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Appearance of Membrane Compromised, Viable But Not Culturable and Culturable Rhizobial Cells as a Consequence of Desiccation

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Appearance of Membrane Compromised, Viable But Not Culturable and Culturable Rhizobial cells as a consequence of Desiccation.

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Abstract

For agricultural purposes, drought related stresses negatively affect the *Rhizobiaceae* in at least three ways. Firstly, rhizobial populations are affected by desertification of agricultural soils. Secondly, the quality of dry-base inocula, also called formula, is negatively affected by a drying step, and thirdly, rhizosphere bacteria protect crop-plants against drought. Although survival of cultivatable bacteria has been studied intensively in dry-base seed inocula and *in-vitro*, thus far research has only marginally addressed the bacterial cell, its cellular structures and physiology. Many questions remain regarding the sensing of and physiological response of rhizobia to desiccation. This review will focus on the three different fractions of cells after desiccation, the membrane compromised cells, the viable but not culturable cells and the culturable cells.

Introduction and Discussion

According to Veron et al. [2006], 40% of the world's surface is threatened by desertification related problems, and consequently the degradation of soil quality due to drought and salinity. Drought and salinity are considered the most important abiotic stresses in many areas in the world, and it is estimated that 1 billion people worldwide populate these lands. Of particular importance to the agricultural industry is the impact of these harsh environmental conditions on the soil-borne endogenous group of proteobacteria, the rhizobia [Zahran, 1999; Fierer et al., 2003; Griffiths et al., 2003; Dardanelli et al., 2012).

The *Rhizobiaceae* is a bacterial family of enormous agricultural importance due to their ability to fix atmospheric nitrogen in an intimate relationship with plants in root or stem nodules enhancing growth under nitrogen limiting conditions. This relationship is negatively impacted by drought related stresses [Jones et al., 2009; Zahran, 1999]. In addition, these bacteria can also improve drought tolerance of agricultural crops [Grover et al., 2011; Zahran, 2010; Dodd and Perez-Alfocea, 2012; Bianco and Defez, 2009]. To make optimal use of the process of nitrogen fixation, seed inoculation companies apply *Rhizobium* strains to the seed surface that are selected for their ability to efficiently colonize the rhizosphere and fix nitrogen. Unfortunately, many inoculants remain unreliable

because of the inability of bacterial cells to persist under adverse conditions, negatively affecting colony-forming units (CFU) of added rhizobia [Kosanke et al., 1992; Deaker et al., 2004; Catroux et al., 2001; Smith, 1992; Bullard et al., 2004; Herridge, 2007]. Furthermore, Ilyas and Bano [2012] provided a nine-point list of characteristics that Plant Growth Promoting Rhizobacteria [PGPR] should have for successful dry-base formulation development. This list includes desiccation resistance.

The ability of selected strains to survive desiccation depends on many factors, for example, the drying method used, such as forced-drying using vacuum versus air-drying, the media used, the speed and severity of drying, the extent and speed of rehydration, the growth-phase, the drying temperature, the availability of solutes and the carrier material [Vriezen et al., 2006; 2007]. In addition, intragenic differences to cope with desiccation stress affect survival. For example, slow growers tend to be more desiccation resistant than fast-growers [Zahran, 2001]. These data suggest that no single trait affects the ability of rhizobia to survive desiccation but that several mechanisms are likely responsible [Vriezen et al., 2006; 2007; 2013].

In a review by Vriezen et al. [2007], the authors provided a hypothetical model for the response of rhizobia to desiccation

(**Figure 1**). The model includes two not mutually exclusive physiological responses. Upon drying, rhizobia sense the consequences of drying, which may be the lowering of wateractivity (A_w), accumulation of solutes and concentration of enzymes. Thus far it remains unclear how desiccation is sensed and resistance is mediated [Vriezen et al., 2005; 2007; Hirsh, 2010). The response likely includes the accumulation of osmoprotectants and compatible solutes known to increase desiccation survival. For example, trehalose increases the ability to survive desiccation [McIntyre et al., 2007]. Also heat shock protein (HSP) and chaperones and reactive oxygen species (ROS) scavenging enzymes accumulate [Feng et al., 2002; Cytryn et al., 2007]. In contrast, when cells are desiccated, cells are not active, thus damage accumulates due to the inability to repair those. Consequently, upon rewetting and regaining of metabolism cells get the opportunity to repair damage accumulated during storage or that acutely appears upon rewetting. These damages include damage to DNA, damage to membranes and the cell wall [Humann et al., 2009; Potts, 1994; Leslie et al., 1995; Vriezen et al., 2012; Salema et al., 1982; Bushby and Marshall, 1977B].

Figure 1: Model representing two hypothetical pathways for responses of rhizobia to desiccation stress and desiccation-

induced damages.

Although improvement of long-term survival and seed inocula storage time has been the focus of desiccation research, relatively little work has focused primarily on the bacterial cell. Most, if not all, research only used culturability as a measure to estimate survival consequently marginalizing often the vast majority of cells not forming colonies. But what is the fate of the cells not forming colonies? Recently, Vriezen et al. [2012] described the appearance of Viable But Not-Culturable (VBNC) *Sinorhizobium meliloti* cells upon desiccation and resuscitation. Because a major target for desiccation stress are the cell membranes, they hypothesized that cells, which lost the ability to form colonies, have compromised cell membranes [Potts, 1994; Leslie et al., 1995]. Thus, it was expected that all cells not forming colonies were membrane compromised. To test this, Vriezen et al. [2012] applied the live/dead stain to cells after desiccation. This stain uses two dyes, syto-9 and propidium iodide, which differ in their ability to cross the cytoplasmic membrane. Syto-9 can always cross the membrane and stains all cells green. However, propidium iodide can only cross the membrane when the permeability is increased and staining the cell red. These red cells are dead. When this stain was used on rhizobial cells prior to and after drying, unexpected results

were obtained. The data presented in **figure 2** shows that two staining methods (crystal violet and live/dead) yielded the same results prior to drying, after three days at 100% RH and after three days at 22% RH. The increase in countable cells at 100% RH conforms to what one expects since an increase in colony forming units was observed over three days at 100% RH in previous studies [Vriezen et al., 2006]. However, a change in the fraction of red (dead) and green (living cells) was observed. Prior to drying, the red fraction of cells was very low (4.4±0.5%) and increased substantially during desiccation to 56.9±10.6%. During this process, culturability decreased to 3.1%. If it is assumed that the colony forming cells are a fraction of the viable cells, we can only conclude that many cells are alive but are not able to form colonies and are in a VBNC state. This VBNC state exists in many microorganisms, including rhizobia [Manahan and Steck, 1997; Alexander et al., 1999; Basaglia et al., 2007; Räsänen et al., 2001; Vriezen et al., 2012; Catroux et al., 2001]. The induction of this physiological state by desiccation is a novel and very relevant observation since only cells able to form colonies can infect plants, as reported for strain *S. meliloti* 41 [Basaglia et al., 2007]. The term VBNC applies directly to the observations presented in **Figure 2**: Cells of *Sinorhizobium meliloti* 1021, that were rehydrated after desiccation, can be divided into

three different fractions of cells after desiccation, the membrane compromised- (MC), the viable but not culturable- (VBNC), and the culturable cells (colony forming units, or CFU) and correspond to fraction III, II and I in Vriezen et al. [2012] respectively.

Figure 2: Survival and recovery of *S. meliloti* cells after drying and rewetting.

The Membrane Compromised (MC) fraction

Fraction III: That cell membranes are a target for desiccation is not novel, however, the extent to which desiccation compromises membranes in *Sinorhizobium meliloti* 1021 is unexpected. In the aforementioned study, 56.9±10.6% of cells stain red and have lost membrane integrity, indicating that of the very substantial number of cells not forming colonies (100%-3.1%=96.9%), 59% of this fraction have non-functional membranes. Thus, the loss of membrane integrity is the main cause of death for *Sinorhizobium meliloti* 1021. Vriezen et al. [2012] hypothesized that this desiccation-induced loss of membrane integrity can be explained by changes in phase transition of phospholipid membranes, due to the removal of water affecting the phospholipid head spacing and due to rehydration and the consequent breakage of the cell wall. Furthermore, lipid

peroxidation and Fe^{3+} catalyzed oxidation also lead to the loss of structure of the membranes when cells are not able to repair damages [Deaker et al., 2004; Potts, 1994]. Under normal wetted conditions, membranes are in the liquid crystalline phase (**Figure 3A**). Upon the extraction of water, phase transition occurs leading to the gel-phase of the membrane. Upon rewetting, phase transition occurs again, leading to leakage of cell constituents from the cell and to the loss of membrane integrity and cell death.

Phase transition can be followed using Fourier Transformation Infra Red [FTIR, Leslie et al., 1995] spectroscopy in which the vibration frequency of the phospholipid head groups is measured (**Figure 3B**). Upon decreasing temperatures, this frequency decreases indicating membrane transition. The temperature at which this transition occurs is the midpoint temperature. Interestingly, if ambient temperature is higher than the membrane midpoint temperature, no phase transition occurs upon drying and rewetting, reducing cell death. However, when drying takes place at an ambient temperature below the midpoint temperature, cell death due to membrane transition is increased. A consequence of a change in membrane midpoint temperature can be seen in the change in vibration frequency (**Figure 3B**). When the midpoint temperature is lowered one expects the temperature at which phase transition

occurs to be lower resulting in a wider window of ambient temperatures higher then the midpoint temperature, increasing survival. Survival data indicates that this is what happens after desiccation of *Sinorhizobium meliloti* 1021. An increase in viability was observed with increasing temperature with a maximum at 37°C indicating that this process may underlie the observations [Vriezen et al., 2006].

