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Identification and Characterization of a NaCl responsive Genetic Locus Involved in Survival During Desiccation in *Sinorhizobium meliloti*

By

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ABSTRACT

The *Rhizobiaceae* are a bacterial family of enormous agricultural importance due to the ability of its members to fix atmospheric nitrogen in an intimate relationship with plants. Their survival as naturally occurring soil bacteria in agricultural soils as well as popular seed inocula is affected directly by drought and salinity. Survival after desiccation in the presence of NaCl is enabled by underlying genetic mechanisms in the model organism *S. meliloti* 1021. Since salt-stress parallels a loss in water activity, the identification of NaCl responsive loci may identify loci involved in survival during desiccation. This enabled identification of the loci *asnO* and *ngg* for their reduced ability to grow on increased NaCl concentrations, likely due to their inability to produce the osmoprotectant N-acetylglutaminylglutamine (NAGGN). In addition, the mutant harboring *ngg*::Tn*5lux*AB was affected in its ability to survive desiccation, and responded to osmotic stress. Desiccation sensitivity may be due to secondary functions of Ngg like cell wall metabolism suggested by the presence of a dAla-dAla domain, and by sensitivity of the mutant to β-lactam antibiotics. *Asn*O::Tn*5lux*AB is expressed during the stationary phase under normal growth conditions. Aminoacid sequence similarity to enzymes producing β-lactam inhibitors and increased resistance to β-lactam antibiotics may indicate *asnO* is involved in the production of a β-lactam inhibitor.

INTRODUCTION

The *Rhizobiaceae* are a bacterial family of enormous agricultural importance due to their ability to fix atmospheric nitrogen in an intimate relationship with plants (1). They occur naturally in most agricultural soils and their survival is affected directly by both drought and salinity (2). Unfortunately, changes in climate patterns are occurring and as a direct consequence salinification and desertification, are some of the major threats to agricultural land use. It is estimated that over 40% of arable land will be affected by desiccation and salinity by 2025 (2). Furthermore, production of seed inocula often includes a drying-phase negatively affecting colony-forming units of added rhizobia (3, 4), potentially resulting in desiccation induced Viable But Non-Culturable cells (5).

A multitude of conditions have been studied affecting survival during desiccation of agriculturally important *Rhizobiaceae* (6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20). These conditions include the intrageneric differences to cope with desiccation stress, which affect survival (6, 9, 10, 21, 22, 18, 5, 13). These data suggest that no single gene affects the ability of rhizobia to survive desiccation but that several mechanisms are likely responsible.

One of the conditions affecting the ability of *S. meliloti* to survive desiccation is NaCl availability (10, 13), hence our study of the response of *S. meliloti* 1021 to drought in conjunction with different salt-stresses (13). We showed that this strain survives desiccation worse when exponentially growing cells were resuspended in water compared to resuspension in water containing 400 mM NaCl. In contrast, increasing amounts of sodium chloride (200 and 400mM) added to standard media like Yeast Manitol Broth and

66 Phosphate Mannitol Medium were found to enhance survival. This effect was mainly
67 caused by the presence of anions in the growth medium rather than cations (10, 13).
68 These observations indicate that the response to desiccation in conjunction with the
69 presence of NaCl is physiological in origin (13, 4), and that the underlying genetic
70 mechanisms are important for the cell's survival during desiccation. The *in-situ*
71 relevance of this physiological effect is further demonstrated by the fact that NaCl and
72 the availability of osmoprotectants affect survival of rhizobia in soil (23, 24), as well as
73 in seed inocula (25).

74 Examples of genetic mechanisms involved in survival during desiccation have
75 been identified previously and include the operon *agl* for trehalose/maltose and sucrose
76 uptake (*smb03060-03065*) (26), or the trehalose/maltose/sucrose operon (*thu*, *smb20324-*
77 *20330*) identified by Jensen (27). The *thu* operon is expressed to a higher level during an
78 osmotic upshift (28), and McIntyre (29) showed that loci involved in trehalose
79 metabolism affect survival during desiccation. The above-described uptake systems
80 involve osmoprotectants, which accumulate during salt stress, thus potentially affect
81 NaCl mediated survival during desiccation.

82 In addition to trehalose uptake, a target of desiccation responses is the cell wall,
83 which is also affected by the presence of NaCl (15, 30, 31). These responses include
84 exopolysaccharide production (32, 33, 34), which is assumed to affect desiccation
85 survival (4). Furthermore, Wei (35) and Miller-Williams (36) have shown that responses
86 to NaCl and osmotic stress affect genes potentially involved in the central metabolism
87 like elongation factors, DNA ligases, chaperones, and cell division proteins. It is well

88 known that DNA is a target for desiccation stress, and recently Humann (37) confirmed
89 this for rhizobia.

90 In this study the hypothesis that certain NaCl responsive loci are involved in
91 survival during desiccation was further tested. We identified Tn*5lux*AB tagged loci that
92 are responsive to increased concentrations of NaCl. Some of these mutants were also
93 tested for their ability to survive desiccation, and NaCl responsive loci involved in
94 survival during desiccation were characterized for their response to water stress. Finally,
95 we also tested their response to β-lactam antibiotics to test for potential involvement in
96 cell wall function.
97

MATERIALS AND METHODS

Materials

S. meliloti 1021 was obtained from our strain collection (38). An *E. coli* strain containing plasmid pRK2013 was obtained from T. Lessie (39), and used for triparental matings. Phage ΦM12, employed to reconstruct Tn*5luxAB* transcriptional fusions, was supplied by Dr. Graham Walker (40). A Tn*5lux*AB transcriptional fusion mutant bank was created for *S. meliloti* strain 1021 as described previously (41). *S. meliloti* strains Sce1-Sce12 were identified and characterized in this study. *S. meliloti* strain CV2, which serves as the positive control for NaCl dependent luciferase expression, was described by Milcamps (41). A negative control for NaCl dependent luciferase expression (strain 1D1) was randomly chosen from the mutant bank. *E. coli* DH5α- and JM109-competent cells were obtained from Invitrogen or prepared following standard protocols (42). All strains were maintained on TY plates with the appropriate antibiotics. Media used were TY (43), GTS (41), LB (42), YMB and PMM (5, 13, 44). Alfalfa seeds were obtained from the seed company Outsidepride (BS-ALFALFA-5;Lot No:A2N-1769-3; Outsidepride, Salem, OR, USA). According to the manufacturer, these seeds have not been treated with any chemicals.

Induction studies using Tn*5lux*AB transcriptional fusions

The screening of the Tn*5lux*AB transcriptional fusion mutant bank was carried out using a photonic camera (Hamamatsu C1966-20 (45)), as described previously by Milcamps (41) and adapted to screen for luciferase fusions induced during exposure to NaCl as

follows: The induction screenings were performed on PMM plates (PMM with 15g/L agar) containing 400 mM NaCl, and luciferase expression was measured after four and eight hours of incubation. Strains were selected based on an increase in luciferase expression when compared to the same strain not exposed to NaCl. Those strains with increased luciferase expression in four replicate screenings were considered further. The luciferase activity assays using a luminometer are described by Phillips (46), with the following modification: PMM was employed. One culture (OD~0.2) was split into six 23 mm culture tubes (5 mL of culture per tube) and diluted with the same amount (1:1) of PMM or PMM with 800 mM NaCl, resulting in PMM media containing 0 and 400 mM NaCl in triplicate. Light emission using a TD 20/20 luminometer was measured by adding a 100 L subsample to a 10 L BSA (2% Sigma Co) aldehyde (n-decanal, 0.2%, Sigma Co.) solution. Strains with a positive response during eight hours in three replicate experiments were explored further.

