstream XAC0100 was DownXAC0100F and DownXAC0100R. On the other hand, for XAC4007 deletion mutant, the pair used for the amplification of the region upstream XAC4007 was UpXAC4007F and UpXAC4007R; the pair used for the amplification of the region downstream XAC4007 was DownXAC4007F and DownXAC4007R, respectively (Supplementary Table 1). All fragments were amplified by PCR, downstream regions were digested with the restriction enzymes *Bam*HI and *Eco*RI and upstream regions were digested with the restriction enzymes *Eco*RI and *Hind*III. Then, digested fragments were cloned onto the pk18*mobsacB* digested with *Bam*HI and *Hind*III. This vector is a derivative plasmid of pK18*mob* and pK19*mob* with a genetically modified *sacB* gene which confers sucrose-sensitivity to Gram- and some Gram+ species. These plasmids are useful tools to force and to detect double cross-over events and to mutate a punctual gene inside an operon (Schafer *et al.* 1994). For bacterial conjugations, plasmids carrying out the constructions were transferred to Xcc by biparental mating from the broad host-range-mobilizing *E. coli* strain S17–1. Bacterial mixtures were spotted onto LB agar plates and incubated for 48 h at 28°C. The bacterial spots were harvested, washed and transferred to selective medium. Transconjugants were selected onto LB agar supplemented with Km and Ap for plasmid integration by a simple recombination event. To select double crossover events a single colony was grown for 24 h in non-selective LB medium at 28°C. About 2×10^6 cells were plated onto LB agar plates containing 10% (w/v) sucrose and incubated for 48 h at 28°C. About 50% of the resulting colonies were sensitive to Km, indicating the excision of the plasmid after selection for kanamycin resistance. XAC0100 and XAC4007 deletion mutant strains were verified by PCR by using oligonucleotides UpXAC0100F and DownXAC0100R to ΔXAC0100 and UpXAC4007F and DownXAC4007R to ΔXAC4007. To perform the double mutant strain, both in XAC0100 and XAC4007, pk18*mobsacB* plasmid carrying out the flanking regions of XAC0100 was transferred to ΔXAC4007 by biparental conjugation from the *E. coli* strain S17–1 and ΔXAC0100/4007 double mutant strain was selected as was described above. Complementation of ΔXAC0100 was performed by amplification of XAC0100 with FXAC0100 and RXAC0100 oligonucleotides while complementation of ΔXAC4007 was performed by amplification of XAC4007 with FXAC4007 and RXAC4007 oligonucleotides (Table S1, Supporting Information). Amplified products were digested with *Hind*III and *Bam*HI restriction enzymes and ligated to pBBR1MCS-2 and pBBR1MCS-5 plasmids (Kovach *et al.* 1995), respectively, digested with the same enzymes. The resulting plasmids were transferred to ΔXAC0100 and ΔXAC4007 by biparental mating, rendering ΔXAC0100c and ΔXAC4007c, correspondingly.

Plant material and inoculations

Citrus sinensis cv. Valencia was used as host plant for plant-pathogen interaction evaluation and tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) were used as non-host plants. All plants were cultivated in a growth chamber in incandescent light at 28°C with a photoperiod of 16 h. Bacteria were grown in SB medium to OD_{600nm} of 1, harvested by centrifugation and resuspended in 15 mM NaCl at 10^7 CFU mL^{-1}. For disease symptoms assays, bacterial suspensions were infiltrated into leaves with needleless syringes. Cankers were counted from 20 citrus leaves inoculated with the different strains at 10^5 CFU mL^{-1} and the areas of infected leaves were measured from digitalized images using Fiji software (Schindelin *et al.* 2012). *In planta* growth assays were performed by grinding 0.5 cm^2 leaf discs from infiltrated leaves in 0.5 mL of 15 mM NaCl, followed by serial dilutions and plating onto SB agar plates. Colonies were counted after 48 h of incubation at 28°C, and the results were presented as CFU cm^{-2} of leaf tissue. Also, bacterial inoculations were made by spraying onto citrus leaves. Epiphytic fitness was evaluated as previously described (Gottig *et al.* 2009). Briefly, bacteria were sprayed onto leaves at 10^9 CFU mL^{-1} resuspended in 15 mM NaCl and 0.01% (v/v) silwet L-77, until both leaf surfaces were uniformly wet. After inoculation, leaves were wrapped in plastic bags (wet-chamber conditions) for 24 h. Three leaf samples (~15 cm^2 per time-point) were taken out at different days after inoculation and transferred to test tubes with 3 mL of 15 mM NaCl. Tubes were submerged in a Digital Ultrasonic Bath Branson model #5510 for 10 min. Subsequently, each tube was vortexed for 5 min, and serial dilutions were plated onto SB agar plates containing Ap. To evaluate bacterial adherence onto leaves surface, 20 μL of each bacteria suspension was incubated onto leaves abaxial surface for 6 h at 28°C, in a humidified chamber. Bacterial adhesion was visualized by staining with crystal violet (CV) (Gottig *et al.* 2009).

Stress-resistance in *Xanthomonas*

Growth of culturable bacterial cells was evaluated under different conditions, Xcc, ΔXAC0100, ΔXAC0100c, ΔXAC4007, ΔXAC4007c and ΔXAC0100/4007 were subcultured in fresh SB medium supplemented with 0.25 M NaCl, 0.25 M KCl, 0.8 M mannitol or 25% (w/v) PEG8000 from saturated cultures grown during 16 h at 28°C, 200 rpm. SB medium without supplementation was used as a control. In all cases, growth of culturable cells

was monitored spectrophotometrically by optical density at λ = 600 nm (OD_{600nm}).

The desiccation assay was adapted from Mattimore and Battista (Mattimore and Battista 1996). Briefly, 10 mL of bacterial cells grown until exponential phase were collected by centrifugation, washed with 10 mL of 15 mM NaCl and resuspended in the same volume of 15 mM NaCl. A 100 μL aliquot of each bacterial suspension, having nearly 10^8 CFU of Xcc, ΔXAC0100, ΔXAC0100c, ΔXAC4007, ΔXAC4007c and ΔXAC0100/4007, was placed into sterile microplate of 24 wells by triplicate and incubated in a desiccator maintained at 30% relative humidity (RH) at room temperature (~25°C). Relative humidity inside the desiccator was continuously monitored with a digital hygrometer and maintained with silica gel balls. One set of bacterial strains was removed at a regular interval of time (0, 1, 2 and 3 days), and cells were recovered by soaking with 200 μL of 15 mM NaCl under sterile conditions. For proteins protection assays, 10 mL of *Xanthomonas* cells were grown until exponential phase, collected by centrifugation, washed with 10 mL of 15 mM NaCl and resuspended in an equal volume of 15 mM NaCl. 100 μL aliquots of bacterial suspension were incubated with 5 μM Xac0100 and Xac4007 recombinant proteins or with bovine serum albumin (BSA) (Sigma–Aldrich); thioredoxin (Trx) as controls and then placed in the desiccator as described above. Appropriate dilutions of these samples were plated onto SB agar plates and incubated at 28°C for 48 h. CFU were counted and Log CFU mL^{-1} was calculated.

