LEA proteins are involved in cyst desiccation resistance and other abiotic stresses in *Azotobacter vinelandii*

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Abstract Late embryogenesis abundant (LEA) proteins constitute a large protein family that is closely associated with resistance to abiotic stresses in multiple organisms and protect cells against drought and other stresses. *Azotobacter vinelandii* is a soil bacterium that forms desiccation-resistant cysts. This bacterium possesses two genes, here named *lea1* and *lea2*, coding for *avLEA1* and *avLEA2* proteins, both containing 20-mer motifs characteristic of eukaryotic plant LEA proteins. In this study, we found that disruption of the *lea1* gene caused a loss of the cysts' viability after 3 months of desiccation, whereas at 6 months, wild-type or *lea2* mutant strain cysts remained viable. Vegetative cells of the *lea1* mutant were more sensitive to osmotic stress; cysts developed by this mutant were also more sensitive to high temperatures than cysts or vegetative cells of the wild type or of the *lea2* mutant. Expression of *lea1* was induced several fold during encystment. In addition, the protective effects of these proteins were assessed in *Escherichia coli* cells. We found that *E. coli* cells overexpressing *avLEA1* were more tolerant to salt stress than control cells; finally, in vitro analysis showed that *avLEA1* protein was able to prevent the freeze thaw-induced inactivation of lactate dehydrogenase. In conclusion, *avLEA1* is essential for the survival of *A. vinelandii* in dry conditions and for protection against hyper-osmolarity, two major stress

factors that bacteria must cope with for survival in the environment. This is the first report on the role of bacterial LEA proteins on the resistance of cysts to desiccation.

Keywords *Azotobacter* · LEA proteins · Cysts desiccation · Osmotic stress · High temperatures

Introduction

Organisms are exposed to abiotic stress conditions, such as high or low temperatures, freezing, salinity and drought (Bray et al. 2000). Abiotic stress has a negative impact on living cells, affecting their sub-cellular structures, enzyme activity, displacement of membrane proteins, and loss of membrane integrity (Zhu et al. 1997). In the face of unfavourable and extreme conditions, organisms experience morphological, physiological, biochemical and molecular changes and develop responses that allow them to adapt to unfavourable situations (Yancey 2005). Late embryogenesis abundant (LEA) proteins are present in many organisms, including plants, bacteria and invertebrates, in which they play a role in resisting environmental conditions, such as freezing, drought or desiccation (Tunnacliffe and Wise 2007; Battaglia et al. 2008; Hand et al. 2011; Toxopeus et al. 2014). Several in vitro assays have shown that some LEA proteins are able to stabilize structures or molecules during freezing and drying (Tunnacliffe and Wise 2007; Battaglia et al. 2008), which is related to their ability to prevent enzyme aggregation under stress conditions (Nakayama et al. 2008; Boucher et al. 2010). Most LEA proteins are highly hydrophilic, with low cysteine, phenylalanine or tryptophan content (Dure et al. 1989; Wise 2003; Shih et al. 2008). LEA proteins are considered as fully disordered proteins with a high percentage of random coils in aqueous solution, but when water is removed, they form secondary

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structures, such as α helices, β sheets and hairpin loops (Tunnacliffe and Wise 2007; Garay-Arroyo et al. 2000). LEA proteins have been classified into six or more groups, according to similarities in their amino acid sequences and conserved motifs (Baker et al. 1988; Tunnacliffe and Wise 2007; Battaglia et al. 2008). Group 1 LEA proteins (G1LEA) possess a 20-amino acid motif (20-mer), which may be repeated up to eight times, as is the case of LEA proteins present in the crustacean *Artemia franciscana* (Campos et al. 2013; Toxopeus et al. 2014). LEA 20-mers are also similar to hydrophilic repeated motifs of general stress proteins of the bacterial GsiB superfamily (Stacy and Aalen 1998). Wu et al. (2011) found that the 20-mer motifs of G1LEA proteins are divisible into two sub-motifs; one sub-motif is conserved among archaea, bacteria and eukaryotes, whereas the other differs among prokaryotes-archaea and eukaryotes. Thus, the 20-mer seems to differ phylogenetically in eukaryotic and bacterial LEA proteins (Wu et al. 2011).

Azotobacter vinelandii is a gram-negative soil bacterium that produces the exopolysaccharide alginate, the intracellular poly- β -hydroxybutyrate (PHB) and a family of phenolic lipids known as alkylresorcinols (ARS). This bacterium has the ability to form metabolically dormant cells called cysts. One of the main features of the cyst is its ability to withstand desiccation, being able to survive storage in dry soil for more than 10 years (Vela 1974). In laboratory conditions, *A. vinelandii* cysts are produced in late stationary phase, or upon induction of vegetative cells with specific reagents, such as *n*-butanol or β -hydroxybutyrate (Lin and Sadoff 1968; Sutherland 1985).