Figure 3: Membrane properties.

The structural adaptations to membrane phospholipids that affect fluidity and midpoint temperature are the following: Non-reducing sugars such as trehalose stabilize the phospholipid head spacing of the membranes, leading to a decrease of the membrane midpoint temperature and increased survival even at lower ambient temperature (**Figure 3A&B**). Also longer fatty acids decrease fluidity leading to an increase in the midpoint temperature. Furthermore, an increase in *cis*-bonding, thus an increase in unsaturation leads to an increased fluidity, thus a lower midpoint temperature [Leslie et al., 1995]. Therefore, a decrease in the unsaturated/saturated (u/s) ratio of membrane phospholipids leads to a lower fluidity and a higher midpoint temperature. Boumahdi et al. [1999] studied survival after desiccation of *S. meliloti*, *B. japonicum* and *B. elkanii* in

relation to growth-phase and the fatty acid u/s ratio. Even though differences in u/s were found depending on the growth-phase, these differences did not correlate with the ability to survive desiccation at many RH's (3-83.5% RH) except under the following conditions: In *B. elkanii*, an increase in saturation lead to a decrease in desiccation survival at 67.8% RH at 30°C, and in *B. japonicum*, the same was seen at 3% and 22%RH. While the expected correlations were seen in Bradyrhizobia under certain conditions, this was not seen in *S. meliloti* RCR2011. In another publication by Boumahdi et al. [2001], growth at decreased water activities (A_w) affected the u/s ratio in *S. meliloti* 2011, *B. elkanii* and *B. japonicum*. This effect was strongest in *B. elkanii*. Surprisingly though, with decreasing A_w a decrease in u/s ratio was found, counter intuitive to what one would expect in order to survive water-stress with the membrane as major target. Why these correlations were not observed across the range of A_w's, RH's, strains and growth-phases tested is unclear. However, it indicates that other mechanism underlie these phenomena.

In addition to the responses described above, hypothetically, an increase in the concentration of hopanoids should increase fluidity and lower the midpoint temperature. Hopanoids are the prokaryotic equivalent of cholesterol in eukaryotes [Kannenberg et al., 1999; Kannenberg et al., 1995].

Their function remains unknown, however, their presence in membranes leads to reduced permeability and increased order of membranes above the midpoint temperature at which molecular disorder threatens membrane stability. These aliphatic compounds have also been identified in *Rhizobium* but to our knowledge have not yet been studied in relation to desiccation survival.

The Viable But Non-Culturable (VBNC) fraction

Fraction II: The second most important fraction in a culture of cells after desiccation are the VBNC cells. Many environmental factors have been identified inducing a VBNC state in bacteria, such as temperature stress, osmotic upshift and oxygen stress, tap water and the VBNC inducing component copper in *A. tumefaciens* and *R. leguminosarum* [Oliver, 2005; Räsänen et al., 2001; Manahan and Steck, 1997; Alexander et al., 1999]. Also desiccation can induce a VBNC state in *E. cloacae* and *S. meliloti* [Pederson and Jacobsen, 1993; Vriezen et al., 2012]. This VBNC fraction can be divided into two sub fractions, those cells for which VBNC is reversible and can be resuscitated (temporarily non-culturable), and those for whom VBNC is a permanent state (permanently non-culturable) (Maraha, 2007). In a paper by Hammes et al. [2011], the authors named these two fractions "Potentially reversible, starved or injured" and "Irreversible, or dying/dead". The demarcation of these two

fractions is a hard to assess amount of DNA- and protein-damage. It remains unclear which level of damage leads to death or the inability to resuscitate.

Several researchers have attempted to understand the conditions modulating the culturability of bacteria. Barry et al. [1956] noted that autoclaving media leads to an increase in H_2O_2 decreasing CFU's. The addition of sodium pyruvate and catalase to the medium can increase resuscitation in many organisms [Mizunoe et al. 1999; Imazaki and Nakaho, 2009]. However, this approach proved unsuccessful in *S. meliloti* 1021 and 41 [Basaglia et al., 2007; Vriezen et al., 2012]. It appears that all desiccation and O_2 limitation induced VBNC cells are in a permanent state of non-culturability under the conditions tested [Basaglia et al., 2007; Toffanin et al., 2000]. In contrast, a very slow supply of oxygen appeared to resuscitate some cells [Basaglia et al., 2007]. These results indicate that resuscitation from the VBNC state differs between *E. coli* and *Rhizobium*, even though O_2 damage may occur in both cases.

One explanation for the occurrence of desiccation induced rhizobial VBNC cells is that these cells are without a functional template for the replication of DNA but have intact membranes. DNA is a major target of desiccation in microorganisms inducing double strand breaks in *E.coli* and *D. radiodurans* [Asada et al., 1979; Mattimore and Battista, 1996].

In support of this hypothesis are the observations by Humann et al. [2009], whom isolated a desiccation sensitive *Sinorhizobium* mutant with a Tn*5* insertion in its *uvr*C locus which is involved in DNA repair. Therefore, the inability to repair desiccation induced DNA damage leads to a decrease in CFU's and likely to an increase in VBNC cells in rhizobia.

To identify additional physiological responses potentially involved in the VBNC state of rhizobia, we consulted three studies addressing the physiological responses of the VBNC state in *Pseudomonas* and *E. coli*, both proteobacteria. The proteins identified in these three studies are summarized in **Table 1** [Maraha, 2007; Asakura et al., 2008; Muela et al., 2008]. Several loci, OmpW, HisJ and ProX were found in both proteobacteria in more than one study. Surprisingly, OmpW, found strongly expressed in VBNC cells in several microorganisms and studies, could not be identified in *S. meliloti*. Actually, only HisJ has significant identity in *S. meliloti* 1021 (>95% of the query sequence, and >30% Identity). Using the same criteria, five more loci were identified and are DdpA, TpiA, LeuD, OppA, and EF-TU, which, together with HisJ are the first set of *S. meliloti* candidate loci affecting the VBNC state. Interestingly, neither the loci identified by Humann et al. [2009], nor those involved in trehalose metabolism [Reina-Bueno et al., 2012; McIntyre et al., 2007; Flechard et al., 2010], nor loci

responsive upon desiccation [Cytryn et al., 2007] were identified, indicating that DdpA, TpiA, LeuD, OppA, and EF-TU represent a novel set of candidate loci for the rhizobial VBNC state.

Table 1: Candidate loci in *S. meliloti* 1021 potentially involved in the VBNC state.

How do these observations relate to Rhizobia? Identification of the proteins mentioned above indicates that damage to amino acid metabolism and protein synthesis, which may result in permanent VBNC cells in *Rhizobium*. For example, Asakura et al. [2008] showed that HisJ, LeuD and OppA were increased in a oxidative- and osmo tolerant *E. coli* strain, while TpiA was decreased compared to a oxidative- and osmo sensitive *E.coli* strain after passing through the GI track. This would indicate that strains not sensitive to oxygenic stress, thus "VBNC resistant", have increased expression of HisJ, LeuD and OppA. In Muela et al. [2008], EF-TU (TufA, involved in recruiting charged tRNA to the a-site on the rhibosome) was found expressed in the VBNC state in phosphate buffered saline (PBS). According to Barcina and Arana [2008], this conforms to the findings by Kong et al. [2004] in *Vibrio vulnificus* and Asakura et al. [2007] in *E. coli*. Hydrogen peroxide sensitive

Vibrio cells, having lost catalase activity, are entering the VBNC state and have increased OmpW and TufA levels.

The hypothesis stated above is further supported by the identification of *rel*A mutants with a desiccation sensitive phenotype by Humann et al., [2009]. RelA (stringent response) is stimulated by aminotriazole (AT), a histidine analog inducing histidine starvation [Wells and Long, 2002; Kroll and Becker, 2011]. Histidine starvation induces *rel*A and therefore the stringent response. HisJ mutants potentially induce histidine starvation, thus these findings support the hypothesis that VBNC cells are affected in amino acid metabolism and translation. A substantial part of the permanently non-culturable rhizobial cells after desiccation may thus be irreversible VBNC, or dying/dead due to substantial amounts of DNA and protein damage.