When the response to water activity and osmotic stress was tested, media with double the amount of the final concentration of NaCl were mixed 1:1 with the growing cell culture. Except in the screening in which luciferase expression is reported as RLU/mL/min, in all other studies, results are reported as RLU/mL/min/OD_{595} (RLU=relative light units). Concentrations to mimic a reduction in water activity were calculated from empirically obtained data presented by Leistner and Rodel (47) and by Brown (48). The addition to PMM of 400 mM NaCl, 520 mM Glycerol, 222 mM Sucrose, or 780 mM Polyethyleneglycol (PEG200) results in a final wateractivity (A_w) of 0.986.

144 **Growth experiments**

145 Initial growth experiments were performed as follows. Five milliliters of PMM and PMM
146 amended with 400 mM NaCl in culture tubes were inoculated with 50 L of a three-day-
147 old TY culture, incubated at 28°C, with shaking at 220 rpm. Over the course of five
148 days, growth was checked twice daily and compared to *S. meliloti* 1021. Growth curves
149 were generated using 30 mL media in 250 ml flasks, inoculated with $^{1}/_{100}$ $^{v}/_{v}$ three-day-
150 old TY culture and incubated at 28°C and agitated at 220 rpm. Antibiotics were used in
151 the following concentrations: kanamycin 25 g/mL, streptomycin 25 g/mL,
152 spectinomycin 25 g/mL, and chloramphenicol 10 g/mL. For the amino acid
153 complementation studies, 5 mL of PMM+400 mM NaCl in culture tubes were inoculated
154 with 50 L full density, 3-day-old TY cultures. Amino acids were added at a final
155 concentration of 50 g/ml. Cultures were incubated at 28°C and agitated at 220 rpm.
156

157 **Molecular methods**

158 All molecular procedures were based on protocols of Sambrook and Russell (2001), or
159 previously described by Wolk (45) and Milcamps (41). The copy number of Tn*5lux*AB
160 was determined using Southern hybridization, and the rescue, sequencing, and insertion
161 site determination were performed as described by Milcamps (41) and Wolk (45).
162 Fragments resulting from *Bgl*II and *Eco*RI restriction digests were separated on 0.7%
163 agarose gels and transferred to nitrocellulose filters using Southern blotting. *Eco*RI
164 digested, DIG* labeled pRL1063a served as the probe (Boehringer Mannheim, Richfield,
165 CT). The insertion site was determined by sequencing outward from the insertion
166 sequence using primers, as described by Milcamps (41) and comparing the sequences to

167 the *S. meliloti* 1021 database

168 (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi/). The Tn*5lux*AB insertions

169 were transduced using ΦM12 to reconstruct the same mutation in a new genetic

170 background to reduce the possibility of secondary mutations (40). Transductants were

171 selected on GTS with kanamycin and streptomycin, and the copy number of Tn*5lux*AB

172 was determined as described above.

173

174 **Molecular sequence analysis**

175 The amino acid sequences from the loci tagged in strain Sce10 and Sce11 were blasted

176 against cDart and the Conserved Domain Database at NCBI

177 (http://www.ncbi.nlm.nih.gov). The promoter prediction programs employed were (i)

178 Neural Networks (http://www.fruitfly.org/seq_tools/promoter.html, 49), (ii) Sequence

179 Alignment Kernel (http://nostradamus.cs.rhul.ac.uk/~leo/sak_demo/, 50) and (iii) Virtual

180 Footprint (http://prodoric.tu-bs.de/vfp/vfp_promoter.php, 51).

181

182 **Antibiotic susceptibility**

183 A full-density TY culture was diluted in sterile water to OD_{595} = 0.05. A volume of 100

184 L of diluted cells was spread on PMM plates, and a filter paper with a defined amount

185 of antibiotics was placed on the plate surface. Antibiotic discs were generated from filter

186 paper or supplied by Benton-Dickinson (Sparks, MD, USA). Plates were incubated for

187 three days at 28^{o}C, and growth was checked twice daily. Antibiotics tested were

188 streptomycin, kanamycin, bacitracin, cycloserine, penicillin, and vancomycin. Inhibition

189 with lysozyme was also tested by dropping 5 L of a 50 mg/ml solution on a lawn of
190 cells. Plates were checked daily for inhibition of the lawn.
191
192 **Survival during desiccation.**
193 This method was previously described by Vriezen (13), and used with the following
194 modifications: Three culture tubes containing 5 mL liquid TY medium with the
195 appropriate antibiotics were inoculated with one isolated colony from fresh TY plates and
196 grown to full OD at 28^{o}C and agitated at 220 rpm. 50 L of these cultures were
197 transferred to three culture tubes containing 5 mL PMM+400 mM NaCl and antibiotics
198 and incubated until OD_{595} values of 0.2 – 0.4 were reached. Equal amounts of cells,
199 estimated using the OD_{595} values, were concentrated in a microcentifuge (13,000 g for
200 two minutes), the supernatant was removed and the pellets washed in one milliliter of
201 PMM+400 mM NaCl. 100 L suspensions were pipetted onto a membrane filter in a
202 microcentrifuge tube. Six tubes were stored in a 450 mL glass jar containing 100 mL
203 oversaturated KCl solution, which resulted in a relative humidity in the airphase of 22%.
204 After a storage time of at least three days at RT in the dark, samples were removed and
205 exposed to 100% RH for one hour. One mL of YMB was added to resuspend the cells
206 and surviving colony forming units were established. The relative survival was calculated
207 using the colony forming units at T=0 as 100%.
208
209
210
211

Symbiotic phenotypes

The mutants selected were tested for their symbiotic phenotype as described by Milcamps (41). Inoculated seedlings were incubated for four weeks at 20^{o}C with a 12 h light and dark cycle. Alfalfa roots were checked for the occurrence of nodules.

RESULTS

Identification of a NaCl-responsive putative operon.

The goal of this work was to identify genetic loci in *S. meliloti* 1021 involved in survival during desiccation. Previous studies have shown that the survival during desiccation of *S. meliloti* increases in the presence of NaCl (10, 13). Therefore, a *S. meliloti* Tn*5lux*AB transcriptional fusion mutant bank (41) was screened for an increase in luciferase expression during exposure to 400 mM NaCl, followed by a screen for loci affecting the desiccation phenotype of mutant strains. After eight hours of exposure to NaCl twelve mutants that were significantly induced ($P<0.001$) in the presence of NaCl were identified (Figure 1) that had a single copy of Tn*5lux*AB integrated (Data not shown). These strains were annotated Sodium chloride expressed-strains, abbreviated as Sce1 to Sce12. Except for Sce5 and Sce12, all strains showed a significant increase in luciferase expression after three hours of incubation in the presence of 400 mM NaCl (Fold increase ≥ 2, $P<0.001$, $N \geq 6$). The genomic sequence of regions immediately adjacent to Tn*5lux*AB was determined for ten strains, and was used to identify the tagged loci in *S.meliloti* 1021 (Table 1). Expression of Sce10 (*asnO*) in response to NaCl was confirmed using RT-PCR (Data not shown).