The genome of *A. vinelandii* contains four genes encoding predicted LEA-like proteins (Campos et al. 2013; Setubal et al. 2009; Wu et al. 2011). These genes are annotated as Avin11010, Avin11020, Avin44390 and Avin02300. The protein encoded by Avin11010 contains four 20-mer repeats with identity to eukaryotic G1LEA proteins; therefore, it was thought to be an example of horizontal gene transfer between the domains of life (Wu et al. 2011). The proteins encoded by the other three genes contain only one 20-mer (Campos et al. 2013). The 20-mer present in the protein encoded by Avin11020 is shown here to contain the sub-motif present in eukaryotic LEA proteins, while the Avin44390 and Avin02300 encoded proteins possess the prokaryotic sub-motif and share a similarity to proteins of the GsiB family. Due to their resemblance to plant LEA proteins that accumulate in seeds during the dormancy period, where they are likely to be involved in desiccation tolerance, in the present study, we chose Avin11010 and Avin11020 to determine their role in desiccation tolerance of dormant cysts. We also determined the role of these genes under different abiotic stress conditions in cyst and vegetative cells of *A. vinelandii*. We demonstrated herein that the LEA protein encoded by Avin11010 is essential for the desiccation resistance (viability) of cysts over

prolonged time, and in coping with abiotic stress conditions in vegetative cells.

Materials and methods

Biological material and growth conditions

Bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. For vegetative growth, *A. vinelandii* was cultured at 30 °C on Burk's nitrogen-free salt medium (Kennedy et al. 1986), supplemented with 2% sucrose (BS), or on Burk's nitrogen-free salt medium with 0.2% *n*-butanol as carbon source (BB), for encystment induction (Sadoff et al. 1971). *Escherichia coli* DH5 α (Hanahan 1983) and BL21(DE3) were grown on Luria-Bertani (LB) medium at 37 °C (Miller 1972). Antibiotic concentrations used for *A. vinelandii* or *E. coli* were as follows: nalidixic acid (30 μ g/mL), kanamycin (2 μ g/mL), gentamycin (0.5 μ g/mL), spectinomycin (100 μ g/mL), tetracycline (30 μ g/mL) and ampicillin (100 μ g/mL).

Determination of cyst resistance to desiccation

For encystment induction and desiccation resistance, vegetative cells grown for 24 h on BS medium were centrifuged, washed and resuspended in Burk's nitrogen-free salt medium. A sample of washed cells was plated on solid BB encysting medium. After 5 days of incubation, samples harvested from these plates containing 10^6 to 10^7 cysts (colony-forming units, CFU) were applied to Millipore membranes (0.2- μ m pore size). The membranes were placed in sterile Eppendorf tubes and placed in sterile petri dishes. After 5 or more days, dried cysts in the filters were resuspended and plated on solid BS medium to determine viable counts.

In silico analysis of protein sequence

Sequence analysis of *lea1* and *lea2* was performed using the BLAST program on the NCBI web server (<http://blast.ncbi.nlm.nih.gov/>). Nucleotide translation and isoelectric point were performed using EXPASY tool (<http://www.expasy.org/tools/protparam.html>). Multiple sequence alignment was performed using CLUSTAL Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Hydropathy plots were constructed with the Kyte and Doolittle algorithm (<http://ipsort.hgc.jp/>) (Kyte and Doolittle 1982). Prediction of naturally disordered regions was done with PONDR (www.pondr.com).

Table 1 Bacterial strains, plasmids and oligonucleotides used in this work

Strain plasmid or oligonucleotide	Description or sequence (5'–3')	Reference ^a
Strains		
<i>A. vinelandii</i>		
AEIV	WT	[1]
<i>lea1</i>	AEIV derivative harbouring pLEA1101 plasmid carrying a Av11010::Gm mutation	This work
<i>lea2</i>	AEIV derivative harbouring pLEA1102 plasmid carrying a Av11020::Km mutation	This work
<i>lea1-lea2</i>	AEIV derivative harbouring pLEADC carrying a Av11010-Av11020::Sp mutation	This work
<i>lea1–2-plea1–2</i>	AEIV derivative with Av11010 and Av11020 genes co-integrated	This work
<i>lea1-gusA</i>	AEIV with transcriptional fusion from pUMATc _{gusA} carrying <i>lea1::gusA</i>	This work
<i>lea2-gusA</i>	AEIV with transcriptional fusion from pUMATc _{gusA} carrying <i>lea2::gusA</i>	This work
AEIV <i>gacA</i>	AEIV with a <i>gacA</i> ::Gm mutation	[2]
<i>E. coli</i>		
DH5α	<i>supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	GIBCO-BRL
BL21(DE3)	<i>F[−] ompT hsdS_B (r[−]_B m[−]_B) gal dcm</i>	Invitrogen
BL(DE3)/pET22b+	BL21 carrying the empty expression vector pET22b+	This work
BL(DE3)/p <i>avLEA1</i>	BL21 carrying the plasmid <i>pavLEA1</i> for <i>avLEA1</i> protein production	This work
Plasmids		
pJET1.2/blunt	Cloning vector Amp ^r	Thermo Scientific
pET22b+	Cloning and expression vector Amp ^r	Novagen
pBLS190	Plasmid used to obtain Tc ^r cassette	[3]
pBSL97	Plasmid used to obtain the Km ^r cassette	[3]
pBSL98	Plasmid used to obtain the Gm ^r cassette	[3]
pLEA11012	pJET1.2/blunt containing a 1.2 kb PCR fragment from both, <i>lea1</i> and <i>lea2</i> genes	This work
pLEA1101	pJET1.2/blunt containing a small fragment of 103 bp of <i>lea1</i>	This work
pLEA1102	pJET1.2/blunt containing a small fragment of 65 bp of <i>lea2</i>	This work
pLEADC	pJET1.2/blunt containing <i>lea1</i> and <i>lea2</i> genes interrupted by Sp ^r cassette	This work
pUMATc	pUMA derivative with a Tc cassette with the <i>gusA</i> gene for transcriptional fusions	[4]
<i>pavLEA1-gusA</i>	pJET1.2/blunt containing 178 bp corresponding to the <i>lea1</i> promoter region	This work
<i>pavLEA2-gusA</i>	pJET1.2/blunt containing 195 bp corresponding to the <i>lea2</i> promoter region	This work
pET22b+	pET22b6XHis-Tag	Novagen
<i>pavLEA1</i>	pET22b6XHis-Tag containing <i>avLEA1</i> protein from <i>A. vinelandii</i>	This work
Oligonucleotide		
Av1020F1	CATGCGTAAAGCCGAAAGC	This work
Av1020R1	GATGGGTTTTTTTTCGATAG	This work
Av10invF1	CAAACAGGAAAACCGCGG	This work
Av10invR1	GATCTCGCCGCCTTTCTG	This work
Av20invF1	CGTGAAGTCATCCAGGAAG	This work
Av20invR1	CGCTTACACTGATCGAACC	This work
AvFTLEA1F1 ^b	GAACCGCGGGGGGCGAAAAATAGGCGG	This work
AvFTLEA1R1 ^b	CACCTGCAGGGAGCCTTTGTCTTGCC	This work
AvFTLEA2F1	CCAGAAAGGCGGCGAGAT	This work
AvFTLEA2R1	TAGCCTTCCGGGCCGAGTT	This work
AvExpLEAF1 ^c	GAAC CATAT GAGCGTACGGGAGGCCGG	This work
AvExpLEAR1 ^c	GAAC TCGAGGCTCTTGCGGCTGCCCT	This work