The Culturable Fraction (CFU)

Fraction 1: The CFU fraction is the smallest of the three recognized fractions of rewetted *Sinorhizobium meliloti* 1021 after desiccation. This fraction is so small [3.1%, Vriezen et al., 2012] that it falls within the error of measurement of the MC fraction (±10.6%). Thus, even a doubling in the fraction of culturable cells would not be reflected in a significant change in dead and living cells. Even though, it is the culturable fraction that counts in formulations of rhizobia since non-

409 growing but living cells do not contribute to nodule formation
410 [Basaglia et al., 2007]. Therefore, understanding the conditions
411 and cellular responses increasing this fraction remain of
412 crucial importance. Even though no data exist on how osmotic
413 stress and temperature affect the appearance of VBNC and MC
414 fractions, more is known about these conditions in relation to
415 desiccation survival.
416
417 Effect of osmotic and salt stress
418 In a review by Vriezen et al. [2007], the authors hypothesized
419 about the effect of NaCl stress on the ability of *S. meliloti* to
420 survive desiccation. Exposure to NaCl during drying may increase
421 the ability to survive desiccation even considering that salt
422 stresses, osmotic stresses and desiccation stress are very
423 different in essence. Osmotic stress is the abundance of
424 solutes, salt stresses, and of (non-) toxic ionic compounds,
425 while desiccation stress results from the lack of water. The
426 reason for the hypothesis is the available data indicating an
427 overlap in response between the stresses reviewed by Vriezen et
428 al. [2007]. The conclusions were that (i) chloride stress
429 induces a response in combination with nutrients from the media
430 that lead to an increase in survival, (ii) the response is
431 strain specific and (iii) the increase in CFU during NaCl
432 mediated desiccation is physiological in origin. Even though the

response of *S. meliloti* to NaCl does increase CFU after desiccation, it does only exclude some aforementioned stresses from inducing these physiological responses. However, in their review Vriezen et al. [2007] argued that screening for loci responsive to NaCl stress would select for loci potentially involved in survival during desiccation. In support of this are the findings by Streeter [2007] that NaCl increases intracellular trehalose content in *Bradyrhizobium japonicum*, and the finding by Humann et al. [2009], who showed that a *rpo*E2 mutant was sensitive to desiccation. RpoE2 is a response regulator for envelop-stress. The hypothesis is further supported with the identification of the *S. meliloti* 1021 mutant with a Tn*5lux*AB transcriptional fusion inserted in a NaCl inducible putative Open Reading Frame (ORF, *ngg*) sensitive to survival during desiccation [Vriezen et al., 2005; 2013]. Ngg is responsive to NaCl stress and also affects the ability of *S. meliloti* 1021 to survive desiccation.

Which NaCl mediated responses affect survival during desiccation? We address four potential responses. Firstly, although certain compatible solutes and osmoprotectants accumulate during NaCl and osmotic stress and have a positive effect on the survival during desiccation of *Rhizobium*, others have not. For example, in rhizobia, the recently identified NaCl induced loci *asnO* and *ngg* involved in the production of the

457 dipeptide NAGGN show differential responses to desiccation
458 [Vriezen et al. 2005; 2013; Sagot et al., 2010]. A Tn*5lux*AB
459 insertion in locus *asn*O does not lead to a decrease in survival
460 during desiccation, while an insertion in locus *ngg* does. This
461 indicates that NAGGN accumulation as a response to NaCl stress,
462 does not affect the ability to survive desiccation since both
463 loci are involved in the synthesis of NAGGN. In contrast,
464 compatible solutes like sucrose and trehalose are known to
465 affect survival by their stabilizing abilities of the cell
466 membrane. Trehalose accumulates in osmo-stressed rhizobia and
467 provides protection against desiccation by maintaining membrane
468 integrity during drying and rewetting. Its presence may explain
469 the increase in desiccation survival during the stationary phase
470 and when rhizobial cells are exposed to NaCl [Welsh and Herbert,
471 1999; Breedveld et al., 1990; 1993; Streeter et al., 2003;
472 Leslie et al., 1995; Potts, 1994; Reina-Bueno et al., 2012;
473 McIntyre et al., 2007; Flechard et al., 2010]. Gouffi et al.
474 [1995; 1998; 1999; 2000] found that trehalose and sucrose are
475 synthesized *de novo* during exponential growth. Uptake mechanisms
476 in rhizobia were also described; an *agl* operon for
477 trehalose/maltose and sucrose uptake (*smb03060-03065*) was
478 identified by Willis and Walker [1999] and Jensen et al. [2002]
479 identified an alternative trehalose/maltose/sucrose operon (*thu*,
480 *smb20324-20330*). Dominique-Ferreras et al. [2006] showed that

the *thu* operon is upregulated during an osmotic upshift and the importance of the osmotic stress responsive loci *ots*A and *tre*S in trehalose accumulation. McIntyre et al. [2007] showed that *ots*A provides resistance to desiccation. Interestingly, trehalose synthesis genes (*ots*AB and *tre*S) are increasingly expressed during drying of *Bradyrhizobium japonicum* [Cytryn et al., 2007] and Sugawara et al. [2010] shows that *tre*S and *tre*Y mutants of this organism have lower survival rates after desiccation.

Wei et al. [2004] and Miller-Williams et al. [2006] also identified *Sinorhizobium* mutants unable to grow at increased NaCl concentrations. The mutations causing these phenotypes were traced to genes involved in the central metabolism, such as elongation factors, chaperones and cell division proteins. Also genes for DNA ligases were higher expressed as well as a putative DNA polymerase, an invertase and a ribonuclease. These observations are most interesting considering VBNC cells may not be able to resume growth after exposure to desiccation conditions due to extensive DNA damage. If these responses affect desiccation resistance or the appearance of desiccation induced VBNC cells in *Rhizobium* remains to be seen.

Polysaccharides are of interest with respect to desiccation since adaptations of the polysaccharide composition have been observed for *S. meliloti* undergoing osmotic stress and are known

to affect survival during dry conditions [Breedveld et al., 1991; Llorett et al., 1998; Chenu, 1993]. Vanderlinden et al. [2011] identified a *R. leguminosarum* Tn*5* mutant in which exopolysaccharide (EPS) production positively correlates with desiccation resistance. The open reading frame mutated is *RL2975*, however, a similar ORF does not exist in *S. meliloti*. The mutant was not sensitive to hyperosmotic stress, nor sensitive to detergents, suggesting the outer membrane was not affected. However, it is naïve to consider polysaccharides a panacea to all desiccation related issues. Vriezen et al. [2007] evaluated many reasons why this is not the case and gave examples of studies resulting in contradictory observations. For example, a decrease in survival of colony forming rhizobia was observed upon the addition of polysaccharides when dried at a RH > 3%, but an increase in survival at 3% relative humidity [Mary et al., 1986]. Polysaccharide-producing variants of *Rizobium trifolii* in sandy soil and under fast drying conditions showed no consistent improvement in survival [Bushby et al., 1977A]. Osa-Afiana and Alexander [1982] showed that, when dried slowly in Collamer silt loam, the production of EPS decreases survival during desiccation of *Bradyrhizobium japonicum* strains, even though polysaccharides did increase survival of *R. trifolii* 412 in a Lima silt loam [Pena-Cabrialis and Alexander, 1979]. The reason for these apparent contradictions is likely due to the

complexity of- and ambient conditions during- desiccation. A polysaccharide may provide protection under one condition while is detrimental under other conditions. The mechanisms by which polysaccharides provide protection are not clear and specific properties of polysaccharides have different effects on a microorganism's ability to survive desiccation. Four of these properties are (i) buffering against changes in water content, (ii) exclusion of toxic compounds, such as Cl^- and O_2, (iii) the final water content of polysaccharides under ambient conditions, and (iv) the effect of hysteresis in the water retention isoterms of polysaccharides [Potts, 1994; Rinaudo, 2004; Chenu, 1993].

Existing data on the environmental conditions affecting polysaccharide production show that an increase in osmotic pressure results in enhanced production of high molecular weight (HMW) succinoglycan over low molecular weight (LMW) succinoglycan [Breedveld et al., 1991] and that the expression of genes involved in EPSI production are up-regulated during salt stress [Ruberg et al., 2003, Jofre and Becker 2009]. These observations suggest that in *S. meliloti* NaCl-dependent EPS production leads to the production of HMW succinoglycan, resulting in an increase in CFU's after desiccation. In addition, structural changes under the influence of osmotic- and salt stress have also been reported for lipopolysaccharides

(LPS) [Bhattacharya and Das, 2003; Llorett et al., 1995]. Interestingly, Llorett et al. [1995] found a different LPS content in EFB1 cells grown on different salts, while polyethylene glycol (PEG) 200, which causes only osmotic stress, does not induce such a change. These differential responses may correlate with the differences in survival during desiccation when exposed to several different salts and argue for a potential role of LPS in survival during desiccation [Vriezen et al., 2006]. Indeed, Vanderlinden et al. (2010; 2011; 2012) showed that a mutation in the *fab*F1 and *fab*F2 gene in *R. leguminosarum*, involved in LPS formation increased sensitivity to desiccation and osmotic stress. Thus, structurally intact LPS are important in protecting *R. leguminosarum* cells against desiccation.

Vriezen et al. [2007] hypothesized that enzymes involved in the production of HMW succinoglycan would positively affect CFU's. For example, mutations in *S. meliloti* ExoP (Smb21506) was found to block polymerization of EPS1, and ExoQ (Smb20944) is required for the production of HMW succinoglycan [Gonzales et al., 1998; Jofre and Becker, 2009]. In support of this theoretical consideration was the ~5 fold induction of *exo*P in desiccated *Bradyrhizobium japonicum* [Cytryn et al., 2007]. Interestingly, in *S. meliloti* depolymerization of HMW leads to the production of LMW succinoglycan, which is ExoK (Smb20955)

and ExsH (Smb20932) mediated [York and Walker, 1998]. However, Cytryn et al. [2007] did not find these genes in their induction studies.

Lastly, Cytryn et al. [2007] found an upregulation of glycogen synthase (*glg*A) during desiccation. Glycogen may assist in restoring cell volume after osmotic shock [Han et al., 2005]. The *glgA2*, *glgB2* and *glgX* genes involved in glycogen metabolism (*smb20704*, *smb21447*, *smb21446* respectively), are higher expressed during exposure to osmotic stress and may have a role in desiccation survival.

The impact of temperature

Theoretically, temperature is involved in survival during desiccation through the phase change of membranes during drying and rewetting leading to the loss of membrane integrity (Leslie et al., 1995). The logical consequences of this process would be that an increase in drying temperature prevents membrane transition. Vriezen et al. [2006] and this manuscript (**Figure 4**) found a positive correlation between survival and temperature with an optimum at 37°C. This indicates a potential physiological response to temperature affecting survival after desiccation.

