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1 **WORKING TITLE:** A Novel Denitrification Regulator, Adr, Mediates Denitrification Under
2 Aerobic Conditions in *Sinorhizobium meliloti*.
3 **RUNNING TITLE:** Aerobic Denitrification in *Sinorhizobium meliloti.*
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24 **Abstract**

25 *Sinorhizobium meliloti* is a soil dwelling bacteria capable of forming a symbiotic
26 relationship with several legume hosts. Once symbiosis is established, *S. meliloti* fixes
27 atmospheric nitrogen into nitrogenated compounds, thus carrying out an important step in the
28 nitrogen cycle. *S. meliloti* is also capable of the reverse process, denitrification, the reduction of
29 nitrate and nitrite to nitrogen gas. In this study we have identified a novel regulator of
30 denitrification in *S. meliloti*, Adr, which affects the expression of the denitrification genes in
31 aerobically grown cultures. Analysis of the Adr sequence reveals a LuxR-like quorum sensing
32 regulator, however, it does not respond to the known quorum sensing signals produced by *S.*
33 *meliloti*. Additionally, we show that FixJ, the major regulator of denitrification and microaerobic
34 respiration in *S. meliloti*, is active under our growth conditions. Comparison of the FixJ
35 microarray to our Adr microarray shows a significant overlap between the two regulons. We also
36 show that while Adr is not necessary for symbiotic nitrogen fixation, a functional copy of this
37 regulator confers a competitive advantage to *S. meliloti* during host invasion. Our findings
38 suggest that Adr is a new type of denitrification regulator and that it acts at the same regulatory
39 level as FixJ.

40 **Importance**

41 Rhizobia contribute to the nitrogen cycle by fixing atmospheric nitrogen to nitrogenated
42 compounds and by denitrification, the reduction nitrate and nitrite to nitrogen gas. Denitrification
43 enhances the survival of *Sinorhizobium meliloti* in the various environments it may encounter,
44 such as free-living conditions in the rhizosphere, during invasion of the plant host, and after a
45 symbiotic relationship has been established. Oxygen concentration is the typical signal for
46 denitrification gene expression. Recent studies of low oxygen cultures of *S. meliloti* have

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47 outlined the regulation structure for denitrification. In this study, we examine the regulation of
48 denitrification in aerobically grown *S. meliloti* cultures. Understanding how *S. meliloti* responds
49 to various oxygen concentrations will result in a more complete picture of denitrification
50 regulation in this agriculturally important organism and the impact of denitrification on the soil
51 microbiome as a whole.
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53 **Introduction**

54 *Sinorhizobium meliloti* is an aerobic soil dwelling α-proteobacteria that is found in a
55 variety of environments in which oxygen concentrations fluctuate, including free-living in the
56 soil or in association with a plant host. Like many *Rhizobiaceae, S. meliloti* contributes to the
57 nitrogen cycle, either by fixing atmospheric nitrogen as bacteroids in conjunction with a legume
58 host (*Medicago sativa*) or by breaking down nitrates and nitrites to nitric oxide, nitrous oxide, or
59 dinitrogen gas (Figure 1) (1). Although these two processes seem incongruous, both play an
60 important role in *S. meliloti* (2, 3). During symbiosis with a legume host, oxygen limitation is
61 intrinsic to the nodule and is required for the expression and function of the nitrogenase enzyme.
62 The first step of denitrification, the reduction of nitrate to nitrite, removes any excess reducing
63 power that may be present in the cell (4). Further steps remove nitrite from the nodule, ensuring
64 the optimal environment for nitrogenase function. Aside from the presence of NO_x (NO_3^-, NO_2^-,
65 NO, or N_2O), the major signal for the expression of the denitrification pathway is oxygen
66 limitation. Denitrification usually occurs under anaerobic or low oxygen tension conditions
67 where oxygen cannot serve as an efficient electron acceptor. However, it is now well established
68 that denitrification can occur aerobically, either with no or partial oxygen limitation, in several
69 bacteria, including *Paracoccus denitrificans*, *Pseudomonas aeruginosa,* and *Agrobacterium sp*.
70 (5). While denitrification is documented in *S. meliloti,* most reports focus on microaerobic
71 conditions such as those found in the nodule (6, 7). The primary role of aerobic denitrification is
72 likely the removal of excess reducing power or detoxification of NO_x found in the environment
73 (5). Since respiratory reduction of NO_x is coupled with energy generation, denitrification even in
74 the presence of oxygen can enhance bacterial survival in environments where the oxygen
75 concentration may fluctuate (5).

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76 Denitrification may also allow *S. meliloti* to remove nitrate or nitrite from the soil near
77 plant roots as these compounds inhibit bacterial attachment to host roots (8). While migrating
78 through the infection thread during root invasion, the ability of *S. meliloti* to denitrify enhances
79 its survival when it encounters plant defense responses such as nitric oxide bursts (9). Once *S.*
80 *meliloti* successfully inhabits the nodules, denitrification is thought to be active to reduce nitrate,
81 as it is inhibitory to nitrogenase activity (10).

82 An overview of the regulation of denitrification and nitrogen fixation in *S. meliloti* is
83 shown in Figure 8. The FixL/FixJ two component system is essential for *S. meliloti* to form a
84 symbiotic relationship with a legume host (11). FixL is a membrane bound sensory kinase that
85 detects oxygen levels in the environment. When the oxygen concentration in the environment
86 drops, FixL autophosphorylates and transfers the phosphate to FixJ (12). Previous studies have
87 demonstrated that phosphorylation of FixJ relieves the weak interaction of the FixJ DNA binding
88 domain (C-terminal) and the signal recognition domain (N-terminal) by triggering a
89 conformational change (13). Once this interaction between domains is abolished, FixJ is able to
90 bind DNA and/or dimerize (14). The FixJ C-terminal domain alone is able to recognize
91 promoters and activate transcription in the absence of phosphorylation, which supports the
92 theory that the N-terminal signal recognition domain is regulatory in nature (15). Though
93 dimerization is not essential for DNA binding or promoter recognition, it is thought to contribute
94 to promoter binding affinity (14).

95 Two component systems similar to FixL/FixJ are fairly ubiquitous in nitrogen fixing
96 bacteria. However, regulation downstream of FixJ can vary greatly (16). In *S. meliloti* FixJ has
97 five direct targets: *nifA*, *fixK1*, *fixK2*, *proB2,* and *SMc03253* (17, 18). NifA is located on the
98 symbiotic plasmid, pSymA, and is essential for nitrogen fixation. It is an enhancer-binding

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99 protein that acts in conjunction with σ^{54} to control the expression of nitrogenase (*nifH, nifDK*)
100 and a high oxygen affinity cytochrome oxidase (*fixABCX*) (11). Two copies of FixK are also
101 found on pSymA and are classified as *crp/fnr*-type regulators that control the expression of the
102 denitrification genes (*nap, nir, nor, nos*) as well genes responsible for the synthesis of an oxidase
103 with high oxygen affinity (*fixNOPQ*) (19). It is unknown why chromosomally located *proB2* and
104 *SMc03253,* both involved in proline metabolism, are direct targets of FixJ (17, 18).

105 Unlike many other FixL/FixJ systems found in rhizobia, FixJ does not directly
106 autoregulate in *S. meliloti* (Figure 8). Instead, FixK controls the expression of *fixT,* an antikinase
107 that represses the FixLJ regulon by preventing the autophosphorylation of FixL (20). Repression
108 by FixT is abolished in the absence of the glutamine-dependent asparagine synthetase, *asnO,*
109 though how this occurs is still poorly understood (21). One possibility suggests that AsnO may
110 serve a regulatory role by monitoring nitrogen balance within the cell via a metabolite, such as
111 glutamine, and controlling nitrogen fixation accordingly (21). However, more study is required
112 to determine how the interaction works, though it is known that neither FixT nor AsnO is
113 required for nitrogen fixation in *S. meliloti* (22).

114 In addition to acting as an oxygen sensor, FixL also detects nitric oxide. Meilhoc et al.
115 demonstrated that FixLJ along with the nitric oxide response regulator (NnrR) are involved in the
116 *S. meliloti* response to nitric oxide exposure (23). The nitric oxide response was observed when
117 lag phase cultures were exposed to a nitric oxide donor under aerobic conditions, as well as
118 inside the nodule.

119 During symbiosis, oxygen concentrations can be as low as 5-30 nM within legume
120 nodules, compared to the aerobic conditions in culture media where oxygen concentrations are
121 approximately 250 μM at the start of growth (22, 24). Since most denitrification studies are

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122 performed under low oxygen tension and/or early in culture growth, we found that there is
123 limited literature regarding denitrification in *S. meliloti* under conditions such as the higher cell
124 population densities, which occur in the rhizosphere prior to root invasion. Previous unpublished
125 work performed in our laboratory has revealed that a LuxR-like regulator, SMc00658, is
126 involved in the regulation of denitrification at high cell population densities in aerobically grown
127 cultures (Patankar AV and González JE, unpublished data). For this reason, we have changed the
128 name of the *SMc00658* locus to the aerobic denitrification regulator, *adr*. Unlike traditional
129 LuxR-like response regulators, Adr does not respond to the *N*-acylhomoserine lactone (AHL)
130 molecules produced by *S. meliloti* (25). It also does not appear to be regulated by the legume
131 symbiosis quorum sensing regulator ExpR. In the absence of Adr, we see a dramatic drop in the
132 expression of *fixK*, *nifA*, and the downstream genes (*nap*, *nir*, *nor*, *nos*) which is remarkably
133 similar to the expression profile of a *fixJ* mutant (17). This change in expression is independent
134 of the presence or absence of *S. meliloti* AHLs (Patankar AV and González JE, unpublished
135 data).

136 In this study, we show that the FixLJ system is actively expressed under aerobic growth
137 conditions and that Adr is necessary for this activity. We also demonstrate that while Adr has a
138 dramatic effect on the expression of FixK and NifA under aerobic conditions, it is not required
139 for nitrogen fixation in the nodule.

140 **Results**

141 **Adr sequence analysis.** Initial sequence analysis suggests that Adr belongs to the LuxR
142 family of transcriptional regulators. These regulators are identified by a carboxyl-terminal helix-
143 turn-helix motif and an amino-terminal signal recognition domain. LuxR family regulators are
144 best known for their role in quorum sensing, where they act as the signal response regulator.

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145 However, the LuxR family is large and loosely conserved, with a reported 18-25% overall
146 sequence identity (26). *S. meliloti* has several characterized LuxR-like regulators, including the
147 quorum sensing regulators ExpR and SinR, the motility regulators VisN and VisR, and the
148 methyl cycle regulator NesR (27-29). Here we report a new LuxR-like transcriptional regulator
149 in *S. meliloti,* Adr, which appears to regulate aerobic denitrification.

150 Though members of the LuxR family generally have very low sequence identity, 95% of
151 known LuxR family members share nine conserved residues that are important for DNA binding
152 and AHL signal recognition (Figure 2) (30). Sequence analysis shows that Adr only has four of
153 these nine conserved residues, three of the DNA binding residues and only one of the signal
154 recognition residues. Based on this analysis, it is unlikely that Adr responds to AHL signals.
155 Additionally, when *S. meliloti* is unable to produce AHLs, there is no effect on the expression or
156 function of Adr when compared to AHL producing cultures (Patankar AV and González JE,
157 unpublished data).

158 Among functionally characterized LuxR-like regulators reported to date, AvhR (a
159 regulator from *Agrobacterium vitis* responsible for plant necrosis) shows the most sequence
160 homology to Adr (28%). Consequently, this regulator also lacks the typically conserved AHL
161 binding residues found in other classical quorum sensing LuxR type regulators (31).

