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Consequences of Elevated Salt Concentrations Expression Profiles in the Rhizobium *S. meliloti* 1021 Likely Involved in Heat and Desiccation Stress

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Chapter 12.1. Consequences of elevated salt concentrations on expression profiles in the rhizobium *S. meliloti* 1021 likely involved in heat and desiccation stress

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12.1.1. Introduction

Microorganisms have developed multiple direct and indirect mechanisms that protect them against the environmental stresses they encounter. One of the most severe and widespread problems facing crop production is the degradation of soil quality due to desiccation and salinity, and almost 40% of the world's land surface is affected by salinity-related problems (Zahran 1999, Veron, 2006). Water, and its availability, is one of the most vital environmental factors to affect the growth and survival of microorganisms (Potts 1994).

Within the soil environment, indirect protection of cellular structures and contents can be provided by cell surface coating with clay minerals or close association with organic substances. Some bacteria (*Bacillus* spp. and Actinobacteria) form heat-resistant spores to withstand dry conditions and high temperatures, while other microorganisms encase individual cells or aggregates of cells with polymeric substances or slime layers to form an extensive exopolymeric matrix or biofilms. These outer structures enable them to adhere to inorganic (*e.g.* soil pore walls, water conduits, mineral surfaces) or organic (e.g. roots) surfaces to insulate the entire microbial community against effects of high temperature and the associated lack of available water (Jozefaciuk et al., 2006; Berendsen et al., 2012; Rolli et al., 2015). Despite the potential physiological

and lifestyle adaptations to desiccation available to soil microorganisms, many microbes yield to heat stress with damages caused by desiccation. When desiccation advances too rapidly, cellular contents including proteins and nucleic acids are irreversibly damaged which will inhibit microbial growth (Raivio 2007). With increasing desiccation, membranes and cell walls are ruptured, effectively killing the cells (Vriezen et al., 2007; de Goffau et al., 2011, Bushby and Marshall 1977, Salema et al., 1982).

Microbes respond differently to heat induced desiccation stress, and the overall microbial diversity in soil changes leading to an imbalanced system due to loss of the functions provided by stress sensitive microbes (Fierer et al., 2003; Griffith et al., 2003). Soil moisture deficit not only affects microbial diversity, it also changes the availability of substances for microbial metabolism (Dominguez-Ferreras et al., 2006; Kremer 2012); similarly, salinity has a direct effect on the composition of microbial communities (Rath and Rousk, 2015).

Rhizobia are rod-shaped, Gram-negative bacteria with the ability to produce nodules on the roots of leguminous plants (Oldroyd et al., 2011). They belong to the family *Rhizobiaceae*, which are part of the α-proteobacteria. Soil environmental conditions are critical factors to the activity, persistence, and survival of rhizobia in the soil. The changes in the

rhizospheric environment can affect both growth and saprophytic competence, which will influence competitiveness and persistence (Dowling and Broughton 1986), and new attempts to remediate this with soil additives are actively pursued (Jiang et al., 2015).

Desiccation is one of the most common stresses soil microorganisms have to face and it produces many stress responses in the microbial cell. The majority of microbes cannot multiply below a water availability of 0.900 (Manzoni et al., 2012; Stevenson et al., 2015), and the responses of bacterial cells to desiccation can be: shrinkage of the bacterial cytoplasm and capsular layers, increase in intracellular salt levels, crowding of macromolecules, damage to external layers (pili, membranes), changes in ribosome structure, and decrease in growth (Vriezen et al., 2007). Reactive oxygen species can also damage proteins and DNA, leading to accumulation of mutations (Potts 1994). Death of rhizobial cells during desiccation was suggested to associate with changes in cell walls and membrane integrity (Bushby and Marshall 1977). It has been hypothesized that during dehydration, the removal of water hydrogen bonds to the phospholipid head groups of the membrane decreases the spacing between adjacent lipids. The membrane is then converted from the liquid crystalline into the gel phase already at room temperature. Subsequent rehydration results in a further phase transition of the membrane back to the liquid

crystalline phase. As a consequence, the membrane barrier is disrupted, leading to leakage of membranes (Potts, 1994, Welsh, 2000, Leslie et al., 1995).

Desiccation tolerance is the ability of cells to undergo nearly absolute dehydration through air-drying, without being irreversibly damaged or killed. This is the most severe water deficit stress since the removal of the cell-bound water imposes such structural, physiological, and biochemical stresses to which cells must adapt or die (Billi and Potts 2002; Reina-Bueno et al., 2012). Of particular importance to crop production is the impact of these harsh environmental conditions on the persistence and survival of rhizobia, and thus understanding rhizobia expression responses to common environmental stressors is paramount (López-Leal et al., 2014), especially in view of a fast changing climate with immediate consequences as seen in agriculture, *e.g.* the current drought in California.

Water availability determines both the vitality and functionality of living systems, and desiccation is among the most significant environmental stresses encountered by terrestrial bacteria. Still, the genetic mechanisms underlying sensing and providing resistance to this stress are still poorly understood (Cytryn et al., 2007). The objective of this review was to obtain a comprehensive understanding of the genetic mechanisms involved in desiccation tolerance by members of the

Rhizobiaceae, and to get closer to a model that can delineate the consequences of elevated salt concentrations on rhizobial expression profiles.

12.1.2. Responses to NaCl elevating desiccation tolerance

The ability of microorganisms to survive or even proliferate in a stressful, nutrient poor environment such as the soil remains poorly understood. Most likely, and as we tried to argue in this review, understanding the desiccation responses in conjunction with NaCl and heat stress, may be crucial to understanding soil microbial ecology and diversity. In particular, quick changes in water availability may be one of the stresses microorganisms are exposed to with a large impact on their ability to survive in soil and thus for the microbial community. Although not a panacea for desiccation related problems, at least some organisms have evolved the ability to respond to osmotic stress induced by the presence of salt or low water-activity (A_w) in order to survive desiccation (Vriezen et al., 2007). One example is the study by Chen and Alexander (1979) in which acclimation in media with low A_w led to a better ability to survive subsequent desiccation. Furthermore, Mary et al., (1986) showed that desiccation survival of *Rhizobium* strain RCR2011 was increased in the presence of LiCl and NaCl. This increase is strain specific. Vriezen et al. (2006) also showed

that model organism *S. meliloti* 1021 increases desiccation survival in PMM medium, a defined minimal medium with phosphate-based buffer, in the presence of 400 mM NaCl. This increase is physiological in origin rather than a protective mechanism of the solutes present. In 2013, Vriezen et al. presented the study of a locus involved in survival after desiccation in the presence of 400 mM NaCl, providing support for NaCl response mechanisms also involved in survival during this stress. This locus (*ngg*) is also found in Pseudomonads (Sagot et al., 2010) with a conserved function on NaCl. RpoE2 (*smc01506)* is a sigma factor with extracytoplasmic function involved in the general stress response of *Sinorhizobium meliloti*. It is also up regulated in the response to NaCl, and *rpoE2::hph* reduces the ability to survive desiccation (Sauviac and Bruand, 2014).

12.1.3. The search for loci conserved in the response to NaCl in *S. meliloti* and *R. etli*

Although the heterogeneity of soil and the many different bacterial species present predicts an uncountable number of strategies to respond to the stresses imposed, related organisms may have stress responses in common. We hypothesized that the beneficial response to NaCl, i.e. an increase in the ability to survive desiccation, is conserved in a range of soil borne microorganisms. To find conserved loci in two kinds of rhizobia,

two lists with NaCl inducible loci were accumulated: (1) a list with *Sinorhizobium meliloti* 1021 (taxonomy ID 266834) genes up regulated in a response to NaCl from three different studies (Ruberg et al., 2003, Dominguez-Ferreras et al., 2006, and Vriezen et al., 2013) and, (2) a list with NaCl responsive loci from *Rhizobium etli* (Lopez-Leal et al., 2014). The compilation of the NaCl inducible list in *S. meliloti* contains a total of 579 loci. Only one locus (*smb20481, asnO*) was identified in all three studies even though the operon in which *asn*O resides was identified in all three studies. All in all, the three studies have some loci in common and appear complementary.