Vriezen et al. [2007] reviewed the conditions in soil and seed inocula and concluded they do not support the *in vitro* observations, because many different researchers obtained

601 contradictory results. They concluded that at least one
602 additional factor must exist applying an unknown, yet overruling
603 stress to dry cells. For example, (i) dry seed inocula have a
604 water activity of 0.45-0.6 thus still contain a relatively high
605 amount of water [Smith, 1992; Deaker et al., 2004]. (ii)
606 Isolated rhizobia show large differences in their ability to
607 respond- and adapt to life at high temperature which is not
608 necessarily linked to their ability to survive desiccation
609 [Trotman and Weaver, 1995]. Therefor, heat-tolerant strains may
610 not have an increased ability to survive desiccation, unless
611 temperature, rather than drought, is the superimposed stress.
612 However, the identification of a *Azorhizobium sesbania* Tn*5*
613 mutant sensitive to drought and temperature reveals a genetic
614 basis for this response in some strains [Rehman and Nautiyal,
615 2002]. In addition, Reina-Bueno et al. [2012] identified an *ots*A
616 mutant of *R. etli* which was affected by drying, but also lost
617 the response to temperature.
618 The molecular responses to stress in Rhizobia were recently
619 reviewed by Alexandre and Oliveira [2012] and include heat
620 inducible small heat shock proteins (HSP) [Ono et al. 2001;
621 Munchbach et al., 1999], the heat shock proteins DnaKJ, GroESL
622 and GroEL [Minder et al. 1997; Rodrigues et al. 2006; Rodriquez-
623 Quinones, 2005; Fisher et al., 1993], transcriptional regulation
624 by RpoH (Narberhaus et al. 2005; Ono et al., 2001), and EPS and

LPS [Nandal et al., 2005]. Potential sensing mechanisms involve *cis*-acting ROSE elements or RNA thermometers [Waldminghaus et al., 2005; Narberhaus et al. 2005; Nocker et al. 2001], and thermo- induced changes in DNA structure and nucleoid associated proteins [Shapiro and Cowen, 2012; Steinman and Dersch, 2013]. However, no papers were found on rhizobial thermosensing by responding to changes in cell membranes.

Several of the aforementioned mechanisms may affect survival of rhizobia after desiccation [Cytryn et al., 2007]. These authors showed an increase in expression of *gro*ESL-related chaperones, indicating a potential involvement of these genes in survival during desiccation in *Bradyrhizobium japonicum.* It is likely that similar mechanisms exist in *Sinorhizobium meliloti*. Furthermore, Dominguez-Ferreras et al. [2006] identified several loci responsive to an increase in osmotic and salinity stress also associated with the temperature response. Most interestingly, RpoE2 (Smc01506), affecting survival during desiccation, also controls 44 genes involved in the heat-shock response [Humann et al., 2009; Sauviac et al., 2007]. Therefore, RpoE2 may regulate the part of the osmotic and temperature response also affecting its ability to survive and grow after desiccation.

NaCl and temperature: An interconnected response to desiccation?

Intellectually it makes sense that microorganisms respond to an increase of solutes or to temperature in order to respond to desiccation. However, how likely is to have a molecular junction of a NaCl inducible gene that, when knocked out, leads to temperature dependent desiccation sensitivity? In addition to some NaCl induced loci described earlier, locus *smb01590*, found by Vriezen et al. [2013], also affects survival during desiccation (**Figure 4**). Interestingly, the ability of the mutant carrying a Tn*5lux*AB insertion in ORF *smc01590* (Sce-1) to survive desiccation is better, albeit not significant (P=0.22), than that of the reference strain at 4°C. While survival of the reference strain increases with increasing temperature, survival of Sce-1 is decreasing with increasing temperature leading to a much better survival of the reference strain at 37°C. There are at least two different not mutually exclusive explanations for the observation. Firstly, this observation suggests the involvement of the membrane in this process, since the reference strain does respond exactly as explained above: If ambient temperature is higher than the membrane midpoint temperature, no phase transition occurs, reducing cell death and increasing culturability. When drying takes place at an ambient temperature below the midpoint temperature, cell death due to membrane transition is increased.

673

674 **Figure 4:** Survival after desiccation of *S. meliloti* 1021 and
675 Sce-1.

676

677 In mutant Sce-1, it appears that the inability to correctly
678 adjust the membrane midpoint temperature at increased
679 temperature leads to the opposite effect. At 4°C, cell death in
680 the reference strain and the mutant strain is comparable. The
681 reference strain can respond to increases in temperature leading
682 to increased survival. At 4°C, both strains experience an ambient
683 temperature lower than the membrane midpoint temperature leading
684 to similar survival rates. However, at increased temperatures,
685 the reference strain experiences an ambient temperature
686 comparable or higher than the midpoint temperature, which
687 increases survival. Due to the inability to lower the midpoint
688 temperature, the Sce-1 mutant still experiences an ambient
689 temperature lower than the midpoint temperature, leading to a
690 reduced survival compared to the reference strain.
691 Alternatively, a defect in the temperature response in
692 strain Sce-1 potentially leads to reduced survival rate with
693 increasing temperature. The lack of the production of heat shock
694 proteins and chaperones may explain this phenomenon. The
695 postulated non-exclusivity of the two hypothetical explanations

allows that one of the responses to NaCl is the decrease of the midpoint temperature.

Interestingly, prodomain analysis of the aminoacid sequence indicates that Smc01590 encodes a 210AA peptide with a leader peptide targeting the cytoplasmic membrane. Smc01590 also contains an SH2/SH3 domain. SH3 domains are called Molecular Velcro [Morton and Campbel, 1994] due to their ability to form strong bonds with other proteins by targeting proline rich areas, which are also found in the sequence. Its location and these domain/motif interactions suggest that Smc01590 can form membrane located proteinaceous structures stabilizing the membrane. Prodomain also predicts several cytoplasmic kinase sites, which are commonly involved in signal transduction directly or indirectly involved in sensing- and maintaining membrane stability. Thus, it appears that Smc01590 is potentially a sensor in a signal transduction pathway, in which changes in membrane fluidity due to temperature and osmotic pressure lead to the expression of downstream loci involved in the lowering of the membrane midpoint temperature. This protein and its locus are not under RpoE2 control, and may be part of a novel signaling network.

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References

Alexander E, Pham D, Steck TR. 1999. The viable-but-nonculturable condition is induced by copper in *Agrobacterium tumefaciens* and *Rhizobium leguminosarum*. Appl. Environ. Microbiol. 65:3754–6.

Alexandre A, Oliveira S. 2012. Response to temperature stress in rhizobia. Crit. Rev. Microbiol. 7:1–10.

Asada S, Takano M, Shibasaki I. 1979. Deoxyribonucleic acid strand breaks during drying of *Escherichia coli* on a hydrophobic filter membrane. Appl. Environ. Microbiol. 37:266–273.

Asakura H, Kawamoto K, Haishima Y, Igimi S, Yamamoto S, Makino S. 2008. Differential expression of the outer membrane protein W (OmpW) stress response in enterohemorrhagic *Escherichia coli* O157:H7 corresponds to the viable but non-culturable state. Res. Microbiol. 159:709–717.

Barcina I, Arana I. 2009. The viable but nonculturable phenotype: A crossroads in the life-cycle of non-differentiating bacteria? Rev. Environ. Sci. Biotechnol. 8:245-255.

Barry V, Conalty M, Denneny JM, Winder F. 1956. Peroxide formation in bacteriological media. Nature 178(4533):596-7.

744 Basaglia M, Povolo S, Casella S. 2007. Resuscitation of viable
745 but not culturable *Sinorhizobium meliloti* 41 prp4-luc: effects
746 of oxygen and host plant. Curr. Microbiol. 54:167–74.
747 Bhattacharya I, Das HR. 2003. Cell surface characteristics of
748 two halotolerant strains of *Sinorhizobium meliloti*. Microbiol.
749 Res. 158:187–194.
750 Bianco C, Defez R. 2009. *Medicago truncatula* improves salt
751 tolerance when nodulated by an indole-3-acetic acid-
752 overproducing *Sinorhizobium meliloti* strain. J. Exp. Bot.
753 60:3097–3107.
754 Boumahdi M, Mary P, Hornez JP. 1999. Influence of growth phases
755 and desiccation on the degrees of unsaturation of fatty acids
756 and the survival rates of rhizobia. J. Appl. Microbiol. 87:611–
757 61.
758 Boumahdi M, Mary P, Hornez JP. 2001. Changes in fatty acid
759 composition and degree of unsaturation of (Brady) rhizobia as a
760 response to phases of growth, reduced water activities and mild
761 desiccation. Ant. v. Leeuwenhoek. 79:73–79.
762 Breedveld M, Zevenhuizen L, Zehnder A. 1990. Osmotically induced
763 oligo-and polysaccharide synthesis by *Rhizobium meliloti* SU-47.
764 J. Gen. Microbiol. 136:2511–2519.
765 Breedveld MW, Dijkema C, Zevenhuizen LP, Zehnder AJ. 1993.
766 Response of intracellular carbohydrates to a NaCl shock in
767 *Rhizobium leguminosarum* biovar trifolii TA-1 and *Rhizobium*

meliloti SU-47. J. Gen. Microbiol. 139:3157–3163.