162 **Disruption of *adr* reduces expression of denitrification and nitrogen fixation genes.**

163 To determine the regulatory role of Adr during normal growth of *S. meliloti*, we conducted a
164 microarray analysis to compare the transcriptomic profiles of wild-type *S. meliloti* Rm8530 to
165 Rm8530 *adr*. Since Adr is similar to quorum sensing related regulators, conditions for the
166 microarray followed those used for other quorum sensing expression analysis microarrays (27,
167 32). Cultures were aerated and grown to early stationary phase (OD_{600} 1.2) before RNA was

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168 harvested. Preliminary analysis performed using the AffyMetrix GCOS software revealed that
169 427 genes were differentially regulated between the wild-type and the *adr* mutant (Supplemental
170 Table S1). Of these, 247 genes were downregulated in the *adr* mutant. Within this set of
171 downregulated genes, we were particularly interested in the genes involved in denitrification
172 (*nap, nir, nor, nos*) and microaerobic respiration (*fix*) (Table 4). Further analysis of the
173 microarray data revealed that the regulatory genes *fixK1, fixK2,* and *nifA* were also
174 downregulated in the absence of Adr. Previous work on the denitrification pathway performed by
175 Bobik et al. revealed that FixJ is the major regulator of limited oxygen response in *S. meliloti*.
176 This response includes activating the denitrification pathway. When comparing our microarray
177 results to those obtained by Bobik et al. we observed an 83% overlap of genes regulated by FixJ
178 and Adr, indicating that Adr is a yet uncharacterized regulator involved in the denitrification and
179 limited oxygen response pathways in *S. meliloti* (Table 4) (17).
180 To verify our microarray results, several genes involved in the denitrification pathway
181 were selected for quantitative real time-PCR analysis (Figure 3). Results are represented as fold
182 change, which is $2^{\Delta Ct}$, where ΔCt equals the difference in expression of the wild-type from the
183 mutant. *napA, nirK, norC,* and *nosZ* are structural subunits of nitrate reductase, nitrite reductase,
184 nitric oxide reductase, and nitrous oxide reductase, respectively. Genes were selected based on
185 M-value (>1.5). The results of this analysis indicate a significant downregulation (5- to 300-fold
186 change) in each step of the denitrification pathway in the absence of Adr (Figure 3a). We also
187 included *fixK* and *nifA* in this analysis since these two regulators also appeared in our microarray
188 data. The reduction of *fixK* (16-fold) and *nifA* (42-fold) expression seen in Figure 3a in the *adr*
189 mutant is typically seen in a *fixJ* mutant, indicating that Adr impacts these genes even in the

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190 presence of FixJ. The decrease of FixK is likely causing the downstream effect in the
191 denitrification genes.

192 We next measured the effect of Adr on other FixK dependent genes, including the two
193 subunits of high oxygen affinity cytochrome oxidases, *fixG* and *fixQ1* (Figure 3b) (33). As was
194 the case with the denitrification pathway genes, the lack of Adr lead to a decrease in *fixG* and
195 *fixQ1* expression (39-fold and 42-fold respectively), confirming that FixK is downstream of Adr
196 when regulating genes related to the denitrification and limited oxygen response pathways.

197 We also measured *nifA* expression since, like FixK, it is controlled by FixJ. As was the
198 case with *fixK,* the removal of Adr leads to a decrease in expression of *nifA* (Figure 3a).
199 However, unlike with FixK, we found that genes downstream of NifA, such as *nifH* or *fixA,* were
200 not affected by the presence or absence of Adr (Figure 3b). This is unsurprising since previous
201 studies have shown NifA to be sensitive to oxygen levels higher than those found in the nodule;
202 though the *nifA* gene is being expressed, its translated form is not active under our aerobic
203 growth conditions (3).

204 Though not seen in our microarray data, we also tested the effect of Adr on *nnrR*, the
205 nitric oxide response regulator. This regulator induces the transcription of nitrite reductase
206 (*nirKV*) and nitric oxide reductase (*norECBQD*) in the presence of nitric oxide. While FixLJ also
207 responds to nitric oxide, NnrR is not in the FixJ regulon (23). Under our conditions, we saw no
208 differential expression of *nnrR* (data not shown).

209 Since the expression of both *nifA* and *fixK* are impacted by the absence of Adr, it appears
210 that Adr is acting in a manner that is either parallel and/or in conjunction with FixJ.

211 **Adr and FixJ regulate denitrification in tandem.** Previous studies of *S. meliloti*
212 denitrification have revealed FixJ as the major denitrification regulator (Figure 8) (17, 34). These

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213 studies approached denitrification regulation by examining expression under microoxic (<2%
214 oxygen) conditions, comparable to those found before the cells enter the nodule as well as after
215 the cells differentiate into nitrogen fixing bacteroids. In our study, we show that many of the
216 same genes regulated by FixJ also show differential expression in the absence of Adr in
217 aerobically grown cells. Though not a direct comparison due to the difference in conditions, we
218 found that 83% of genes reported by Bobik, et al. to be regulated by FixLJ are also differentially
219 expressed in the Adr mutant (17). Additionally, it was also noted that the five genes directly
220 regulated by FixJ (*fixK1*, *fixK2*, *nifA*, *proB2*, and *SMc03253*) were also found to be regulated by
221 Adr (Table 4) (18).

222 In light of these findings, we generated a *fixJ* mutant to determine if the FixLJ system is
223 active under the same conditions as Adr. qRT-PCR analysis of the *fixJ* mutant revealed that the
224 FixJ is actively influencing denitrification expression in aerated stationary phase cultures (Figure
225 4a). Direct targets of FixJ such as *fixK* and *nifA* were downregulated (approximately 1000-fold)
226 in the *fixJ* mutant; downregulation was also see in the indirect targets including the
227 denitrification (100- to 1000-fold) and microoxic respiration genes (65- to 70-fold) (Figure 4b
228 and 4c). However, as with the *adr* mutant, we did not see a large change in expression of genes
229 controlled by NifA (a direct target of FixJ) such as the nitrogenase structural gene *nifH* or a
230 subunit of another oxidase, *fixA* (Figure 4c). This supports data from other laboratories that
231 found that nitrogenase and its auxiliary proteins are only expressed in bacteroids (3).

232 The presence of an active FixJ revealed the possibility that *adr* is either regulated by the
233 FixLJ system or regulated independently but affecting the same genes. To this end, we measured
234 the expression of *adr* in the absence and presence of *fixJ*. We also tested the reverse to determine

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235 if *fixJ* expression depends on the presence of *adr.* In both cases we saw no differential expression
236 between the wild-type and mutant backgrounds (data not shown).

237 A *fixJ adr* double mutant was also constructed to determine if there is an additive effect
238 on expression by Adr and FixJ. Expression levels of *fixK, napA, nirK, norC,* and *nosZ* were
239 compared between the *adr*, *fixJ*, and *fixJ adr* double mutant. Results show that there is no
240 compounding effect by introducing an *adr* mutation into a *fixJ* mutant (Figure 4a).

241 To verify that Adr is working at the FixJ level, we measured the expression of the
242 denitrification genes in a *fixK* double mutant. The results confirm that when FixK is not
243 expressed, which we suspect occurs in an *adr* mutant, the denitrification genes are not expressed
244 (Figure 4a).

245 **Expression of *adr* during growth.** Past studies have focused on the activity of FixJ and
246 as a consequence experiments were performed under conditions that lead to FixLJ activation.
247 Therefore, previous work was generally done early in the growth cycle (OD_{600} 0.2-0.5) and
248 under microoxic (2% O_2) conditions. To determine if growth phase had an effect on Adr
249 expression, we performed additional expression measurements during lag (OD_{600} 0.2) and mid
250 exponential (OD_{600} 0.8) growth which was compared to our stationary phase data (OD_{600} 1.2).
251 Results show that there is very little change in expression of the *napA*, *nirK*, *norC*, and *nosZ*
252 genes during lag growth when wild-type was compared to the *adr* mutant. However, activation
253 of denitrification gene expression occurs during mid exponential phase and continues into
254 stationary phase (Figure 5). We also analyzed the expression of *adr* over time and found that
255 there is no difference in expression of the gene in wild-type Rm8530 between the three time
256 points tested (Table 5).

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257 Since differential expression due to Adr was detected during exponential growth, we next
258 attempted to determine if a media soluble signal was responsible for this change. Spent media
259 was harvested from early stationary phase cultures and used to grow fresh cultures of Rm8530
260 and Rm8530 *adr* to OD_{600} 0.2. While no difference in expression was seen between cultures
261 using fresh versus spent media, we do not rule out the possibility of a signal produced by the cell
262 involved in the activation of Adr (data not shown).

263 **Effect of the absence of FixL.** As discussed previously, FixL is the oxygen sensing
264 component of the FixLJ system. Our data presented here shows that there is a relationship
265 between Adr and FixJ under aerobic conditions. To determine if FixL has a role in this
266 relationship by interacting with Adr, a *fixL* mutant was generated and expression of *adr* was
267 measured. Expression of *adr* was not affected by the absence of FixL in stationary phase (data
268 not shown).

269 **The Adr mutant shows decreased survival under oxygen limitation in the presence**
270 **of nitrite.** Since Adr enhances expression of the denitrification pathway under aerobic
271 conditions, we measured the effect of Adr during aerobic growth. Rm8530 (wild-type) and
272 Rm8530 *adr* were grown in TYC media supplemented with either 10 mM KNO_3, or 5 mM
273 $NaNO_2$ was. The growth rate was similar between all strains under all three conditions (Figure
274 6a). Viable counts were performed on aerobically grown cultures after 72 hours to determine
275 survival of *S. meliloti* under conditions listed above. No difference in survival was observed
276 (Figure 6b).

277 Since no difference in growth or survival was observed during aerobic growth conditions,
278 we also conducted the same growth and survival assay under microaerobic conditions. Cultures
279 were grown in LB/MC for two days then diluted to OD_{600} 0.2 in TYC before microaerobiosis

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280 was induced by filling the headspace of the vial with a mixture of 2% oxygen and 98% argon
281 gas. Figure 6c shows that, while growth appeared to be similar between the wild-type and mutant
282 under all three conditions, the number of viable colony forming units dropped significantly when
283 the *adr* mutant was grown in media containing nitrite (Figure 6d). Anaerobic conditions were
284 also tested but no growth or denitrification was seen after seven days of incubation (data not
285 shown).

286 **The nitrate reductase activity in *S. meliloti***. The *S. meliloti* nitrate reductase is encoded
287 by the *nap* genes, which are controlled by FixJ, though expression of these genes in *S. meliloti*
288 was thought to be constitutive regardless of oxygen concentration (2, 35). The microarray data
289 and qRT-PCR analysis presented here indicate otherwise. We have shown that the *nap* genes are
290 expressed by wild-type *S. meliloti* during aerobic growth, but that the expression is controlled by
291 both FixJ (through FixK) and Adr (Figure 4a). Previous studies have demonstrated that under
292 microaerobic conditions, the *S. meliloti* strain Rm2011, a strain very similar to Rm8530, has an
293 active nitrate reductase and can convert nitrate to nitrite during growth (36). To determine if the
294 transcripts of the *nap* genes are being translated into a functional enzyme in our strains, we
295 performed assays using methyl viologen to determine the Rm8530 and Rm8530 *adr* nitrate
296 reductase activities. Cells grown aerobically or microaerobically in nitrate free minimal media
297 were tested. In both the aerobically grown wild-type and *adr* mutant samples, no change in
298 absorbance was observed after adding 10 mM of KNO_3, indicating that the conditions tested,
299 these strains do not produce detectable levels of nitrate reductase. The same results were seen in
300 cells grown in 2% oxygen (data not shown). However, when the cells were grown for 72 hours
301 such as in Figure 6, low amounts of nitrite were detected qualitatively (via nitrite detection

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302 strips) in cultures of both Rm8530 and the *adr* mutant grown in 10 mM KNO_3, indicating that
303 there is a low level of nitrate reductase activity.

304 **The Adr mutant has reduced nitrite reductase activity.** *S. meliloti* encodes a copper-
305 containing nitrite reductase, *nirK*, whose enzyme product is found in the periplasm (35, 37). As it
306 appears that *S. meliloti* is incapable of utilizing nitrate for the first step of denitrification, we next
307 tested whether nitrite could be used to initiate the reaction. Under microaerobic conditions, wild-
308 type Rm8530 showed a growth and survival deficiency (Figure 6c and 6d). However, this growth
309 defect was not as dramatic as that seen in the *adr* mutant. This led us to believe that Rm8530 is
310 capable of using nitrite to initiate denitrification, and that the inability to do so prevents the
311 mutant from removing this toxic compound from the environment, resulting in slower growth
312 and poor survival. To test the nitrite reductase activity, we performed the same methyl viologen
313 assay as described above, using 10 mM sodium nitrite as the substrate. We found the nitrite
314 reductase activity of wild-type cells to be ten times higher than that of the *adr* mutant when the
315 cells were grown aerobically (Table 6). This observation matches our qRT-PCR data in that the
316 expression of *nirK* is reduced in the Adr mutant when grown under aerobic conditions, but not as
317 reduced as the expression levels of *nirK* in the FixJ mutant (Figure 4).

318 Microaerobically grown wild-type cells also exhibited higher nitrite reductase activity
319 than the *adr* mutant, though the mutant was still capable of reducing nitrite (Table 7). We also
320 observed that under microoxic conditions, cells lacking *adr* can express nitrite reductase at a
321 level comparable to wild-type under aerobic conditions (Table 6). We predict that this change in
322 expression of nitrite reductase from aerobic growth to microaerobic growth is due to the
323 activation of the FixLJ system under microoxic conditions. However, it appears that for optimal

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324 denitrification under these conditions using nitrite as the substrate, both FixJ and Adr are
325 necessary.