The list with *R. etli* NaCl responsive loci was compiled by using the aminoacid sequence of every translated Open Reading Frame (ORF) of NaCl inducible loci within the *R. etli* genome retrieved from NCBI (*R. etli* CFN42, Taxonomy ID 347834) as query in the *S. meliloti* 1021 database search at the INRA in Toulouse (Institut National de la Recherche Agronomique). The original list with NaCl up regulated loci in *R. etli* contains the 102 NaCl-only inducible loci and 106 (51% of total) NaCl- and Heat inducible loci (Lopez-Leal et al., 2014). Searching for conserved loci in the *S. meliloti* 1021 genome yielded 76 and 84 (53% out of total of 160 NaCl up regulated loci) sequences with similarity to the *R. etli* CFN42 query sequence, respectively.

The list with 519 *S. meliloti* and 160 *R. etli* loci were

compared and 38 NaCl responsive loci in *R. etli* (38/160=23.8%, or 38/5963=0.59%; Gonzales et al., 2006) are also NaCl responsive in *S. meliloti* and represented 35 different loci in this organism (35/519=6.7%, or 35/6218=0.56%; Galibert et al., 2001, Barnet et al., 2001, Finan et al., 2001, Capela et al., 2001) (Table 12.1.1.).

Table 12.1.1. here…….

Sequence similarity to Smb21031, Smb20587, and Smc02365 was found twice. Striking is the similarity of the number of selected loci relative to the total number of protein coding genes in both organisms. We did not find a significant bias of the number of NaCl responsive loci located on the symbiotic plasmid pSymB as reported by Dominguez-Ferreras et al., (2006).

Two additional studies identified loci important for growth at increased NaCl concentrations (Wei et al., (2004) and Miller-Williams et al., (2006), but none of these loci were significantly up regulated in response to NaCl stress in genomics-studies for *S. meliloti* and *R.etli*.

12.1.4. Conserved responses to NaCl in *S. meliloti* and *R. etli*

The list of conserved NaCl responsive loci in *S. meliloti* and *R. etli* contains loci that are NaCl inducible and are

potentially important for NaCl mediated desiccation survival in soil. In this list, two loci, *sma0934* and *smb21445*, were found targeting DNA. The first locus is a conjugal transfer protein found on rhizobial plasmids (Stiens et al., 2007). The second locus encodes for a DNA topoisomerase and is found just downstream of glgX2, a locus most likely involved in glycogen de-branching. DNA targeting enzymes are well known for their involvement in desiccation survival (Humann et al., 2009), and finding conserved, NaCl-inducible DNA targeting proteins is thus not surprising. Locus *smb21445* is under control of NtrR, a negative regulator of nodulation and nitrogen fixation genes, and under microoxic conditions (Puskas et al., 2004). A high amount of NaCl reduces the solubility of oxygen, and Vriezen et al., (2013) found several NaCl responsive loci also responsive to low oxygen. A candidate sRNA coding gene (B42) between Smb21444 and Smb21445 was identified by de Val et al., 2005. Locus S*mb21446* (glgX2), involved in glycogen metabolism may aid in restoring cell-volume after osmotic shock in *Yersinia pestis* as hypothesized by Han et al., (2005). This locus is also up regulated as a response to a shift to acidic pH (Hellweg et al., 2009). Schlüter et al., (2013) identified three asRNAs (smb_asRNA2938-40) in the smb21446 ORF indicating expression of this locus is under complex control by sRNAs. In addition, expression of one of these RNAs (smb_asRNA2938) may be

controlled by RpoD, RpoH1, and RpoH2. Interestingly, Dominguez-Ferreras (2009) proposed to rename Smb20447, a locus just upstream of Smb20446, to TreZ for its involvement in trehalose metabolism. When all five loci, smb21444 - smb21448, are drawn out with their respective stress responses, a short regulatory mechanism can be proposed (Figure 12.1.1.).

Figure 12.1.1. here…….

Increased NaCl concentrations, microoxic and acidic conditions positively regulate transcription from a NtrR regulated promoter, bringing to expression an amylase that may be involved in restoring cell volume and a DNA topoisomerase. Assuming the intragenic region between smb21446 and smb21447 contains a bidirectional promoter under the same control mechanisms (Schlüter et al. 2013), TreZ would also be transcribed in cohort with a putative DNA polymerase. The accumulation of trehalose and the simultaneous stimulation of DNA repair or replication functions would be an expected response to NaCl mediated desiccation.

It appears that many loci involved in nutrient uptake are up regulated. These loci are *smb20227*, a nutrient deprivation induced locus (NdiA1), and *smb20574*, a glucosyl mutase (TreY, Flechard et al., 2010). Smb20229 (NdiB, strain C22 in Milcamps

et al., 1998) was first found in a search for loci induced by a lack of nutrients. Further characterizations and regulation studies indicate that this gene is carbon- and nitrogen-limitation induced, NaCl induced, with increased expression during stationary phase, and under TspO control (Davey et al., 2000). *Tsp*O is up regulated during desiccation in *B. japonicum* (Cytryn et al., 2007). Smb20227 is an ORF immediately upstream of *ndi*B, and is most likely regulated in the same way. At least, *ndi*A1 responses in the same manner to NaCl (Vriezen et al., 2013, Sauviac et al., 2007), carbon-, and nitrogen starvation (Djordjevic et al., 2003, Sauviac et al., 2007), and is expressed during the stationary phase (Sauviac et al., 2007). Smb20227 is also responsive to a decrease in pH (Hellweg et al., 2009), is under RelA control (Kroll and Becker 2011) and mutants have an attenuated symbiotic phenotype. *Rel*A mutants are sensitive to desiccation (Humann et al., 2009) and it has been hypothesized that an incomplete stringent response is responsible for the lack of a restart of metabolism, which leads to the appearance of Viable But Non Culturable cells after desiccation (Vriezen et al., 2012, Vriezen et al., 2015).

The locus *smb20574* (TreY) is involved in the biosynthetic accumulation of the compatible solute trehalose under hyperosmolar conditions. *Smb20574* is under RpoE2 control and stationary growth-phase induction is abolished in the mutants

(Flechard et al, 2010). Even though McIntyre et al., (2007) reported very relevant phenotypes for *tre*Y mutants in *R. leguminosarum* bv trifolii, Dominguez-Ferreras et al., (2009) found only very minor phenotypes in *S. meliloti* 1021.

The locus *smb21587*, a putative ABC transporter for pentoses is the first gene in an operon for pentose ABC uptake, possibly under control of a *Gnt*R type regulator. Smb21587 is induced by fucose, arabinose, and talose (Mauchline et al., 2006), which is surprising considering that Smb21587 is a putative pentose ABC transporter. Most interestingly, just upstream of smb21587 is the gene for a putative glutathione synthetase (*gsh*B2) located and transcribed in the same direction. Glutathione is involved in the stress response to radical oxygen species and therefore may have a role in survival during desiccation in plants and bacteria (Pukackwa et al., 2006, Fredrickson et al., 2008).

Smc01642 encodes for the uptake of proline-betain under low or high osmolarity. Proline, proline-betain, betain, and stachydrine are taken up by osmotically stressed cells (Alloing et al., 2006, Phillips et al., 1998). Yurgel et al., (2013) studied the N-response of *S. meliloti*. *Smc01642* and *his*X (*smc00672*) are expressed when NtrC is phosphorylated when nitrogen is limited. Expression of these loci is also up regulated as a consequence of the general stress response. Vriezen et al., (2015) hypothesized that HisX may have a

function in the formation of Viable But Non Culturable cells upon desiccation. Expression of Smc01642 is also dependent on AbcR1 and AbcR2, sRNA's that directly target Ribosome Binding Sites (RBS) with anti-Shine-Dalgarno motifs on the RNAs (Torres-Quesada et al., 2014). A change in temperature has an instant effect on translation, and regulatory RNA's may function as thermometers (Shapiro and Cowen, 2012). However, this locus has not been found in attempts to find heat up regulated loci in either *R. etli* nor in *S. meliloti*. Therefore, if these loci are involved in the heat response, regulation may occur on the translational rather then transcriptional level.