^a References: [1] Svein Valla (Norwegian University of Science And Technology), [2] Manzo et al. 2011, [3] Alexeyev et al. 1995, [4] Muriel-Millán et al. 2015

^b Restriction sites for *SacII* and *PstI* are cursive and underlined respectively

^c Restriction sites for *XhoI* and **NdeI** are cursive underlined and bold respectively

Construction of *lea* mutants

The *lea2* gene is located immediately upstream of the *lea1* gene. We used primers Av1020F1/Av1020R1 and AEIV wild-type strain (AEIV) chromosomal DNA as a template to amplify by PCR (Phusion DNA Polymerase, Thermo Scientific, USA) a fragment of 1.12 kb containing *lea1* and *lea2* genes; this fragment was ligated into the pJET1.2/blunt vector (Thermo Scientific, USA) to produce pLEA11012 plasmid. Primers Av10invF1/Av10invR1 were used to delete by inverse PCR a 299-bp fragment of the *lea1*-coding region from plasmid pLEA11012. The linearized plasmid thus obtained was ligated to a Gm^r cassette to produce plasmid pLEA1101 in which the *lea1* gene was replaced by the Gm^r cassette. Similarly, primers Av20invF1/Av20invR1 and plasmid pLEA11012 were used to remove by inverse PCR 89 bp of *lea2* and were replaced by a Km^r cassette to produce pLEA1102. To remove both *lea1* and *lea2* genes from plasmid pLEA11012, a fragment of 636 bp was deleted by inverse PCR using primers Av10invF1/Av20invR1. In this case, the *lea1-lea2* genes were replaced by a spectinomycin resistance cassette (Sp^r) yielding the plasmid pLEADC. A physical map of the *lea1-lea2* region present in the four plasmids constructed is shown in Fig. 1S. *A. vinelandii* AEIV competent cells were transformed with plasmids pLEA1101, pLEA1102 and pLEADC, as described previously (Bali et al. 1992). Gentamycin-, kanamycin- and spectinomycin-resistant transformants were isolated and named *lea1*, *lea2* and *lea1-lea2* respectively; DNA from these mutant strains and Av1020F1/Av1020R1 primers was used to confirm by PCR the presence of the *lea1*-Gm^r, *lea2*-Km^r and *lea1-lea2*-Sp^r mutations in the respective mutants. To construct the strain *lea1-2-plea1-2*, plasmid pLEA11012 that is unable to replicate in *A. vinelandii* was transformed into strain *lea1-lea2* for integration into the chromosome. A transformant resistant to tetracycline was isolated and confirmed by PCR to carry plasmid pLEA11012 co-integrated into the chromosome as a result of a single-homologue recombination event. A schematic representation of the integration of plasmid pLEA11012 into the *lea1-lea2* chromosome is shown in Fig. S1.

Osmotic stress tolerance assays

A. vinelandii strains were grown at 30 °C in 50 mL of BS medium in 250-mL Erlenmeyer flasks, supplemented with different concentrations of NaCl and sorbitol (Sigma-Aldrich, CA, USA) ranging from 0.1 to 1 M. After 48 h of growth, appropriate dilutions were plated onto BS plates to determine CFU.

E. coli BL(DE3)/pET22b+ and BL(DE3)/pavLEA1 strains were cultured at 37 °C in 20 mL of LB medium containing the appropriate antibiotics. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM was added when

the cultures reached an OD_{600nm} of 0.6 and were further incubated for 2 h. One hundred-microlitre samples from these cultures were spread on LB plates containing increasing concentrations of NaCl (0.1–0.5 mM). Cell viability was plotted as the percentage of CFU on the stressed plates, relative to the colony numbers of control appearing on the non-stressed plates (Soto et al. 1999) after overnight incubation at 37 °C.

Thermotolerance experiments

Test tubes containing 1 mL sample of *A. vinelandii* vegetative cells or cysts were incubated in water baths adjusted to temperatures ranging from 40 to 60 °C. After 15 min of incubation, the tubes were removed and cooled immediately; dilutions were plated onto BS plates. CFU were counted after 48 h of incubation at 30 °C.