326 **Absence of Adr results in a decrease in competitiveness during *Medicago sativa***
327 **symbiosis.** Successful symbiosis and nitrogen fixation requires *S. meliloti* to not only perform
328 the appropriate symbiotic functions at the right time, but it must also compete against other
329 organisms in the rhizosphere for resources and hosts. To determine the symbiotic and
330 competitive characteristics of an *adr* mutant, plant nodulation assays were performed. *Medicago*
331 *sativa* inoculated with either Rm8530 or Rm8530 *adr* were equally proficient at forming
332 nitrogen fixing nodules (Figure 7a). Since it has been shown that FixJ is a regulator of symbiosis,
333 we also included a *fixJ* mutant and the *fixJ adr* double mutant in the nodulation assay to ensure
334 that any effect that Adr has on nodulation was not obscured by an active FixJ. However, as
335 shown in Figure 7a, there is no significant difference between plants inoculated with Rm8530
336 *fixJ* and those inoculated with the double mutant. Both sets of plants exhibited the classic Fix$^-$
337 phenotype of poor growth and a severe decrease in the number of nitrogen fixing nodules, which
338 is typical of FixJ mutants.

339 While there appears to be no difference between wild-type Rm8530 and Rm8530 *adr*
340 invasion efficiency in monoculture, we also assessed the ability of these strains to compete for
341 invasion when co-inoculated. Wild-type Rm8530 and the *adr* mutant were mixed in a range of
342 ratios (100:1, 10:1, 1:1, 1:10, and 1:100) and applied to plants. After harvesting bacteria from the
343 root nodules, we found that that Rm8530 was able to out-compete the *adr* mutant during
344 nodulation (Figure 7b). This indicates that while Adr is not essential for nitrogen fixing nodules
345 to form, it is beneficial to *S. meliloti* during growth in the rhizosphere.

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346 **Discussion**

347 Understanding the process of denitrification and its regulation is an ongoing task. While
348 the machinery of denitrification and nitrogen fixation have been known for some time,
349 determining how these pieces fit together under a complex regulatory web has proved to be a
350 challenging undertaking. Though most denitrification regulatory elements are very similar, such
351 as a sensory two-component system and *crp/fnr*-type regulators, interactions between these
352 components vary between denitrifiers (2). Members of the same regulatory family perform
353 different roles in various genetic backgrounds and target different respiratory systems while
354 regulating denitrification (2). For example, in *S. meliloti* and *Bradyrhizobium japonicum,* FixL
355 senses low oxygen tension and activates a regulatory cascade through FixJ that leads to the
356 expression of the denitrification and nitrogen fixation pathways (38). Additionally, both *S.*
357 *meliloti* and *B. japonicum* possess duplicated copies of the *fixK* gene, a *crp/fnr*-type regulator.
358 However, in *S. meliloti* both copies of *fixK* activate the same set of promoters, while in *B.*
359 *japonicum* $FixK_2$ is essential for denitrification and nitrogen fixation, as well as regulating the
360 expression of $FixK_1$ (16). In *Pseudomonas* species, a variant of FixLJ, the NarXL system,
361 controls denitrification and NarL acts as the transcriptional activator of the nitrate reductase
362 genes (39, 40).

363 Unlike the typical model denitrifiers, genes for the denitrification pathway in *S. meliloti*
364 are located on the symbiotic plasmid pSymA instead of the chromosome (2, 33). Generally,
365 rhizobia are considered to have acquired nitrogen fixing capabilities (*fix* and *nif* genes) by
366 horizontal gene transfer of the symbiotic plasmid (35). It is reasonable to assume that the same is
367 true for the denitrification genes as they are interspersed among the *fix* genes on pSymA and both
368 the symbiotic nitrogen fixation genes and the denitrification genes share the same regulator

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369 (FixJ). The origin of the denitrification genes may also explain the differences in denitrification
370 efficiency observed between various strains of *S. meliloti*. For example, in a study of 13 *S.*
371 *meliloti* strains, three failed to denitrify when nitrate was used as a terminal electron acceptor
372 under anaerobic conditions, despite the presence of the appropriate reductases (1). However,
373 when some amount of oxygen was present, all strains tested were able to reduce nitrate to either
374 nitrous oxide or dinitrogen gas (1).

375 In most bacteria the regulators of the denitrifying pathway (*fixK*, *nosR*, *nnr*) lie in close
376 proximity to the genes that code for the reductase enzymes; this is also true of *S. meliloti*, these
377 regulators are found on the symbiotic plasmid pSymA (33). Other denitrification regulators that
378 have been observed are typically global transcriptional activators found outside the
379 denitrification loci (2). In this study we analyze Adr, a LuxR-like protein located on the
380 chromosome of *S. meliloti*. LuxR family proteins affect a broad number of cell functions that
381 typically act on multiple operons; both FixJ and NarL belong to this superfamily due to the helix-
382 turn-helix homology they share. Adr also appears to play a role in the expression of many cell
383 functions including denitrification, ornithine catabolism (*arc*), sugar metabolism (*smo*), motility,
384 and genes that appear to be involved in respiration (*pnt* and *cyo*) (Supplemental Table S1).

385 As Adr is a predicted quorum sensing regulator, we examined whether population size or
386 AHLs play a role in how this regulator functions. We observed that the presence or absence of
387 the native AHLs synthesized by *S. meliloti* had no effect on the expression of the denitrification
388 genes. Upon further analysis of the Adr sequence, we found that though this regulator contains a
389 LuxR-like AHL binding domain, key residues for AHL binding are absent. This makes it
390 unlikely that Adr is a traditional AHL-dependent LuxR-like quorum sensing regulator. However,
391 when we examined the expression of the denitrification genes at different growth phases in the

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392 presence and absence of Adr, we found that Adr begins to affect expression in between early
393 growth (OD_{600} 0.2) and mid-exponential growth (OD_{600} 0.8). This allows for the possibility that
394 Adr is responding to an environmental or secreted signal that is not traditionally associated with
395 quorum sensing, such as environmental oxygen concentration. In an attempt to induce expression
396 during early growth, we grew cells in spent media that should contain the putative signal
397 molecule, but we were unable to detect any difference in expression between cells grown in fresh
398 media and spent media. However, we do not rule out the possibility of a cell produced molecule
399 triggering the expression of denitrification genes by Adr.

400 Various factors such as nitrate concentration, oxygen concentration, metal ion
401 availability, and moisture influence denitrification in the laboratory as well as the external
402 environment (2). Denitrification can occur under a range of oxygen concentrations and is no
403 longer considered a strictly anaerobic process. In this study we examined how oxygen influences
404 the expression of the denitrification system in *S. meliloti*. In our genome wide transcriptomic
405 study, we compared the *adr* mutant to wild-type Rm8530. We found that the denitrification
406 pathway is expressed in aerobically grown wild-type cells during stationary phase, but
407 expression is decreased in the absence of *adr*. Previously, Bobik et al. *s*howed that FixJ is the
408 major regulator of denitrification and low oxygen response in *S. meliloti.* When our data is
409 compared side by side with that obtained by Bobik et al., there is a remarkable overlap between
410 the results (17). With this in mind, we examined the relationship between Adr and FixJ. Under
411 our conditions we did not expect the low oxygen dependent regulator of denitrification, FixJ, to
412 be active. However, we observed that when FixJ was removed, the expression of the
413 denitrification genes was reduced 100- to 1000-fold when compared to wild-type levels of
414 transcription. There are several possible explanations for this 1) the oxygen content of our

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415 aerobic cultures was low enough to activate FixJ via FixL, 2) unphosphorylated FixJ is sufficient
416 to induce transcription of the denitrification genes, or 3) an unknown factor allows for enhanced
417 aerobic activity of FixJ. Considering the high degree of similarity between the Adr data and the
418 expression data gathered by Bobik, *et al* on FixJ, we consider the third option to be most likely,
419 with the unknown facilitating factor being identified here as Adr. We do acknowledge that
420 oxygen concentrations in stationary phase media are decreased when compared to fresh media.
421 However, it is clear that FixJ and Adr each have an effect on denitrification expression without
422 intentional oxygen removal. Further research is required to determine the mechanism by which
423 the above observed phenomena occur.

424 Due to the tiered expression control of the denitrification genes, we also included FixK in
425 our study. Under aerobic growth conditions, the presence of both FixJ and Adr, acting through
426 FixK, are required for the optimal expression of the denitrification genes (*nap*, *nir*, *nor*, *nos*) in *S.*
427 *meliloti*. While the absence of Adr decreases the expression of these genes 5- to 300-fold,
428 removing FixJ has a larger effect on expression. This indicates that the effect of Adr on
429 denitrification is indirect; as proposed above, Adr likely acts to facilitate FixJ-dependent
430 expression when oxygen is present. Since neither FixJ nor Adr affect the other's transcription,
431 we suspect that a protein-protein interaction may be occurring. Whether Adr functions to
432 stabilize FixJ under free-living oxygenated conditions or help FixJ bind to the DNA is unknown
433 at this time. Further study is required to elucidate the potential interaction occurring between
434 these two proteins.

435 In addition to transcriptomic studies, we also assessed the ability of Rm8530 and the
436 Rm8530 *adr* mutant to denitrify in a variety of conditions, including different oxygen
437 concentrations and in the presence and absence of nitrate and nitrite. Nitrate represents one of the

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438 fixed forms of nitrogen in the environment and an essential component in the biosphere, serving
439 as a nutrient for plants and microorganisms and acting as a final electron acceptor in several
440 bacteria, archaea, and eukaryotes (41). This heavily sought after resource is utilized by microbes
441 involved in performing reactions that contribute to the nitrogen cycle, such as denitrification and
442 dissimilatory nitrate reduction (42). The reduction of nitrate to nitrite is a key reaction in the
443 nitrogen cycle and is the first step of denitrification. Nitrite is found in water and soil
444 environments as a result of the first step of denitrification and as an intermediate of microbial
445 nitrification (43).

446 Under anaerobic conditions, no growth and no evidence of denitrification was observed
447 in either strain of *S. meliloti*, even after seven days of incubation. Since *S. meliloti* is an aerobe,
448 we surmise that it is not capable of transitioning from respiration to denitrification when there is
449 a complete lack of oxygen. It has also been suggested that most rhizobial denitrification is energy
450 inefficient, since they are observed to grow slowly in comparison to other denitrifiers (44). This
451 observation serves to emphasize the variation in denitrification ability seen between strains of *S.*
452 *meliloti*. In the previously mentioned study of thirteen *S. meliloti* strains, ten strains were capable
453 of anaerobic growth using nitrate as a terminal electron acceptor (1).

454 Under aerobic conditions we observed limited denitrification by qualitatively monitoring
455 nitrite levels in the media using nitrite test strips; cells grew normally in both nitrate and nitrite
456 with no survival defects (Figure 6a and 6b), though the *adr* mutant was not as efficient at
457 reducing nitrate and nitrite as the wild-type strain. When cells were grown under microaerobic
458 conditions, a decrease in growth and survival in relation to the aerobic cultures was observed
459 (Figure 6). This growth defect may be explained in several ways. When growing aerobically, *S.*
460 *meliloti* does not have to rely on denitrification for energy generation. While the process is

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461 occurring, it is simply not necessary for the production of proton motive force and ATP because
462 the electrons will pass to oxygen instead of a NO_x. Once the oxygen concentration in the
463 environment drops, the cells require high oxygen affinity cytochrome oxidases and NO_x
464 reductases in order to maintain energy production. Therefore, it is possible that this decrease in
465 growth is due to the lower amount of energy that *S. meliloti* is able to produce under limited
466 oxygen conditions (45).

467 While nitrite is known to be toxic to *S. meliloti*, we saw no evidence of this during
468 aerobic growth. When the cells were grown microaerobically in the same concentration of nitrite,
469 growth and survival were both reduced (Figure 6c and 6d). In the *adr* mutant, it is reasonable to
470 assume that this reduction in survival is due to the impaired ability of the strain to reduce nitrite,
471 leading to lower energy production and longer exposure to the nitrite. The wild-type strain also
472 has reduced survivability which may be attributable to the closed environment of the growth vial
473 that would allow the accumulation nitric oxide (toxic to *S. meliloti*) before complete reduction to
474 nitrogen gas can occur.