Freezing and oxidative stress survival assays were performed by culturing Xcc, ΔXAC0100, ΔXAC0100c, ΔXAC4007, ΔXAC4007c and ΔXAC0100/4007 until early exponential growth phase (OD_{600nm} = 1, equivalent to 1 × 10^9 CFU mL^{-1}). Aliquots of 1 mL of each bacterial strain were treated with 0 and 30 mM H_2O_2 for 20 min, for oxidative stress; and aliquots of 0.2 mL of each bacterial strain were incubated at −20°C for 2 h, for freezing stress and at room temperature, as control. After incubation time, samples were centrifuged, washed once with 15 mM NaCl and serial dilutions were plated onto SB agar plates. Colonies were counted after incubation for 48 h at 28°C and the percentage of culturable bacteria was calculated as CFU mL^{-1} after treatment divided by the CFU mL^{-1} at room temperature x 100.

Expression and purification of *Xanthomonas* recombinant proteins

The full-length XAC0100 and XAC4007 genes were amplified by PCR from Xcc genomic DNA using the oligonucleotides FXAC0100 and RXAC0100 and FXAC4007 and RXAC4007 to express Xac0100 and Xac4007 proteins, respectively (Table S1, Supporting Information). PCR products were cloned into pET28a (+) vector (Novagen, Merck KGaA, Darmstadt, Germany) previously digested with the restriction enzymes *NdeI* and *SalI*. After transformation into *E. coli* strain BL21-CodonPlus-RIL the synthesis of recombinant proteins was induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) for 5 h at 28°C. The proteins were purified by affinity chromatography from the soluble fraction of the bacterial lysates using Ni^{2+}-nitrilotriacetate (Ni-NTA) agarose (Qiagen, Hilden, Germany) and dialyzed overnight with several changes of 25 mM Tris-HCl (pH 7.5). The purity of recombinant proteins was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

In vitro L-lactate dehydrogenase freeze-thaw assays

L-lactate dehydrogenase (L-LDH) from rabbit muscle was obtained from Sigma (Roche Diagnostics GmbH, Germany) and diluted in 25 mM Tris–HCl buffer (pH 7.5) and 50% (v/v) glycerol following the manufacturer's recommendations. In a microfuge tube, 0.15 μM L-LDH was added to a final volume of 50 μL of reaction buffer (25 mM Tris-HCl, pH 7.5) with or without stabilizing agents (at molar ratios of 1:1), among them: BSA, Trx, Xac0100 and Xac4007 recombinant proteins. Mixtures were frozen for 15 min at −70°C and thawed for 15 min in a water bath at 25°C, this constituted one freeze-thaw cycle, which was repeated three times. At the end of the treatment, samples were kept on ice until enzyme activity was determined. L-LDH activity was measured in a final reaction volume of 200 μL, containing 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 200 μM NADH, 2 mM pyruvate and 0.034 μM L-LDH, monitoring the NADH oxidation at 340 nm at 25°C every 10s for 4 min (Synergy 2 Reader (BioTek)). All assays were carried out in triplicate and values were expressed as percentage of residual L-LDH activity relative to unfrozen samples activity.

Abiotic stress tolerance in *E. coli* transformants

Abiotic stress tolerance was evaluated *in vivo* in *E. coli* strain BL21-CodonPlus-RIL expressing recombinant empty vector pET28a (*E. coli*/EV), pET28a-XAC0100 (*E. coli*/Xac0100) and pET28a-XAC4007 (*E. coli*/Xac4007). IPTG induction was made for 2 h at 28°C. Bacterial cultures were adjusted to OD_{600nm} = 0.3 (equivalent to 1 × 10^8 CFU mL^{-1}) in fresh LB medium supplemented with 50 μg mL^{-1} Km, 30 μg mL^{-1} Cm and 0.5 mM IPTG. Additional 0.25 M NaCl was used for salt tolerance and 0.8 M mannitol and 10% (w/v) PEG8000, for osmotic stress. Growth curves in microplates of 96 wells were carried out and OD_{600nm} value was plotted for 18 h. Freezing tolerance was assessed by incubating *E. coli* strains at −20°C for 2 h and at room temperature, as control and, serial dilutions were plated onto LB agar plates. Colonies were counted after incubation for 24 h at 37°C and Log CFU mL^{-1} was calculated. Bacterial survival under oxidative stress was assessed by incubating induced *E. coli* strains with 0 and 30 mM H_2O_2 for 20 min. Then, treated bacteria were centrifuged, washed once with 15 mM NaCl and serial dilutions were plated onto LB agar plates. After overnight incubation at 37°C, colonies were counted and cultured cells were calculated as CFU mL^{-1} after treatment divided by the CFU mL^{-1} without treatment x 100. For desiccation tolerance assays, 10 mL of induced *E. coli* strains were collected by centrifugation, washed with 10 mL of 15 mM NaCl and resuspended in an equal volume of 15 mM NaCl. A 100 μL aliquot of each bacterial suspension at 10^8 CFU of *E. coli* strain BL21-CodonPlus-RIL expressing recombinant empty pET28a (*E. coli*/EV), pET28a-XAC0100 (*E. coli*/Xac0100) and pET28a-XAC4007 (*E. coli*/Xac4007), was placed into sterile microplate of 24 wells by triplicate and incubated in desiccators maintained at 30% RH at room temperature (~25°C). One set of bacterial strains was removed at a regular interval of time (0, 1, 2, and 3 days), and cells were recovered by soaking with 200 μL of 15 mM NaCl under sterile conditions. The appropriate dilutions of these samples were spread onto LB agar plates and incubated at 37°C for 24 h.

Statistical analysis

In all figures, bars show the mean of the data, the error bars show the standard deviation and asterisks (*) indicate significant difference ($P < 0.05$) analyzed by one-way analysis of variance (ANOVA).