Bullard G, Roughley R, Pulsford D. 2005. The legume inoculant industry and inoculant quality control in Australia: 1953–2003. Anim. Prod. Sci. 45:127–140.

Bushby H, Marshall K. 1977A. Some factors affecting the survival of root nodule bacteria on desiccation. Soil Biol. Biochem. 9:143 – 147.

Bushby H, Marshall K. 1977B. Desiccation induced damage to the cell envelope of root nodule bacteria. Soil Biol. Biochem. 9:149 – 152.

Catroux G, Hartmann A, Revellin C. 2001. Trends in rhizobial inoculant production and use. Plant and Soil. 230:21–30.

Chenu C. 1993. Clay- or sand-polysaccharide associations as models for the interface between micro-organisms and soil: Water related properties and microstructure. Geoderma. 56:143 – 156.

Cytryn EJ, Sangurdekar DP, Streeter JG, Franck WL, Chang W, Stacey G, Emerich DW, Joshi T, Xu D, Sadowsky MJ. 2007. Transcriptional and physiological responses of *Bradyrhizobium japonicum* to desiccation-induced stress. J. bac. 189:6751–6762.

Dardanelli M, Medeot D, Paulucci N, Bueno M, Vicario J, Garcìa M, Bensi N, Niebylski A. 2012. Biochemical processes of rhizobacteria and their application in biotechnology. In: Malik A, Grohmann E, editors. Environmental Protection Strategies for Sustainable Development. The Netherlands: Springer. p. 379–396.

792 Deaker R, Roughley RJ, Kennedy IR. 2004. Legume seed inoculation
793 technology–a review. Soil Biol. Biochem. 36:1275 – 1288.
794 Dodd IC, Pérez-Alfocea F. 201. Microbial amelioration of crop
795 salinity stress. J. Exp. Bot. 63:3415–3428.
796 Domìnguez-Ferreras A, Pérez-Arnedo R, Becker A, Olivares J, Soto
797 MJ, Sanjuán J. 2006. Transcriptome profiling reveals the
798 importance of plasmid pSymB for osmoadaptation of *Sinorhizobium*
799 *meliloti*. J. bac. 188:7617–25.
800 Feng L, Roughley RJ, Copeland L. 2002. Morphological changes of
801 rhizobia in peat cultures. Appl. Environ. Microbiol. 68:1064–
802 1070.
803 Fierer N, Schimel JP, Holden PA. 2003. Influence of drying-
804 rewetting frequency on soil bacterial community structure.
805 Microb. Ecol. 45:63–71.
806 Fischer H, Babst M, Kaspar T, Acuna G, Arigoni F, Hennecke H.
807 1993. One member of a GroESL-like chaperonin multigene family in
808 *Bradyrhizobium japonicum* is co-regulated with symbiotic nitrogen
809 fixation genes. EMBO 12:2901-2912.
810 Flechard M, Fontenelle C, Blanco C, Goude R, Ermel G,
811 Trautwetter A. 2010. RpoE2 of *Sinorhizobium meliloti* is
812 necessary for trehalose synthesis and growth in hyperosmotic
813 media. Microbiol. 156:1708–1718.
814 González JE, Semino CE, Wang LX, Castellano-Torres LE, Walker
815 GC. 1998. Biosynthetic control of molecular weight in the

816 polymerization of the octasaccharide subunits of succinoglycan,
817 a symbiotically important exopolysaccharide of *Rhizobium*
818 *meliloti*. PNAS. 95:13477–13482.
819 Gouffi K, Pichereau V, Rolland JP, Thomas D, Bernard T, Blanco
820 C. 1998. Sucrose is a nonaccumulated osmoprotectant in
821 *Sinorhizobium meliloti*. J. bac. 180:5044–5051.
822 Gouffi K, Pica N, Pichereau V, Blanco C. 1999. Disaccharides as
823 a new class of nonaccumulated osmoprotectants for *Sinorhizobium*
824 *meliloti*. Appl. Environ. Microbiol. 65:1491–1500.
825 Gouffi K, Bernard T, Blanco C. 2000. Osmoprotection by pipecolic
826 acid in *Sinorhizobium meliloti*: Specific effects of d and l
827 isomers. Appl. Environ. Microbiol. 66:2358–2364.
828 Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ. 2003.
829 Physiological and community responses of established grassland
830 bacterial populations to water stress. Appl. Environ. Microbiol.
831 69:6961–6968.
832 Grover M, Ali SZ, Sandhya V, Rasul A, Venkateswarlu B. 2011.
833 Role of microorganisms in adaptation of agriculture crops to
834 abiotic stresses. World J. Microbiol. Biotechnol. 27:1231–1240.
835 Hammes F, Berney M, Egli T. 2011. Cultivation-independent
836 assessment of bacterial viability. In: Müller S, Bley T,
837 editors. High Resolution Microbial Single Cell Analytics.
838 Berlin: Springer. p. 123–150.
839 Han Y, Zhou D, Pang X, Zhang L, Song Y, Tong Z, Bao J, Dai E,

840 Wang J, Guo Z, et al. 2005. Comparative transcriptome analysis
841 of *Yersinia pestis* in response to hyperosmotic and high-salinity
842 stress. Res. Microbiol. 156:403–415.
843 Herridge D. 2007. Inoculation technology for legumes. In:
844 Dilworth MJ, James EK, Sprent JI, Newton WE, editors. Nitrogen-
845 fixing leguminous symbioses. The Netherlands: Springer. p. 77-
846 115.
847 Hirsch AM. 2010. How rhizobia survive in the absence of a legume
848 host, a stressful world indeed. In: Seckbach J, Grube M,
849 editors. Symbioses and Stress. The Netherlands: Springer. p.
850 375–391.
851 Humann JL, Ziemkiewicz HT, Yurgel SN, Kahn ML. 2009. Regulatory
852 and DNA repair genes contribute to the desiccation resistance of
853 *Sinorhizobium meliloti* 1021. Appl. Environ. Microbiol. 75:446-
854 53.
855 Ilyas N, Bano A. 2012. Potential use of soil microbial community
856 in agriculture. In: Maheshwar DK, editor. Bacteria in
857 Agrobiology: Plant Probiotics. Berlin: Springer. p. 45–64.
858 Imazaki I, Nakaho K. 2009. Temperature-upshift-mediated revival
859 from the sodium-pyruvate-recoverable viable but nonculturable
860 state induced by low temperature in *Ralstonia solanacearum*:
861 Linear regression analysis. J. Gen. Plant Path. 75: 213–226.
862 Jensen JB, Peters NK, Bhuvaneswari TV. 2002. Redundancy in
863 periplasmic binding protein-dependent transport systems for

trehalose, sucrose, and maltose in *Sinorhizobium meliloti*. J. bac. 184:2978–86.

Jofré E, Becker A. 2009. Production of succinoglycan polymer in *Sinorhizobium meliloti* is affected by Smb21506 and requires the n-terminal domain of ExoP. MPMI. 22:1656–1668.

Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC. 2007. How rhizobial symbionts invade plants: The *Sinorhizobium–Medicago* model. Nat. Rev. Microbiol. 5:619–33.

Kannenberg EL, Perzl M, Härtner T. 1995. The occurrence of hopanoid lipids in *Bradyrhizobium* bacteria. FEMS Microbiol. Lett. 127:255–262.

Kannenberg EL, Poralla K. 1999. Hopanoid biosynthesis and function in bacteria. Naturwissenschaften. 86:168–176.

Kong I, Bates TC, Hülsmann A, Hassan H, Smith BE, Oliver JD. 2004. Role of catalase and OxyR in the viable but nonculturable state of *vibrio vulnificus*. FEMS Microbiol. Ecol. 50:133–142.

Kosanke JW, Osburn RM, Shuppe GI, Smith RS. 1992. Slow rehydration improves the recovery of dried bacterial populations. Can. J. Microbiol. 38:520–525.

Krol E, Becker A. 2011. Ppgpp in *Sinorhizobium meliloti*: Biosynthesis in response to sudden nutritional downshifts and modulation of the transcriptome. Mol. Microbiol. 81:1233–1254.

Leslie SB, Israeli E, Lighthart B, Crowe JH, Crowe LM. 1995. Trehalose and sucrose protect both membranes and proteins in

intact bacteria during drying. Appl. Environ. Microbiol. 61:3592–7.

Lloret J, Bolanos L, Lucas MM, Peart JM, Brewin NJ, Bonilla I, Rivilla R. 1995. Ionic stress and osmotic pressure induce different alterations in the lipopolysaccharide of a *Rhizobium meliloti* strain. Appl. Environ. Microbiol. 61:3701–3704.

Lloret J, Wulff BB, Rubio JM, Downie JA, Bonilla I, Rivilla R. 1998. Exopolysaccharide II production is regulated by salt in the halotolerant strain *Rhizobium meliloti* EFB1. Appl. Environ. Microbiol. 64:1024–1028.

Manahan SH, Steck TR. 1997. The viable but nonculturable state in *Agrobacterium tumefaciens* and *Rhizobium meliloti*. FEMS Microbiol. Ecol. 22:29–37.

Maraha N. 2007. Physiological status of bacteria used for environmental applications. PhD thesis. Karolinska Institutet. Stockholm.

Mary P, Ochin D, Tailliez R. 1986. Growth status of rhizobia in relation to their tolerance to low water activities and desiccation stresses. Soil Biol. Biochem. 18:179–184.

Mattimore V, Battista JR. 1996. Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. J. bac. 178:633–637.

McIntyre HJ, Davies H, Hore TA, Miller SH, Dufour JP, Ronson CW.