Smc01827 is a putative uracil or uridine ABC transporter. Uridine is important in transcription, which may have a role in the startup of metabolism after cells have adapted to the new condition. Uracil and uridine induce this locus (Mauchline et al., 2006), and Smc01827 transcription is under strong negative regulation by Hfq (Sobrero et al., 2012, Torres-Quesada et al., 2010).

Other ABC transporters for sugars are *smc02774 and smc02471*. *Smc02471* encodes for a putative periplasmic ABC type transporter of sugars. It has an upstream ROK type negative regulator, and a gene encoding for a structural lipoprotein downstream. *Smc01774* is in an operon for the uptake of fucose and pyruvate, and is located between the two transposable

elements Trm19 (*smc02779*) and Trm20 (*smc02762*). All genes in this stretch of the *S. meliloti* genome are in the same orientation with the exception of *smc02777*, a HTH type transcriptional regulator targeting DNA. Smc02774 is induced by fucose and pyruvate (Mauchline, 2006) and is used as a biosensor for these compounds (Bourdes et al., 2012). Smc02774 is ~four fold up regulated by exogeneous cAMP (Tian et al., 2006) leading to the proposal that expression of *smc02774* is σ^{54} (NtrA) dependent as well as dependent on the transcriptional modulator NtrR under microoxic conditions (Puskas et al., 2004). The sigma factor σ^{54} is higher expressed in desiccated *Bradyrhizobium japonicum* strains (Cytryn et al., 2007)

In the paragraphs above, regulatory mechanisms for expression of these genes were briefly mentioned. The Ntr, Hfq, RelA, and TspO mediated NaCl responses conserved between *S. meliloti* and *R. etli* underly expression of these loci. Humann et al. (2009) identified more regulators involved in survival during desiccation. The regulators found are RelA (Smb02659), RpoE2 (Smc01506), and Hrp (Smc02754). Smc01506 controls expression of smc01505 (RsiA1), an anti-sigma factor for σ^{24}, a response regulator of the response to increased extracellular stress such as NaCl (Sauviac et al., 2007). This locus is also found conserved between the *S. meliloti* and *R. etli* genome

responsive to NaCl and leads to a desiccation sensitive phenotype in a smc01506 mutant (Sauviac et al., 2014). In addition, *smc01504* (*rsi*B1) is up regulated 1.6-7 fold over 72 hours of desiccation in *Bradyrhizobium japonicum* (Cytryn et al., 2007). Clearly, this RpoE2 regulated pathway in the response to NaCl mediated desiccation resistance is conserved in several rhizobia. Expression of *smc01505,* however, is independent of RelA (Kroll and Becker 2011), and expression of *smc01504* is decreased in a TolC mutant (Santos et al., 2010).

Other conserved NaCl induced regulators are Sma1493 a LysR type DNA targeting regulator located just upstream of uncharacterized oxidoreductases, and Smc02844, an uncharacterized TetR type regulator. None of these loci have been studied in detail however, Sma1493 mutants have a slight reduction in the number of pink nodules, thus have a symbiotic phenotype (Luo et al., 2005). Yet another NaCl responsive regulator is Smc02366 (RagA), with a putative histidine kinase sensor immediately downstream. This regulatory system may be involved in the response to heavy metals, however was not found in a screen for heavy metal responsive loci by Rossbach et al., (2008). VBNC cells are a product of desiccation as shown by Vriezen et al., (2012) and copper is a metal known to be toxic and to induce a VBNC state in *Rhizobium* (Manahan and Steck 1997, Alexander et al., 1999). Smc02366 is also up regulated upon an

acidic shift (Hellweg et al., 2009). Therefore, this locus may have a function in acidic environments rich in heavy metals and maybe useful for remediation of acidic, metal contaminated sides such as acid mine drainage (Becerra et al., 2009).

Upstream of *rag*A, DegP1 (Smc02365), a periplasmic serine protease involved in oxygen-, heat-, and envelope stress (Raivio et al., 2005) was also found up regulated in both organisms during NaCl stress. Due to these features, Vriezen et al., (2007) had hypothesized the involvement of DegP1 in survival during desiccation in these organisms. Later, DegP1 was found to be significantly down regulated in a *hfq* mutant (Torres-Quesada et al., 2010), and up regulated in *bac*A and *tol*C mutants (Karunakaran et al., 2010, Santos et al., 2010).

Oxygen stresses caused by the accumulation of radical oxygen species and during desiccation are a substantial cause of damage and a reduction of viability. Expression of *deg*P1 and also *sod*C (*smc02597*) seem appropriate responses when cells are being desiccated. Other superoxide dismutases have been found up regulated during drying: *e.g.* SodF in desiccated *B. japonicum* (Cytryn et al., 2007) cells as well as long term desiccated *Nostoc commune* (Shirkey et al., 2000). Valverde et al., (2008) predicted that expression of *smc02597* is sRNA mediated, but is induced during infection (Ampe et al., 2003).

Finally, finding a cold-shock protein (Smc04319, O'Connor

and Thomashow, 2000) indicates that the extraction of water by hyperosmotic stress has a similar effect as the immobilization of water when cooling. The extraction of free water due to drying, increasing osmotic stress or to cooling may be the common signal and mechanism in the response to these stresses and explain the finding of a NaCl up regulated locus involved in the cold shock response. The expression of Smc03419 is reduced under microoxic conditions in a *ntr*R mutant (Puskas et al., 2004).

12.1.5. NaCl responsive hypothetical proteins

Eye-catching is the vast amount of (conserved) hypothetical proteins this list represents (15/35=43%), indicating many possible loci yet to be studied for their involvement in these stress responses. Three of these loci are reviewed here. Under carbon starvation *smc00800* and *smc00885* are RelA independent targets of RpoE2. Under nitrogen starvation, however, expression of these loci is RelA dependent (Krol and Becker, 2011). Both proteins exhibit increased expression levels during stationary phase and at 40˚C (Sauviac et al., 2007). The Smc00885 contains a PRC-H barrel domain, as described by Anantharaman and Aravind (2002). These domains are found in the photosynthetic complex in purple bacteria, and have been hypothesized to be involved in non-photosynthetic electron transfer reactions in rhizobia

(Anantharaman and Aravind 2002). Expression of *smc00885* is increased in *Medicago sativa* nodules, in the reference strain as well in a *bac*A mutant (Capela et al., 2006), is under RpoE2 control (Bastiat et al., 2010), and is up regulated in the presence of Nitric Oxide (NO), a former of reactive oxygen species (Lindermayr and Durner, 2015, DeBruijn et al., 2006).

The third locus in this group is smc01590. Vriezen et al. (2013) first reported that exposure to NaCl up regulates this locus in *S. meliloti* and *R. etli*, but no phenotype on NaCl was found. Computational approaches to find promoter sequences identified an upstream promoter for RpoE2 (Schlüter et al., 2013). This is in conflict with what Flechard et al. (2010) reported. This group did not find expression of *smc01590* RpoE2 dependent. However, Vriezen and deBruijn (2015) reported that this locus is involved in the survival during desiccation. This raises the following question: Is RpoE2 mediated desiccation resistance directly due to transcriptional activation of *smc01590* or is there another target for RpoE2 involved in the response to desiccation? Most interestingly, the ability of the mutant strain containing the transcriptional fusion *smb01590::tn5lux*AB (Sce1) is reduced with increasing temperature, while survival of the reference strain is increased with increasing temperature. It appears that the mutant is not able to correctly adjust membrane stability resulting in a

decrease in survival at higher temperatures compared to the reference strain (Vriezen and deBruijn, 2015).

12.1.6. The reduced ability of strain Sce1 to survive desiccation at 37˚C is inversely related to growth at this temperature.