Four of the ten identified Tn*5lux*AB tagged loci in strains Sce2, Sce3, Sce10, and Sce11, mapped to the same part of the genome, and had inserted in what appears to be one operon (Figure 2A). The first four genes are involved in dipeptide uptake, the fifth is a potential regulator of this operon, and the last two are hypothetically involved in the

239 synthesis of NAGGN, a compatible solute known to result in growth at increased
240 osmolarity upon osmotic shock (52, 53).

241

242 **_Ngg_::Tn_5lux_AB affects the ability to respond to water stress.**

243 To test if the tagged mutants are indeed affected in their ability to grow on increased
244 concentrations of NaCl, eight mutants (Sce1, Sce2, Sce3, Sce4, Sce6, Sce9, Sce10,
245 Sce11) were grown in PMM and in PMM amended with 400 mM NaCl. No mutant
246 showed differences in growth compared to the reference strain in PMM, indicating the
247 absence of auxotrophs under this condition. However, in the presence of 400 mM NaCl,
248 strains Sce10 and Sce11 were reduced in their ability to grow (Figure 3A). The Tn*5*
249 mutations in these two strains were reconstructed in the reference strain and their geno-
250 and phenotypes confirmed. Furthermore, complementation by a plasmid borne *asnO* in
251 Sce10 was verified (Data not shown).

252 To determine if locus *asnO* and *ngg* also affect the ability of *S. meliloti* 1021 to
253 survive desiccation, strain Sce10 and Sce11 were dried at 22% RH for three days in
254 PMM in the presence of 400 mM NaCl. As controls, the survival of *S. meliloti* 1021 and
255 strain Sce3 were also determined. As the data indicate, strains Sce3 and Sce10 were able
256 to survive desiccation to a similar degree as the reference strain ($P>0.05$), while strain
257 Sce11 was 15.4-fold reduced relative to the reference strain ($P=3.5x10^{-3}$; Figure 3B). The
258 ability of strains Sce3 and Sce10 to survive desiccation shows that neither the presence of
259 the transposon, the expression of luciferase in the presence of NaCl, the ability to grow in
260 the presence of NaCl, the presence of NAGGN, nor polar effects can account for the
261 reduced ability to survive desiccation of strain Sce11.

To determine if a reduction in water activity affects expression of *ngg::*Tn*5lux*AB, strain Sce11 was exposed to several compounds in concentrations that lead to the same reduction in water activity (A_w). The presence of 400 mM NaCl, 520 mM Glycerol, 222 mM Sucrose, or 780 mM PEG-200 in PMM, all give an A_w of 0.986 (47, 48). The results depicted in figure 3C indicate that a reduction in A_w does not affect expression of *ngg::*Tn*5lux*AB since in that case luciferase expression levels would be similar in all four conditions. Although *ngg::*Tn*5lux*AB does not respond to a reduction in water activity, the response to the same osmotic stress caused by 400 mM NaCl or 800 mM sucrose, a non-accumulating osmoprotectant in rhizobia (54), is similar in both conditions. This indicates that *ngg::*Tn*5lux*AB responds to osmotic stress and that the response to NaCl is more likely caused by osmotic stress than by ionic stress. Thus, a reduction in A_w and an increase in ionic stress do not affect expression of *ngg::*Tn*5lux*AB.

Glutamine and glutamate complementation of growth in the presence of NaCl .

AsnO and *ngg* are genetically linked in a diverse range of microorganisms and are involved in the production of NAGGN, a dipeptide that accumulates during osmotic stress (13, 44, 52, 53). The possibility that strains Sce10 and Sce11 are limited in their response to grow in the presence of glutamine under inducing and growth-limiting conditions was tested since biosynthesis of NAGGN uses glutamine as substrate. The response of strain Sce11 resembles that of the reference strain, indicating that disruption of *ngg* does not lead to malfunction in the response to NaCl in the presence of glutamine (Figure 4). However, strain Sce10 had lost its ability to grow on 400 mM NaCl in the

285 presence of glutamine, suggesting that disruption of *asn*O leads to the inability to use
286 glutamine in the response mechanism to increased NaCl concentrations. Although the
287 addition of glutamate did not affect growth in *S. meliloti* 1021 (P=0.32), it stimulated
288 growth slightly in Sce11, and reduced growth slightly in Sce10 (P=0.02, and P=0.04
289 respectively).

290

291 ***AsnO::*Tn*5luxAB* and *ngg::*Tn*5luxAB* are differentially expressed during the**
292 **stationary phase.**

293 Previous work indicated that the survival of *S. meliloti* increases 2.9 fold in the stationary
294 phase compared to the exponential phase (13). To test if *asnO*::Tn*5luxAB* and
295 *ngg*::Tn*5luxAB* are expressed during the stationary phase, luciferase activity levels were
296 measured during a growth curve (Figure 5). When growing in PMM, the growth curves
297 for strains Sce10 and Sce11 were similar to those of *S.meliloti* 1021, suggesting the
298 absence of auxotrophs under permissive conditions, although exponential growth started
299 earlier in Sce11. When luciferase induction levels were measured during the course of
300 growth, a differential response of *asnO*::Tn*5luxAB* and *ngg*::Tn*5luxAB* was found during
301 lag-, exponential-, and stationary phase. Expression of *asnO*::Tn*5luxAB* drastically
302 decreased during exponential growth of strain Sce10 and increased again in the stationary
303 phase. If responsive at all, expression of *ngg*::Tn*5luxAB* is slightly increased very early
304 during exponential growth, however, *asnO*::Tn*5luxAB* shows a decrease in luciferase
305 activity in the early stages of growth, while activity increases again during the stationary
306 phase.

307

Domain structure *AsnO* and Ngg relate to antibiotic resistance.

A blast analysis of the amino acid sequence against the cDart and the Conserved Domain Database at NCBI revealed that $AsnO_{S.meliloti\ 1021}$ contains a GnAT domain between amino acid 2-224 (Evalue=$6e^{-32}$) and is associated with a conserved Asn synthase domain located between amino acid 246-524 (Evalue=$1.7e^{-55}$) (Figure 2B). $AsnO_{S.meliloti\ 1021}$ is in a class of sequences most closely related to $AsnB_{E.coli}$ (Evalue=$5.6e^{-118}$), a glutamine hydrolyzing asparagine synthase as reported earlier (53, 55, 56). More diverse members of this group of proteins include $AsnO_{B.subtillis}$ and $LtsA_{C.glutamicum}$ (55). Although previously not appreciated, $AsnO_{S.meliloti}$ also has similarity to β-$LS_{S.clavuligerus}$, a protein with the ability to synthesize clavulanic acid, an inhibitor of β-lactamases (57).

An amino acid sequence comparison of Ngg against cDart and the Conserved Domain Databases revealed two conserved domains. The first is an acetyl transferase domain between amino acids 170-240 (Evalue=$9e^{-6}$). The second domain has highest sequence similarity to RimK RelE ligase (Evalue=$5.2e^{-7}$), cyanophycin synthase (CphA, Evalue=$1.6e^{-56}$) and dAla-dAla ligase (Ddl domain; peptidoglycan synthesis) between amino acids 287-595 (Evalue=$4.2e^{-47}$) (Figure 2B). Potential involvement in dAla-dAla ligase activity and peptidoglycan synthesis suggests that strain Sce11 is cycloserine sensitive (58, 59) and/or penicillin sensitive (60).