912 2007. Trehalose biosynthesis in *Rhizobium leguminosarum* bv.
913 trifolii and its role in desiccation tolerance. Appl. Environ.
914 Microbiol. 73:3984–3992.
915 Miller-Williams M, Loewen PC, Oresnik IJ. 2006. Isolation of
916 salt-sensitive mutants of *Sinorhizobium meliloti* strain Rm1021.
917 Microbiol. 152:2049–2059.
918 Minder A, Narberhaus F, Babst M, Hennecke H, Fischer HM. 1997.
919 The DnaKJ operon belongs to the sigma32-dependent class of heat
920 shock genes in *Bradyrhizobium japonicum*. Mol. Gen. Genet. MGG.
921 254:195–206.
922 Mizunoe Y, Wai SN, Takade A, Yoshida S. 1999. Restoration of
923 culturability of starvation-stressed and low-temperature-
924 stressed *Escherichia coli* O157 cells by using H_2O_2-degrading
925 compounds. Arch. Microbiol. 172:63–67.
926 Morton C, Campbell I. 1994. SH3 domains: Molecular 'Velcro'.
927 Curr. Biol. 4:615–617.
928 Muela A, Seco C, Camafeita E, Arana I, Orruno M, Lopez JA,
929 Barcina I. 2008. Changes in *Escherichia coli* outer membrane
930 subproteome under environmental conditions inducing the viable
931 but non culturable state. FEMS Microbiol. Ecol. 64:28-36.
932 Münchbach M, Nocker A, Narberhaus F. 1999. Multiple small heat
933 shock proteins in rhizobia. J. bac. 181:83–90.
934 Nandal K, Sehrawat AR, Yadav AS, Vashishat R, Boora K. 2005.
935 High temperature-induced changes in exopolysaccharides,

936 lipopolysaccharides and protein profile of heat-resistant
937 mutants of *Rhizobium* sp. cajanus. Microbiol. Res. 160:367–373.
938 Narberhaus F, Waldminghaus T, Chowdhury S. 2006. RNA
939 thermometers. FEMS Microbiol. Rev. 30:3–16.
940 Nocker A, Krstulovic NP, Perret X, Narberhaus F. 2001. Rose
941 elements occur in disparate rhizobia and are functionally
942 interchangeable between species. Arch. Microbiol. 176:44–51.
943 Oliver J. 2005. The viable but nonculturable state in bacteria.
944 J. Microbiol. 43:93–100.
945 Ono Y, Mitsui H, Sato T, Minamisawa K. 2001. Two RpoH homologs
946 responsible for the expression of heat shock protein genes in
947 *Sinorhizobium meliloti*. Mol. Gen. Genet. 264:902–912.
948 Osa-Afiana L, Alexander M. 1982. Differences among cowpea
949 rhizobia in tolerance to high temperature and desiccation in
950 soil. Appl. Environ. Microbiol. 43:435–439.
951 Pederson J, Jacobsen C. 1993. Fate of *Enterobacter cloacae* JP210
952 and AEO106 (pro101) in soil during waterstress: Effect on
953 culturability and viability. Appl. Environ. Microbiol. 59:1560–
954 1564.
955 Pena-Cabriales J, Alexander M. 1979. Survival of *Rhizobium* in
956 soils undergoing drying. Soil Sci. Soc. Am. J. 43:962–966.
957 Potts M. 1994. Desiccation tolerance of prokaryotes. Microbiol.
958 Rev. 58:755–805.
959 Räsänen LA, Elväng AM, Jansson J, Lindström K. 2001. Effect of

960 heat stress on cell activity and cell morphology of the tropical
961 *Rhizobium*, *Sinorhizobium arboris*. FEMS Microbiol. Ecol. 34:267-
962 278.
963 Rehman A, Nautiyal CS. 2002. Effect of drought on the growth and
964 survival of the stress-tolerant bacterium *Rhizobium* sp. NBRI2505
965 sesbania and its drought-sensitive transposon Tn*5* mutant. Curr.
966 Microbiol. 45:368–377.
967 Reina-Bueno M, Argandoña M, Nieto JJ, Hidalgo-Garcìa A,
968 Iglesias-Guerra F, Delgado MJ, Vargas C. 2012. Role of trehalose
969 in heat and desiccation tolerance in the soil bacterium
970 *Rhizobium etli*. BMC Microbiol. 12:207.
971 Rinaudo M. 2004. Role of substituents on the properties of some
972 polysaccharides. Biomacromol. 5:1155–1165.
973 Rodrigues CS, Laranjo M, Oliveira S. 2006. Effect of heat and pH
974 stress in the growth of chickpea mesorhizobia. Curr. Microbiol.
975 53:1–7.
976 Rodrìguez-Quiñones F, Maguire M, Wallington E, Gould PS, Yerko
977 V, Downie J, Lund P. 2005. Two of the three GroEL homologues in
978 *Rhizobium leguminosarum* are dispensable for normal growth. Arch.
979 Microbiol. 183:253–265.
980 Rüberg S, Tian ZX, Krol E, Linke B, Meyer F, Wang Y, Pühler A,
981 Weidner S, Becker A. 2003. Construction and validation of a
982 *Sinorhizobium meliloti* whole genome DNA microarray: Genome-wide
983 profiling of osmoadaptive gene expression, J. Biotechnol.

106:255–268.

Sagot B, Gaysinski M, Mehiri M, Guigonis JM, LeRudulier D, Alloing G. 2010. Osmotically induced synthesis of the dipeptide N-acetylglutaminylglutamine amide is mediated by a new pathway conserved among bacteria. PNAS. 107(28):12652-12657.

Salema M, Parker C, Kidby D, Chatel D, Armitage T. 1982. Rupture of nodule bacteria on drying and rehydration. Soil Biol. Biochem. 14:15–22.

Sauviac L, Philippe H, Phok K, Bruand C. 2007. An extracytoplasmic function sigma factor acts as a general stress response regulator in *Sinorhizobium meliloti*. J. Bac. 189:4204–16.

Shapiro RS, Cowen LE. 2012. Thermal control of microbial development and virulence: Molecular mechanisms of microbial temperature sensing. mBio. 3(5).

Smith R. 1992. Legume inoculant formulation and application. Can. J. Microbiol. 38:485–492.

Steinmann R, Dersch P. 2013. Thermosensing to adjust bacterial virulence in a fluctuating environment. Fut. Microbiol. 8:85–105.

Streeter J. 2003. Effect of trehalose on survival of *Bradyrhizobium japonicum* during desiccation. J. Appl. Microbiol. 95:484–491.

Sugawara M, Cytryn EJ, Sadowsky MJ. 2010. Functional role of

Bradyrhizobium japonicum trehalose biosynthesis and metabolism genes during physiological stress and nodulation, Appl. Environ. Microbiol. 76:1071–1081.

Toffanin A, Basaglia M, Ciardi C, Vian P, Povolo S, Casella S. 2000. Energy content decrease and viable-not-culturable status induced by oxygen limitation coupled to the presence of nitrogen oxides in *Rhizobium hedysari*. Biol. Fert. Soils. 31:484–488.

Trotman AP, Weaver RW. 1995. Tolerance of clover rhizobia to heat and desiccation stresses in soil. Soil Sc. Soc. Am. J. 59:466–470.

Vanderlinde EM, Harrison JJ, Muszyński A, Carlson RW, Turner RJ, Yost CK. 2010. Identification of a novel ABC transporter required for desiccation tolerance and biofilm formation in *Rhizobium leguminosarum* bv. viciae 3841. FEMS Microbial. Ecol. 71:327–340.

Vanderlinde EM, Magnus SA, Tambalo DD, Koval SF, Yost CK. 2011. Mutation of a broadly conserved operon (*rl3499-rl3502*) from *Rhizobium leguminosarum* biovar viciae causes defects in cell morphology and envelope integrity. J. bac. 193:2684–2694.

Vanderlinde EM, Yost CK. 2012 Genetic analysis reveals links between lipid a structure and expression of the outer membrane protein gene, RopB, in *Rhizobium leguminosarum*. FEMS Microbiol. Lett. 335:130–139.

Verón SR, Paruelo JM, Oesterheld M. 2006. Assessing

1032 desertification. J. Arid Environ. 66:751-763.
1033 Vriezen JAC. 2005. Responses of *Sinorhizobium meliloti* 1021 to
1034 water stress, PhD thesis. University of Massachusetts. Amherst.
1035 Vriezen JAC, de Bruijn FJ, Nüsslein KR. 2006. Desiccation
1036 responses and survival of *Sinorhizobium meliloti* USDA 1021 in
1037 relation to growth phase, temperature, chloride and sulfate
1038 availability. Lett. Appl. Microbiol. 42:172–178.
1039 Vriezen JAC, de Bruijn FJ, Nüsslein KR. 2007. Responses of
1040 rhizobia to desiccation in relation to osmotic stress, oxygen,
1041 and temperature. Appl. Environ. Microbiol. 73:3451–3459.
1042 Vriezen JAC, de Bruijn FJ, Nüsslein KR. 2012. Desiccation
1043 induces viable but non-culturable cells in *Sinorhizobium*
1044 *meliloti* 1021. AMB Express. 2:1–9.
1045 Vriezen JAC, de Bruijn FJ, Nusslein KR. 2013. Identification and
1046 characterization of a genetic locus involved in survival during
1047 desiccation of *Sinorhizobium meliloti*. Submitted to Appl.
1048 Environ. Microbiol.
1049 Waldminghaus T, Fippinger A, Alfsmann J, Narberhaus F. 2005. RNA
1050 thermometers are common in alpha and gamma proteobacteria. Biol.
1051 Chem. 386:1279–1286.
1052 Wei W, Jiang J, Li X, Wang L, Yang SS. 2004. Isolation of salt-
1053 sensitive mutants from *Sinorhizobium meliloti* and
1054 characterization of genes involved in salt tolerance. Lett.
1055 Appl. Microbiol. 39:278–283.