Construction of transcriptional fusions

For the construction of transcriptional fusions of *lea1* and *lea2* genes with the *gusA* reporter gene, primers AvFTLEA1F1/AvFTLEA1R1 and AvFTLEA2F1/AvFTLEA2R1 were used to amplify PCR fragments of 178 and 195 bp, containing the promoter regions of *lea1* and *lea2* genes respectively. The amplified fragments were cloned into the *SacII* and *PstI* restriction sites of the pUMATgusAT vector (Muriel-Millán et al. 2015), to generate pavLEA1-*gusA* and pavLEA2-*gusA* plasmids respectively. These plasmids were transformed into AEIV strain; tetracycline-resistant derivatives were selected. The strains, containing the gene fusions integrated into the chromosome as a result of a double recombination event, were named *lea1-gusA* and *lea2-gusA*. The presence of the fusions into the chromosomes of *A. vinelandii* was confirmed by PCR (data not shown). β-Glucuronidase activities of at least three independent cultures were determined following the method described by Romero et al. (2013).

Expression and purification of avLEA1-6His protein

For construction of the expression vector pavLEA1, which contains avLEA1 fused with a C-terminal 6xHis-Tag (Merck, NJ, USA), primers AvExpLEAF1/AvExpLEAR1 (containing *NdeI* and *XhoI* restriction sites) and AEIV chromosomal DNA were used to amplify by PCR a DNA fragment of 396 bp corresponding to the *lea1* gene lacking the ATG start codon and the stop codon; this fragment was cloned into the pET22b+ vector at *NdeI* and *XhoI* restriction sites to produce plasmid pavLEA1. *E. coli* strain BL21(DE3) was used to express the avLEA1 protein. Strain BL(DE3)/pavLEA1 was inoculated in LB medium supplemented with ampicillin (Sigma-Aldrich, CA, USA) and grown overnight at 37 °C. A sample of the overnight culture was used to inoculate 100 mL of fresh medium to 0.05 OD_{600nm}. After 2 h at 37 °C, IPTG

was added to a final concentration of 1 mM and further incubated for 2 h. Cell cultures were harvested by centrifugation. The recombinant protein was purified according to “The handbook for high-level expression and purification of 6xHis-Tagged proteins, 5th edition” (Qiagen, Hilden, Germany). Samples of the purified *av*LEA1 protein were analysed in SDS-PAGE using 12% acrylamide gels (Sambrook and Russell 2001) with Tris-glycine SDS running buffer. Detection of 6xHis-Tag purified protein was performed by immunoblotting, utilizing the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo-Scientific, Rockford, USA) (Fig. S2). Protein was quantified by the Bradford assay.

L-Lactate dehydrogenase activity assay

L-Lactate dehydrogenase (LDH) from rabbit muscle (Sigma-Aldrich, MO, USA) was dissolved (0.4 mg mL^{-1}) in assay buffer, containing 100 mM dibasic sodium phosphate buffer pH 7.5, 2 mM pyruvate (Sigma-Aldrich, MA, USA) and 100 mM NADH (Roche, NJ, USA). LDH enzymatic activity was determined as follows. One hundred microlitres of the LDH solution was added to 500 μL demineralized water with or without purified *av*LEA1 protein. The mixture was frozen for 15 min in dry ice and thawed for 15 min in a water bath at 25 °C. This freeze thaw cycle was repeated up to 10 times, after that. The LDH activity was then determined by NADH absorbance at OD_{340nm} for 1 min due to the conversion of NADH into NAD at 25 °C using an Ultrospec 3300 Pro UV/Visible Spectrophotometer (Amersham-Biosciences, Freiburg, Germany). All of the values given were expressed as the percentage of the rate of the reaction measured for the untreated samples. Enzyme activity for each sample shown was determined in at least three independent tests. Bovine serum albumin (BSA), (Sigma-Aldrich, CA, USA) was used as a positive control because it is a protein with known cryoprotective activity.

Statistics

Each experiment was performed with three replicates. Statistical relevance was determined by Student's *t* test or one-way ANOVA using Prism version 6 (GraphPad Software, USA).

Results

In silico analysis of *A. vinelandii* *av*LEA1 and *av*LEA2 proteins

The genome of *A. vinelandii* contains four genes encoding predicted LEA-like proteins (Campos et al. 2013). The protein

encoded by *Avin11010* contains four repeats of the 20-mer; the protein *Avin11020* contains only one 20-mer (Campos et al. 2013). The 20-mer sequences of the protein encoded by *Avin11010* are present in eukaryotic LEA proteins, but not in prokaryotes (Wu et al. 2011). To analyse the sequence of *A. vinelandii* LEA proteins, we performed a multiple alignment analysis of the 20-mers present in the four LEA proteins from *A. vinelandii* and LEA proteins from other prokaryotes, archaeae and eukaryotes (Fig. 1). As reported by Wu et al. 2011, the protein encoded by *Avin11010* possesses eukaryotic sub-motifs (Fig. 1). Here, we found that the 20-mer present in the protein encoded by *Avin11020* contains the sub-motif present in eukaryotic LEA proteins. The *Avin44390*-encoded LEA has an archaeae sub-motif, while *Avin02300* possesses the prokaryotic sub-motif (Fig. 1). The 20-mer sequence of *Avin02300* shares the highest identity to putative LEA proteins present in bacteria belonging to the genus *Pseudomonas* that are phylogenetically closely related to *A. vinelandii* (Setubal et al. 2009). The *Avin11010* and *Avin11020* genes were named *lea1* and *lea2* and their protein products *av*LEA1 and *av*LEA2 respectively. The full-length open reading frame (ORF) of *lea1* is 402 bp, which encodes a protein of 133 amino acid residues with a calculated molecular mass of 14.3 kDa and a theoretical pI of 6.87. The *lea2* gene encodes a 55-amino acid protein, with a calculated molecular mass of 5.9 kDa, and a theoretical pI of 9.9. The *lea2* gene is