Because of these similarities, we hypothesized that strains Sce10 and Sce11 are affected in their ability to deal with β-lactam antibiotics since β-lactam antibiotics target the cell wall (60). To put these hypotheses to the test, Kirby-Bauer experiments were performed (Table 2). Both strains show the expected sensitivities to several antibiotics including streptomycin (all insensitive), and kanamycin (Tn5*luxAB* carries kanamycin

resistance in Sce10 and Sce11 making them less sensitive to kanamycin). Sce10 is less sensitive to the β-lactam antibiotic penicillin, and in contrast, Sce11 is more sensitive to penicillin than the reference strain *S. meliloti* 1021.

Symbiotic characterization.

To further characterize the mutants, alfalfa seedlings were infected with the Tn5*lux*AB mutant strains and the ability to nodulate was recorded. All strains formed nodules, thus no locus is essential for nodulation. When strains Sce10 and Sce11 were used to infect alfalfa roots, the strains formed pink nodules, indicating that they are nitrogen fixation proficient. Furthermore, alfalfa plants appeared the same when inoculated with *S. meliloti* 1021 or with Sce10 and Sce11, and did not appear nitrogen limited.

DISCUSSION

To address our hypothesis that a genetic mechanism is available in *S. meliloti* 1021 inducible by NaCl and affecting this organism's ability to survive desiccation (13), a genetic screen for NaCl inducible loci tagged by Tn*5lux*AB was performed. Twelve mutants harboring single transcriptional fusions that express luciferase at higher levels of NaCl than without NaCl were found (Figure 1). Four of these Tn*5lux*AB tagged loci in strains Sce2, Sce3, Sce10, and Sce11, form what appears to be one operon (Figure 2A). Parts of this operon were previously identified and described (14, 53, 55, 56). Genetic loci part of this operon are known to be responsive to a decrease in oxygen availability (56), as well as to an increase in NaCl (61), and to PhoB independent phosphate limitation (62).

Our hypothesis that certain NaCl responsive loci are involved in survival during desiccation was supported by the results that two of the mutants (Sce10 and Sce11, with *asnO* and *ngg* tagged respectively) were reduced in their ability to grow at increased NaCl concentrations (Figure 3A), and the *ngg* locus was also involved in NaCl mediated survival during desiccation (Figure 3B). Induction of this locus is mainly osmotic stress related, and not by a reduction in water activity, nor by ionic stress (Figure 3C). Other loci in *S. meliloti* must exist that affect survival during desiccation, since *ngg::*Tn*5lux*AB is not predominantly responsive to NaCl. This conclusion is supported by the fact that survival of the reference strain *S. meliloti* 1021 is mainly affected by the presence of the chloride anion rather that its cation (13). AsnO however, is not involved in NaCl mediated desiccation resistance.

366 Although the loci tagged in Sce2, Sce3, Sce10, and Sce11 may form one large
367 operon, experimental data supporting one large transcriptional unit are still lacking. Our
368 expression data do indicate different dynamics between *asn*O and *ngg* during the
369 stationary phase. If the dipeptide uptake system and *asn*O and *ngg* do not form one
370 operon we would expect promoter sequences just upstream of *asn*O, which were not
371 found. Differential expression of *asn*O::Tn*5lux*AB and *ngg*::Tn*5lux*AB also indicate
372 regulatory sites between these two ORF's, which were not found either, nor were
373 terminator sites as indicated by Kingsford (63). Therefore, we expect these genes to form
374 one large operon unless unknown genetic elements are present in this locus.
375 AsnO and Ngg are both involved in a pathway for the production of NAGGN, a
376 powerful osmoprotectant. Its biochemical production involves two steps as reported by
377 Sagot (53). In this pathway, Ngg (N-acetylglutaminylglutamine synthetase) produces the
378 dipeptide N-acetylglutaminylglutamine (NAGG) from glutamine. AsnO is a glutamine
379 dependent amidotransferase transferring the amide nitrogen from glutamine to N-
380 acetylglutaminylglutamine to produce NAGGN. The disruption of one of both steps
381 leads to slower growth in the presence of NaCl. However, the fact that mutant Sce11
382 shows the same response to glutamine addition in the presence of NaCl as the reference
383 strain, and mutant Sce10 does not, suggests that even in the absence of functional Ngg,
384 substrate for AsnO is still present. Thus, (i) another pathway for NAGG production must
385 be present in *S. meliloti* 1021 or, (ii) enough NAGG is present to ensure production of
386 NAGGN for at least a couple of generations. Although unlikely due to the reduced
387 growth on NaCl and the postulated lack of NAGGN accumulation, a possible alternative
388 is that (iii) the Ngg::Tn*5lux*AB fusion protein in Sce11 is only partially disrupted, *e.g.*

389 the acetyl transferase domain may still function. The argument in interpretation (ii) has
390 some merit since growth complementation takes place at OD<0.6, but is reduced with
391 higher OD's (data not shown). Regardless, potential accumulation of NAGG in the *asn*O
392 mutant does not lead to increased growth and from the data it is clear that the postulated
393 reduced levels of NAGGN do not explain the reduced ability to survive desiccation of
394 strain Sce11. It is most likely that as yet unknown functions are associated with AsnO
395 and Ngg, *e.g.* involvement in cell wall metabolism for the possible reasons explained
396 next.

397 The aminoacid sequence of Ngg is most similar to cyanophycin synthase, and
398 contains two domains, NAT, or GCN-5 domain, and a Ddl domain. Ddl domains are
399 involved in the synthesis of peptidoglycan, and cell wall integrity is one of the main
400 factors affecting the ability to survive desiccation in rhizobia (15, 30, 31). Furthermore,
401 Ddl domains are found in dAla-dAla ligases, targets for cycloserine, and peptidoglycan
402 crosslinking enzymes, and for β-lactam antibiotics like penicillin. Our observations are
403 that strain Sce11 is indeed more sensitive to penicillin than the reference strain, however,
404 it is not affected by cycloserine (Table 2). The reduced ability to survive desiccation of
405 strain Sce11is explained by a weaker cell wall, which is less able to withstand the
406 extreme hypo-osmotic stress upon rehydration. That this may occur was shown
407 previously by Salema (30), and by Bushby and Marshall (31) who found the cell wall and
408 envelop to be a major target upon rehydration.

409 The aminoacid sequence of AsnO is most similar to AsnB, or asparagine
410 synthase, and contains a GnAT and Asn domain (Figure 2B). Previously not appreciated,
411 the similarity of AsnO to β-LS allows for the hypothesis that AsnO may be involved in

412 the production of a β-lactamase inhibitor. In this case, dysfunction of the production of
413 inhibitor leads to an increased resistance to antibiotics such as penicillin. The data in
414 Table 2 shows exactly that: Strain Sce10 is less sensitive to the β-lactam antibiotic.
415 Even though strains Sce10 and Sce11 form effective nodules on alfalfa, it is
416 unclear if both strains are effective during competition under conditions more closely
417 matching natural environments. Soil is a harsh environment and cells are continuously
418 exposed to challenging environmental conditions (64). Soils frequently undergo drying
419 and rewetting cycles. During drying salts accumulate which further reduces the microbes
420 ability to grow. Also, many soil organisms produce antimicrobials, which may have
421 negative effects on strains lacking locus *ngg*. The plants may reduce competitiveness of
422 strains lacking locus *ngg* by excreting toxic secondary plant metabolites. On the other
423 hand, a slight growth advantage of strain Sce11 may allow faster invasion of hair roots
424 and have a positive effect of nodule occupancy since growth may be a primary factor
425 during infection.
426 In conclusion, the function of AsnO and Ngg is not limited to the production and
427 accumulation of NAGGN alone. Firstly, NAGGN accumulation is not involved in the
428 survival during desiccation. Secondly, AsnO and Ngg may have functions like the
429 production of a β-lactamase inhibitor and involvement in cell wall metabolism. Thirdly,
430 they are involved in responses to β-lactam antibiotics, and fourthly, a regulatory function
431 has been attributed to AsnO during nodule development (14, 55), although regulation
432 remains obscure. Alternatively, it is interesting to speculate about physical location and
433 other roles, *e.g.* the opposite effects of AsnO and Ngg on antibiotic resistance may