1056 Wells DH, Long SR. 2002. The *Sinorhizobium meliloti* stringent
1057 response affects multiple aspects of symbiosis. Mol. Microbiol.
1058 43:1115–1127.
1059 Welsh DT, Herbert RA. 1999. Osmotically induced intracellular
1060 trehalose, but not glycine betaine accumulation promotes
1061 desiccation tolerance in *Escherichia coli*. FEMS Microbiol. Lett.
1062 174:57–63.
1063 Welsh DT. 2000. Ecological significance of compatible solute
1064 accumulation by micro-organisms: From single cells to global
1065 climate. FEMS Microbiol. Rev. 24:263-290
1066 Willis LB, Walker GC. 1999. A novel *Sinorhizobium meliloti*
1067 operon encodes an alpha-glucosidase and a periplasmic-binding-
1068 protein-dependent transport system for alpha-glucosides. J. bac.
1069 181:4176–4184.
1070 York GM, Walker GC. 1998. The *Rhizobium meliloti* ExoK and ExsH
1071 glycanases specifically depolymerize nascent succinoglycan
1072 chains. PNAS. 95:4912–4917.
1073 Zahran HH. 1999. *Rhizobium*-legume symbiosis and nitrogen
1074 fixation under severe conditions and in an arid climate.
1075 Microbiol. Mol. Biol. Rev. 63:968–89.
1076 Zahran HH. 2001. Rhizobia from wild legumes: diversity,
1077 taxonomy, ecology, nitrogen fixation and biotechnology. J.
1078 Biotechnol. 91:143–153.
1079 Zahran HH. 2010. Legumes–microbes interactions under stressed

environments. In: Khan MS, Musarrat J, Zaidi A, editors. Microbes for Legume Improvement. Vienna: Springer. p. 353–387.

Figure legends

Figure 1

Model representing two hypothetical pathways for responses of rhizobia to desiccation stress and desiccation-induced damages. The "preceding-storage induction" pathway (A) implies a response to water, osmotic, or salt stress, and the "post-storage induction" pathway (B) implies a response to the desiccation-induced damages upon rewetting. Reprinted with permission from Vriezen et al. [2007].

Figure 2

Survival and recovery of *S. meliloti* cells after drying and rewetting. Quantitative recovery of cells (direct count) after three days of storage under 100% or 22% RH on nitrocellulose filters (white bar = cristal violet, light grey bar = Live/dead, dark grey bar = %red, black bar = %green). All error bars represent the SEM. Reprinted with permission from Vriezen et al. [2012].

Figure 3

Membrane properties: (A) Phase transition from liquid crystalline to gel phase and the prevention by trehalose [amended from Welsh, 2000]. (B) Vibration frequencies of the

phosphate head-groups and the effect of trehalose on the Midpoint Temperature (Tm) [amended from Leslie et al., 1995].

Figure 4

Survival after desiccation of *S. meliloti* 1021 (WT, grey bar) and the *smc01590*::Tn*5lux*AB mutant (Sce-1, white bar) and the fold difference in survival of Sce-1 relative to WT (open circle) at 4, 20 and 37°C.

Table 1

Table 1: Candidate loci in *S. meliloti* 1021 potentially involved in the VBNC state.

Information query sequences[a]								Information results from blast agains *S. meliloti* 1021 database[d]		
Gene Name	Organism[b]	Reference	Query NCBI acc#[c]	Score[e]	E-value	% Query	% Identity	Locus	Gene Name	Description
ddpA	Pf	Maraha	YP_002870490	1349	$1.7e^{-176}$	97	50	Smc00786	*dppA1*	Oligopeptide ABC transporter
hisJ	Pf/Ec	Maraha/Asakura	AP_002909	472	$7.7e^{-45}$	99	42	Smc00140		Putative amino acid binding protein
livK	Pf	Maraha	YP_002870995	231	$4.6e^{-15}$	96	24	Smc02355		Putative branched chain amino acid binding ABC transporter
proX	Pf/Ec	Maraha/Asakura	AP_003252	209	$1.5e^{-10}$	96	26	Smc00672	*hisX/hutX*	Histidine ABC transport
ompW	Pf/Ec	Maraha/Asakura/ Muela	AP_001882	NI						
pstI	Ec	Asakura	P08839	189	$6.2e^{-14}$	36	31	Smc00025	*ppdK*	Putative pyruvate phosphate dikinase
serA	Ec	Muela	NP_417388	319	$1.9e^{-28}$	60	31	Smc02849	*gyaR*	Probable glyoxylate reductase
thrC	Ec	Muela	NP_414545	541	$2.1e^{-49}$	88	33	Smc00077	*thrC1*	Probable threonine synthase
leuD	Ec	Asakura	P30126	517	$9.9e^{-48}$	95	52	Smc03795	*leuD*	Probable 3-isopropylmalate dehydrolase
dps	Ec	Asakura	CAA49169	NI						
oppA	Ec	Asakura	P23843	912	$4.4e^{-90}$	97	38	Smb21192	*oppA*	ABC transporter tri/tetra peptides
dnaK	Ec	Asakura	P23869	194	$1.5e^{-15}$	84	39	Smc01700	*ppiA*	Putative peptidyl-prolyl cis-trans isomerase
znuA	Ec	Asakura	P39172	388	$2.6e^{-51}$	61	40	Smc04245	*znvA*	Probable Zinc uptake ABC transporter
tpiA	Ec	Asakura	P04790 (obsolete number)	472	$2.5e^{-43}$	97	42	Smc01023		Triose phosphatase isomerase
tig	Ec	Asakura	P22257 (obsolete number)	596	$4.7e^{-55}$	99	33	Smc02050	*tig*	Probable trigger factor
EF-Tu	Ec	Muela	BAE77952	1559	$7.2e^{-157}$	100	75	Smc01312	*tufA*	Probable EF-Tu

(a) Query sequence obtained from several studies in related microorganisms [Maraha, 2007; Muela et al. 2008; Asakura et al. 2008].

(b) Pf=*Pseudomonas fluorescens* SBW25, EC=*Escherichia coli* K12

(c) Where available, the *E. coli* sequence was used and obtained from NCBI.

(d) Blasted against the *S. meliloti* database http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi using tblastnt

(e) NI = Not Identified

Figure 1

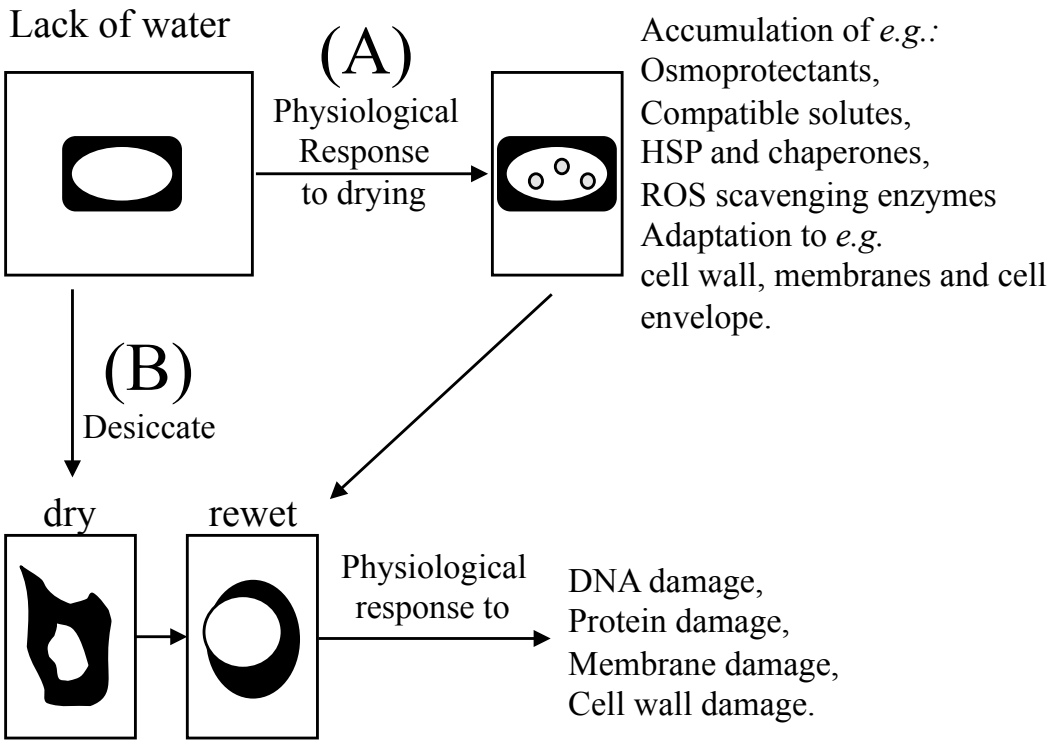

Lack of water
(A)
Physiological
Response
to drying
Accumulation of *e.g.:*
Osmoprotectants,
Compatible solutes,
HSP and chaperones,
ROS scavenging enzymes
Adaptation to *e.g.*
cell wall, membranes and cell
envelope.
(B)
Desiccate
dry
rewet
Physiological
response to
DNA damage,
Protein damage,
Membrane damage,
Cell wall damage.