RESULTS

In silico analysis of Xcc proteins possibly involved in stress response

To search for Xcc proteins involved in stress responses, YjbJ protein sequence from *E. coli* (strain K12) (Pineda-Lucena *et al.* 2002), classified as a hydrophilin protein on the basis of the criteria of small size, high glycine content (>6%) and high hydrophilicity (>1.0) (Garay-Arroyo *et al.* 2000, Reyes *et al.* 2005), was used as query protein. Blastp searches retrieved two Xcc proteins named Xac0100 and Xac4007, encoded by XAC0100 and XAC4007 genes, respectively. Alignment between YjbJ and Xac0100 showed 42% identity (59% sequence similarity) (Fig. 1A), while the comparison between YjbJ and Xac4007 showed 50% identity (66% sequence similarity) (Fig. 1B). Moreover, alignment between Xac0100 and Xac4007 showed 35% identity (55% sequence similarity) (Fig. 1C). Molecular weight, instability index, hydrophilicity, structural information availability and prediction of intrinsically unstructured/disordered domains were evaluated *in silico*. Both proteins displayed small sizes and were predicted to be hydrophilic: Xac0100 with 62 amino acids, molecular weight of 6541.3 Da, pI of 9.40 and hydropathy index of -0.873 and Xac4007 with 69 amino acids, molecular weight of 7981.7 Da, pI of 5.01 and hydropathy index of -1.181. Besides, glycine content analysis showed that Xac0100 and Xac4007possess 11.3% and 8.7%, respectively (Table 1). Sequence analyses in Uniprot database indicated that Xac0100 and Xac4007 were classified as CsbD family members (Table 1). However, sequences comparison between CsbD from *B. subtilis* and Xac0100 and Xac4007 showed low sequence similarity, 28% and 30%, respectively (data not shown). Taking into consideration the higher sequence similarity to YjbJ than to CsbD, in addition to the general characteristics shared with other proteins belonging to the family (Battaglia *et al.* 2008), both Xac0100 and Xac4007 proteins could be considered as hydrophilins.

XAC0100 and XAC4007 are overexpressed both in abiotic stress and during plant-pathogen interaction

To analyze if XAC0100 and XAC4007 play a role under abiotic stress conditions, Xcc was subjected to different stress challenges such as saline, osmotic and oxidative stress, water deficit, and bacterial growth to stationary phase. Differential expression of both genes was evaluated by qRT-PCR. XAC0100 was induced 5 times and XAC4007 30 times under saline stress, compared to the control condition corresponding to Xcc grown in SB medium without salt supplementation (Fig. 2A). In the presence of the osmotic stressor mannitol, XAC0100 expression was induced 2.3 times while XAC4007, 15 times related to the control (Fig. 2A). In Xcc subjected to oxidative stress, XAC0100 expression was induced 7 times and XAC4007 increased 24 times, relative to the SB control (Fig. 2A). Desiccation conditions increased 20 times the expression levels of XAC0100 and 218 times for XAC4007 relative to the control without water deficit. During stationary growth phase, expression levels of XAC0100 and XAC4007 increased 5 and 108 times, respectively compared to the exponential growth phase (Fig. 2A). On the other hand, to assess if XAC0100 and XAC4007 were differentially expressed during plant-pathogen interaction, expression levels in XVM2 medium, a nutrient poor medium that simulates the plant apoplastic space, were analyzed compared to gene expression in SB medium (control condition). Expression levels of XAC0100 and XAC4007 were significantly induced in XVM2 ($P < 0.05$), with induction levels of 18 and 290 times, respectively (Fig. 2B). Then, to evaluate whether XAC0100 and XAC4007 were expressed during the pathogenic process, RNA was obtained from Xcc recovered from infected *C. sinensis* leaves at 1 and 6 days post bacterial inoculation. qRT-PCR results showed that XAC0100 expression was induced 17 times and XAC4007 38 times after 6 days, compared to expression levels obtained after 1 day post bacterial inoculation (Fig. 2C).

Characterization of XAC0100 and XAC4007 Xcc mutant strains during plant-pathogen interaction

With the aim to evaluate the participation of XAC0100 and XAC4007 in plant–pathogen interaction, mutant strains named ΔXAC0100 (lacking XAC0100 gene), ΔXAC4007 (lacking XAC4007 gene), ΔXAC0100/4007 (lacking both XAC0100 and XAC4007 genes) and the complemented strains (ΔXAC0100c and ΔXAC4007c) were constructed. *C. sinensis* plants were inoculated through different methods. Wild type Xcc, ΔXAC0100, ΔXAC0100c, ΔXAC4007, ΔXAC4007c and ΔXAC0100/4007 strains infiltrated by pressure at 10^7 CFU mL^{-1} triggered similar disease symptoms in citrus leaves and no differences were observed in the time of onset of lesion formation, lesion size and number of culturable bacteria recovered from infected tissue (Suppl. Fig. 1A and 1B). These results suggest that the expression of these genes does not affect bacterial virulence while the pathogen is inside the apoplast. Moreover, no differences were observed in the number of cankers after bacterial infiltrations at lower concentrations of 10^5 CFU mL^{-1} (Fig. 1C) or in cell attachment onto leaves (Fig. 1D). Then, the hypersensitive response (HR), used by plants to limit pathogen growth and propagation (Wright and Beattie 2004) was evaluated. No differences were observed in the behavior of ΔXAC0100, ΔXAC4007 and ΔXAC0100/4007 to induce HR in pepper and tomato leaves compared to wild type Xcc (Fig. 1E). However, inoculation by spraying bacteria on *C. sinensis* leaf surfaces, a method that resembles natural infection, showed impaired virulence of mutants strains compared to Xcc. Canker lesions on leaves infected with wild type Xcc were 2.12, 2.97 and 3.13 times higher compared to those inoculated with ΔXAC0100, ΔXAC4007, ΔXAC0100/4007 strains, respectively (Fig. 3A). The epiphytic fitness of all strains was analyzed and important differences were observed between the mutants and wild type strain. After 14 days, the culturable population of wild type Xcc was 6.2, 9.5 and 12.6 times higher than ΔXAC0100, ΔXAC4007 and ΔXAC0100/4007, respectively. After 21 days, differences were considerably pronounced and the culturable wild type Xcc was 23.4, 31.5 and 31.6 times higher than ΔXAC0100, ΔXAC4007, ΔXAC0100/4007, respectively (Fig. 3B). Complemented strains ΔXAC0100c and ΔXAC4007c reversed the phenotypes observed in cankers number and epiphytic fitness (Fig. 3A and 3B).

XAC0100 and XAC4007 participation in abiotic stress

Both XAC0100 and XAC4007 genes were shown to have an important role on epiphytic fitness, when bacteria are on the leaf epidermis and water accessibility is low. Therefore, Xcc, ΔXAC0100,

(A)

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YjbJ        MNKDEAGGNWKQFKGKVKEQWGKLTDDDMTIIEGKRDQLVGKIQERYGYQKDQAEKEVVDWETRNEYRW 69
Xac0100     MDKNRIDGAAKQVKGSVKEAIGRVTGDKSTELEGAAEKNVGKVQRKAGEVADDVRDAVKSTK------- 62
            *:*:. .*  **.**.***  *::*.*. * :**  :: ***:*.: *   *:... * . :
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(B)

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YjbJ        MNKDEAGGNWKQFKGKVKEQWGKLTDDDMTIIEGKRDQLVGKIQERYGYQKDQAEKEVVDWETRNEYRW 69
Xac4007     MNSDIISGKWTQLKGKAQAKWGDLTDDDFKVAEGNAEYLQGKLQERYGWDRDRAQTEVRAFEKSLRDDT 69
            **.*  .*:*.*:***.: :**.*****:.: **: : * **:*****:::*:*:.**  :*.  .
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(C)