475 We next explored whether the *nap* and *nir* translation products are functional in *S.*
476 *meliloti*. Both the nitrate (*nap*) and nitrite (*nir*) reductases in *S. meliloti* are localized in the
477 periplasm. *S. meliloti* only possesses the periplasmic *nap* variant of nitrate reductase; the
478 respiratory nitrate reductase is not present in the genome. Typically, periplasmic nitrate
479 reductases are expressed aerobically in order to remove excess reducing power and provide
480 nitrite for the next step of aerobic denitrification (46). Conversion of nitrite to nitric oxide can be
481 carried out by two types of reductases: the cytochrome cd_1-type reductase or the copper
482 containing nitrite reductase. *S. meliloti* encodes *nirKV*, a copper containing periplasmic nitrite
483 reductase and an accessory protein which is required for reductase activity (45). The function of

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484 both the nitrate and nitrite reductases can be assayed with the addition of the artificial electron
485 donor methyl viologen. When added to whole cells, methyl viologen can donate electrons to
486 enzymes located in the periplasm, but cannot cross the bacterial membrane, allowing for a
487 specific assay of reductases localized in the periplasm (37).

488 Since the nitrate and nitrite reductases are not oxygen sensitive, we assayed the reductase
489 activities in aerobically grown cells as well as microaerobically grown cells. No denitrifying
490 activity was seen when nitrate was used as a substrate in either growth conditions. This
491 corroborates the results seen in our growth assays. If *S. meliloti* were capable of reducing nitrate
492 to nitrite, we would expect to see the same microaerobic growth defect in cultures with nitrate as
493 those results seen when nitrite was present (Figure 6d). These results lead to the conclusion that
494 the denitrification pathway in Rm8530 is truncated and likely begins at the second step, the
495 reduction of nitrite to nitric oxide. Many denitrifiers are capable of bypassing this low energy
496 reduction of nitrate to nitrite since nitrite is present in the environment (43). For example, similar
497 results were seen in a *napA* gene analysis in *Pseudomonas* isolates; several strains were positive
498 for *nap* gene expression but were incapable of denitrification when nitrate was provided as a
499 substrate (47).

500 When nitrite was provided as a substrate, we found nitrite reductase activity in both
501 aerobic and microaerobic conditions in wild-type Rm8530. There is limited activity of the nitrite
502 reductase in the *adr* strain under aerobic conditions, supporting our previous data showing a
503 reduction in transcription of these genes in the mutant. Additionally, when the *adr* mutant was
504 grown under microaerobic conditions, there was increased activity of the nitrite reductase
505 compared to aerobic conditions, though levels were not restored to wild-type activity. This
506 increase may be linked to the microaerobic environment. Previous studies have shown that under

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507 low oxygen concentrations, FixJ is responsible for *nirK* expression (48). This leads to the
508 possibility that Adr mainly functions when the cells are growing in oxic conditions.

509 To determine if Adr is necessary for symbiosis with a plant host, we performed symbiosis
510 assays. We found no significant difference between wild-type and the *adr* mutant symbiotic
511 ability, which indicates that Adr is not essential for symbiosis or nitrogen fixation. This suggests
512 that under microoxic conditions, prior to bacteroid differentiation, FixJ in conjunction with FixL
513 are sufficient for mediating expression of the denitrification genes. However, when co-inoculated
514 on the same plant, we observed that the Rm8530 wild-type strain has a competitive advantage in
515 forming nitrogen fixing nodules.

516 Taken together with our growth and reductase activity results, we propose that a
517 functional Adr, along with FixJ, helps prepare *S. meliloti* for symbiosis during the free-living
518 phase when oxygen concentrations are either too high for FixL to effectively increase
519 phosphorylated FixJ levels, or during the infection stage when oxygen levels fluctuate and begin
520 to decrease as the cells enter the nodule. As seen in Figure 8, we have added Adr to the existing
521 model for denitrification regulation, with a tentative interaction proposed between FixJ and Adr.
522 Whether Adr is influenced by an effector molecule has yet to be elucidated. We observed
523 expression of the denitrification genes in wild-type *S. meliloti* in the presence of oxygen and the
524 absence of denitrification substrates, which can be abolished by the removal of either Adr or
525 FixJ. Aerobic expression of the denitrification genes may serve multiple purposes, such as
526 removal of excessive reducing power or reducing toxic NO_x from the environment around the
527 cell. Whether directly or indirectly, Adr influences the aerobic expression of the denitrification
528 genes in *S. meliloti* in conjunction with FixJ. Further study should include elucidation of the

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529 possible interaction between FixJ and Adr and the identification and/or isolation of the Adr
530 effector molecule.

531 **Materials and Methods**

532 **Bacterial strains and media.** Strains and plasmids used in this study are listed in Table
533 1. *S. meliloti* strains were grown at 30º C, 250 rpm in Luria-Bertani media supplemented with 2.5
534 mM $MgSO_4$ and 2.5 mM $CaCl_2$ for routine cultures (referred to as LB/MC). *Escherichia coli*
535 cultures were grown in Luria-Bertani media with the appropriate antibiotics at 37º C, 250 rpm.
536 For RNA isolation, *S. meliloti* cultures were grown in tryptone-yeast extract medium
537 supplemented with 3.6 mM calcium chloride (TYC) or minimal low phosphate (19 mM glutamic
538 acid, 55 mM mannitol, 0.1 mM K_2HPO_4/KH_2PO_4 mix, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 0.004
539 mM biotin, pH 7).

540 To screen for recombinant mutants, strains were grown on minimal glutamate media
541 (MGM) plates (11 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g glutamate, 10 g mannitol, 1.5%
542 agar, 1 mg/ml biotin, 27.8 mg $CaCl_2$, and 246 mg $MgSO_4$); plates were supplemented with 5%
543 sucrose when appropriate. Media for growth curves was supplemented with various nitrogen
544 sources at the following concentrations: 10 mM KNO_3, or 5 mM $NaNO_2$.

545 Antibiotics were used in the following concentrations when appropriate, streptomycin
546 (Sm) 500 µg/ml, neomycin (Nm) 200 µg/ml, trimethoprim (Tp) 200 µg/ml, spectinomycin (Sp)
547 100 µg/ml, kanamycin (Km) 25 µg/ml, and chloramphenicol (Cm) 20 µg/ml.

548 **Construction of the *adr* mutant.** *adr* was amplified from *S. meliloti* Rm8530
549 chromosomal DNA and cloned into the EcoRV site of pPCR-Script, creating the vector p658.
550 The EZ::TN insertion kit (Epicenter) was used to disrupt the cloned *adr* by transposon
551 mutagenesis to create p658Tp. The disrupted *adr* was then cloned into the SpeI site of the suicide

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552 vector pJQ200SmSp and the resulting recombinant plasmid (pJQ658Tp) was transformed into
553 DH5α (25). Triparental mating was performed with DH5α pJQ658Tp, MT616 (helper strain),
554 and Rm8530 (49). *S. meliloti* carrying the disrupted copy of *adr* was selected by plating on
555 minimal media supplemented with Tp and 5% sucrose (50). Mutations were confirmed by PCR
556 and phage ϕM12 was used to transduce the *adr* mutation into subsequent strains (51). Primers for
557 mutant construction and confirmation are listed in Table 2.

558 **Construction of the denitrification mutants.** Internal fragments of *fixJ* and *fixK2* were
559 cloned into pK19mobΩHMB creating recombinant vectors harbored in *E. coli* S17-λ*pir* (Table
560 1). These vectors were provided by Dr. Anke Becker from the Philipps University of Marburg,
561 Germany. The vector carrying *fixK2* was modified by inserting a hygromycin cassette cloned
562 from pMB419 into the Km cassette of the pK19fixK2 backbone. Vectors were transferred via bi-
563 parental mating into *S. meliloti* Rm8530 and recombinants were selected by plating on minimal
564 media with the appropriate antibiotics. Mutations were confirmed by PCR and phage ϕM12 was
565 used to transduce the *fixJ* mutation in Rm8530 *658*::Tp (51).

566 To construct the *fixK1 fixK2* double mutant, an internal fragment of *fixK1* was cloned into
567 pVIK112 to create pVIKfixK1. This vector was transferred via tri-parental mating into the
568 Rm8530 *fixK2*::Hy strain. Mutations were confirmed by PCR.

569 *S. meliloti* Rm2011 containing a Tn5 mutation in *fixL* was also provided by Dr. Anke
570 Becker. This mutation was transferred into Rm8530 by phage ϕM12 and plated on LB agar with
571 the appropriate antibiotics (51). Mutations were confirmed by PCR.

572 **RNA purification and cDNA synthesis.** Bacterial cultures were grown for two days in
573 LB/MC and appropriate antibiotics. A 1:100 dilution was used to inoculate 20 ml of TY media
574 supplemented with Sm. Cultures were grown aerobically to OD_{600} 0.2 (lag), 0.8 (mid log), or 1.2

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575 (stationary). After reaching the appropriate growth stage, 1.5 ml aliquots of culture were
576 harvested by centrifugation (14,500 rpm for 2 minutes at 4º C), immediately frozen in liquid
577 nitrogen, and stored at -80º C for future use. RNA purification was performed using the RNeasy
578 Mini Kit (Qiagen) with slight modifications. Briefly, cells were thawed on ice then resuspended
579 in 10 mM Tris HCl (pH 8) and RLT buffer provided from the Qiagen kit (supplemented with β-
580 mercaptoethanol). The cells were transferred to FastProtein tubes (Qbiogene) and disrupted using
581 an MP FastPrep-24 ribolyser (40s, speed 6.5). Spin column purification was performed
582 according to the RNeasy Mini Kit RNA purification protocol. After the first round of
583 purification, samples were treated with Qiagen on column RNase-free DNase. The RNA samples
584 were eluted and DNase treated a second time with the Ambion TURBO RNase-free DNase
585 which was followed by an RNA clean up step. Concentration of RNA was determined by
586 Nanodrop and DNA contamination was assessed by qRT-PCR. cDNA for each strain was
587 synthesized with the Ambion RETROscript kit according to the manufacturer's protocol. 1 µg of
588 total RNA was used per cDNA synthesis reaction.

589 **Affymetrix GeneChip hybridization and expression analysis.** The cDNA synthesis
590 was performed using 10 µg of RNA harvested from cells grown to OD_{600} 1.2. Hybridization of
591 the cDNA to the GeneChip Medicago Genome Array (Affymetrix, Santa Carla, CA) was
592 performed at the Core Microarray facility at UT Southwestern Medical Center (Dallas, TX) as
593 previously described (27, 32). The GeneChip Scanner 3000 was used to measure the signal
594 intensity of each array. Affymetrix GeneChip Operating Software, (GCOS v 1.4) was used to
595 generate the .CEL files. Comparative analysis of the control and experimental expression were
596 represented in terms of M-value (signal log ratio) which also indicated an increase, decrease, or

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597 lack of change in expression of a gene in the mutant with respect to the wild-type. An M-value ≥
598 1 (2-fold change) with a *p*-value of ≤ 0.05 were considered significant.

599 **Quantitative real-time PCR.** Oligonucleotide sequences used for qRT-PCR are listed in
600 Table 3. The reaction mixture for qRT-PCR analysis contained 0.3 µM of sense primer, 0.3 µM
601 of antisense primer, 0.5X of SYBR green 1 (Sigma), 0.5 Omni Mix HS PCR bead (contains 1.5
602 U *Taq* DNA polymerase, 10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM MgCl2, 200 µM
603 deoxynucleotide triphosphate, and stabilizers), and 1 µl of cDNA. Total reaction volume was 25
604 µl. Analysis was performed using a Cepheid Smart Cycler, version 2.0c as previously described
605 (32). Expression analyses were conducted in triplicate. The expression of *SMc00128* was used as
606 an internal control and for normalization, as described previously (52, 53). Expression analysis
607 were performed as three independent experiments.

608 **Growth analysis.** Cells for all growth curves were grown to saturation in LB/MC with
609 appropriate antibiotics. Aerobic growth curves were performed using a Tecan plate reader (29º
610 C, shaking at 250 rpm). Cells were diluted 1:100 in TYC media, TYC with 10 mM KNO_3, or
611 TYC with 5 mM $NaNO_2$ and added to a 96 well plate. OD_{600} readings were taken every 30
612 minutes.

613 Microaerobic conditions were also tested. Starter cultures were diluted to an initial OD_{600}
614 of 0.2, then 2 ml of each culture was added to each vial. Once sealed, the vials were sparged with
615 a mixture of 2% oxygen, 98% argon for one minute. Vials were incubated at 30º C, shaking at
616 250 rpm and OD_{600} was measured every 24 hours. We refer to aerated cultures to differentiate
617 between aerobic cultures grown with free gas exchange with the environment and intentionally
618 oxygen restricted (microaerobic) cultures.