Figure 2

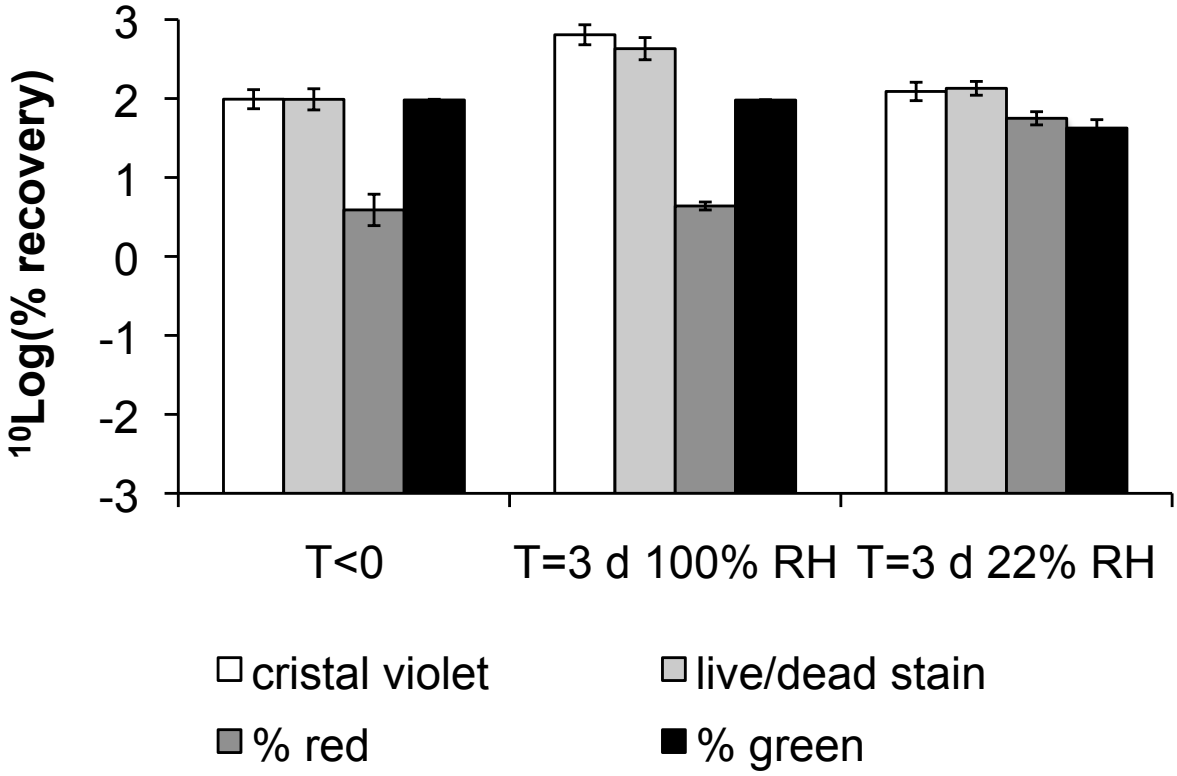
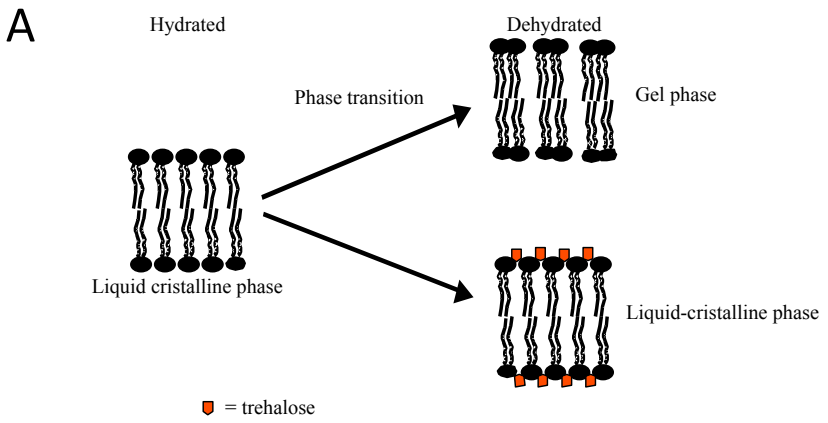

3
2
1
0
-1
-2
-3
10Log(% recovery)
T<0
T=3 d 100% RH
T=3 d 22% RH
cristal violet
live/dead stain
% red
% green

Figure 3


A
Hydrated
Dehydrated
Phase transition
Gel phase
Liquid cristalline phase
Liquid-cristalline phase
= trehalose

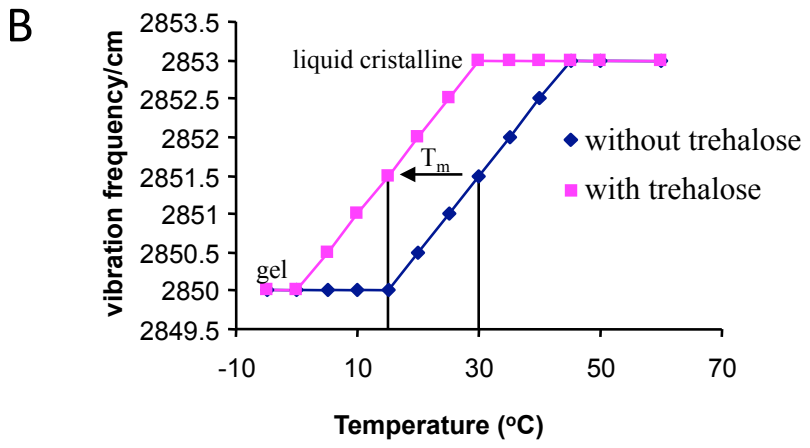
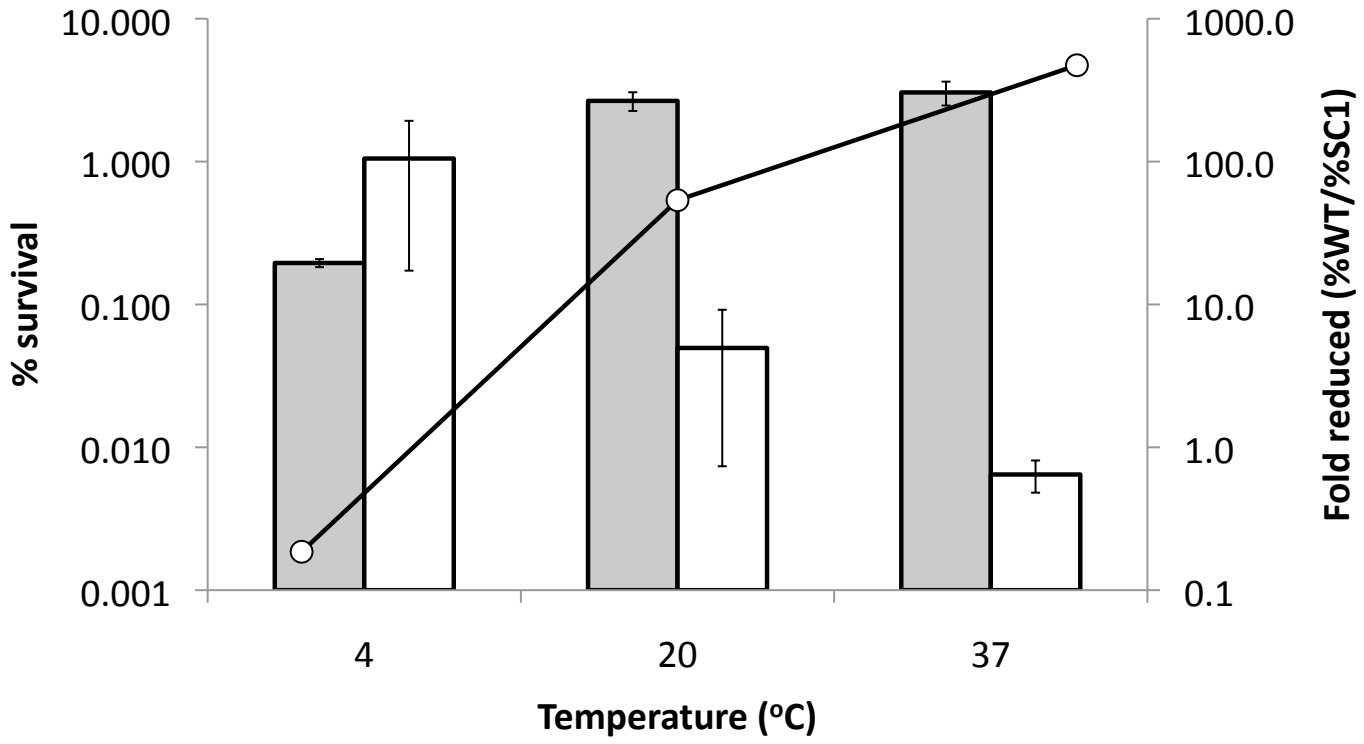

B
vibration frequency/cm
2853.5
2853
2852.5
2852
2851.5
2851
2850.5
2850
2849.5
liquid cristalline
T_m
gel
without trehalose
with trehalose
-10
10
30
50
70
Temperature (°C)

Figure 4


10.000
1.000
0.100
0.010
0.001
% survival
1000.0
100.0
10.0
1.0
0.1
Fold reduced (%WT/%SC1)
4
20
37
Temperature (°C)