The ability to live or even proliferate in soil is an art in survival. In a recent publication the authors (Vriezen et al., 2013) describe the effect of a *Tn5lux*AB insertion in locus *smb20482* in *S. meliloti* 1021 strain Sce11. This mutant strain is reduced in its ability to grow at elevated NaCl concentrations and survival during desiccation. It appears that sce11 is not able to produce the powerful osmoprotectant NAGGN, however, the reduced ability to survive desiccation is not related to the accumulation of osmoprotectants but rather to a secondary function such as cell wall synthesis. In the same publication the authors describe the effect of a *tn5lux*AB insertion in locus *smc01590* in *S. meliloti* 1021 strain Sce1. In contrast to the effect of the *tn5lux*AB insertion in strain Sce11, this mutant strain has no reduced ability to grow at 400 mM NaCl, however, this mutant is sensitive to desiccation relative to the reference strain at increased temperature (37˚C), but not when dried at 4˚C (Vriezen and de Bruijn 2015). Therefore, we hypothesized that *smc01590::tn5lux*AB, although responsive to a

NaCl increase, causes a phenotype when growing at elevated temperature rather than through the presence of NaCl. To test this, mean generation times were estimated when growing in PMM with and without NaCl and at 28˚C and 37˚C (Table 12.1.2.).

Table 12.1.2. here…….

Not surprisingly, and similar to the reported observation by Vriezen et al., (2013), *S. meliloti* 1021 and Sce1 have similar mean generation times when growing at 28˚C in the presence and in the absence of NaCl, albeit Sce1 grows marginally slower. In contrast, at 37˚C, Sce1 grows significantly faster (P=0.04) than the reference strain. Therefore, rather than being reduced in the ability to grow at elevated temperature, an increase in growth was observed indicating a conflict between the ability to survive desiccation and the ability to grow at elevated temperature.

12.1.7. The inability of strain Sce1 to survive desiccation is related to stationary phase expression

To test if *smc01590::tn5luxAB* is also expressed during the stationary phase, luciferase activity was measured during growth in TY medium (Figure 12.1.2.).

Figure 12.1.2. here…….

A decrease in luciferase activity was measured during exponential growth, followed by an increase in luciferase activity during the stationary phase. Thus, the ability of strain Sce1 to survive desiccation may be related to slow growth and stress-resistance during this phase. To put this hypothesis to the test, the ability of strain Sce1 to survive desiccation was determined in relation to time spent in the stationary phase. Both the reference strain and strain Sce1 were grown on TY plates for one to seven days prior to initiating the desiccation experiment. At days one, three, and seven, culture tubes containing 5 ml of liquid TY medium were inoculated with single colonies from TY-plates, and incubated for three days at 28˚C with agitation at 220 rpm. On the third day, culture tubes containing 5 ml of YMB medium were inoculated with 50 µl from the TY-culture tubes, and incubated at 28˚C with agitation at 220 rpm. At an appropriate OD of 0.1-0.2, samples were washed in fresh YMB medium and dried at 22% relative humidity at 20˚C for seven days. In addition, survival of colonies from three-day old TY plates that were incubated for 1, 3, and 7 days in liquid TY medium prior to the desiccation experiment was also determined. Upon rehydrating, the resulting CFU’s were determined and expressed as percentage survival.

Figure 12.1.3. here…….

The data presented in Fig. 12.1.3A. and 12.1.3B. show that Sce1 inoculum taken from the aging liquid TY cultures lead to YMB cultures increasingly less able to survive desiccation relative to the reference strain (Figure 12.1.3A.). Obviously, residing longer in the stationary phase in TY liquid decreases survival of strain Sce1, while the reference strain does not show such a phenotype. The variation in survival seen in the reference strain is of proportions normal for these tests (Vriezen et al., 2006). In contrast, aging in a structured environment such as the agar plate, results in no correlation of survival with culture age (Figure 12.1.3B.). Therefore we conclude that the desiccation phenotype of strain Sce1 is related to the time this strain resided in the stationary phase in liquid TY medium.

12.1.8. Heat- and desiccation stress and stationary phase expression

To further explore the possibility of a conserved response to increased NaCl, increased temperature, desiccation and stationary phase, we evaluated several genomic studies utilizing rhizobia addressing the identification of loci who's expression is affected during the stationary phase (Sauviac et al., 2007,

Chen et al., 2003, using *S. meliloti* 1021), increased temperature (Sauviac et al., 2007, Lopez-Leal et al., 2014) and desiccation (Cytryn et al., 2007, using *B. japonicum* USDA110). In order to make a direct comparison, all loci up regulated during desiccation in *B. japonicum* were first retrieved from NCBI and compared to the *S. meliloti* genome database at INRA using tblastnt. This comparison resulted in only two loci that are up regulated in response to NaCl in both organisms. These loci are the putative DNA topoisomerase (Sm21445), and the putative pentose monosaccharide ABC transporter (Smb21587).

When this list of loci was compared with expression data in response to increased temperature, 11 of the 38 loci (29%) in *R. etli* responded with increased expression to elevated temperature. This is substantially less than the 51% of total NaCl responsive loci up regulated by heat stress. Similarly, eight of 35 loci (23%) in *S. meliloti* were increased by temperature stress. Based on random chance, one would expect an overlap of two to three loci between *R. etli* and *S. meliloti* respectively, however, only one locus (*smb20227*, *ndi*A) was found. In support of the fact that smb20227 is expressed during heat- and saline stress, Schlüter et al., (2013) found in a bioinformatics analysis the promoter sequences RpoH and RpoE2 upstream of this locus. Therefore it appears that, except for *ndi*A, the combined response to NaCl and heat stress is different

in both organisms. This supports the observations by Trotman and Weaver (1995) that isolated rhizobia show large differences in their response to desiccation, which is not related to the ability to respond to increased temperature.

When stationary phase expression is added to the list (*S. meliloti* only), 12 loci (32%) of the NaCl responsive loci were up regulated in the stationary phase. Of these, eight were also up regulated in response to heat. These eight loci are mainly those loci encoding for six hypothetical proteins, Smc01505 and NdiA1. The four loci expressed during the stationary phase that are not responsive to temperature are the locus presented in this manuscript (*smc01590*), and the loci involved in uracil-, fucose-, and proline-betain- uptake. Although not conclusive, it appears that genes up regulated during the stationary phase fall into two groups; those that are heat independent and involved in small molecule uptake, and those that are heat dependent (mainly hypothetical proteins).

12.1.9. Smc01590 is conserved among a wide range of α-proteobacteria associated with soil and high salinity environments

To determine if the Smc01590 mediated stress response to desiccation is conserved, the amino acid sequence of Smc01590 was used as a query against the non-redundant bacterial genomes

database of representative genomes using the function tblastn at NCBI. Besides *S. meliloti* 1021, sixteen more organisms were found with more than 80% overlapping sequence. In addition to many *Rhizobium*-related microorganisms, this gene was also present in *Chelativorans* BNC1 (formerly *Mesorhizobium* BNC1, an organism isolated from EDTA enriched soil (Nörtemann 1993, 1999, Bohuslavek et al., *2001*). *Pelagibacterium halotolerance* B2, an α-proteobacterium isolated from the South Chinese sea (Xu et al., 2011, Huo et al., 2011), *Leisingera methylohalidivorans*, an α-proteobacterium from a tidal pool in California, which grows on methyl bromide (Schaefer et al., 2002), and *Rhodobacter sphaeroides*, an α-proteobacterium usually found in soil and fresh water (Choudhari et al., 2007, Porter et al., 2011). It appears, only *Sinorhizobium meliloti* and *S. medicae* have a full-length copy of this gene. All organisms are associated with soil environments or saline waters.

To determine if topologies made with the aminoacid sequence of this gene are congruent with topologies made with 16S rRNA sequences, all 17 16S rRNA sequences from these organisms were obtained from NCBI, and aligned using Clustal Omega at the EMBL website using default settings (http://www.ebi.ac.uk/Tools/msa/clustalo/). A γ-proteobacterium, *E. coli* K12 functioned as an out-group for this phylogeny of

selected α-proteobacteria. To create trees from the Smc01590 homologs, the same alignment procedure was followed with the exception to use protein sequences. The Smc01590 sequence aligned well at the C-terminal end of the protein, but was less conserved in the center as well as at the N-terminus. In particular, the sequences of the four organisms described above aligned poorly in the center of the protein. These alignments in conjunction with a search for conserved domains using Smc01590 indicate that the SH3 domain (AA 41-93, Vriezen and de Bruijn 2015) and the DUF1236 domain (AA 151-210) are conserved in all sequences used. Even though the alignments are highly variable at the N-terminus and in the center of the alignment, it appears Smc01590 function is conserved among the known strains.