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619 Viable counts were performed for both aerobic and microaerobic cultures. Several
620 dilutions were plated on LB agar with the appropriate antibiotics. Plates were incubated for
621 several days at 30º C before colonies were counted by hand. Nitrite levels of cultures were
622 qualitatively assessed using nitrite test strips (0-80 mg/L or 0-10 mg/L, EMD Millipore).
623 **Media complementation assay.** Cells were grown in the same manner as those grown
624 for RNA pellets. Cultures were grown to OD_{600} of 1.2 in TYC media with Sm. Cells were
625 removed from the media by centrifugation (3 x 6000 rpm, 30 minutes, 4º C) and the resulting
626 supernatant was passed through a 0.22 µm filter (Fisher) and stored at 4º C. Glucose (100 mM)
627 and glutamate (19 mM) were added to the spent media to replace depleted nutrients. To
628 determine if the spent media contained effector molecules that are detected by or that activate
629 *adr*, cultures were grown to OD_{600} of 0.2 and then harvested for RNA in the manner described
630 above.
631 **Methyl viologen assay for nitrate and nitrite reductase activity.** Starter cultures were
632 grown aerobically in TYC media with appropriate drugs for two days. Cells were then spun
633 down (2 minutes, 14,000 rpm), and diluted to an initial OD_{600} of 0.2 in MLP supplemented with
634 Sm and 0.5 µM $NaMoO_4$. Cells grown aerobically were harvested at OD_{600} of 1.2 by spinning
635 down 1 ml of culture (two minutes, 14,000 rpm, 4º C), removing the supernatant, and
636 immediately freezing the cell pellet in liquid nitrogen. Microaerobically grown cells were
637 harvested at OD_{600} of 0.5-0.6 (stationary phase for microaerobic cultures).
638 Samples were assayed for either nitrate or nitrite reductase activity using methyl viologen
639 as the electron donor as previously described (37, 54). Cell pellets were resuspended in 10 mM
640 Hepes buffer (pH 7) and 1 mM methyl viologen to a final volume of 1 ml. The cuvettes were
641 sealed and sparged for five minutes with nitrogen gas. Methyl viologen was reduced by adding

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642 aliquots of freshly prepared aqueous sodium dithionite until a steady state absorbance of 1-1.5 at
643 600 nm was obtained. Substrate (10 mM nitrate or nitrite) was added, and the rate of absorbance
644 decrease measured. The rate of substrate-dependent methyl viologen oxidation was calculated
645 using 13 mM-1 cm-1 as the extinction coefficient of reduced methyl viologen (55). Protein
646 concentrations were measured by the Bradford method (Bio-Rad) and used to determine specific
647 activities. All strains were measured in triplicate.

648 **Plant symbiosis assays.** Infection assays of *Medicago sativa* were performed to
649 determine nodulation and nitrogen fixation efficiency of the *S. meliloti* mutant strains Rm8530
650 *adr,* Rm8530 *fixJ*¸ and Rm8530 *fixJ adr* compared to wild-type Rm8530. *M. sativa* was
651 inoculated with *S. meliloti* strains on Jensen's agar plates as previously described (56). Plants
652 were grown in a 16-hour light cycle at 20º C and in 65% humidity. Weekly inspections of roots
653 and plant health were performed beginning the second week post inoculation. Nitrogen fixing
654 nodules, empty nodules, and plant height were recorded for approximately 60 plants per strain
655 tested. Data shown was collected fourth week post inoculation.

656 **Plant symbiosis competition assay.** Five dilutions (100:1, 10:1, 1:1, 1:10, and 1:100) of
657 Rm8530 to Rm8530 *adr* were tested for competitive nodulation of *M. sativa.* Strains were grown
658 for two days in LB/MC with antibiotics, washed three times with sterile water, then diluted 1:100
659 in water. These dilutions were then mixed to obtain the appropriate ratio of wild-type to mutant.
660 A portion of the mixed inoculum was diluted and plated on the appropriate antibiotics to
661 determine cell viability. *M. sativa* seedlings were inoculated with 1 ml of the dilution and plants
662 were grown in the same conditions discussed above. Harvested nodules were washed in 50%
663 bleach for five minutes and rinsed three times with sterile water. After rinsing, each nodule was
664 added to a well of a microtiter plate and crushed in LB/MC supplemented with 0.3 M glucose.

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665 The bacteria released from the crushed nodules were diluted, divided in half, and plated on
666 LB/MC Sm and LB/MC Tp. Colony PCR was used to confirm the genetic background of the
667 strains. Nodulation competitiveness was tested twice for each dilution.

668 **Acknowledgements**

669 We thank Anke Becker and Jacques Batut for providing strains. We also thank Stephen
670 Spiro for his assistance with the methyl viologen and nitrite detection assays and for reviewing
671 this manuscript. Finally, we thank Nymisha Avadhanam for reading and giving feedback on the
672 manuscript. Our work was supported by National Science Foundation grant MCB-9733532 and
673 National Institutes of Health grant 1R01GM069925 to J.E.G.

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847 **Table 1. Strains and Plasmids**

848

Strains or plasmids	Relevant characteristics	Reference or source
S. meliloti		
Rm8530	Su47 *str-21*, *expR*$^+$, Sm^R	(57)
Rm8530 *adr*	*SMc00658*::Tp	This work
Rm8530 *fixJ*	*fixJ*::Nm	This work
Rm8530 *fixJ adr*	*SMc00658*::Tp *fixJ*::Nm	This work
Rm8530 *fixL*	*fixL*::Nm	This work
E. coli		
DH5α	See source	Life Technologies
MT616	MT607(pRK600)	(49)
S17-*ʎpir*	See source	(58)
Plasmids		
pPCR-Script	See source, Amp^R	Stratagene
pVIK112	*lacZY* for transcriptional fusions, Km^R	(59)
pJQ200SmSp	Suicide vector, *sacB*, Gm^R	(25)
pMB419	Vector carrying Hygromycin cassette	(60)
p658	pPCR-Script carrying *SMc00658*	This work
p658Tp	pPCR-Script carrying *SMc00658* disrupted with Tp	This work
pJQ658Tp	pJQ200 SmSp carrying *SMc00658*::Tp	This work
pVIKfixK1	pVIK112 carrying *fixK1* fragment	This work
pK19fixK2	pK19mobΩHMB carrying *fixK2 fragment*	Anke Becker
pK19fixK2Hy	pK19mobΩHMB carrying *fixK2*::Hy	This work

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849 **Table 2. Primers used for mutant construction**

850

Gene	Forward Primer	Reverse Primer
658-SpeI	GCACTAGTCGCAGACGCGGCG GGCGTCGTC	CGACTAGTGTGGCGATATTGCT GGATGCGCGTC
Hy-BspHI	ATATATATCATGAGCTGCAGAA AGGAATTACCAC	ATATATATCATGACTAGTAACA TAGATGACACCGCGC
fixK2	CGAAGCGGCTAAGTAGTT	TCCTTCAGTTTCGTCACC
fixK1	TCCTTCAGTTTCGTCACC	GATATTACACGGAATCCTACGA
fixJ	GCCATCAACCAGCAGTA	CACATTGTTGATGACGAGAG
fixL	TTCTTCACGGGCTTCATC	CTTCAACGACGACTGCTA

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852 **Table 3. qRT-PCR Primers**

853

Gene	Forward Primer	Reverse Primer
norC	CACCGCTTCCACACATTG	ACTATTACATGAAGACCGAATC
nirK	CCTTGTCATAGGTAATTGAATTGC	CCTCCAGGCATGGTTCCG
nosZ	GCATCCCTCGTTTACAGAC	AGTGTGCTGGTTCGGAAG
napA	ACGCCTTGTAGAGTTCCG	ATCTCCCGACGACGAATAC
nifA	GCCGAAGAGTCCGATTATGAT	CTGCTGATTGTGCGATGAAG
fixQ1	CTCGCAATGACGTTGTTCTT	TCCTCCTTTAACGGGATGAC
fixJ	CACATTGTTGATGACGAAGAG	TTCAGATCGCCGAGATTG
nnrR	ACTCAGGAAGTGGAGCGGCG	TGCAGCGTGGTTCCGGTCAT
fixK	CGGAATATGCAGGAGCGTTCG	CACATTGCCTGCCGATTACCA
adr	TGCCGGTATGGGTCACTTCG	TAGCGCCAGATAGAGCGTCG
nifH	ATATCGTTCAGCACGCAG	GTCGCTCTTCATGATTCC
fixG	TACGTCTTCGGCGGACTGAT	GTTCTCGTCTAGCATGGCCG
fixA	GGCGTGCCGACCATTATCAA	AAATGGCGGTCGGTCAAGAG

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855 **Table 4. Comparison of the FixJ by Bobik, et al. and the Adr microarray**

856

Group and Gene Name	Gene product or description
A.	**Overlap of Adr and FixJ regulation**
SMa0630	conserved hypothetical transmembrane protein
SMa0631	hypothetical protein
SMa0633	hypothetical protein
SMa0636	conserved hypothetical protein
SMa0661	conserved hypothetical protein
SMa0667	conserved hypothetical protein
SMa0669	hypothetical transmembrane protein, HlyD-family
SMa0678	putative amino acid transporter
SMa0680	decarboxylase (lysine, ornithine, arginine)
SMa0682	decarboxylase (lysine, ornithine, arginine)
SMa0684	amino acid transporter
SMa0687	hypothetical protein
SMa0689	conserved hypothetical protein
SMa0690	hypothetical protein
SMa0693	*arcA1*; arginine deiminase
SMa0695	*arcB*; ornithine carbamoyltransferase, catabolic
SMa0697	*arcC*; carbamate kinase
SMa0760	*fixT2*; anti-kinase protein
SMa0762[a]	*fixK2*; transcriptional regulator
SMa0763	hypothetical protein
SMa0765	*fixN2*; cytochrome c oxidase subunit I
SMa0771	hypothetical protein
SMa1013	*actP*; Copper translocating P-type ATPase
SMa1082	hypothetical protein
SMa1084	probable phosphoketolase
SMa1086	conserved hypothetical protein
SMa1087	cation transport ATPase
SMa1089	hypothetical protein
SMa1091	hypothetical protein
SMa1093	hypothetical protein
SMa1095	hypothetical protein
SMa1100	conserved hypothetical protein
SMa1101	hypothetical protein
SMa1118	*hspC*; heat shock protein
SMa1120	ABC transporter, ATP-binding protein
SMa1126	putative protease, transmembrane protein
SMa1128	*degP*; protease like protein

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SMa1131	metallo-beta-lactamase superfamily protein
SMa1132	hypothetical protein
SMa1134	conserved hypothetical protein
SMa1136	hypothetical protein
SMa1146	conserved hypothetical protein
SMa1147	conserved hypothetical protein
SMa1149	conserved hypothetical protein
SMa1151	conserved hypothetical protein
SMa1153	conserved hypothetical protein
SMa1154	conserved hypothetical protein
SMa1155	cation transport P-type ATPase
SMa1156	alcohol dehydrogenase, Zn-dependent class III
SMa1158	conserved hypothetical protein
SMa1163	cation transport P-type ATPase
SMa1166	protein containing an alpha/beta hydrolase fold
SMa1168	dehydrogenase, FAD-dependent
SMa1169	hypothetical protein
SMa1170	conserved hypothetical protein
SMa1176	hypothetical protein
SMa1179	*nosR*; regulatory protein
SMa1182[a]	*nosZ*; nitrous oxide reductase
SMa1183	*nosD*; nitrous oxidase accessory protein
SMa1184	*nosF*; ATPase (Should most if these be italics?
SMa1185	*nosY*; permease
SMa1186	*nosL*; copper chaperone
SMa1188	*nosX*; accessory protein
SMa1191	*hmp*; Flavohemoprotein
SMa1195	conserved hypothetical protein
SMa1200	conserved hypothetical protein
SMa1201	hypothetical protein
SMa1207	transcriptional regulator, CAP/Crp family
SMa1208	*fixS*; nitrogen fixation protein
SMa1209	*fixI1*; ATPase
SMa1210	*fixH*; nitrogen fixation protein
SMa1211[a]	*fixG*; iron sulfur membrane protein
SMa1213	*fixP1*; di-heme c-type cytochrome
SMa1214[a]	*fixQ1*; nitrogen fixation protein
SMa1216	*fixO1*; cytochrome C oxidase subunit
SMa1220	*fixN1*; cytochrome c oxidase subunit 1
SMa1223	*fixM*; flavoprotein oxidoreductase
SMa1225[a]	*fixK1*; transcriptional regulator
SMa1226	*fixT1*; antikinase protein
SMa1231	conserved hypothetical protein