434 indicate a physical interaction between the proteins with antagonistic effects on the cell
435 wall like changes in permeability.
436 Finally, identification of four out of ten loci that are responsive to NaCl and
437 oxygen deprivation is at least curious and was mentioned before (65). A possible
438 explanation may be the reduced solvability of oxygen in media high in salt. It may also
439 be explained by being part of the environmental niche of this organism. In the
440 rhizosphere salts accumulate and available oxygen may be in short supply. Most
441 interestingly, evidence that *asn*O and *ngg* have undergone lateral gene transfer has been
442 presented (J. A. C. Vriezen, unpublished data, 66). Nevertheless, we have identified a
443 system that warrants further investigation on both the molecular as well as the ecological
444 level.
445

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FIGURE LEGENDS

Figure 1:

Identification of *S. meliloti* 1021 mutants carrying NaCl responsive Tn*5lux*AB transcriptional fusions after eight hours of exposure to 400 mM NaCl. RLU = Relative Light Units, White bar = PMM, black bar = PMM+400 mM NaCl, Sce = Sodium chloride expressed. Error bars represent the Standard Error of the Mean (SEM, N=9) except for CV2 (N=6) and 1D1 (N=12). Using the one sided T-test with equal variance, all luciferase fusions are significantly expressed on the $P<0.001$ level.

Figure 2:

Physical map of four of the twelve identified Tn*5lux*AB tagged loci responsive to NaCl, which may form one continuous operon. (A) Large arrows indicate an identified Open Reading Frame (ORF), *smb*-number designates the *Sinorhizobium meliloti* SymB ORF reference number. The insertion sites of the Tn*5lux*AB in the respective strains are indicated. Location is approximate, but sizes are to scale. Smb20476-Smb20479 are involved in ABC transport of dipeptides. Smb20476 is a putative periplasmic dipeptide binding protein, Smb20477 a putative permease, Smb20478 the putative ATP binding protein of the permease, Smb20479 is a putative ATP binding protein, Smb20480 a putative transcriptional regulator, presumable *cis*-acting, Smb20481 is asparagin synthase, and Smb20482 a hypothetical protein. (B) Domain structure of AsnO and Ngg. Only domains with the highest blast scores are given. Abbreviations for the different domains are: Gn_At for glutamine aminotransferase, Asn_Synthase for asparagine

682 synthase, NAT_SF for A-acetyltransferase, cya_phycin_syn for cyanophycin synthase,
683 and DdlA for dAla-dAla ligase.
684
685 Figure 3:
686 Responses of strains *S. meliloti* 1021, Sce3, Sce10, Sce11, and *ngg::*Tn*5lux*AB to water
687 stresses. (A) Growth of *S. meliloti* 1021 (Ref=Reference strain), Sce10 and Sce11 in the
688 presence of 400 mM NaCl. (B) Survival during desiccation of strains *S. meliloti* 1021,
689 Sce3, Sce10, and Sce11. The error bars represent the Standard Error of the Mean (SEM)
690 measured twice in three independent incubations (N=6 for all cases except WT 1021 for
691 which N=12). (C) Responses of *ngg::*Tn*5lux*AB to a decrease in water activity
692 (A_w=0.986, 400 mM NaCl, 520mM glycerol, 222mM sucrose and 780mM PEG200), and
693 osmotic stress (Osm=800 mOsmol; 400 mM NaCland 800 mM sucrose). Luciferase
694 activity was measured after three hours of incubation. The error bars represent the
695 Standard Error of the Mean (SEM) in one independent incubation measured trice (N=3).
696
697 Figure 4
698 Growth of *S. meliloti* 1021, Sce10, and Sce11 in the presence of 400 mM NaCl with and
699 without amino acid supplementation. Data are expressed as the ratio of the OD_{595} in
700 PMM+400 mM NaCl. Error bars represent the SEM of three growing cultures measured
701 once (N=3). White bars = PMM+400 mM NaCl, grey bars = PMM+400 mM
702 NaCl+Glutamate, black bars = PMM+400 mM NaCl+Glutamine.
703
704

Figure 5

(A) Response of *asnO*::Tn*5lux*AB and *ngg*::Tn*5lux*AB during growth of strains *S. meliloti* 1021, Sce10, and Sce11 in liquid PMM. Error bars for OD measurements represent the Standard Error of the Mean (SEM) of three independent cultures (N=3). Error bars for RLU's represent the SEM of three independent cultures in duplicate (N=6). Black square = Growth of *S. meliloti* 1021 in Optical Density (OD_{595}), Black circles = growth of Sce11 in OD, Black triangles = growth of Sce10 in OD, Open circles = Luciferase activity of *ngg*::Tn*5lux*AB in RLU/min/mL/OD, Open triangles = Luciferase activity of *asnO*::Tn*5lux*AB in RLU/min/mL/OD.

716 **TABLES**

717 Table 1: Selected NaCl responsive Tn*5lux*AB transcriptional fusions in *S. meliloti* 1021 after eight hours of incubation.

	Sequencing and blast results				
Strain	Description[a]	Locus	Gene/Locus name[b]	Location Tn*5lux*AB[c]	References
S. meliloti 1021					38
Sce1	Hypothetical protein with SH3 region	*smc01590*		256-265	This study
Sce2	Putative dipeptide transporter permease and ATP binding protein	*smb20478*		572-580	This study
Sce3	Putative dipeptide ABC transporter ATP binding protein	*smb20479*		124-133	This study
Sce4	Putative nutrient deprivation Induced	*smb20227*	*ndiA1*	299-307	67, 68
Sce5	Hypothetical protein HAD-superfamily	*smc04299*	*loe3**	-1-7	56
Sce6	Conserved hypothetical protein	*smc01445*		4-12	This study
Sce7	NS				This study
Sce8	Putative methyl transferase	*smb20238*	*loe2**/C1	17-26	41, 56
Sce9	NS				This study
Sce10	Asparagine synthase	*smb20481*	*loe4**/*asnO*	979-987	14, 44, 53, 55, 56, 69
Sce11	GCN5 related N-acetyltransferase ATP grasp	*smb20482*	*loe6**/*ngg*	1058-1066	14, 44, 53, 56, 69
Sce12	Short chain alcohol dehydrogenase	*smb20073*		273-281	This study

(a) NS=not sequenced.

(b) *=Low Oxygen Expressed.