Fig. 1 Alignment of the 20-mer sequence of the *A. vinelandii* LEA proteins and LEA proteins from other prokaryotes, archaeae and eukaryotes, and sub-motifs present in all organisms (grey shading), Archaea sub-motif (underlined), Bacteria sub-motif (bold) and Eukarya (black shading). Accession numbers for LEA proteins used in this alignment are *Artemia franciscana* ADE45145; *Arabidopsis thaliana* NP_190749; *A. vinelandii* *Avin11010*, WP_012699753; *Avin11020*, WP_012699754; *Avin44390*, WP_012702924; *Avin02300*, WP_012698919; *Anaeromixobacter* sp. WP_011985696; *Methanocella paludicola* BAI62068; *Methylocystis parvus* WP_026016492; *Pseudomonas aeruginosa* NP_250880; *Pseudomonas fluorescens* WP_003174480; *Pseudomonas mendocina* WP_003246794; *Pseudomonas stutzeri* WP_021209286; *Pseudomonas syringae* EGH33010

located immediately upstream the *lea1* gene separated by an intergenic region of 165 nucleotides.

Kyte and Doolittle hydropathy plots revealed that both the *avLEA1* and *avLEA2* proteins are strongly hydrophilic, with a grand average of hydropathicity value of -1.6 and -1.3 respectively (GRAVY; Kyte and Doolittle 1982) (Fig. S1). In common with other LEA proteins, disordered prediction programs suggest that both *avLEA1* and *avLEA2* lack a fixed tertiary structure, being partially unfolded (Fig. S2).

***avLEA1* is necessary to withstand desiccation on *A. vinelandii* cyst**

LEA proteins are associated with desiccation tolerance in prokaryotic and eukaryotic organisms. Cysts produced by *A. vinelandii* are resistant to desiccation. We therefore determined the role of *avLEA1* and *avLEA2* on the resistance to desiccation of *A. vinelandii* cysts. Strains carrying mutations in *lea1* or *lea2* genes, as well as a double *lea1-lea2* mutant, were constructed as described in the “Materials and methods” section. These mutants and AEIV strain were induced for encystment by incubation of vegetative cells in solid BB medium plates over 5 days as described in the “Materials and methods” section. Samples of these cells (cysts) were harvested and used for light microscopy visualization after ARS staining. Cyst samples were also applied to Millipore membranes that were placed in sterile petri dishes for drying. After 5 days and for up to 6 months, the dried cysts on the filters were resuspended in BS medium, allowing the cysts to germinate; CFU were counted to determine the percentage of cysts resistant to desiccation. Both *lea* mutants and the double mutants were able to form cysts; no morphological differences between cysts formed by these strains and the wild-type strain were observed by optical microscopy (Fig. 2). Desiccation resistance assays were carried out in the cyst samples applied to Millipore membranes after 5 days and 1, 2, 3 and 6 months. Similarly to the cysts formed by the wild-type strain, cysts developed by all three *lea* mutants were resistant to desiccation after 5 days; however, cyst resistance to desiccation was diminished at 2 months and was lost at 3 months in the strain carrying the *lea1* mutation. In contrast, the cysts formed by wild type and the *lea2* mutant remained resistant to desiccation after 6 months (Table 2). We conclude that *avLEA1* and *avLEA2* are not necessary for the encystment process in

A. vinelandii but *avLEA1* is necessary for the cysts to withstand long periods of desiccation.

Expression of *avLEA1* and *avLEA2* in vegetative and encysting cells

As shown above, *avLEA1* but not *avLEA2* contributes to the resistance of cyst to desiccation. Transcriptional *lea1-gusA* and *lea2-gusA* fusions were used to determine the expression of *lea1* and *lea2* genes in vegetative cells and in cells under encystment induction conditions. We observed that transcription of *lea1* increased during encystment and was higher under this condition than in vegetative cells (Fig. 3a), while transcription of *lea2* is similar in the two conditions tested and was threefold to fourfold lower than that of *lea1* during the 5 days of the encystment process (Fig. 3b). These data indicate that the expression of the *lea1* gene is induced under encystment conditions, a feature in agreement with its role in cyst resistance to desiccation.