Neighbor joining (NJ) trees were generated using the software Seaview vs4 (Guoy et al., 2010).

Figure 12.1.4. here…….

When comparing the resulting phylogenetic trees (Figure 12.1.4.), their overall topology is largely similar, an indication that Smc01590 may be part of the core genome in these organisms. Branch-length is longer in the Smc01590 tree, which means that evolutionary rates are higher than in the 16S rRNA tree. The more distantly related α-proteobacteria are also deep-

branching in the Smc01590 tree. In the *Rhizobium/Agrobacterium* clade, topology is the same between the 16S rRNA and Smb01590 topologies, however, bootstrap values are low for the deeper branching nodes. Similarly, even though the topology of the *Ensifer*-group is the same in both trees, the Smc01590 homolog in *S. fredii* evolves faster than the 16S rRNA, resulting in a weakly supported node with a bootstrap value of only 63%. This is even more obvious in the *Mesorhizobium/Chelativorans* group. The nodes in this clade are well supported in the 16S rRNA topology. In contrast, in the Smc01590 topology, the *Chelativorans* is deep branching while the Mesorhizobia are closely related resulting in lesser supported nodes. Interestingly, the four deep-branching organisms, *Rhodobacter sphaeroides*, *Leisingera methylohalidivorans*, *Pelagibacterium halotolerance,* and *Chelativorans* sp., all aligned poorly and have a ~40 Amino Acid insertion between positions 122-169 in the alignment. It appears that the fast evolution of Smc01590 homologs is mainly due to the N-terminus and an inter-domain sequence in the center.

Most interestingly, the insertion in the inter-domain sequence in some organisms correlates with the deep-branching organisms, while the variation at the N-terminus separates *S. meliloti* and *S. medicae* from the rest of the rhizobia. The fact that Smc01590 is only found in the α-proteobacteria associated

to soil or high salinity may suggest Smc01590 homologs have a conserved function in these environments and are likely important for survival in these environments. However, this also raises the question why certain Smc01590 homologs evolve faster than others. For example, Smc01590 in *Chelativorans* evolves substantially faster than any of the *Mesorhizobium* alleles. The reason is unlikely the ability to induce the formation of rootnodules since that is one of the characteristics that separates the *Chelativorans* from the Mesorhizobia (Doronina et al., 2010). It is more likely that the occurrence of the previously mentioned insertion is related to the absence of the nodulation ability of the deep-branching organisms studied. A second example is *Sinorhizobium fredii*, which induces nodule formation on *Glycine max*, while the other Sinorhizobia induce nodules on a wider range of legumes, including *Medicago* species, however not on *Glycine max*. It looks like the differences in evolutionary rates appear to be related to the host-plants and absence of nodulation abilities, however it may relate to secondary factors such as geographical distributions of the host plant and local environment.

12.1.10. Summarizing statements and future direction

By identifying conserved mechanisms to NaCl and desiccation stress, we make an attempt to understand molecular and cellular

mechanisms that are important in survival during desiccation and changing environmental conditions. To this end, we compared two different lists with loci up regulated during NaCl stress in *S. meliloti* 1021 and *R. etli*. We identified 35 loci up regulated in both organisms. Reviewing those loci, potential regulatory mechanisms were identified that are important in the NaCl mediated response to desiccation. At least two loci (Smc01506 (RpoE2) and Smc01590) are involved in survival during this stress. Interestingly, expression of smc01590 may be RpoE2 mediated despite conflicting reports on this possibility.

All data considered, it appears the genetic locus smc01590 is conserved in a wide range of α-proteobacteria, and is expressed during exposure to NaCl and during the stationary phase in *S. meliloti* 1021. Exposure to stationary phase dramatically affects the ability to survive desiccation at temperatures higher than 20˚C. Growth phenotypes at 37˚C indicate that the presence of low concentrations of NaCl is not detrimental to growth, but rather an increase in temperature allows strain Sce1 to grow faster and suggests that *smc01590* encodes for a protein that is involved in the negative regulation of growth and is important in stationary phase survival. This leads to a conflict: An increase in growth-rate at increased temperature leads to a reduced ability to survive desiccation in *S. meliloti*. Current global changes in weather

patterns lead to an increase in short and intense precipitation events (Dore, 2005) and subsequently the re-drying of soil in intermittent periods. The inability of responding to this change will be detrimental to the survival of organisms lacking a functional Smc01590 homolog, which is countered by the ability to grow quicker at increased temperature. The fact that *smc01590* is well integrated into the genome of *S. meliloti* and *R. etli* is an indication that the ability to grow at increased temperature is offset by the ability to withstand periods of drought or other stresses in soil. It can be expected that with current changes in weather patterns, bacteria containing a functional Smc01590 have a competitive advantage, and will increase in abundance in soil, independent of temperature. Determining the shift in bacterial communities under NaCl and heat stress can test this hypothesis. To further elucidate the underlying mechanisms leading to a desiccation sensitive mutant, transcriptional profiling of the reference strain as well as mutant Sce1 will reveal the physiological differences between these strains giving insight into the cellular structures affected.

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Figure Legends

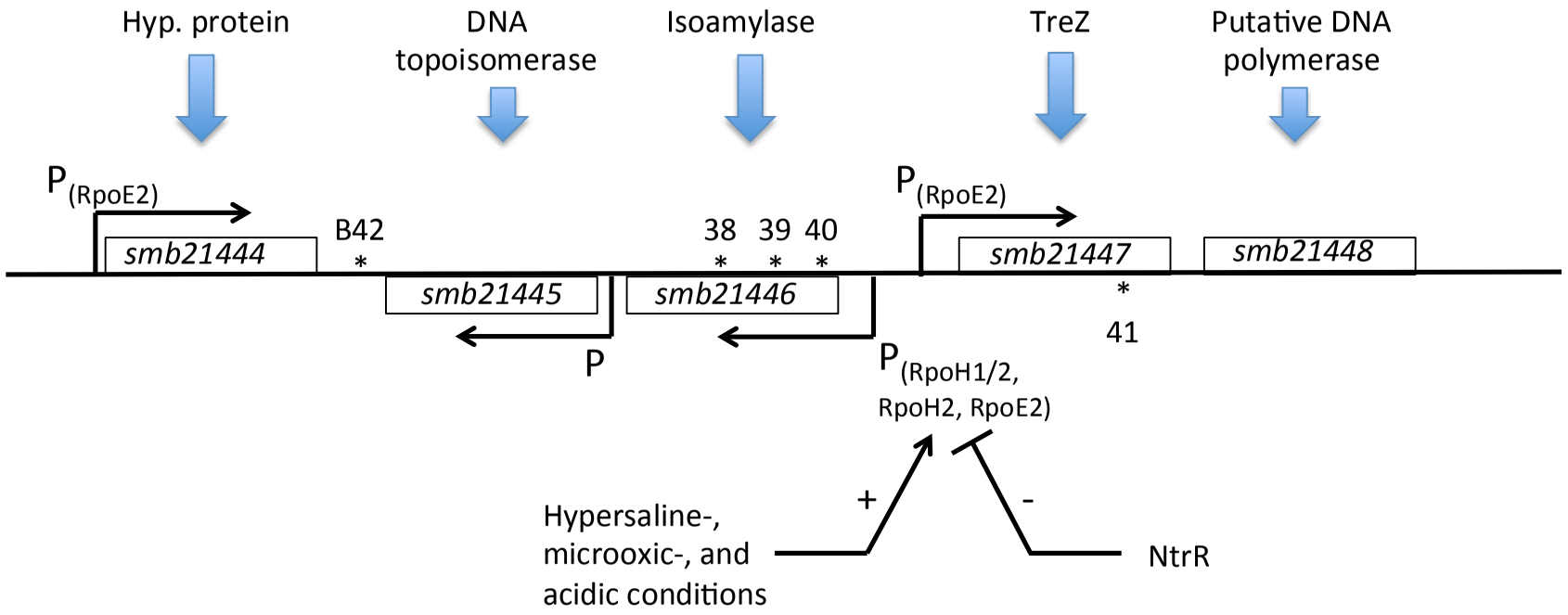
12.1.1. Regulatory model for the sequence of the five loci *smb21445 – smb21448* (Genomic region 1372331-1379386). Promoter regions (P) with possible regulatory mechanisms (RpoE2,H2, H1/2-promoters) according to Schlüter et al., 2013. The asterisk indicates the approximate location of *cis*-encoding sRNA's Smb_asRNA29**38**-29**41**. Location of sRNA B42 was found by del Val et al. (2007).

