Disrupting hierarchical control of nitrogen fixation enables carbon-dependent regulation of ammonia excretion in soil diazotrophs

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Abstract

The energetic requirements for biological nitrogen fixation necessitate stringent regulation of this process in response to diverse environmental constraints. To ensure that the nitrogen fixation machinery is expressed only under appropriate physiological conditions, the dedicated NifL-NifA regulatory system, prevalent in Proteobacteria, plays a crucial role in integrating signals of the oxygen, carbon and nitrogen status to control transcription of nitrogen fixation (*nif*) genes. Greater understanding of the intricate molecular mechanisms driving transcriptional control of *nif* genes may provide a blueprint for engineering diazotrophs that associate with cereals. In this study, we investigated the properties of a single amino acid substitution in NifA, (NifA-E356K) which disrupts the hierarchy of *nif* regulation in response to carbon and nitrogen status in *Azotobacter vinelandii*. The NifA-E356K substitution enabled overexpression of nitrogenase in the presence of excess fixed nitrogen and release of ammonia outside the cell. However, both of these properties were conditional upon the nature of the carbon source. Our studies reveal that the uncoupling of nitrogen fixation from its assimilation is likely to result from feedback regulation of glutamine synthetase, allowing surplus fixed nitrogen to be excreted. Reciprocal substitutions in NifA from other Proteobacteria yielded similar properties to the *A. vinelandii* counterpart, suggesting that this variant protein may facilitate engineering of carbon source-dependent ammonia excretion amongst diverse members of this family.

Significance

The NifL-NifA regulatory system provides dedicated signal transduction machinery to regulate nitrogen fixation in diverse Proteobacteria. Understanding how the balance of nitrogen and carbon resources is signalled via NifL-NifA for precise

26   control of nitrogen fixation may lead to broadly applicable translational outputs. Here,

27   we characterize a NifA variant that bypasses nitrogen regulation but is still dependent

28   on the carbon status to enable ammonia excretion in soil diazotrophs. Disruption of

29   the regulatory hierarchy in response to nitrogen and carbon suggests how the

30   integration of environmental stimuli could be harnessed to engineer conditional

31   release of fixed nitrogen for the benefit of cereal crops.

32

Introduction

33   **Introduction**

34   Biological nitrogen fixation requires diversion of reducing equivalents and ATP

35   derived from carbon metabolism to support the high energetic demands of the enzyme

36   nitrogenase, which converts dinitrogen to ammonia. Tight regulatory control provides

37   the means to balance energy metabolism with nitrogen fixation and ammonia

38   assimilation so that fixed nitrogen is not limiting under diazotrophic growth conditions.

39   Achieving an appropriate balance between carbon and nitrogen metabolism is

40   particularly important for diazotrophic bacteria in order to meet the energetic cost of

41   nitrogen fixation, while thriving in competitive environments. While many studies on

42   the regulation of nitrogen fixation have focused on intricate signalling mechanisms

43   responding to the presence of oxygen and fixed nitrogen (1–3), regulation in response

44   to the carbon status has not been extensively studied, despite its significance for the

45   energetics of diazotrophy and the interplay required to balance the carbon : nitrogen

46   ratio.

47   The nitrogen fixation specific, NifL-NifA, regulatory system provides a very

48   sophisticated signal transduction complex for integration and transmission of various

49   environmental cues to the transcriptional apparatus in *Azotobacter vinelandii* to

50   regulate biosynthesis and expression of nitrogenase (reviewed in 8, 9). The anti-

51   activator NifL is a multidomain protein carrying an N-terminal PAS domain (PAS1) that

52   senses the redox status via a FAD co-factor (6, 7). A second PAS domain (PAS2)

53   appears to play a structural role in relaying the redox changes perceived by the PAS1

54   domain to the central (H) and C-terminal (GHKL) domains of NifL (8, 9). The latter is

55   responsible for ADP binding (10, 11) and is probably the site of interaction for the GlnK

56   signal transduction protein, allowing integration of the nitrogen input into NifL-NifA

57   regulation (12, 13). The protein partner of NifL, the prokaryotic enhancer binding

58   protein NifA, which activates *nif* transcription, is comprised of an N-terminal regulatory

59   domain (GAF), a central AAA+ sigma-54 activation domain and a C-terminal DNA

60   binding domain. The regulatory GAF domain of *A. vinelandii* NifA binds 2-oxoglutarate

61   (14), a TCA cycle intermediate at the interface of carbon and nitrogen metabolism

62   (15). NifA can only escape inhibition by NifL, when the GAF domain is saturated with

63   2-oxoglutarate, thus potentially providing a mechanism for the NifL-NifA system to

64   respond to the carbon status.