***avLEA1* confers tolerance to high temperatures in vegetative cells and in cysts**

LEA proteins have been implicated in tolerance to high and low temperatures. To investigate the contribution of the *avLEA1* and *avLEA2* proteins in tolerance to high temperatures in *A. vinelandii*, we evaluated survival of vegetative cells and cysts of *lea1* and *lea2* mutants subjected to high temperatures. For vegetative cells, strains were grown for 24 h in BS medium at 30 °C and exposed during 15 min to temperatures ranging from 37 to 60 °C. As shown in Fig. 4a, survival diminished considerably as the cells were incubated at higher temperatures. The viability for the *lea1* mutant was considerably affected at 45 and 50 °C. For the AEIV strain and the *lea2* mutant, viability was completely lost at 55 °C. Similarly, when wild-type and *lea* mutant cysts were incubated for 15 min at temperatures ranging from 37 to 60 °C, a considerable loss of viability was observed, although cysts seem to be more resistant than vegetative cells. The strain carrying the *lea1* mutation was not able to survive at 55 °C, whereas cysts produced by the wild type and *lea2* mutant were able to germinate at 55 °C, but lost viability at 60 °C (Fig. 4b). These data indicate that, as was expected, encysted cells are more tolerant to high temperatures than vegetative cells and that the

Fig. 2 Light microscopy (bright field) of Fast Blue B-stained cysts of *A. vinelandii* AEIV strain and *lea* mutants. In all cases, cysts were developed after 5 days of incubation on BB medium plates

Table 2 Cysts survival to desiccation of *A. vinelandii* *lea1* and *lea2* mutants

Strain	Genotype	Desiccation resistance (%)			
		5 days	1 month	3 months	6 months
AEIV	WT	9.7 ± 5.3	11.2 ± 4.1	13.1 ± 8.4	17.5 ± 3.2
<i>lea1</i>	Avin11010::Gm	10.1 ± 3.9	4.2 ± 4.2	1.5 ± 2.3	<0.0001
<i>lea2</i>	Avin11020::Km	15.6 ± 3.5	10.3 ± 3.1	9.5 ± 6.1	18.3 ± 2.6
<i>lea1-lea2</i>	Avin11010-11020::Sp	7.4 ± 4.6	3.5 ± 2.3	2.5 ± 1.3	<0.0001
<i>lea1-2-plea1-2</i>	Avin11010-11020::Sp+pUMA	11.5 ± 3.8	17.1 ± 6.5	21.4 ± 3.2	15.4 ± 4.1
AEIV- <i>gacA</i>	AEIV with a <i>gacA</i> ::Gm mutation	<0.0001	<0.0001	<0.0001	<0.0001

lea1 gene contributes to the tolerance of *A. vinelandii* to high temperatures.

*av*LEA1 is required for osmotic stress tolerance in *A. vinelandii*

We determined the effect of the *lea1* and *lea2* mutations on the growth and survival of *A. vinelandii* under osmotic stress

conditions. Mutant strain cultures were grown at different NaCl or sorbitol concentrations, and viable counts were performed after 48 h. The *lea1* mutant was significantly more sensitive to osmotic stress when grown at 0.15 M NaCl than the AEIV strain and the *lea2* mutant exposed to the same conditions (Fig. 5a). When the concentration of NaCl in the medium was increased to 0.3 M, *lea1* cells were unable to survive, while the *lea2* strain exhibited a tolerance similar to that of the AEIV strain. Similar results were obtained when an osmotically equivalent concentration of sorbitol (1 M) was present in the growth medium as the strains carrying the

Fig. 3 **a** Expression of *lea1* and *lea2* under vegetative growth conditions (white bars) or under encystment induction conditions (black bars), determined as β-glucuronidase activity in AEIV derivatives containing *lea1-gusA* and *lea2-gusA* transcriptional fusions. **b** Expression of *lea1-gusA* (closed squares) and *lea2-gusA* (closed circles) in BB medium during the 5 days of the encystment process. One unit of β-glucuronidase activity corresponds to 1 nmol of substrate (X-Gluc) hydrolyzed per minute per milligram of protein. Error bars represent standard deviations of three independent experiments. Significant differences between *lea1-gusA* and *lea2-gusA* expression are indicated with asterisk evaluated with the Student's *t* test at $p < 0.05$

Fig. 4 Thermo tolerance of *A. vinelandii* cysts and vegetative cells developed by the *lea1* and *lea2* mutants. AEIV (open squares), *lea1* mutant (closed squares), *lea2* mutant (closed circles), *lea1-lea2* double mutant (open circles) and *lea1-2-plea1-2* complemented strain (closed triangles). Cells were grown at 30 °C in BS (a) or BB medium (b). One millilitre of these cultures was incubated during 15 min at temperature ranges from 30 to 60 °C. Cell viability of these samples was determined. Error bars indicate standard deviation of three independent experiments. Asterisk indicates significant difference at $p < 0.05$ with one-way ANOVA analysis

Fig. 5 Osmotic stress tolerance of *A. vinelandii* *lea1* and *lea2* mutants. Growth of AEIV (open squares), *lea1* mutant (closed squares), *lea2* mutant (open circles), double mutant *lea1-lea2* (open triangles) and *lea1-2plea1-2* complemented strain (closed triangles), in the presence of NaCl (a) or sorbitol (b). Cells were grown on liquid BS medium supplemented with increasing concentrations of NaCl or sorbitol; aliquots were plated onto BS agar plates. Error bars represent standard deviations of three independent experiments. Significant differences are indicated with asterisk evaluated with one-way ANOVA analysis at $p < 0.05$

lea1 mutant were more sensitive to inhibiting concentrations of sorbitol than the wild type and the *lea2* mutant (Fig. 5b). These results indicate that the *lea1* gene is necessary for survival of *A. vinelandii* in high concentrations of osmotic agents such as NaCl and sorbitol.