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XAC0100     MDKNRIDGAAKQVKGSVKEAIGRVTGDKSTELEGAAEKNVGKVQRKAGEVADDVRDAVKSTK------- 62
XAC4007     MNSDIISGKWTQLKGKAQAKWGDLTDDDFKVAEGNAEYLQGKLQERYGWDRDRAQTEVRAFEKSLRDDT 69
            *:.: *.*  .*:**..:   * :*.*. .  ** **   **:*.: *   * .:  *::
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Figure 1. Sequence alignments. Protein sequence alignments were computed with Clustal Omega. Asterisks (*) represent identical amino acids, colons (:) conservative replacements, full stops (.) semi-conservative replacements. **(A)**, Alignment between full-length sequences of YjbJ from *E. coli* (strain K12) and Xac0100 from Xcc. **(B)**, Alignment between full-length sequences of YjbJ from *E. coli* (strain K12) and Xac4007 from Xcc. (C) Alignment between full-length sequences of Xac0100 and Xac4007 from Xcc.

Table 1. General characteristics of hydrophilins-like proteins from *Xanthomonas*.

	Xac0100	Xac4007
Amino acids content	62	69
pI	9.40	5.01
Mw (Da)	6541.3	7981.7
Family and domain databases	CsbD family	CsbD family
Glycine percent	11.3	8.7
Instability index	−2.12 (stable)	4.89 (stable)
Grand average of hydropathicity (GRAVY)	−0.873 hydrophilic	−1.181 hydrophilic
Aliphatic index	69.19	56.67
Structure	ND	NMR in YjbJ (Pineda-Lucena, *et al.* 2002)
IUPred	disordered protein	ordered protein

ΔXAC0100c, ΔXAC4007, ΔXAC4007c and ΔXAC0100/4007 culturability under different stress conditions, was analyzed. First, bacterial growth was evaluated in SB, demonstrating that all strains reached the same population under control growth conditions (Fig. 2A). Next, bacterial strains were cultured in SB medium supplemented with NaCl, KCl, mannitol and PEG8000. Both salts and mannitol impaired bacterial culturability similarly in all strains (Fig. 2B, C and D) compared to the bacterial behavior in non-supplemented SB medium (Fig. 2A). However, with PEG8000 supplementation, a non-ionic polyether compound, ΔXAC0100, ΔXAC4007 and ΔXAC0100/4007 cultures were more affected than Xcc or the complemented strains (Fig. 4A). Then, to investigate the role of XAC0100 and XAC4007 on desiccation tolerance, culturability of Xcc, ΔXAC0100, ΔXAC4007 and ΔXAC0100/4007 and the corresponding complemented strains under desiccation stress conditions was analyzed. Mutant strains showed greater susceptibility to desiccation stress compared to Xcc. After 1 day of desiccation, culturability of ΔXAC0100, ΔXAC4007 and ΔXAC0100/4007 dropped 5.2, 1.9 and 3.2 times compared to Xcc, respectively (Fig. 4B). After 3 days of desiccation, ΔXAC0100, ΔXAC4007 and ΔXAC0100/4007 culturable bacteria declined 2.7, 2.2 and 2.7 times compared to Xcc, respectively (Fig. 4B). Comparing the initial populations with those obtained after 3 days exposure to desiccation stress, Xcc culturable population decreased 37 times while ΔXAC0100 77 times, ΔXAC4007 83 times and ΔXAC0100/4007 93 times, highlighting the effect that XAC0100 and XAC4007 in overcoming desiccation stress. Complemented strains showed a similar performance as the wild type Xcc (Fig. 4B). Considering that desiccation stress is also caused by freezing, the performance was analyzed under freezing at −20 °C for 2 h. Strains lacking both XAC0100 and XAC4007 were negatively affected, showing a rate of culturability of 37.4%, 44.4% and 45% for ΔXAC0100, ΔXAC4007 and ΔXAC0100/4007, respectively. Meanwhile, under

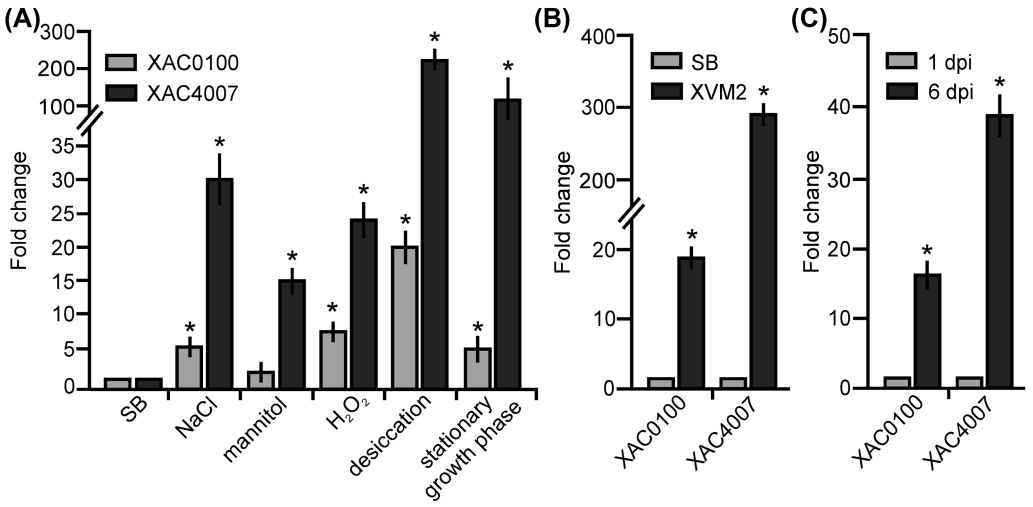

Figure 2. Transcriptional analysis. qRT-PCR assays of XAC0100 and XAC4007 genes with RNA obtained from Xcc **(A)**, cultured in SB without and with 0.25 M NaCl, 0.8 M mannitol, 0.25 mM H_2O_2, bacteria recovered from desiccation conditions and from stationary growth phase, **(B)**, cultured in SB and XVM2 medium and **(C)**, recovered from inoculated *Citrus sinensis* leaves at 1 or 6 days post-inoculation. Bars show the gene expression levels, relative to Xcc cultured in SB medium. As reference gene, *rpoB* was amplified. Values represent the means of three biological replicates and error bars show standard deviations. The data were analyzed by one-way ANOVA ($P < 0.05$) and asterisks indicate significant difference.