65   Understanding how the NifL-NifA system integrates diverse regulatory inputs

66   may allow new strategies for engineering diazotrophs with enhanced ability to fix

67   nitrogen and release ammonia to benefit crop nutrition. Ammonia excretion can be

68   achieved in *A. vinelandii* by engineering constitutive expression of genes required for

69   nitrogenase biosynthesis through inactivation of *nifL* (16–19). However, in the absence

70   of active NifL, all the regulatory signal inputs that control NifA activity are removed,

71   and as consequence, the resulting bacterial strain may be severely disadvantaged in

72   the environment and even unstable under laboratory conditions as already reported

73   (17, 19). Isolation of insertion mutants in *nifL* appears to be conditional upon second

74   site mutations that may alter the level of *nif* gene expression. One such suppressor

75   was identified in a promoter-like sequence upstream of *nifA*, presumably leading to a

4

76   permissive reduction in *nifA* transcript levels (19). A full deletion of *nifL* has also been

77   reported (18), but it is unclear if second site mutations occurred during its isolation.

78   One approach to potentially minimize the energetic burden associated with

79   constitutive expression of nitrogenase is to ensure that regulatory control of NifA

80   activity is maintained in energy-limiting environments. Random mutagenesis of *nifA*

81   followed by screening the activity of the *A. vinelandii* NifL-NifA system in *E. coli*

82   identified various NifA variants able to escape regulation by NifL under nitrogen excess

83   conditions (20). One of these mutations, resulting in a charge-change substitution,

84   E356K, located in the central catalytic domain of NifA, (hereafter named NifA-E356K),

85   was found to require binding of 2-oxoglutarate to the GAF domain to escape NifL

86   repression in response to excess fixed nitrogen (14, 21).

87   In this study we demonstrate that both expression and activity of nitrogenase

88   are insensitive to the nitrogen status when the *nifA-E356K* mutation is introduced into

89   *A. vinelandii*, resulting in excretion of ammonia at millimolar levels during exponential

90   growth, a phenomenon correlated with feedback regulation of glutamine synthetase

91   when nitrogenase is constitutively active. However, unregulated expression of *nif*

92   genes and ammonia excretion by the *nifA-E356K* mutant is conditional on the nature

93   of the carbon source indicating dependency on carbon status signalling and

94   supporting previous biochemical observations that this NifA-E356K variant is

95   dependent on the levels of 2-oxoglutarate to escape nitrogen regulation by NifL *in vitro*

96   (21). Finally, we demonstrate that reciprocal substitutions in NifA proteins of other

97   Proteobacteria lead to similar regulatory phenotypes when assayed in *E. coli* as a

98   chassis and also when the substitution is engineered in the endophytic diazotroph

99   *Pseudomonas stutzeri* A1501 (22, 23). In principle, this single amino acid substitution

100   in NifA (E356K) provides a regulatory switch capable of activating *nif* gene expression

101   under nitrogen excess conditions only when certain carbon sources are available in

102   the environment, thus setting the foundation for engineering a synthetic symbiosis to

103   deliver fixed nitrogen to cereal crops.

104

105   **Results**

106   **The activity of NifA-E356K is not regulated in response to the nitrogen status in**

107   ***A. vinelandii,* resulting in ammonia excretion**

108   Previously the NifA-E356K variant protein was characterized either *in vivo*

109   using *E. coli* as a chassis, or *in vitro* using purified protein components (20, 21). To

110   evaluate if this variant protein would bypass NifL regulation in the original *A. vinelandii*

111   DJ background, we introduced the *nifA-E356K* mutation into the chromosome and

112   examined its influence on transcriptional regulation of the nitrogenase structural genes

113   using RT-qPCR. As anticipated, no *nifH* transcripts were detected in the wild type

114   strain (DJ) in the presence of either 25 or 5 mM of ammonium acetate, whilst increased