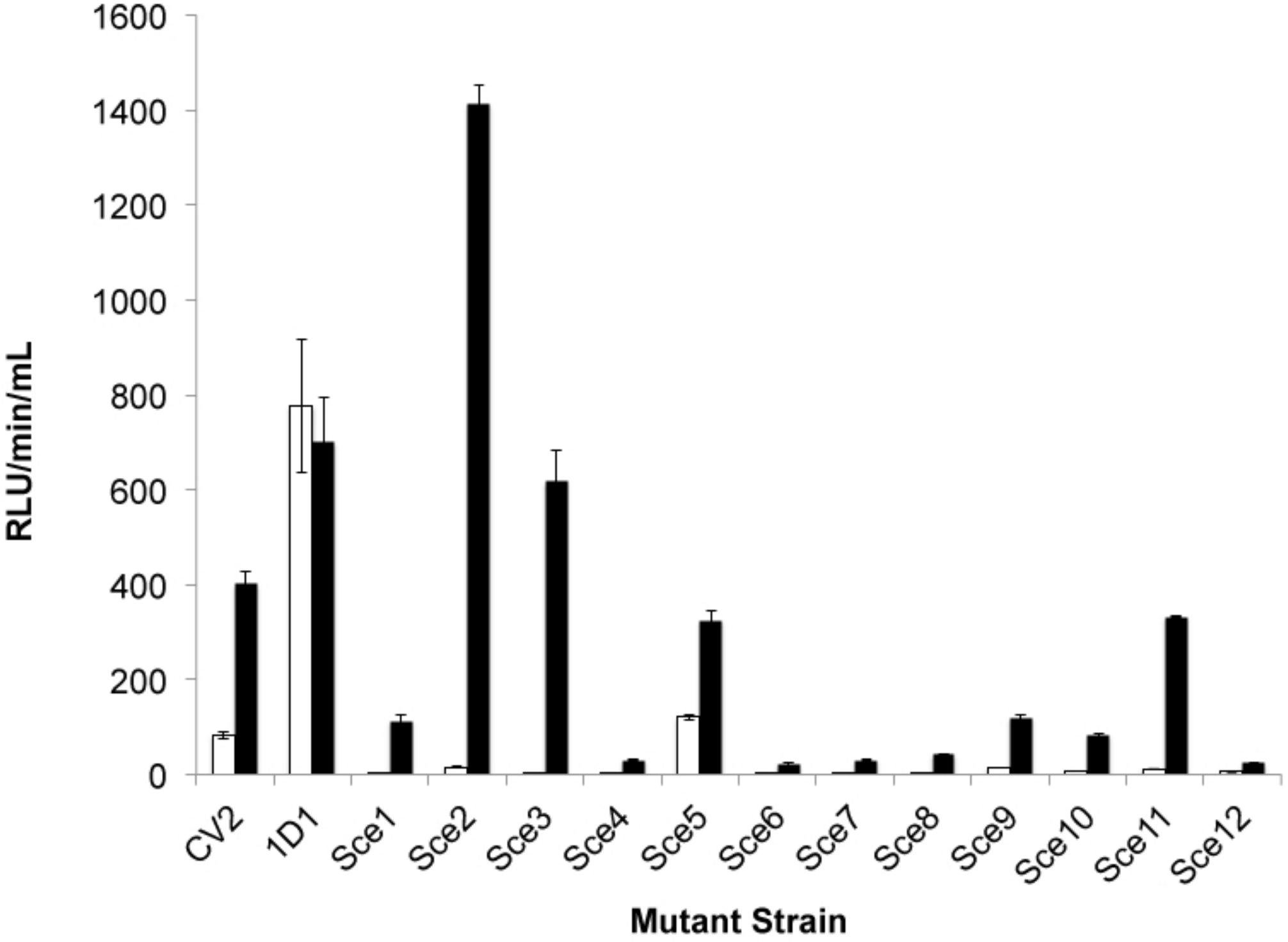
(c) Location of the Tn*5luxAB* insertion relative to upstream ATG.

Table 2: Antibiotic sensitivity assay. Presented are the diameters (mm) of clearance around the disc (7 mm across) together with the associated Standard Error of the Mean (SEM). For each experiment the average of three plates is presented (N=3).

	Amount	*S. mel* 1021		Sce10		Sce11	
Antibiotic	(IU or g)/disc	Diameter (mm)	SEM	Diameter (mm)	SEM	Diameter (mm)	SEM
Streptomycin	10	7.0	0.0	7.0	0.0	7.0	0.0
Kanamycin	30	11.3	0.3	7.0*	0.0	7.0*	0.0
Bacitracin	2 IU	7.0	0.0	7.0	0.0	7.0	0.0
Lysozyme	250	7.0	0.0	7.0	0.0	7.0	0.0
Cycloserin	100	9.7	0.3	10	0.6	9.7	0.7
Penicillin	10 IU	14.3	0.9	7.7*	0.7	22.7*	1.5
Vancomycin	5	21.3	1.2	19	0.0	22.7**	0.3

* Significant differences ($P<0.01$, two-sided T-test with equal variance) between the reference strain and strains Sce10 and Sce11.