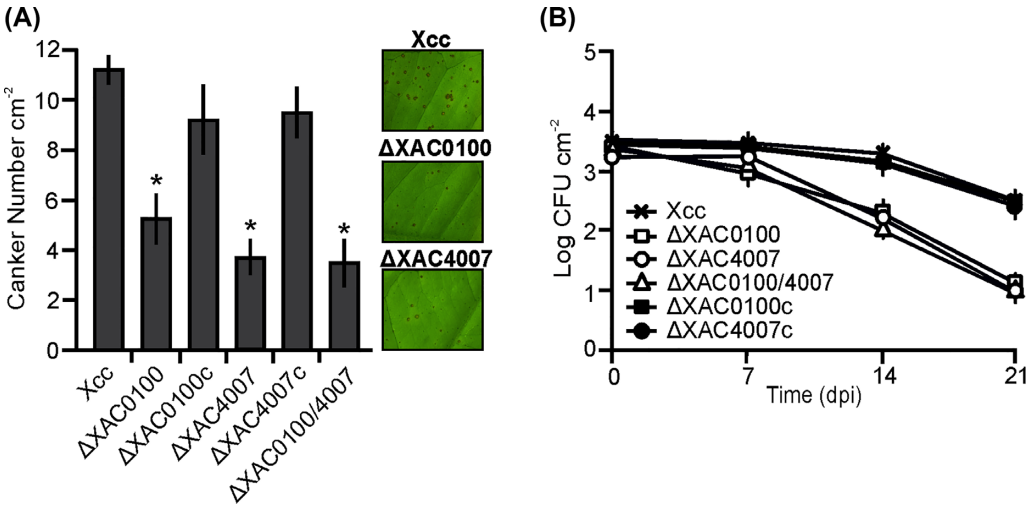

Figure 3. Plant-pathogen interaction of bacterial variants on *Citrus sinensis* leaves. (A), Quantification of canker number in *C. sinensis* leaves after one month of spray treatment with Xcc, ΔXAC0100, ΔXAC4007, ΔXAC0100/4007, ΔXAC0100c and ΔXAC4007c strains at 10^9 CFU mL^{-1}. Bars are the means of 15 leaves tested and error bars are standard deviations, results represent three independent experiments. The data were statistically analyzed using one-way ANOVA ($P < 0.05$). Asterisks denote significant differences between Xcc and mutant strains. On the right, representative images of leaves sprayed with Xcc, ΔXAC0100, ΔXAC4007 strains after one month are shown. **(B)**, Bacterial epiphytic survival calculated as Log CFU cm^{-2} per each set time. Results are representative of three independent experiments, and the average of four leaves tested for each strain was used. Error bars are standard deviations.

the same conditions, Xcc showed a culturability of 65.4%. Complemented strains, ΔXAC0100c and ΔXAC4007c, showed a similar behavior as the wild type Xcc (Fig. 4C).

Also, the role of XAC0100 and XAC4007 in oxidative stress was evaluated. Bacterial strains exposed to hydrogen peroxide showed that ΔXAC4007 and ΔXAC0100/4007 were 26 and 20 times less tolerant than Xcc, respectively, whereas ΔXAC0100 and the corresponding complemented strains showed a similar performance to Xcc (Fig. 4D).

Xac0100 and Xac4007 recombinant proteins partially rescue *Xanthomonas* strains under desiccation

Purified Xac0100 and Xac4007 recombinant proteins were assessed for their ability to overcome stress imposed by desiccation. Therefore, Xcc was incubated with 50 mM Tris-HCl (buffer) as a control and 5 μM of recombinant Xac0100 or Xac4007. Additional controls were made with 5 μM BSA (as a protective control) and Trx at the same concentration, as another protein control. After 1 day of desiccation, slight decreases in bacterial population were observed for all the treatments without significant differences between them (Fig. 5A). After 2 and 3 days under desiccation, culturable Xcc continued to decrease, showing 37 times less population at 3 days in control conditions. Noticeably, supplementation with Xac0100 and Xac4007 recombinant proteins

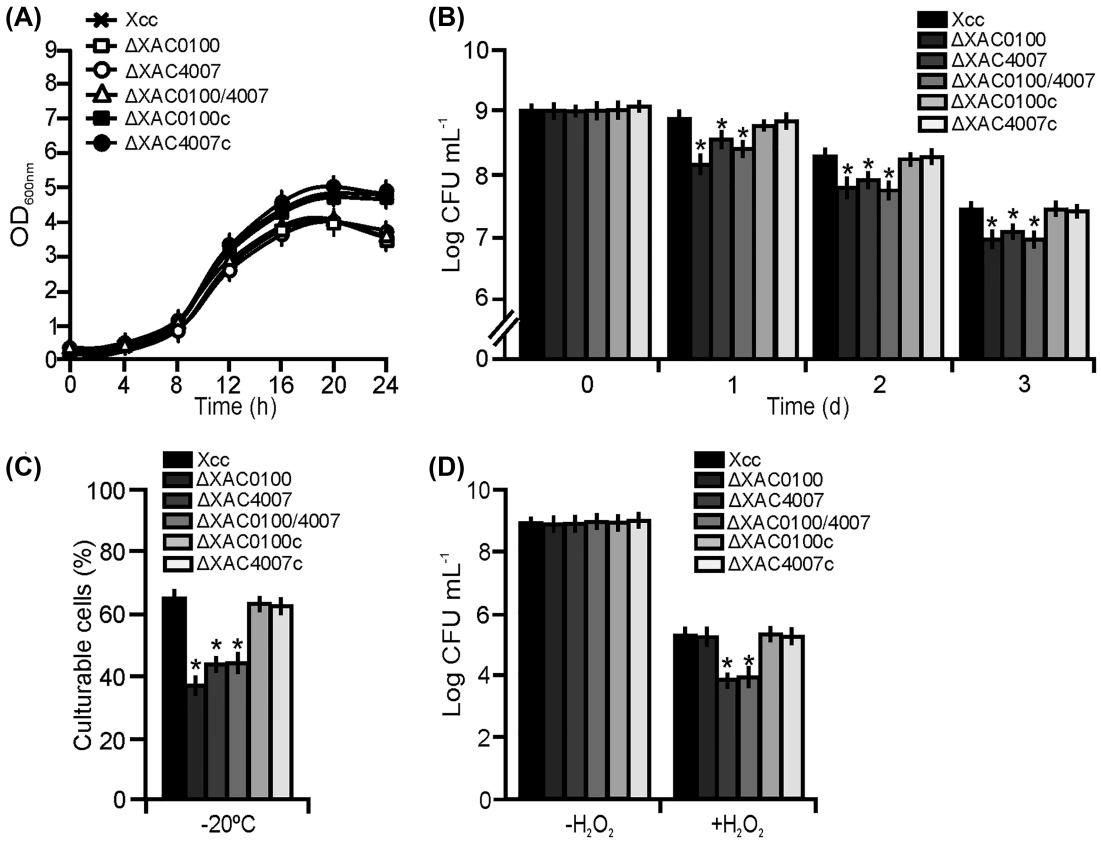

Figure 4. Bacterial fitness. Wild type Xcc, ΔXAC0100, ΔXAC4007, ΔXAC0100/4007, ΔXAC0100c and ΔXAC4007c strains subjected to **(A)**, osmotic stress in SB medium supplemented with 25% (w/v) PEG8000, **(B)**, recovered from 0, 1, 2 and 3 days under desiccation conditions, **(C)** freezing for 2 h at −20 °C and **(D)**, oxidative stress by incubation with 30 mM H_2O_2 for 20 min. Bars represent the mean of three independent experiments and error bars the standard deviations. Asterisks indicate significant differences between Xcc and mutant strains using ANOVA test ($P < 0.05$).