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	SMa1236[a]	*napA*; periplasmic nitrate reductase
	SMa1240	*napF*; component of periplasmic nitrate reductase
	SMa1241	*napE*; component of periplasmic nitrate reductase
	SMa1243	*azu1*; pseudoazurin
	SMa1256	conserved hypothetical protein
	SMa1259	conserved hypothetical protein
	SMa1266	*hemN*; coproporphyrinogen III oxidase
	SMa1273	*norB*; nitric oxide reductase, large subunit
	SMa1276[a]	*norC*; nitric oxide reductase, small subunit
	SMa1279	*norE*; accessory protein for nitric oxide reductase
	SMa1283	*nnrU*; NnrU-like transmembrane protein
	SMa1296	*adhA1*; Alcohol dehydrogenase, Zn-dependent class III
	SMb21487	*cyoA*; Putative cytochrome o ubiquinol oxidase chain II
	SMb21488	*cyoB*; Putative cytochrome o ubiquinol oxidase chain I
	SMb21489	*cyoC*; Putative cytochrome o ubiquinol oxidase chain III
	SMc01169	*ald*; Probable alanine dehydrogenase oxidoreductase
	SMc03252	*proB2*; Putative glutamate 5-kinase
	SMc03253	L-proline cis-4-hydroxylase
B.		**Genes regulated by FixJ and not Adr**
	SMa0128	hypothetical protein
	SMa0130	fatty acid desaturase
	SMa0132	hypothetical protein
	SMa0625	hypothetical protein
	SMa0657	cytochrome c binding protein, amino terminus
	SMa1198	copper binding protein
	SMa1232[b]	*napC*; membrane bound nitrate reductase subunit
	SMa1233[b]	*napB*; periplasmic nitrate reductase
	SMa1239[b]	*napD*; component of periplasmic nitrate reductase
	SMa1288	carboxy-lyase
	SMb20139	conserved hypothetical transmembrane protein
	SMb20433	*eutC*; probable ornithine cyclo-deaminase
	SMb20654	hypothetical protein
	SMb20704	*glgA2*; putative glycogen synthase
	SMb20934	*exsF*; putative two component response regulator
	SMb21490	putative SUR1-like protein
	SMc00063	conserved hypothetical protein
	SMc00739	conserved hypothetical protein
	SMc00784	*fbpA*; iron transport protein
	SMc03254	*fixT3*; putative antikinase

857 Highlighted genes are directly involved in the low oxygen or denitrification pathways.

858 Underlined genes are the FixJ direct targets found by Bobik, et al. (*nifA* not shown as it was not

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859 seen in the FixJ microarray data) (17). [a]Gene expression verified by qRT-PCR in this

860 study.[b]Indicates genes that were confirmed by qRT-PCR to be regulated by Adr but did not

861 appear in the Adr microarray. It should be noted that the FixJ microarray was carried out under

862 different conditions than those used for the Adr microarray (17). For the complete Adr

863 microarray, see Supplemental Table S1.

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864 **Table 5. Relative transcript levels of *adr* over time, represented as Ct value***

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Strain	Lag (OD_{600} 0.2)	Mid-exponential (OD_{600} 0.8)	Early Stationary (OD_{600} 1.2)
Rm8530	34.74 ± 0.48	33.34 ± 0.46	34.64 ± 0.34

866 *Ct value represents the arbitrary cycle where PCR product is detected. Values closer to 40

867 indicate lower amounts of starting transcript.

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868 **Table 6. Specific activity of nitrite reductase estimated by methyl-viologen reduction.**

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Strain	**Nitrite reductase activity Aerobic**	**Nitrite reductase activity Microaerobic**
	μmol/min/mg of protein	μmol/min/mg of protein
Rm8530	0.372 ± 0.031	0.935 ± 0.076
Rm8530 *adr*	0.042 ± 0.004	0.442 ± 0.049

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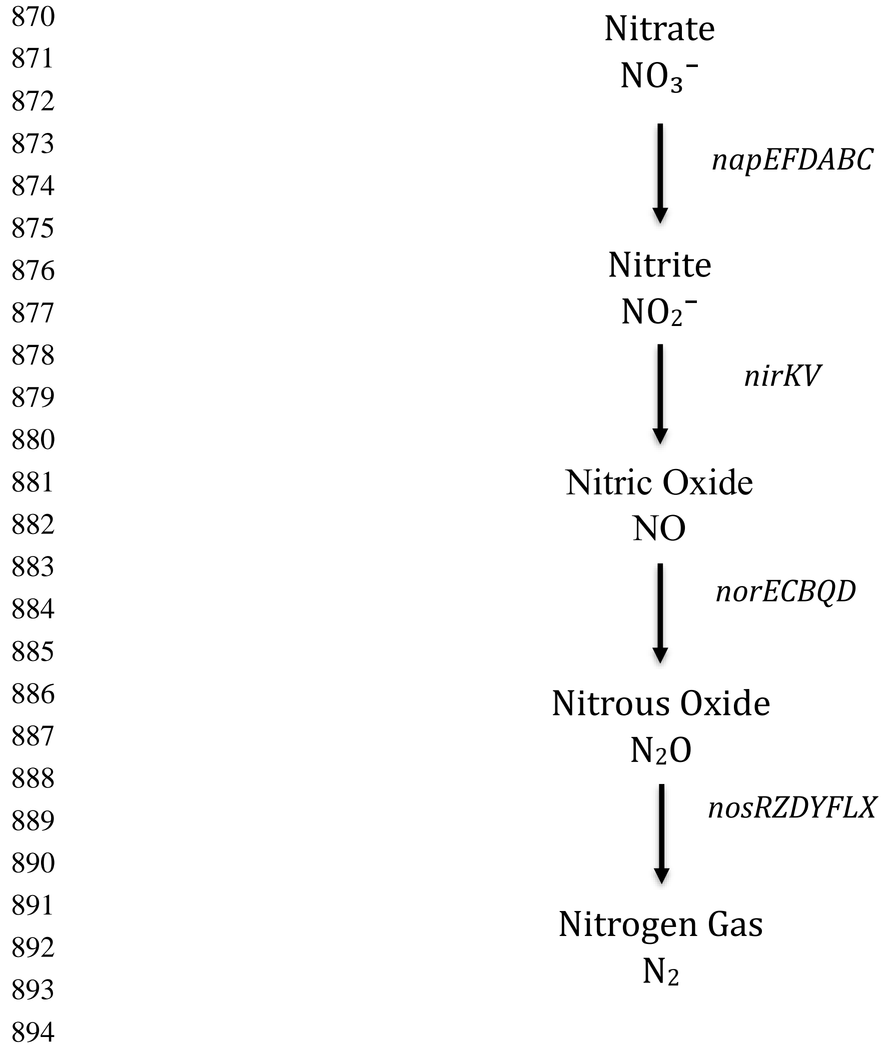

895 **Figure 1. The denitrification pathway in *S. meliloti* and its associated genes.**

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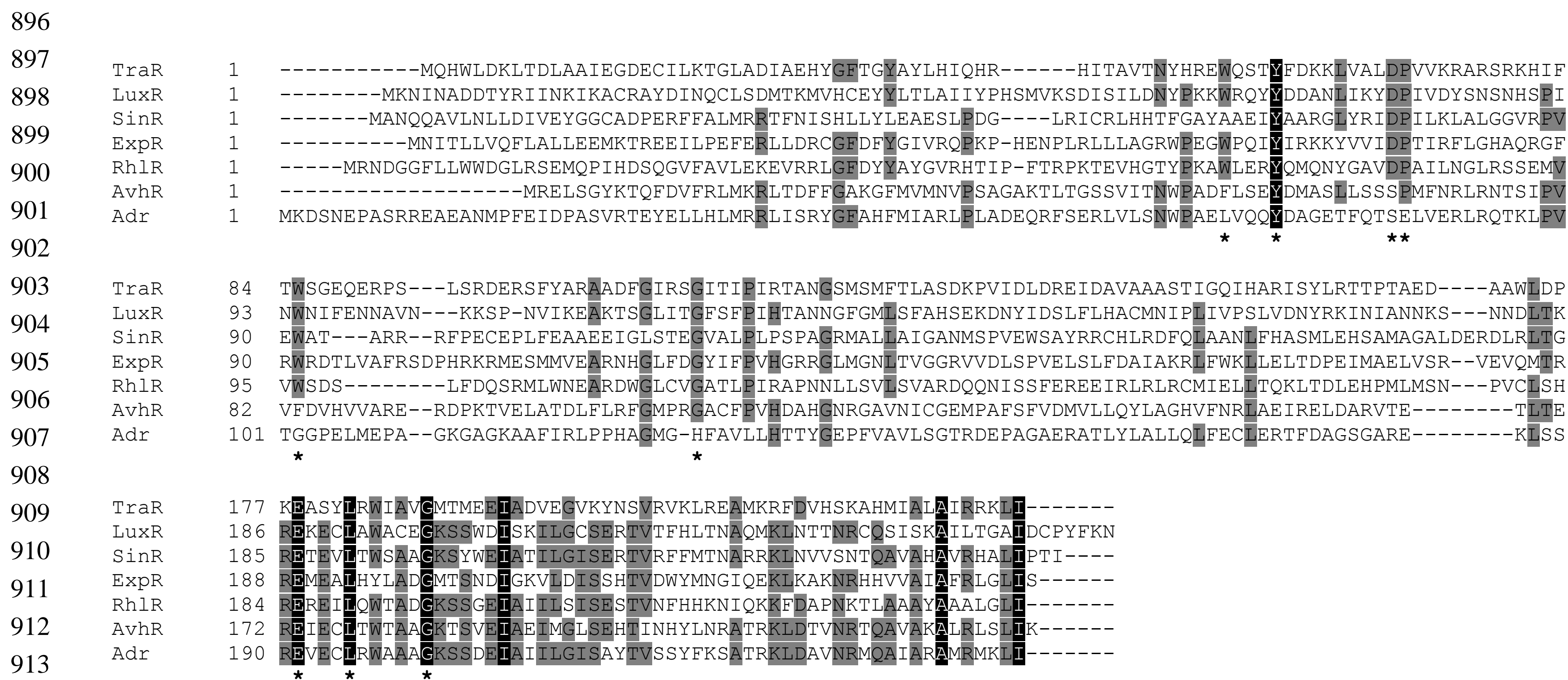

916 **Figure 2. Comparison of Adr (SMc00658) to LuxR.** Sequence alignment of TraR from *Agrobacterium tumefaciens,* LuxR from
917 *Alovibrio fischeri,* SinR and ExpR from *S. meliloti,* RhlR from *Pseudomonas aeruginosa*, and AvhR from *Agrobacterium vitis* with
918 Adr from *S. meliloti.* Grey shaded residues are highly similar between proteins and identical proteins are shaded in black. Asterisks
919 indicate the nine highly conserved residues found in <95% of LuxR-type proteins. The alignment was performed using Invitrogen's
920 Vector NTI 11 software.