** Significant differences ($P<0.01$, two-sided T-test with equal variance) between strains Sce10 and Sce11.


1600
1400
1200
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800
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0
RLU/min/mL
CV2
1D1
Sce1
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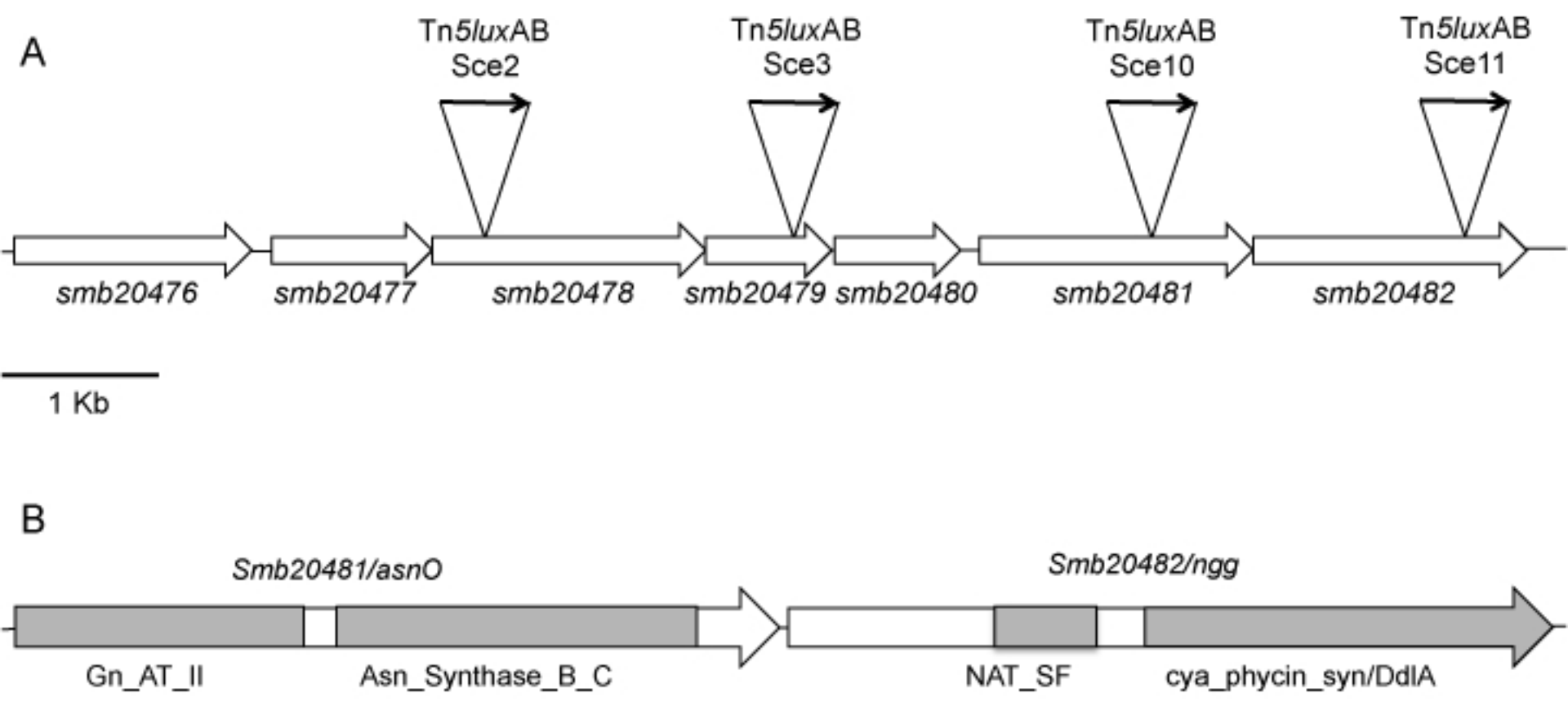
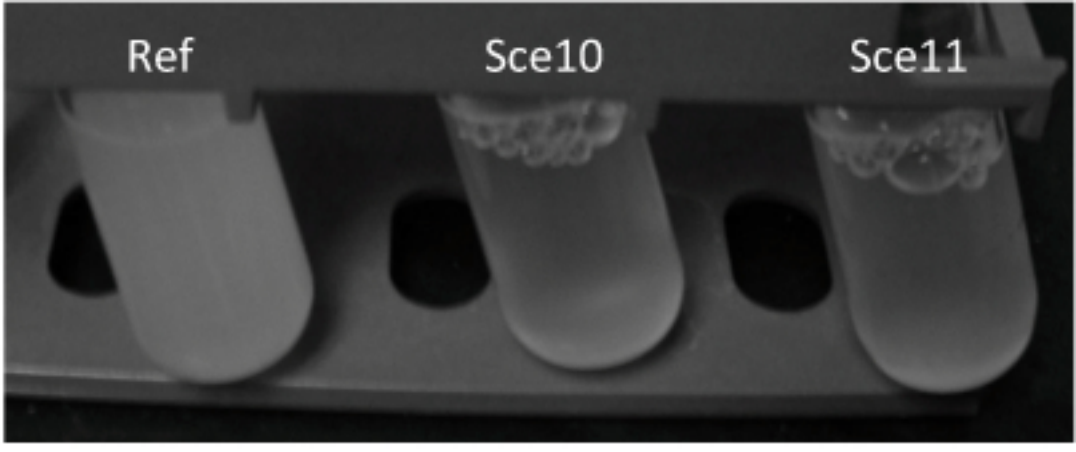
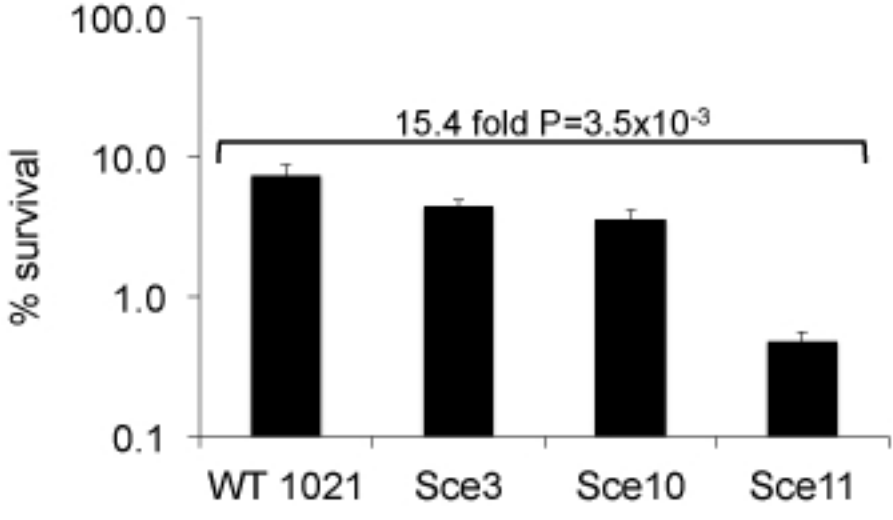
Sce8
Sce9
Sce10
Sce11
Sce12
Mutant Strain


A
Tn5luxAB
Sce2
Tn5luxAB
Sce3
Tn5luxAB
Sce10
Tn5luxAB
Sce11
smb20476
smb20477
smb20478
smb20479
smb20480
smb20481
smb20482
1 Kb
B
Smb20481/asnO
Smb20482/ngg
Gn_AT_II
Asn_Synthase_B_C
NAT_SF
cya_phycin_syn/DdlA