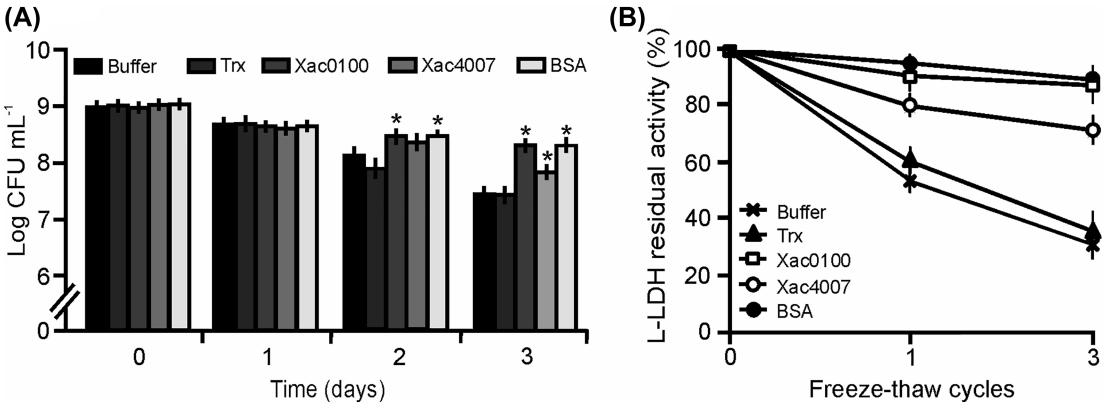

Figure 5. Functional evaluation of *Xanthomonas* recombinant proteins. (A), Wild type Xcc culturability under desiccation conditions, supplemented with 50 mM Tris-HCl pH 8 (buffer) (black), Trx (dark grey), Xac0100 (middle grey), Xac4007 (light grey) and BSA (white) at 5 μM. Bars represent the mean of 4 independent experiments and error bars represent standard deviation. Asterisks indicate significant differences between the control and the stabilizing agents by using ANOVA test ($P < 0.05$). **(B)**, L-LDH activity determined immediately after once and three freeze-thaw cycles from samples containing buffer (crosses), Trx (triangles), Xac0100 (squares), Xac4007 (empty circles) and BSA (full circles). Values represent percentage of residual activity compared to untreated samples. Values represent the mean of five independent experiments. Error bars represent standard deviation.

showed that the population of culturable bacteria at this time declined 4.6 and 17 times, respectively (Fig. 5A). Similar results were observed when ΔXAC0100, ΔXAC4007 and ΔXAC0100/4007 strains, subjected to desiccation, were supplemented with both recombinant proteins (Fig. 3A, B and C).

Xac0100 and Xac4007 recombinant proteins protect L-Lactate dehydrogenase activity against freeze-thaw inactivation

L-Lactate dehydrogenase (L-LDH), which enzymatic activity is lost by freeze-thaw cycles, was used to evaluate Xac0100 and Xac4007 recombinant proteins as enzymatic protective agents.

Activity quantifications of L-LDH after freeze-thaw cycles, in presence and absence of these proteins, were performed. Both Xac0100 and Xac4007 were able to protect L-LDH activity until three freeze-thaw cycles when they were used at molar ratios of 1:1 (enzyme: recombinant protein). Specifically, after three freeze-thaw cycles, 87% of L-LDH activity was recovered when the enzyme was supplemented with Xac0100 and 71% when Xac4007 was used (Fig. 5B). BSA was used as a protective control (Goyal, Walton and Tunnacliffe 2005) (1:1 ratio) by recovering 89% of L-LDH activity after three freeze-thaw cycles. The remaining enzymatic activity after three freeze-thaw cycles on non-supplemented L-LDH samples fell down to 30% (Fig. 5B) and similar results were obtained when Trx recombinant protein (1:1 ratio) was used.

Effects of Xac0100 and Xac4007 proteins in *E. coli*

E. coli strain BL21-CodonPlus-RIL harboring the empty vector pET28a (*E. coli*/EV) or the constructions pET28a-XAC0100 (*E. coli*/Xac0100) and pET28a-XAC4007 (*E. coli*/Xac4007) were subjected to saline, osmotic, freezing, oxidative and desiccation stress. Under standard culture conditions, *E. coli*/Xac4007 showed a significant shorter lag phase ($P < 0.05$) compared to *E. coli*/EV and *E. coli*/Xac0100. *E. coli*/Xac4007 achieved the maximum growth in 8 h while *E. coli*/EV and *E. coli*/Xac0100 in 13 h without differences in reaching maximum OD_{600nm} values (Fig. 6A). Similarly, *E. coli*/Xac4007 cultured in medium supplemented with 0.25 M NaCl showed a shorter lag phase and attained the maximum OD_{600nm} value approximately 6 h earlier than the other bacterial strains. The maximum value reached for *E. coli*/Xac4007 was 1.9, significantly higher than for *E. coli*/EV that was 1.4 ($P < 0.05$) (Fig. 6B). Also, osmotic stress was evaluated and bacterial strains cultured in medium supplemented with 0.8 M mannitol. *E. coli*/Xac4007 showed a slight advantage compared to the other strains (Fig. 6C). Then, freezing tolerance in which a dehydration process also takes place was evaluated. The presence of Xac4007 in *E. coli* strains incubated at −20°C for 2 h was significantly beneficial showing 1.8 times higher population of culturable bacteria compared to *E. coli*/EV (Fig. 6D). However, even if both Xac0100 and Xac4007 demonstrated to have a critical role in tolerance to water deficit in Xcc, *E. coli* cells overexpressing these proteins showed no differences in the number of recovered bacteria after desiccation stress exposition compared to *E. coli*/EV (Suppl. Fig. 4).

Finally, to evaluate if these recombinant proteins may confer any advantage to *E. coli* under another stress source, *E. coli*/EV, *E.coli*/Xac0100 and *E. coli*/Xac4007 were subjected to H_2O_2, as oxidative stress inducer. *E. coli*/Xac4007 showed 3.2 times more culturable population than *E. coli*/EV (Fig. 6E). In all stress conditions assayed, *E. coli*/Xac0100 had a similar performance to *E. coli*/EV.