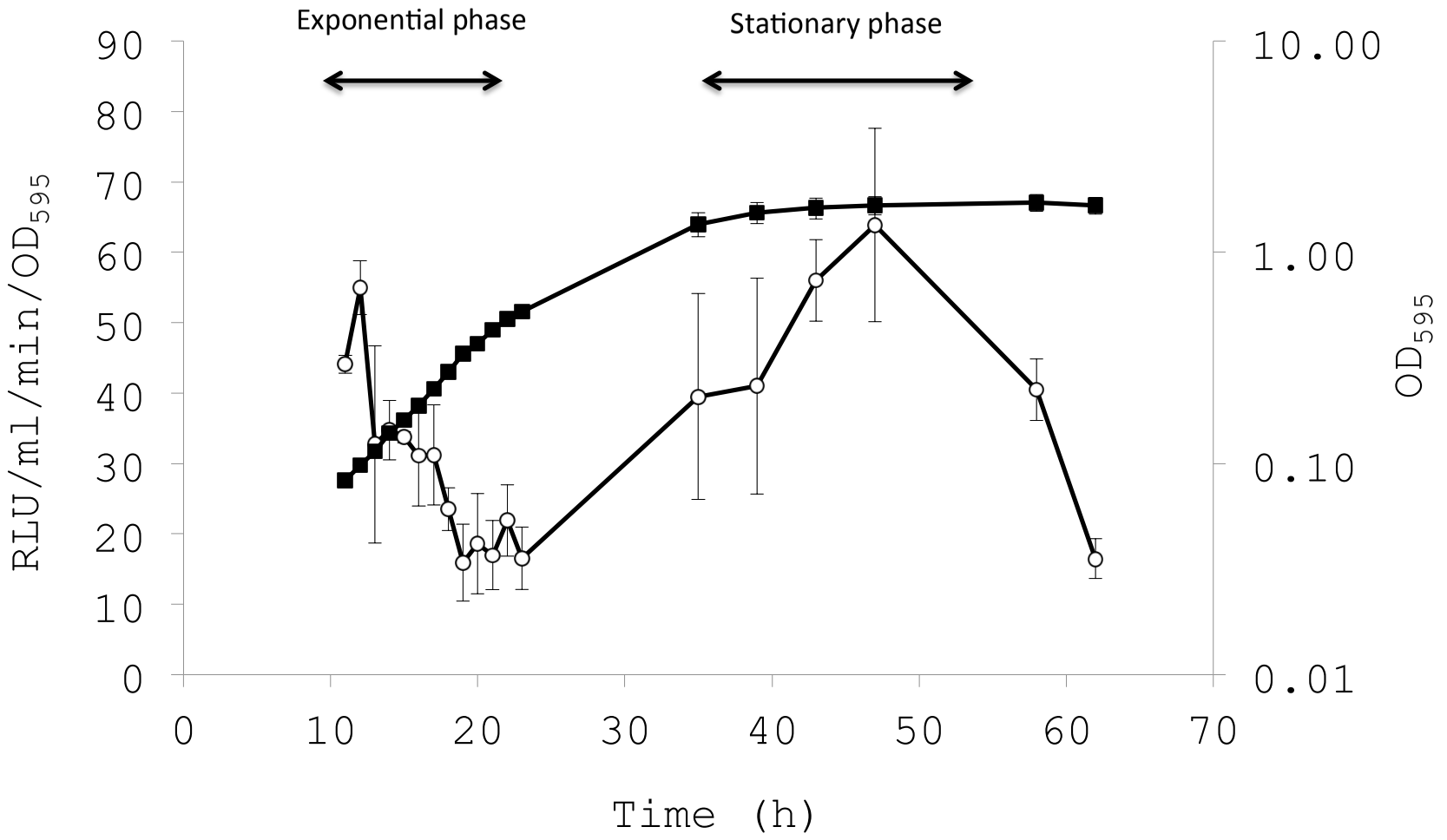
12.1.2. Increased expression during stationary phase of the transcriptional fusion *smc01590::tn5luxAB.* Black squares represent the OD at 595 nm measured during growth of strain Sce1 in TY-medium at 28˚C. White circles represent the Relative Light Units (RLU) during the growth period. Error bars represent the standard deviation of three measurements of a growing culture.

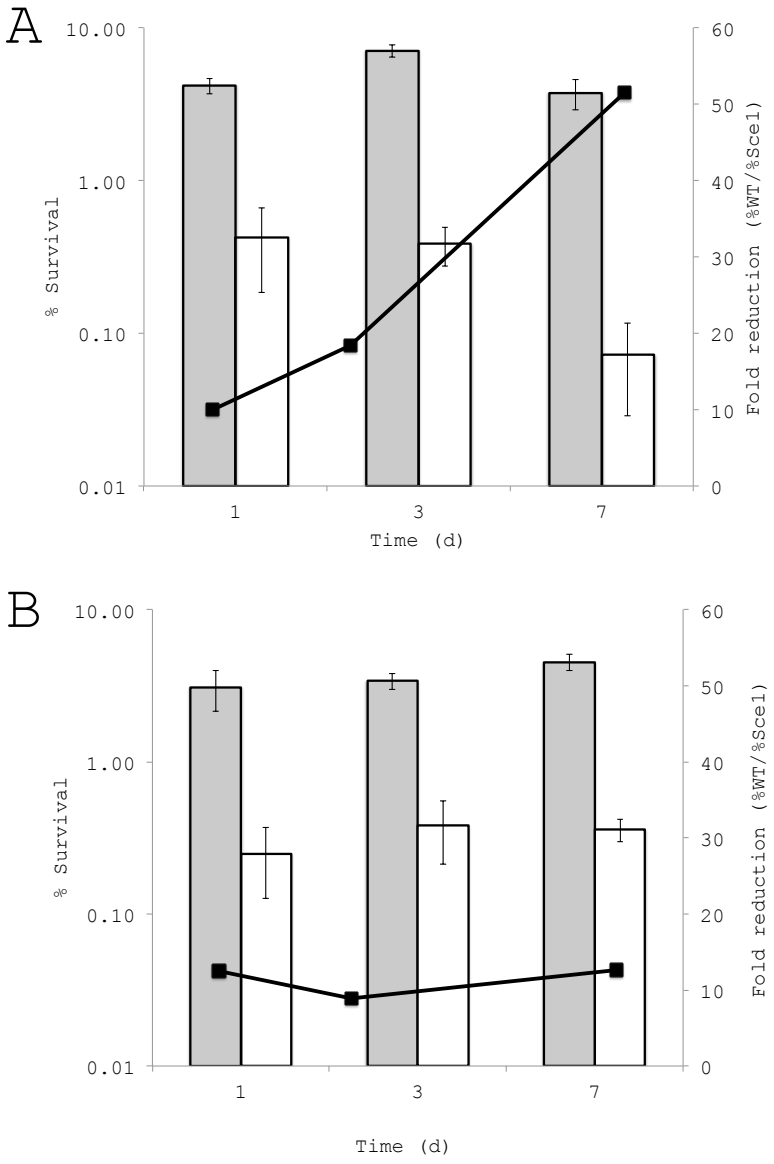
12.1.3. Relative survival after desiccation of *S. meliloti* 1021 (grey bars) and strain Sce1 (white bars), **(A)** after residing in liquid TY medium, and **(B)** after residing on an agar plate. Black squares indicate the fold reduction of strain Sce1 relative to *S. meliloti* 1021. Error bars represent the Standard Error of the Mean (SEM) with n=3 and n=6, respectively.