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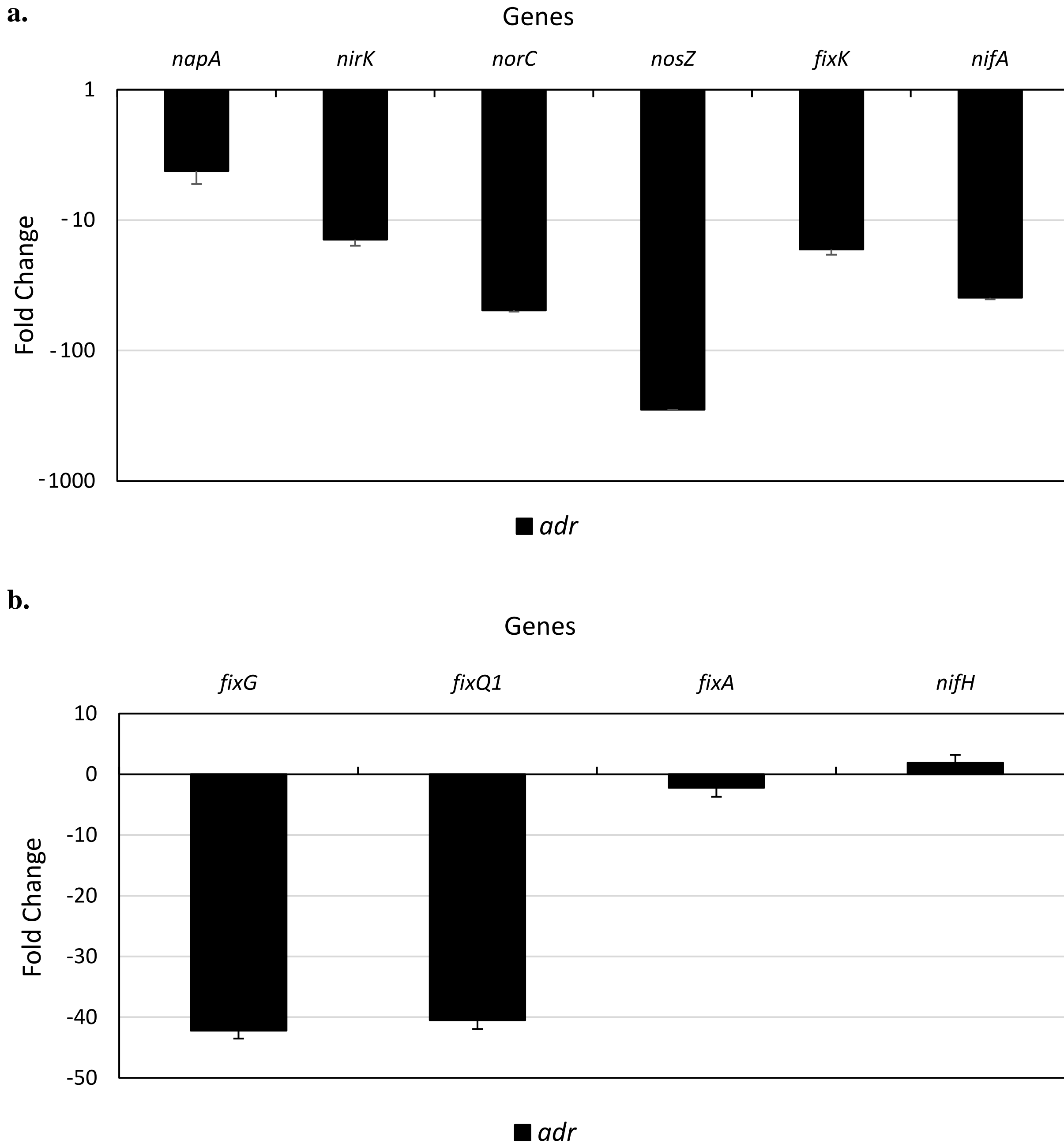

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923 **Figure 3. Relative expression of the *adr* mutant compared to wild-type strain Rm8530.**

924 Expression was measured using qRT-PCR and relative transcript levels are displayed as fold

925 change between the wild-type Rm8530 and the *adr* mutant. Negative values indicate

926 downregulation of the denoted gene in the mutant strain in comparison to the wild-type, while

927 positive values represent the inverse. Results are the average of three independent biological

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928 replicates, error bars are present and represent the standard deviation between three samples.

929 *SMc00128* was used as an internal control (52).

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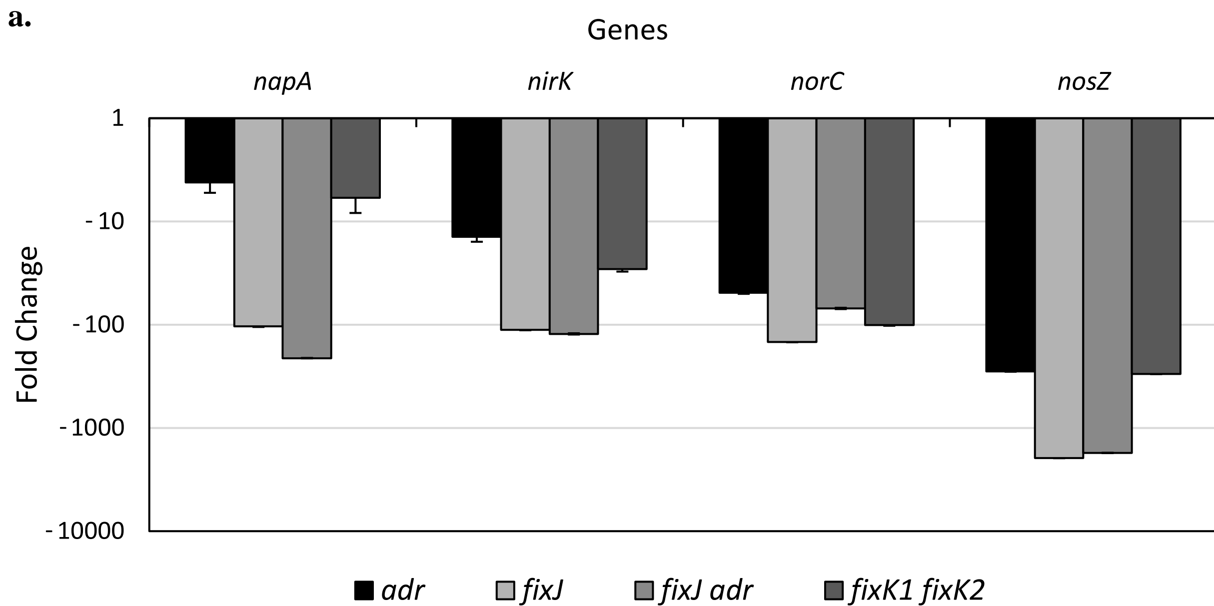

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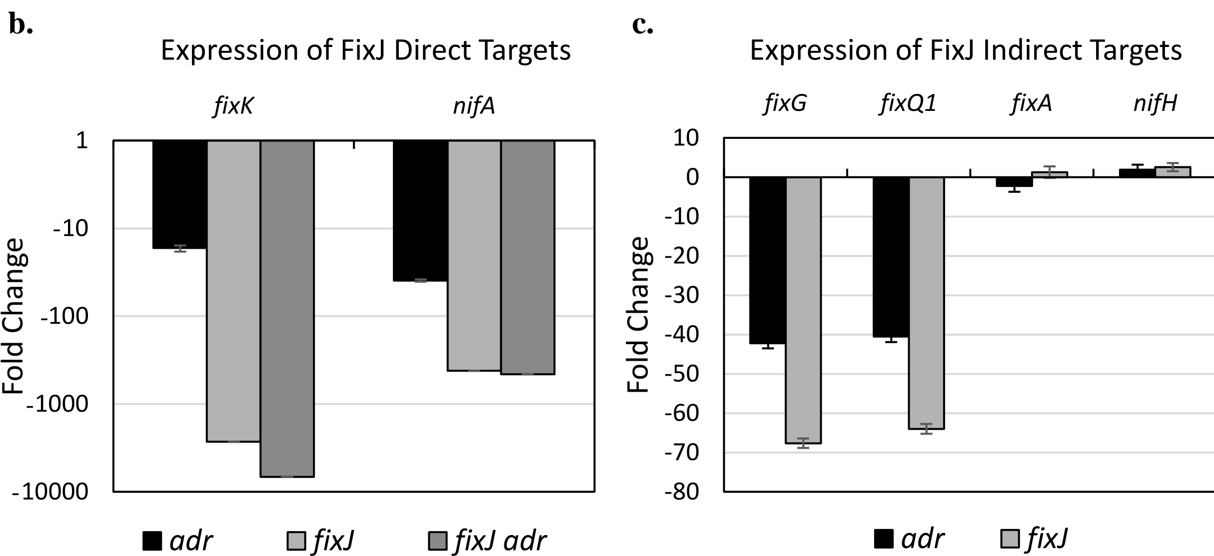

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933 **Figure 4. Aerobic expression of denitrification and other FixJ regulated genes. a)** The
934 expression of denitrification genes from four mutant strains (*adr, fixJ, fixJ adr*, and *fixK1 fixK2*)
935 compared to wild-type Rm8530 expression levels. **b)** Direct targets of FixJ, *fixK* (total
936 expression of both genes) and *nifA*. **c)** Comparison of expression between four indirect FixJ
937 targets. *adr* mutant data is the same as that seen in Figure 3, repeated for clarity. Results are the

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938 average of three independent biological replicates. Error bars are present and represent standard

939 deviation. *SMc00128* was used as an internal control (52).

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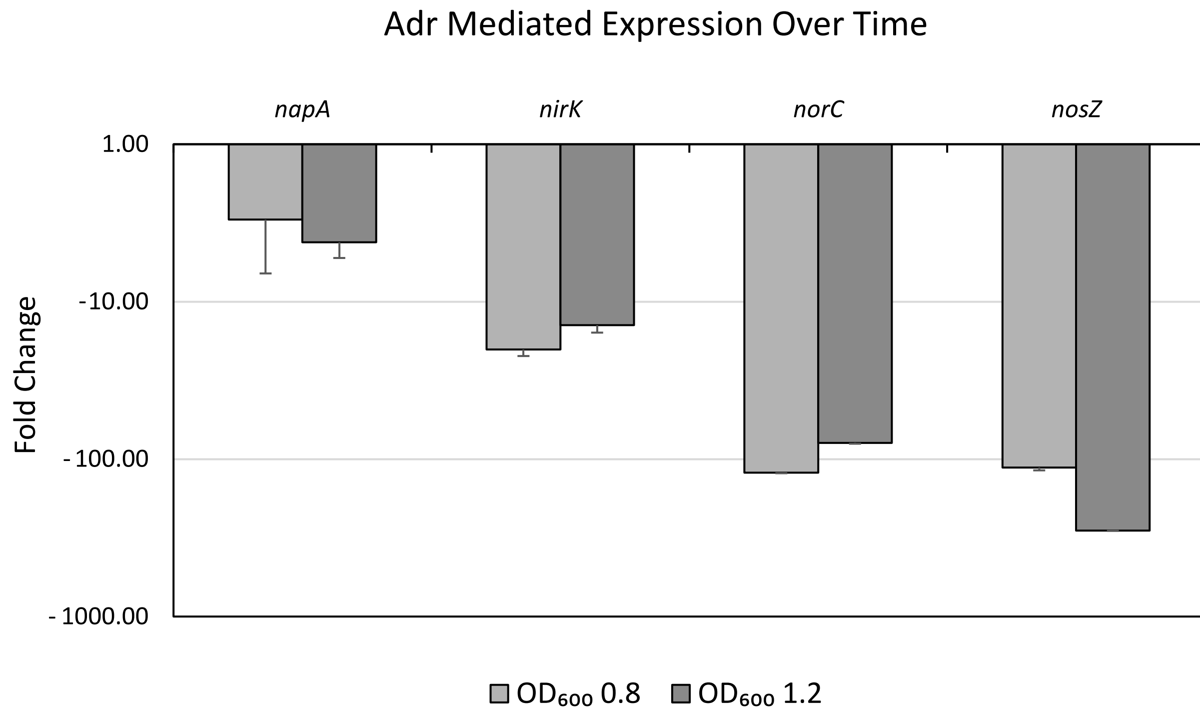

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943 **Figure 5. Expression of denitrification genes in an *adr* mutant over time.** Cells were grown
944 to the indicated optical densities (measured at 600 nm). Expression was measured in three
945 independent biological replicates per density and all are compared to the wild-type expression
946 value. Error bars represent standard deviation. No difference in expression was observed at
947 OD_{600} 0.2. *SMc00128* was used as an internal control to normalize values (52).
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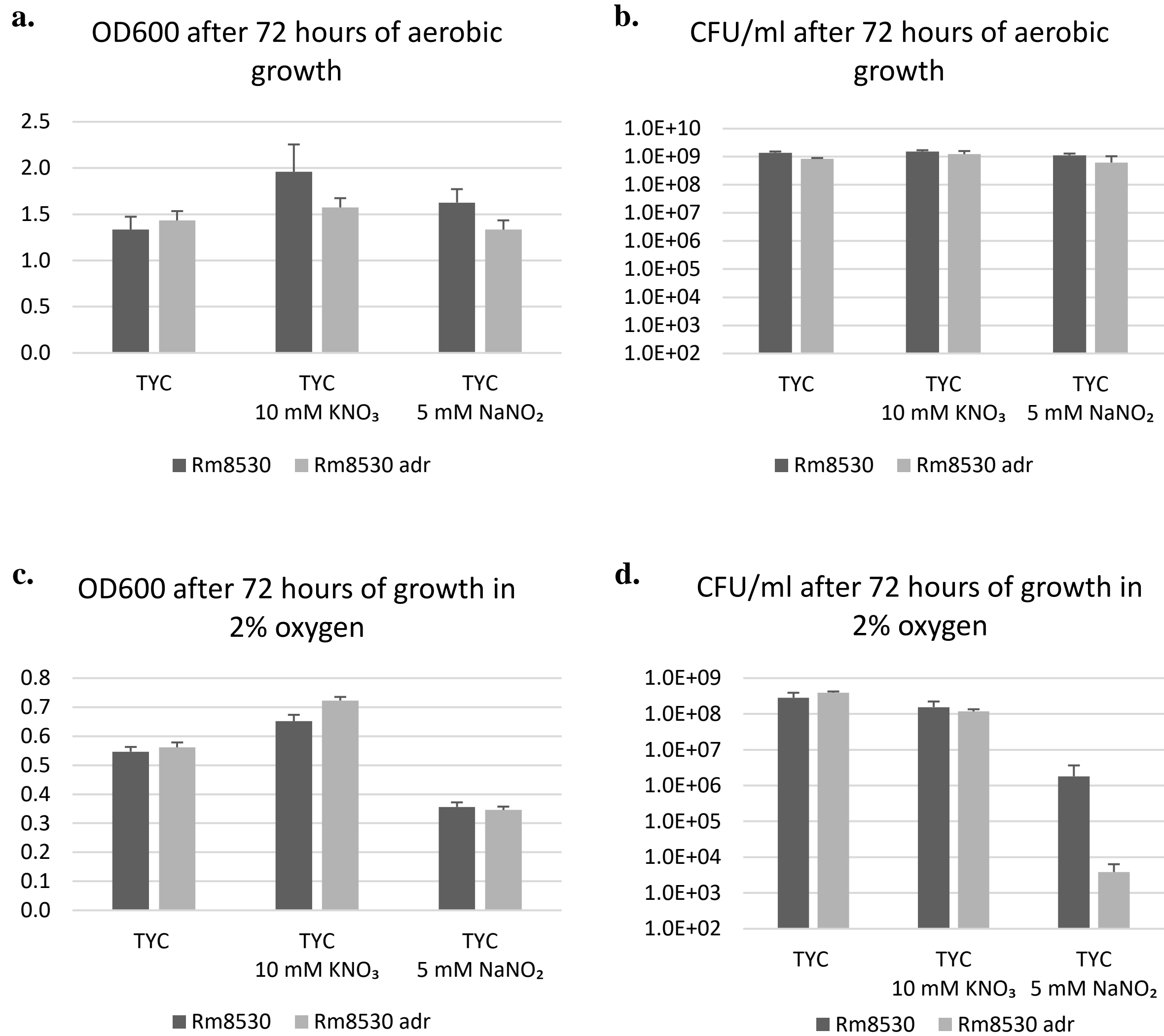