DISCUSSION

This study was designed to further characterize the plant bacterial pathogen Xcc, particularly searching for proteins that may assist Xcc in coping with harsh environmental conditions such as desiccation, a limiting condition during pathogen virulence cycle. Searching for proteins with homology to proteins with functions in tolerance to desiccation in Xcc, two proteins were found: Xac0100 and Xac4007. The expression levels of XAC0100 and XAC4007 were significantly induced under osmotic, oxidative, saline stress, desiccation and starvation. Highest expression levels were obtained under water limitation on microplate and from epiphytic bacteria recovered from sprayed leaves, suggesting a relevant role of XAC0100 and XAC4007 when Xcc needs to survive in an environment under water deprivation. Our results are in agreement with the increased synthesis of YjbJ from *E. coli* and of proteins classified as hydrophilins from *S. cerevisiae* during osmotic stress (Garay-Arroyo *et al.* 2000).

In this work, a functional characterization of Xac0100 and Xac4007 in desiccation tolerance, oxidative and osmotic stresses *in vitro* and *in vivo* was performed. Xcc mutant strains in XAC0100 or XAC4007 infiltrated in the apoplastic space of citrus leaves showed no differences in the time of onset of lesion formation, lesion size or the number of bacteria recovered from inoculated tissue, suggesting that the expression of these genes does not affect the bacterial virulence while the pathogen is inside the apoplast. Noticeably, during the life cycle stage as epiphyte on citrus leaves, Xac0100 and Xac4007 conferred to Xcc an increased capacity to tolerate water deprivation. The lack of XAC0100 or XAC4007 caused lower bacterial epiphytic survival on leaves that correlates with lower number of cankers observed, indicating the importance of both genes in plant-pathogen interaction. Moreover, our results demonstrate that both XAC0100 and XAC4007 have a crucial role in Xcc survival under disadvantageous environmental conditions. In this regards, the hydrophilin protein Psyr_3782 from *P. syringae*, which shares significant amino acid identity with YjbJ and thus with Xac0100 and Xac4007, has been proposed to have an important role in bacterial epiphytic survival (Freeman *et al.* 2013). The ability of two *P. syringae* strains, B728a and DC300, to survive and to tolerate stresses on leaf surfaces was evaluated showing that B728a strain is better adapted for epiphytic survival than DC3000. Both genomes encode 12 putative hydrophilins though it was revealed that Psyr_3782 expression levels were higher in B728a strain compared to DC3000 in the presence of NaCl. In agreement with our results, the improved epiphytic survival was associated with hydrophilin presence and expression (Freeman *et al.* 2013). The ecological niche of a microorganism provides a description about how it responds to the allocation of resources and to the presence of competing species. In essence, the niche is a complex description of the ways in which microbial populations use its environment (Escalas *et al.* 2019). The phyllosphere is colonized by a diverse microbiota, with some microorganisms living on plant surfaces as epiphytes and others inhabiting inside leaves as endophytes (Beattie and Lindow 1999, Lindow and Brandl 2003). When microbial ecosystem is disturbed by different factors, severe consequences for the host health are established (Santillan *et al.* 2019). Taking into account these concepts, where the microbiota living as epiphyte is exposed to several environmental stress conditions we propose that XAC0100 and XAC4007 contribute in maintaining Xcc on the host plant environment, co-existing with microorganisms present in the phyllosphere.

Several stresses imposed to Xcc increased the expression levels of XAC0100 and XAC4007 genes, suggesting their participation in coping with stress sources. Nevertheless, no differences were observed between Xcc and mutant strains when they were subjected to saline stress imposed by NaCl and KCl, or osmotic stress imposed by mannitol. This behavior can be explained by the fact that bacterial performance is a complex response of a whole cell, so different factors, among them multiple proteins and protector osmolytes play a key role to maintain the cell homeostasis (Barcarolo *et al.* 2019). Both XAC0100 and XAC4007 were also involved in desiccation tolerance and the mutants ΔXAC0100, ΔXAC4007 and ΔXAC0100/4007 were more affected than Xcc while subjected to this stress. In this

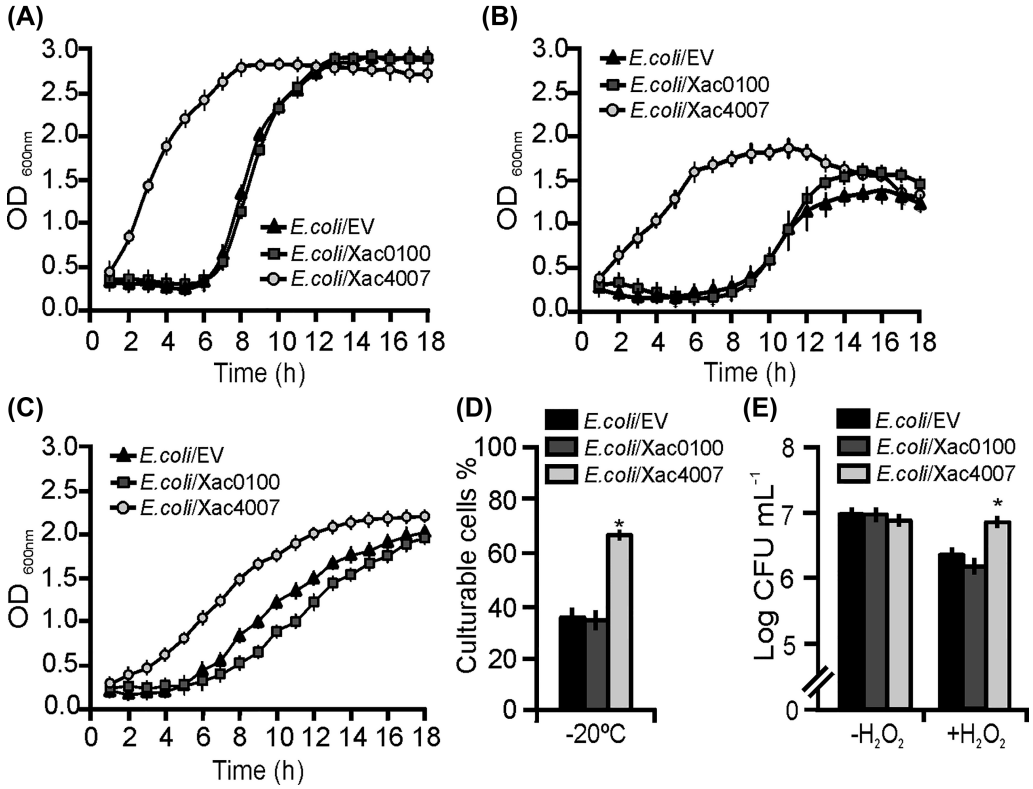

Figure 6. *E. coli* overexpressing Xac0100 and Xac4007. *E. coli*/EV, *E. coli*/Xac0100 and *E. coli*/Xac4007 strains were subcultured until OD_{600nm} reached 0.8, then 0.5 mM IPTG was added and incubated for 2 h at 28°C. Bacterial growth curves were carried out and OD_{600nm} value was plotted for 18 h in **(A)**, LB medium, **(B)**, LB supplemented with 0.25 M NaCl, **(C)**, LB supplemented with 0.8 M mannitol. Besides, induced bacterial cells were submitted to **(D)**, freezing for 2 h at −20°C and **(E)**, oxidative stress by incubation with 0 and 30 mM H_2O_2 for 20 min. Each curve/bar represents the average of three biological experiments with three replicates each. Error bars represent the standard deviation. Asterisks indicate significant differences analyzed by ANOVA test ($P < 0.05$).