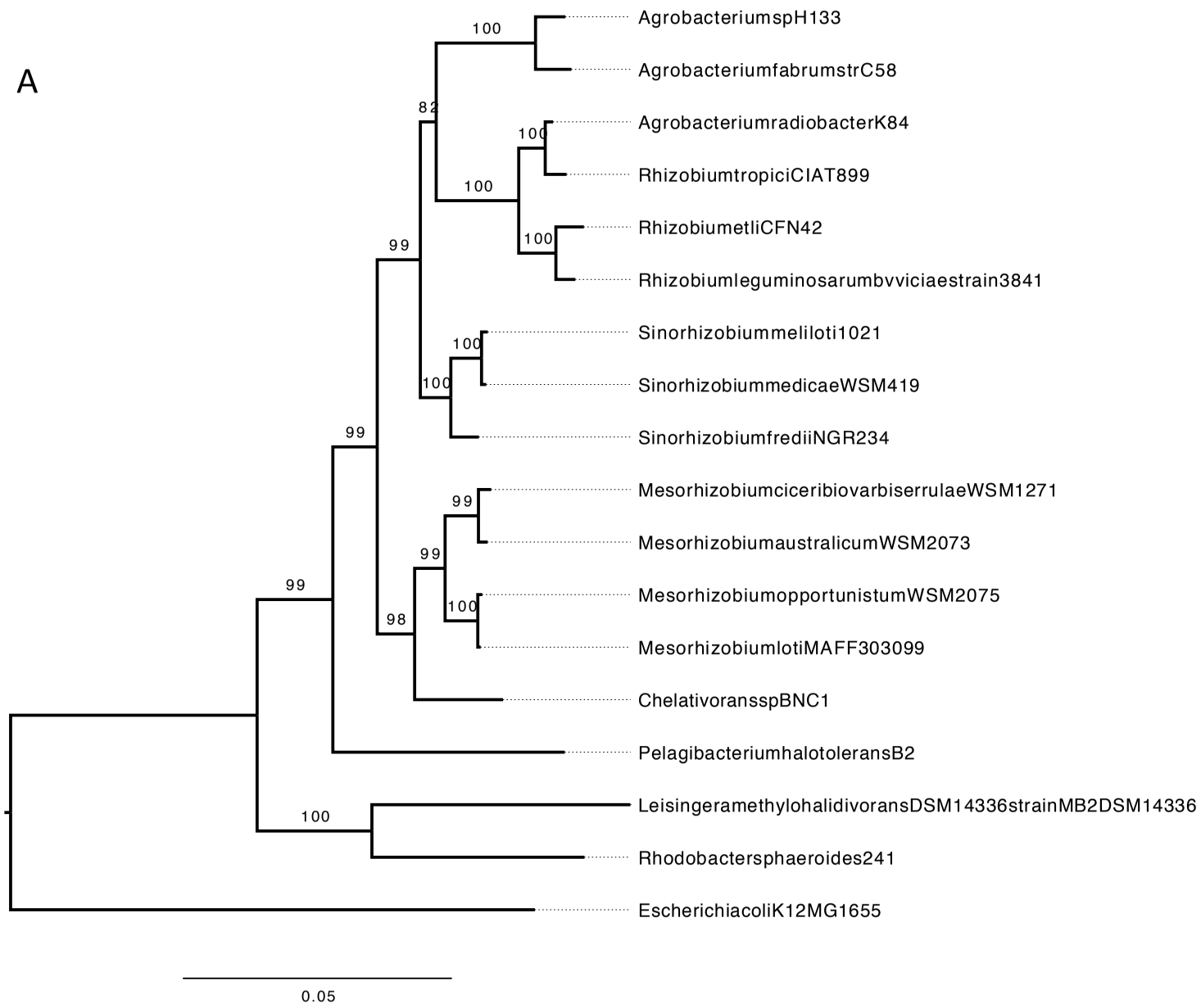
12.1.4. (A) Phylogenetic tree based on 16S rRNA with *E. coli* K12 as an out-group; **(B)** Tree topology based on Smc01590. Phylogenetic trees were constructed based on neighbor joining algorithms using using observed distances while ignoring gaps.

Bootstrap values (%) are from 100 replicates.


Hyp. protein
DNA topoisomerase
Isoamylase
TreZ
Putative DNA polymerase
P(RpoE2)
smb21444
B42
*
smb21445
38 39 40
* * *
smb21446
P
P(RpoE2)
smb21447
smb21448
*
41
P(RpoH1/2, RpoH2, RpoE2)
+
-
Hypersaline-, microoxic-, and acidic conditions
NtrR


Exponential phase
Stationary phase
RLU/ml/min/OD595
OD595
90
80
70
60
50
40
30
20
10
0
10.00
1.00
0.10
0.01
0
10
20
30
40
50
60
70
Time (h)


A
10.00
1.00
0.10
0.01
% Survival
60
50
40
30
20
10
0
Fold reduction (%WT/%Sce1)
1
3
7
Time (d)
B
10.00
1.00
0.10
0.01
% Survival
60
50
40
30
20
10
0
Fold reduction (%WT/%Sce1)
1
3
7
Time (d)


A
AgrobacteriumspH133
AgrobacteriumfabrumstrC58
AgrobacteriumradiobacterK84
RhizobiumtropiciCIAT899
RhizobiumetliCFN42
Rhizobiumleguminosarumbvviciaestrain3841
Sinorhizobiummeliloti1021
SinorhizobiummedicaeWSM419
SinorhizobiumfrediiNGR234
MesorhizobiumciceribiovarbiserrulaeWSM1271
MesorhizobiumaustralicumWSM2073
MesorhizobiumopportunistumWSM2075
MesorhizobiumlotiMAFF303099
ChelativoransspBNC1
PelagibacteriumhalotoleransB2
LeisingeramethylohalidivoransDSM14336strainMB2DSM14336
Rhodobactersphaeroides241
EscherichiacoliK12MG1655
100
82
100
100
100
99
100
100
99
99
99
99
98
100
99
100
0.05