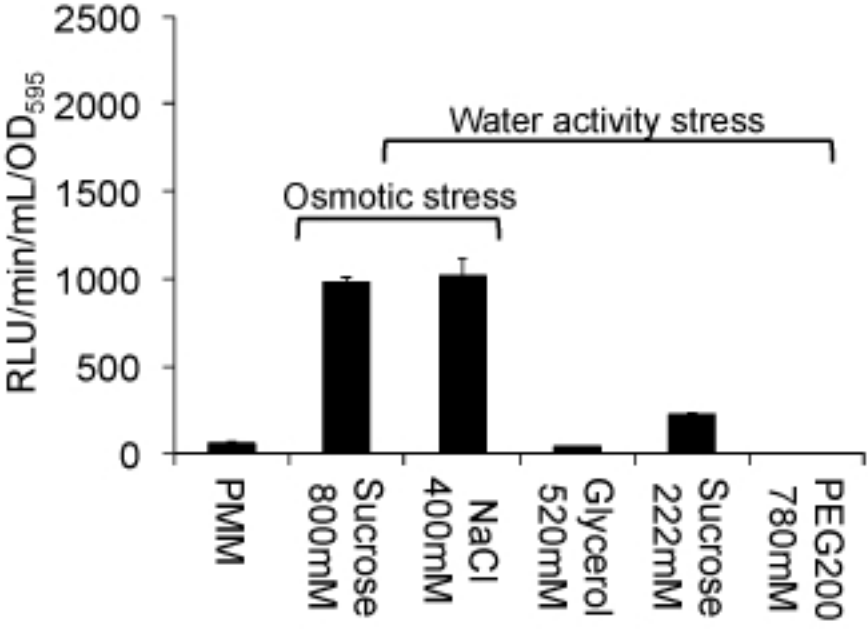
A

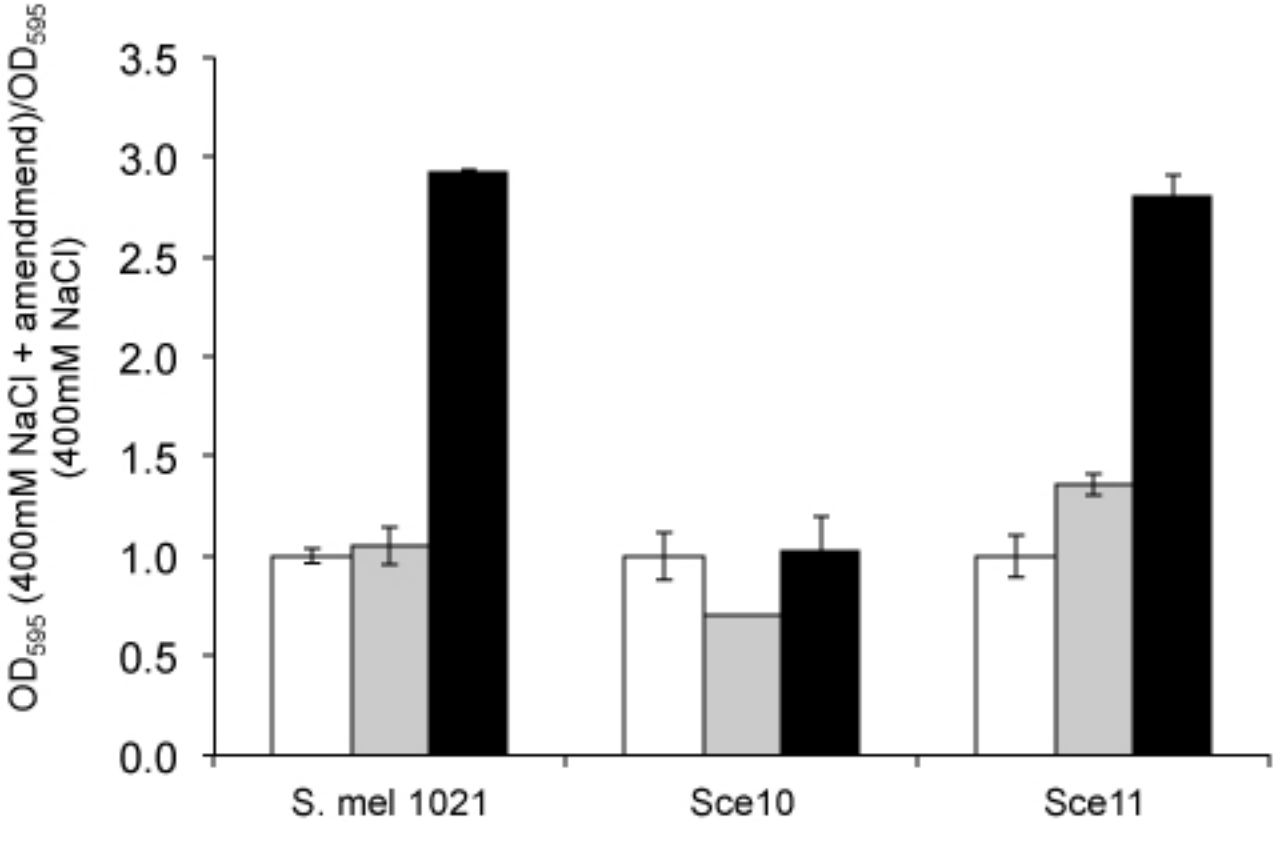

Ref
Sce10
Sce11

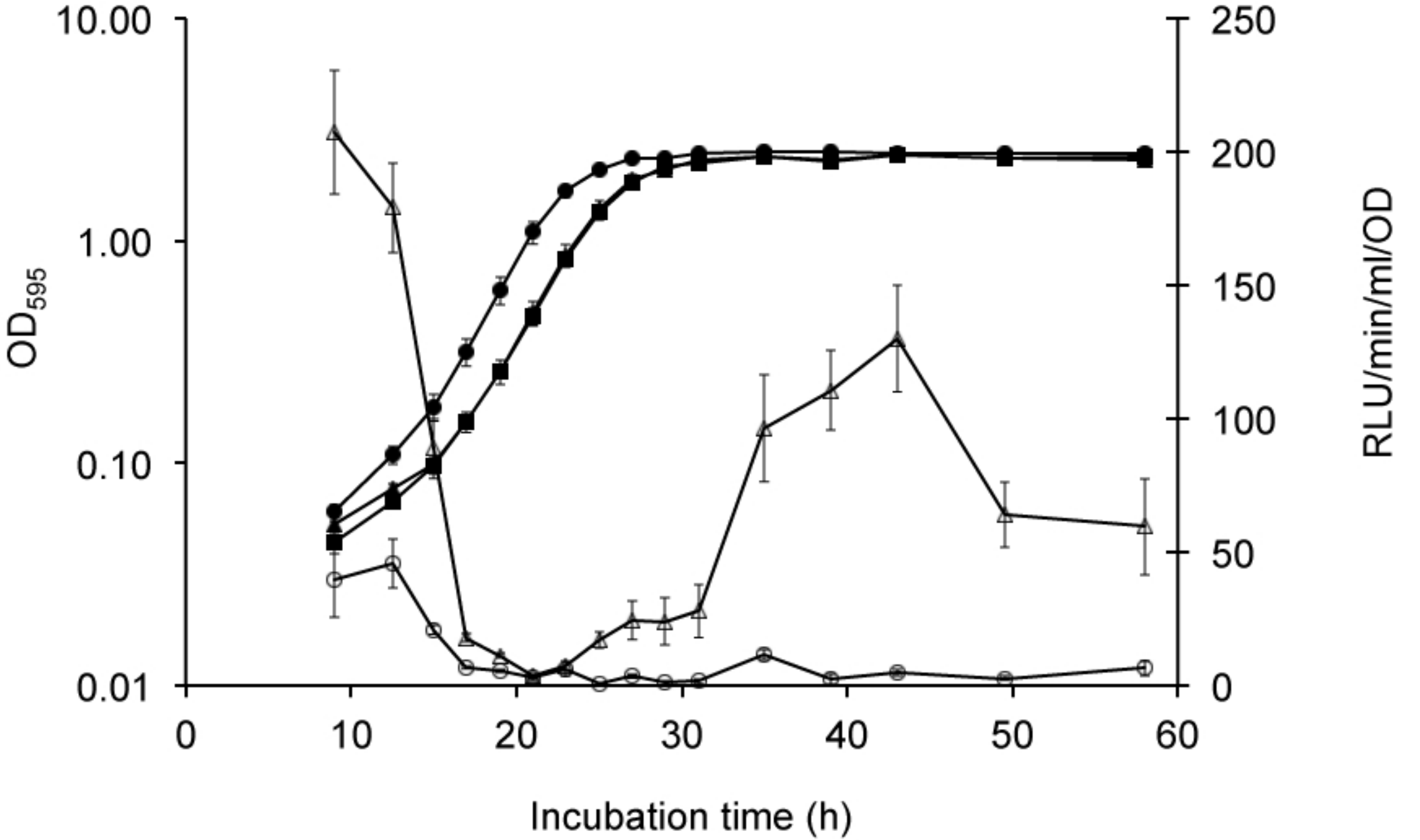
B


100.0
10.0
1.0
0.1
% survival
15.4 fold P=3.5x10-3
WT 1021
Sce3
Sce10
Sce11

C


2500
2000
1500
1000
500
0
RLU/min/mL/OD595
Water activity stress
Osmotic stress
PMM
Sucrose 800mM
NaCl 400mM
Glycerol 520mM
Sucrose 222mM
PEG200 780mM


OD595 (400mM NaCl + amendmend)/OD595 (400mM NaCl)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
S. mel 1021
Sce10
Sce11


10.00
1.00
0.10
0.01
OD595
250
200
150
100
50
0
RLU/min/ml/OD
0
10
20
30
40
50
60
Incubation time (h)