regards, *Deinococcus radiodurans*, a bacterium deeply studied due to its desiccation and radiation resistance, possesses several proteins classified as LEA proteins (Battista, Park and McLemore; Rajpurohit and Misra 2013) which are important for the culturability of desiccated cultures. Besides, in *S. cerevisiae*, five strains overexpressing the hydrophilins SIP18, STF2, GRE1, YJL144w or NOP6 were identified as dehydration tolerant (López-Martínez *et al.* 2012, Rodriguez-Porrata *et al.* 2012) showing an important role for hydrophilins in desiccation tolerance. Accordingly, both XAC0100 and XAC4007 showed the highest differences in transcription levels when Xcc was subjected to desiccation compared to other imposed stresses, highlighting their importance in this stress condition. Moreover, both ΔXAC4007 and ΔXAC0100/4007 strains were significantly affected by oxidative stress compared to Xcc and ΔXAC0100. The differential behavior between ΔXAC4007 and ΔXAC0100 may be explained considering the relative transcript abundances. XAC4007 expression levels were higher than XAC0100, in Xcc under oxidative stress. Accordingly, the five above mentioned *S. cerevisiae* hydrophilin expressing strains were considered as dehydration tolerant, however only the one expressing STF2 showed a better antioxidant capability (López-Martínez *et al.* 2012), suggesting different activities for this type of proteins that may lead to a better microbial performance while subjected to changes in the environment.

Recombinant proteins were tested *in vitro* for their capability to protect bacterial cells subjected to desiccation. Xac0100 showed a similar behavior to BSA, used as bacterial cell protector. Xac4007, even though to a lesser extent than BSA, was also able to protect bacterial strains under desiccation stress. Bacterial cells in stationary phase, unlike cells used in these experiments, are structurally and physiologically different from those in the log phase and usually show an improvement in air-drying resistance (Potts 1994). The presence of Xac0100 or Xac4007 recombinant proteins enabled to preserve the population of culturable bacteria under desiccation stress condition. Besides, recombinant proteins from Xcc exhibited a protective effect in keeping L-LDH enzymatic activity after freeze-thaw cycles. Although the function of hydrophilins has not been clearly understood, many studies have demonstrated that hydrophilins play important roles in abiotic stress tolerance. This is the case for several hydrophilins expressed in *E. coli* such as avLEA1 from *Azotobacter vinelandii* (Rodriguez-Salazar, Moreno and Espin 2017) and OsLEA4, a LEA protein from rice (Hu *et al.* 2012). The protein avLEA1 is essential for the survival of *A. vinelandii* both in dry conditions and for protection against hyper-osmolarity. *In vitro* analysis showed that avLEA1 recombinant protein was able to prevent the freeze thaw-induced inactivation of L-LDH (Rodriguez-Salazar, Moreno and Espin 2017). Also, proteins classified as hydrophilins from plant, fungal and bacteria were able to protect enzymatic activities from malate dehydrogenase (MDH) and L-LDH in an *in vitro* system (Reyes *et al.* 2005). Here, recombinant proteins from Xcc exhibited a protective effect on keeping L-LDH enzymatic activity after freeze-thaw cycles. Therefore, Xac0100 and Xac4007 were able to partially protect biological structures against the effects of water deficit, even if how this function is carried out remains unclear.

In vivo analyses of Xac4007 in *E. coli* showed an improved performance both in standard culture, saline and osmotic stress conditions. The benefit was observed in shortening the culture lag phase compared to *E. coli* bearing the empty vector. Interestingly, *E. coli*/Xac4007 was more tolerant to freezing stress compared to *E. coli*/EV and *E. coli*/Xac0100 and the same behavior was observed in oxidative stress. Most of stress sources used in this work have the capability to promote the formation of reactive oxygen species, which are detrimental to all living organisms. Accordingly, under oxidative stress conditions, *E. coli* cells overexpressing Xac4007 were more tolerant to the presence of hydrogen peroxide, suggesting that this hydrophilin-like protein may play an important role in protecting *E. coli* cells. The differential behavior observed between Xac4007 and Xac0100 is probably attributable to the higher sequence identity percentage between Xac4007 and YjbJ from *E. coli*, fulfilling more similar functions. Consistently with our results, expression of a recombinant LEA3 protein from soybean conferred tolerance to low (4°C) and high (48°C) temperatures and high-salinity stress in *E. coli*. In this regards, overexpression of BnLEA4–1 (LEA4) from *Brassica napus* also conferred salt and extreme temperature tolerance to *E. coli* (Dalal *et al.* 2009). OsLEA4 (LEA4) from *Oryza sativa* L., enhanced the tolerance of *E. coli* to high salinity, heat, freezing, and UV radiation (Hu *et al.* 2012). Similar results were observed for a predicted LEA-like protein (LeamA) from *Neosartorya fischeri* expressed in *E. coli* (van Leeuwen *et al.* 2016). Again, in support of the more active participation of XAC4007 in stress responses, the levels of induction of this gene can explain the differences observed with XAC0100. Even when both Xac0100 and Xac4007 demonstrated to have a critical role in tolerance to water deficit in Xcc, *E. coli* cells overexpressing these proteins showed no differences in the population of culturable bacteria in desiccation compared to *E. coli*/EV. The differential behavior observed between Xcc and *E. coli* in desiccation tolerance, may be attributed to the fact that Xcc, as a phytopathogen, has acquired strategies to maintain itself on plant surfaces or soil, and to survive under changing climate conditions (Yu *et al.* 2013). In conclusion, our results strongly support the important role for these bacterial proteins in different cellular processes such as water stress through desiccation or freezing, oxygen reactive species, osmotic and saline stress conditions. Further investigation about protein structural properties during different stress conditions will be required to elucidate the function of the Xac0100 and Xac4007 proteins in more detail.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

ACKNOWLEDGMENTS

We thank Diego Aguirre for plant technical assistance.

FUNDING

This work was supported by Grants from Agencia Nacional de Promoción Científica y Tecnológica (s) and Agencia Santafesina de Ciencia, Tecnología e Innovación (IO2017–00011 to BSG). MVB is a fellow and JO, NG and BSG are staff members of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina).

Conflicts of interest. None declared.

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