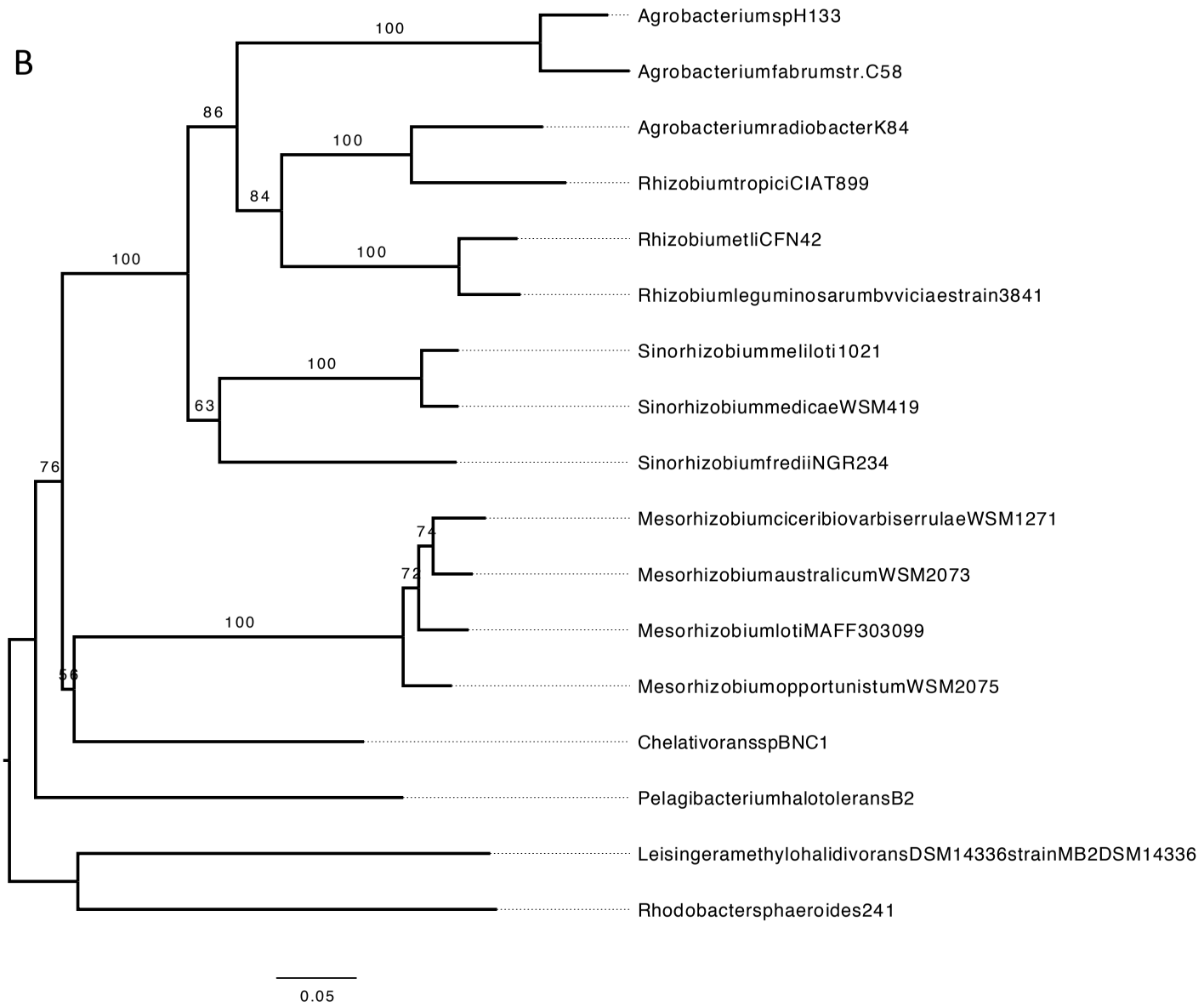

B
AgrobacteriumspH133
Agrobacteriumfabrumstr.C58
AgrobacteriumradiobacterK84
RhizobiumtropiciCIAT899
RhizobiumetliCFN42
Rhizobiumleguminosarumbvviciaestrain3841
Sinorhizobiummeliloti1021
SinorhizobiummedicaeWSM419
SinorhizobiumfrediiNGR234
MesorhizobiumciceribiovarbiserrulaeWSM1271
MesorhizobiumaustralicumWSM2073
MesorhizobiumlotiMAFF303099
MesorhizobiumopportunistumWSM2075
ChelativoransspBNC1
PelagibacteriumhalotoleransB2
LeisingeramethylohalidivoransDSM14336strainMB2DSM14336
Rhodobactersphaeroides241
100
86
100
84
100
100
100
63
76
74
72
100
56
0.05

Table 12.1.1.: NaCl responsive loci found in both *R. etli* and *S. meliloti* 1021

In *R. etli*[a]		Results from database comparison [c]			Condition leading to up regulation in *S. meliloti*[de]		
Locus identifier	**Up regulated by[b]**	**Locus**	**Gene Name**	**Description in database**	**Temp.[f]**	**Stat.**	**Des[g].**
RHE_PA00159		Sma0934	*tra*A1	Conjugal transfer protein			
RHE_CH00796		Sma1493		LysR transcriptional regulator family			
RHE_CH03743	T	Sma1521		Conserved hypothetical protein			
RHE_CH00965		Sma2071		Hypothetical protein	Sau	Sau	
RHE_PF00082		Smb20041		Hypothetical Protein			
RHE_PF00248	T	Smb20093		Hypothetical Protein			
RHE_CH01083	T	Smb20227	*ndi*A1	Putative nutrient deprivation induced protein	Sau	Sau, Dav	
RHE_PB00051	T	Smb20246		Putative oxidoreductase			
RHE_CH00479		Smb20522		Conserved hypothetical protein			
RHE_PE00008		Smb20574		1-4 a D-glucan 1-a D-glucosylmutase			
RHE_CH01335		Smb20879		Protein required for attachment to host cells	Sau	Sau	
RHE_CH00990 RHE_CH00499	T	Smb21031		Hypothetical membrane anchored protein			
RHE_PE00209		Smb21445		Putative DNA topoisomerase I			Cyt
RHE_PE00208		Smb21446	*glg*X2	Isoamylase			
RHE_CH02652		Smb21518		Conserved hypothetical protein signal peptide			
RHE_CH01237		Smb21568	*usg*2	Conserved hypothetical protein			
RHE_CH02085 RHE_PC00171	T	Smb21587		Putative pentose monosaccharide ABC transporter			Cyt
RHE_CH02172		Smc00063		Hypothetical protein	Sau	Sau	
RHE_CH00268		Smc00371		Conserved hypothetical protein	Sau	Sau	
RHE_CH00180		Smc00800		Hypothetical transmembrane protein	Sau	Sau	
RHE_CH00851		Smc00885		Hypothetical transmembrane protein	Sau	Sau	
RHE_CH00563	T	Smc01148		Hypothetical Protein			
RHE_PB00046	T	Smc01447		Hypothetical Protein			
RHE_CH03274		Smc01505	*rsi*A1	anti-sigma factor	Sau	Sau	

RHE_CH03012		Smc01590		Hypothetical protein	This study
RHE_PC00130		Smc01642	*prb*A	ABC transporter, uptake of proline betain	Sau
RHE_PF00307	T	Smc01827		Putative uracil and uridine ABC transporter	Sau
RHE_CH01293 RHE_CH01120		Smc02365	*deg*P1	Probable serine protease	
RHE_CH01294		Smc02366	*rag*A	Probable response regulator	
RHE_CH02053	T	Smc02471		Putative ABC transporter	
RHE_CH01400		Smc02597	*sod*C	Putative cu/zn superoxide dismutase	
RHE_CH00492		Smc02774		Putative fucose or pyruvate ABC transporter	Sau, Chen
RHE_CH03672		Smc02844		Putative transcriptional regulator	
RHE_CH02708	T	Smc04236		Putative glycine rich cell wall protein	
RHE_CH01170		Smc04319		Putative cold shock protein	

a) Data from Lopez-Leal et al. (2014). All loci in *R. etli* are up regulated by NaCl.

b) Loci up regulated by an increase in temperature from 30 to 40˚C are indicated with T.

c) Results from the comparative analysis of *R. etli* aminoacid sequences (from NCBI database) with respective sequences in the *S. meliloti* 1021 database at https://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi

d) Temp., temperature shift; Stat., stationary phase ; Des., desiccation. Annotations for references: Sau=Sauviac et al., (2007), Dav=Davey et al., (2000), Chen=Chen et al., (2003), Vri=Vriezen and deBruijn (2015), Cyt=Cytryn et al., (2007).

e) All loci in *S. meliloti* are up regulated by NaCl. Dominguez-Ferreras et al., (2014) used 300 and 400 mM NaCl, Vriezen et al., (2013) used 400 mM NaCl, and Ruberg et al., (2007) used 380 mM NaCl.

f) Sauviac et al. (2007) compared expression levels between cultures at 28˚C and 40˚C.

g) Up regulated during desiccation in *Bradyrhizobium japonicum.*

Table 12.1.2.: Growth rates of *S. meliloti* and strain Sce1 in hours per generation.

	1021			Sce1			Statistics		
Growth Condition	Mean	SEM	N	Mean	SEM	N	P (F-test)	P (T-test)	
28˚C PMM	5.7	0.4	12	6.2	0.5	12	0.47	0.39	*
37˚C PMM	5.5	1.2	6	4.6	0.1	6	0.05	0.04	**
28˚C PMM + 400 mM NaCl	9.1	0.2	3	9.6	0.2	3	0.94	0.08	*

* Return of the homoscedastic two-sided T-test

** Return of the heteroscedastic two-sided T-test