***av*LEA1 protein has a protective effect on LDH activity under freeze thaw cycles in vitro**

LDH enzymatic activity is known to be sensitive to water limitation upon freeze thaw cycles (Goyal et al. 2005). Some GLEA proteins have been shown to protect the enzymatic activity of LDH when subjected to freeze thaw treatments (Reyes et al. 2008). An *av*LEA1 protein containing 6xHis-Tag was expressed in *E. coli* BL21(DE3) cells and purified as described in the “Materials and methods” section and Fig. 2S. Samples of the purified *av*LEA1 protein were used to evaluate its protective effect on the LDH enzymatic activity in vitro after freeze thaw cycles. Activity of LDH was monitored in the presence or absence of purified *av*LEA1. The

*av*LEA1/LDH molar ratio used was 1:1. The LDH enzymatic activity was determined by the conversion of pyruvate, measured by a decrease in absorbance at 340 nm due to the oxidation of NADH. In the presence of *av*LEA1, the LDH activity remained at 52% after 2 cycles and 21% after 6 cycles, while in its absence, the remaining activity corresponded to 20 and 10% respectively (Fig. 6). In the presence of BSA, the LDH activity was totally protected. The ratio of BSA/*av*LEA1 was 1:1. This result indicates that like other LEA proteins, *av*LEA1 has a protective effect on LDH activity under repeated freeze thaw cycles.

Heterologous expression of *av*LEA1 improves salt tolerance but not heat resistance of *E. coli*

As shown above, *av*LEA1 protein is necessary to withstand osmotic stress caused by 0.3 M of NaCl in *A. vinelandii*. To determine the in vivo effect of *av*LEA1 overexpression in the tolerance of *E. coli* to ionic stress proper dilutions of *E. coli* cultures, BL(DE3)/*pav*LEA1 and control strain BL(DE3)/pET22b+ were spread on plates containing increasing concentrations of NaCl (from 0.2 to 0.6 M). As shown in Fig. 7, BL(DE3)/*pav*LEA1 and BL(DE3)/pET22b+ strains lost viability as the concentration of NaCl in the plates was increased; viability was completely lost for the two strains at 0.6 M of NaCl. However, viability of BL(DE3)/*pav*LEA1 was higher than that of the BL(DE3)/pET22b+ strain in NaCl concentrations 0.3 and 0.4 M. The survival of BL(DE3)/pET22b+ was 70 and 30% respectively, while in the BL(DE3)/*pav*LEA1 strain, 91 and 60% of the cells remained viable (Fig. 7). These results indicated that the overexpression of the *av*LEA1 protein enhances the tolerance of *E. coli* to high-salinity conditions.

Fig. 6 Effect of *av*LEA1 on LDH activity after freeze thaw cycles. The LDH activity after 10 freeze thaw cycles was determined in the presence of purified *av*LEA1 (grey bars) or BSA protein (black bars); buffer (white bars) was used as a control. The data were expressed as percentage (%) of activity measured before the stress in the presence of the corresponding protein. The average of the enzyme activity and standard deviations were based on three independent experiments. Asterisk indicates significant difference evaluated with the Student's *t* test at $p < 0.05$

Fig. 7 Effect of *av*LEA1 expression on *E. coli* viability under osmotic stress (NaCl). **a** Growth of *E. coli* BL(DE3)/*pav*LEA1 and BL(DE3)/*pET22b+* strains on LB plates with or without different NaCl concentrations ranging from 0.2 to 0.6 M. **b** Survival (%) of *E. coli* BL(DE3)/*pav*LEA1 (closed circles) and BL(DE3)/*pET22b+* (closed squares). Survival was calculated as the percentage of CFU on the plates containing NaCl relative to CFU present in the control LB plates. The standard deviations were calculated from three independent experiments. Asterisk indicates significant difference evaluated with the Student's *t* test at $p < 0.05$

The tolerance of *E. coli* strains BL(DE3)/*pET22b+* and BL(DE3)/*pav*LEA1 subjected to heat stress was also determined by spreading dilutions of cultures on LB plates containing 1 mM IPTG and incubating them at 37, 45, 50 and 55 °C. The number of colonies grown at 37 °C was considered as the 100% viability control. No colonies were observed at 55 °C, and a similar loss of viability was observed for both strains at 45 and 50 °C, indicating that the *av*LEA1 protein did not confer tolerance to high temperatures (Data not shown).

Discussion

LEAs are highly hydrophilic, intrinsically unstructured proteins that, in vitro, reduce protein aggregation caused by desiccation and freezing. These proteins were first identified in plants (Dure et al. 1989; Dure 1993), but LEA homologues are also present in other eukaryotes, prokaryotes and archaea (Hand et al. 2011; Wu et al. 2011; Campos et al. 2013). LEA proteins are characterized by a 20-mer motif

that possesses a sub-motif that differs among prokaryotes and eukaryotes. There are extensive correlative data linking the expression of LEA proteins with tolerance to water stress caused by desiccation, salinity or low temperatures in plants (Tunnacliffe and Wise 2007; Olvera-Carrillo et al. 2010). A relationship between LEA homologues and water deficit stress has also been reported in eukaryote organisms other than plants and in some prokaryotes (Garay-Arroyo et al. 2000; Solomon et al. 2000; Browne et al. 2002; Gal et al. 2004; Stacy and Aalen 1998; Battista et al. 2001; Denekamp et al. 2010; Hand et al. 2011; Wu et al. 2011; Hatanaka et al. 2013).