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953 **Figure 6. Growth and survival of the Adr mutant.** Growth measurements of wild type
954 Rm8530 and the mutant Rm8530 *adr* were conducted in triplicate under aerobic and
955 microaerobic conditions as described previously

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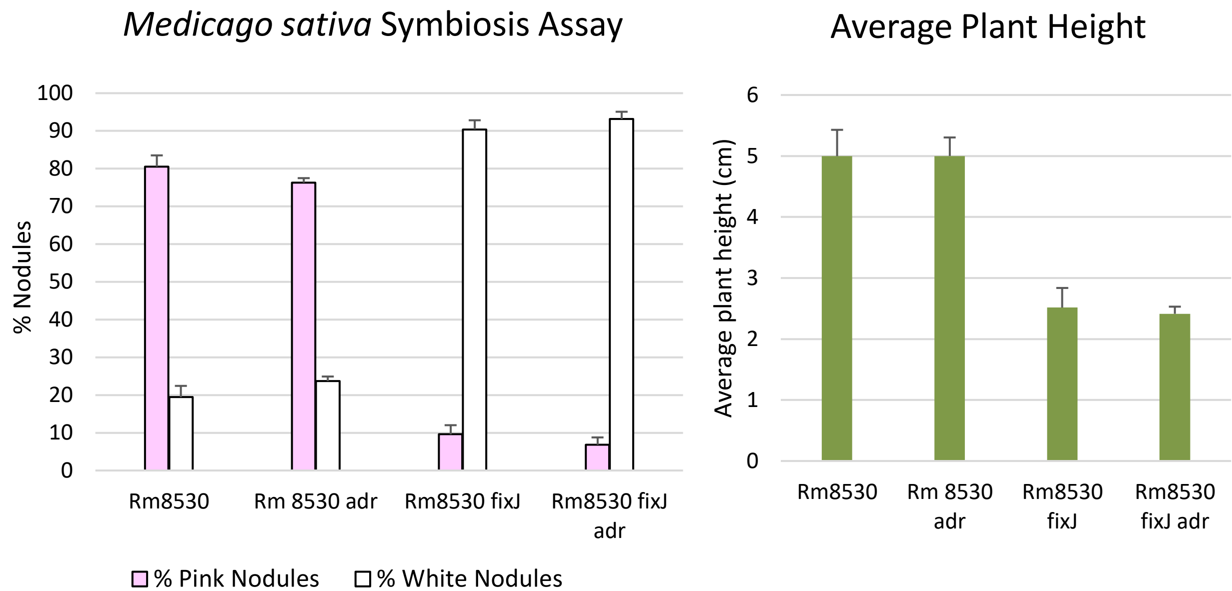
956 **a.**



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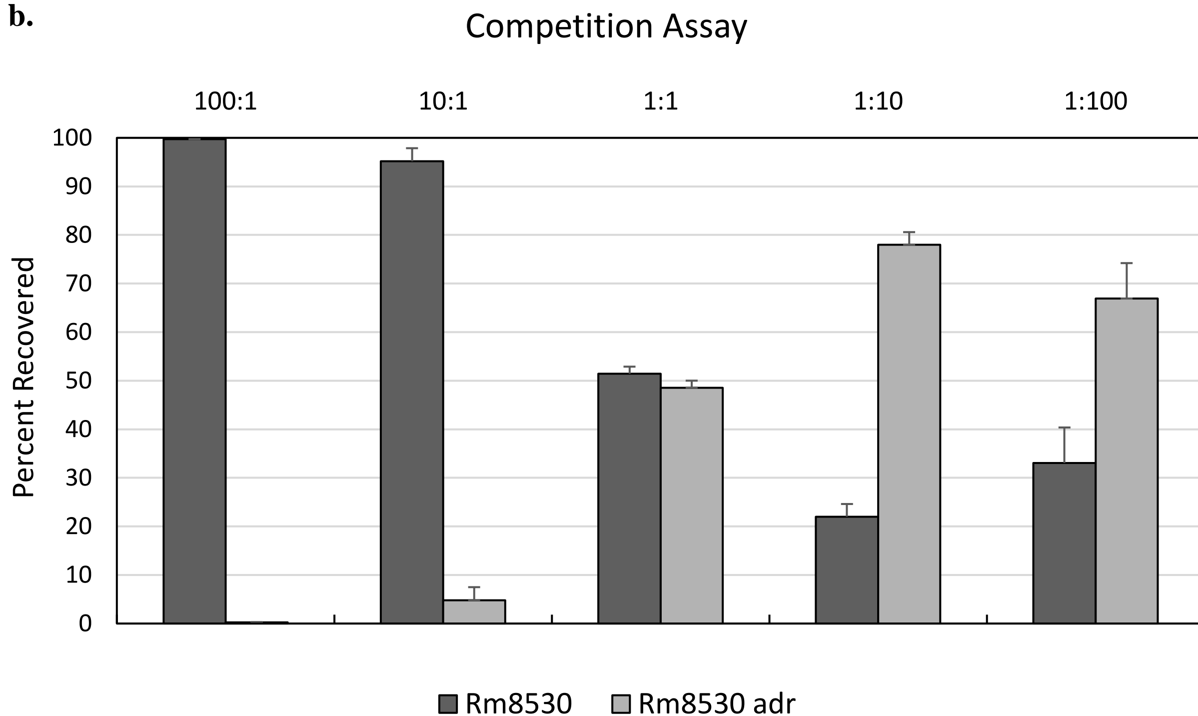
b.



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959 **Figure 7. Symbiosis and competition assays.** The *adr* mutant was capable of establishing

960 symbiosis at levels comparable to the wild-type Rm8530, but was unable to compete with wild-

961 type for nodule occupancy when both strains were co-inoculated. **a)** *Medicago sativa* cv.

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962 Iroquois inoculated with wild-type and the *adr* mutant were proficient at forming nitrogen fixing
963 nodules and the plants were healthy. Strains deficient in *fixJ* were unable to establish nitrogen
964 fixing nodules and resulted in plants that were not as healthy as the wild-type and *adr* mutant
965 plants. The results are the average of three independent experiments and the standard deviations
966 are shown. **b)** Plants were co-inoculated with varying proportions of Rm8530 and the *adr*
967 mutant, represented on the X-axis. The percentage of bacteria recovered is shown on the Y-axis.
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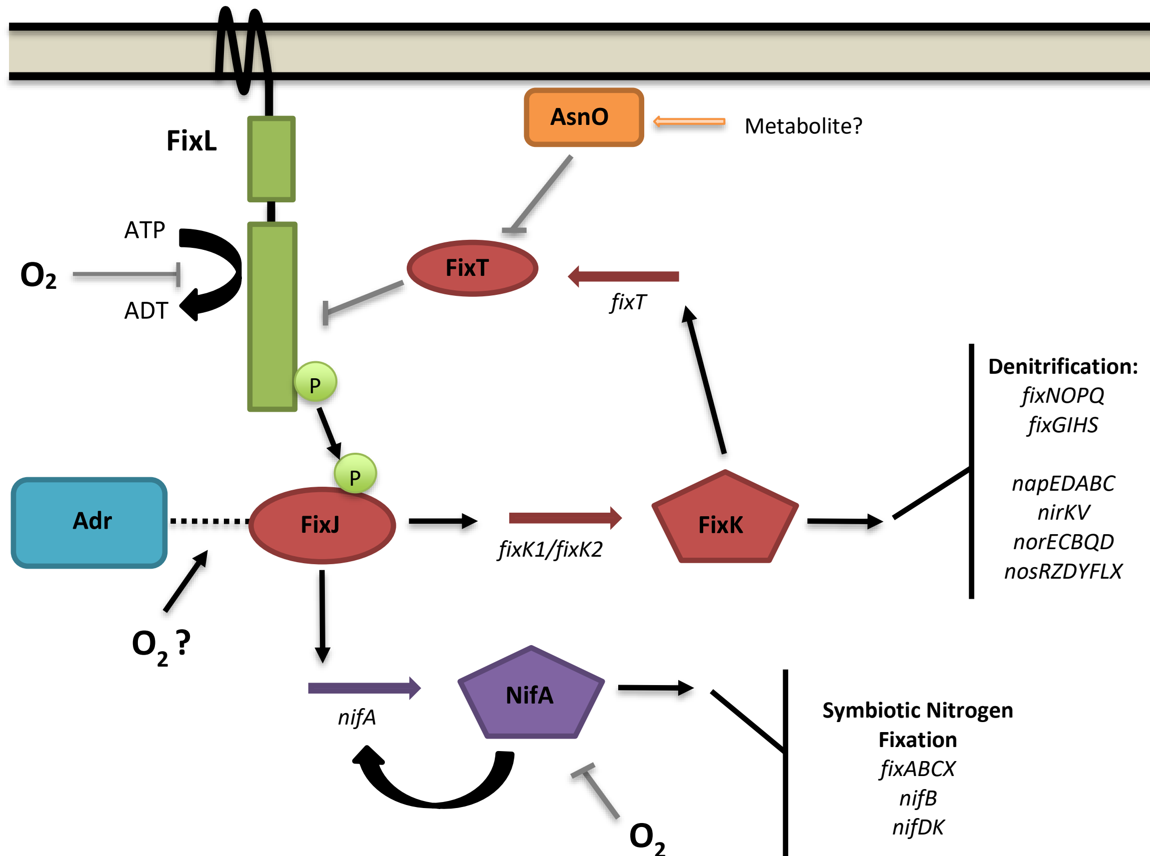

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970 **Figure 8. Model for regulation of denitrification and nitrogen fixation *S. meliloti*.** Revised
971 comprehensive model for the regulation of denitrification and nitrogen fixation in the presence
972 and absence of oxygen. Grey lines indicate an inhibitory effect while black arrows indicate a
973 positive effect. In the presence of oxygen, the autophosphorylation of FixL is inhibited, leading
974 to a low level of phosphorylated FixJ in the cell. Adr interacts with FixJ (dotted line) and
975 promotes the expression of the denitrification regulator FixK and the nitrogen fixation regulator
976 NifA. FixK goes on to induce expression of the denitrification genes, as well as the antikinase
977 FixT, which further inhibits the autophosphorylation of FixL. NifA is oxygen sensitive and is
978 deactivated, therefore preventing the expression of the symbiotic fixation genes. When the
979 oxygen concentration is low, FixL can autophosphorylate and transfer the phosphate group to
980 FixJ. In the phosphorylated form, FixJ can activate the expression of the denitrification and

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981 nitrogen fixation genes without the help of Adr. AsnO acts to inhibit the antikinase activity of
982 FixT, allowing this feedback loop to continue until environmental conditions change. Figure
983 adapted from Terpolilli, et al. (16).