A cyst is a resting or dormancy stage of a microorganism, usually a bacterium, a protist or an invertebrate, that helps the organism to survive in unfavourable environmental conditions. Cysts allow these organisms to remain in a state of dormancy for long periods. In *A. franciscana*, a crustacean that produces cysts that survive desiccation and temperatures below −20 °C (Crowe et al. 1981; Hengherr et al. 2011), LEA proteins belonging to group 1 have been shown to contribute to the desiccation and freeze

tolerance of encysted embryos (Toxopeus et al. 2014). They also play an important role on the response to salt stress, as LEA-coding genes were found highly expressed under cyst conditions and in response to hyper saline stress in embryonic development (Wu et al. 2011).

A. vinelandii, the soil bacterium studied here, produces cysts that are resistant to desiccation, able to survive storage in dry soil for more than 10 years (Vela 1974). This bacterium also possesses four genes encoding proteins with identity to G1LEA proteins, two of them, *lea1* and *lea2*, encoding for *avLEA1* and *avLEA2* proteins, which possess 20-mer sequences with the sub-motif present in LEA proteins from eukaryotic organisms. In this study, strains carrying deletion mutations of *lea1* and *lea2* were constructed to investigate their role in cyst tolerance to desiccation. We found that the *avLEA1* protein contributes to the tolerance of the cysts to desiccation since the viability of cysts developed by the *lea1* mutant was completely lost after 6 months of drying, while the cysts developed by the wild type or the *lea2* mutant strain remained viable. As in *A. vinelandii* cysts, the contribution of LEA proteins to survival in drying conditions was also observed in *Deinococcus radioduran*, as the inactivation of two genes encoding for LEA proteins caused a loss of viability of 75% when cultures were exposed to drying for a period of 2 weeks (Battista et al. 2001). Also in *Caenorhabditis elegans* when the expression of LEA genes is silenced by RNA interference, there is a decrease in survival during induction of desiccation, osmotic and heat stress in this nematode (Gal et al. 2004).

The expression levels of *lea1* during the encystment process were higher than in vegetative cells; moreover, *lea2* was not highly induced on cysts or during the encystment process. Similarly to what we observed in *A. vinelandii*, a differential expression of LEA transcripts during development and cyst formation of *A. franciscana* was reported (Denekamp et al. 2010). Moreover, in *C. elegans* LEA genes, transcription is induced during dehydration at the stress-resistant juvenile stage (Gal et al. 2004).

This study also showed that *avLEA1* but not *avLEA2* conferred *A. vinelandii* resistance to osmotic and high-temperature condition stresses, suggesting that the difference in the number of 20-mers and/or the size in these two LEA proteins may cause a significant difference in their protective activity of subcellular structures and membranes. However, there are no reports that correlate the number of 20-mers present in a protein and their protective effects.

AEIV and OPA *A. vinelandii* reference strains were isolated from cold soils from Wisconsin, USA, and from Trondheim, Norway, respectively. BLAST using the 20-mer of the *avLEA1* or *avLEA2* revealed the presence (in addition to *Azotobacter croccocum* and *A. vinelandii*) of genes encoding

putative LEA proteins having the eukaryotic sub-motif in two other species of bacteria, *Methylocystis parvus* and *Anaeromyxobacter* sp. (Fig. 1). Interestingly, *M. parvus* belongs to *Methylocystaceae* taxa, associated with cold soils (Wunderlin et al. 2016), and both *M. parvus* and *Anaeromyxobacter* have been reported to be able to form cysts.

In general, the denaturation and dysfunction of enzymes or different proteins occur when cells are subjected to low- or high-temperature stress or dehydration (Goyal et al. 2005; Reyes et al. 2008). LEA proteins have been proposed to act as “molecular shields” (Goyal et al. 2005; Wise and Tunnacliffe 2004) avoiding the aggregation of proteins from irreversible interactions, protecting functions or enzyme activity. In the present study, the *avLEA1* protein was shown to protect in vitro LDH activity against the inactivation induced by freeze thaw cycles; after 2 and 10 freeze thaw cycles, LDH activity remained twice that when LDH was freeze-thawed without the purified *avLEA1*. In *Chlorella vulgaris*, LEA proteins were found to protect a freeze-labile enzyme against freeze inactivation (Honjoh et al. 2000). Thus, we hypothesised that *avLEA1* protein would play an important role in protecting the proteins and enzymes of *A. vinelandii* cells in vivo under high-temperature stress and in vitro when subjected to freeze thaw cycles.

Finally, the functional expression of LEA proteins in vivo in *E. coli* under abiotic stresses has been extensively investigated. One *LEA4* gene from *Brassica napus* was overexpressed in *E. coli* and shown to confer salt and extreme temperature tolerance to this bacterium (Dalal et al. 2009). More recently, the heterologous expression of an atypical hydrophobic LEA protein from *Oryza sativa* L. in *E. coli* improved resistance against diverse abiotic stresses: high salinity, osmotic, freezing, heat and UV radiation (He et al. 2012). This study showed that overexpression of *avLEA1* protein improved the tolerance of *E. coli* to NaCl osmotic stress; however, we did not observe protection of *E. coli* to high temperatures by *avLEA1* protein. This unexpected result is at present difficult to explain, since other LEAs expressed in *E. coli* confer tolerance to high temperatures. One explanation is that the *avLEA1* folding needed for the protective activity occurs at high temperatures in *A. vinelandii* but not in *E. coli*.

In conclusion, we demonstrated that *avLEA1* protein is a key determinant on the tolerance of *A. vinelandii* towards abiotic stress such as desiccation, osmolarity and high temperatures since the survival of a *lea1* mutant was severely impaired when the organism was exposed to stress conditions. This is the first report on the functional properties of an LEA protein in *A. vinelandii*.

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