115   transcript levels were observed in the absence of ammonium (Fig. 1A). In contrast,

116   high levels of *nifH* transcripts were observed in all conditions tested for the *nifA-E356K*

117   mutant strain (EK) (Fig. 1B), confirming that *nifA-E356K* is able to escape nitrogen

118   regulation in *A. vinelandii*. Comparison of the levels of *nifH*, *nifL* and *nifA* transcripts

119   in the EK strain relative to the wild type (DJ) (Fig. 1C) revealed that whilst *nifH* levels

120   are higher in the EK mutant, this is not correlated with increased levels of *nifL* and *nifA*

121   transcripts. This is in line with previous reports establishing that *nifLA* expression is

122   not subject to autoactivation by NifA in *A. vinelandii* (24, 25) and suggests that

123   constitutive expression of the nitrogenase structural gene operon in the EK mutant is

124   intrinsic to the *nifA-E356K* mutation itself, rather than a consequence of

125   overexpression of this mutant gene. The increase in *nifH* transcripts correlated with

126   higher nitrogenase activity in the EK strain, which was not regulated in response to

127   excess fixed nitrogen, in contrast to the wild-type strain (Fig. 1D). As anticipated from

128   the energetic constraints associated with constitutive expression and activity of

129   nitrogenase, the EK mutant had an apparent growth deficiency in liquid media

130   (Supplementary Material Fig. S1). However, when an insertion replacing the *nifH*

131   structural gene was introduced into the EK strain to generate strain EKH (EK,

132   *ΔnifH::tetA)* this growth penalty was alleviated (Fig. S1-D-F) suggesting that it results

133   from unregulated expression and activity of nitrogenase.

134   When cultivated under diazotrophic conditions in nitrogen-free media

135   supplemented with sucrose as carbon source, the EK strain excreted millimolar levels

136   of ammonia (Fig. 1E). The onset of ammonia excretion occurred at the early stages of

137   growth (O.D$_{600nm}$ 0.3 - 0.6) reaching a peak at mid to late-exponential phase (O.D$_{600nm}$

138   1.4 - 2.0) and declined upon entry into stationary phase, potentially as a consequence

139   of oxygen limitation and lower nitrogen fixation rates.

140

Nitrogen fixation and ammonium assimilation are uncoupled in the *nifA-E356K* mutant strain

143   It is perhaps surprising that a single amino acid substitution in NifA enables

144   ammonia excretion, since lowering the flux of ammonia assimilation through

145   the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway is expected to

146   be an additional pre-requisite for high level release of ammonia. The enzyme

147   glutamine synthetase (GS) is a key component for ammonia assimilation in

148   proteobacteria and is subject to post-translational regulation via adenylylation in

149   response to the nitrogen status (26, 27). Under excess nitrogen conditions, GS is

150   adenylylated reducing its biosynthetic activity (3, 27). Comparison of the Mg^{2+}, ATP-

151   dependent glutamine synthetase biosynthetic (GSB) and the Mn^{2+}, AsO4-, ADP-

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152   dependent glutamine transferase (GST) activities can provide a snapshot of GS

153   adenylylation states *in vivo*, given that GSB activity can only be detected in the non-

154   adenylylated enzyme subunits (28–30). We observed that GSB activity was higher in

155   wild type (DJ) when compared to the mutant (EK) in all growth phases when cells were

156   cultivated under diazotrophic conditions (green shaded plots in Fig. 2A). Conversely,

157   GST activity was higher in the *nifA-E356K* strain (EK) than in the wild type (DJ) (Fig.

158   2B). A comparison of GST/GSB ratios in both strains (Fig. 2C) demonstrated that GS

159   in the wild type (DJ) is likely to be entirely non-adenylylated under diazotrophic

160   conditions (GST/GSB ratio ≦ 1), whilst the NifA-E356K mutant (EK) had much higher

161   GST/GSB ratios (ranging from 15 to 30-fold) suggesting that in this mutant strain, GS

162   is more heavily adenylylated. Under fixed nitrogen excess conditions (yellow shaded

163   plots in Fig. 2), as anticipated, a marked reduction of GSB activity was observed in the

164   DJ strain (Fig. 2A, yellow plot) followed by an increase in the GST activity (Fig. 2B,

165   yellow plot). For the EK strain however, relatively minor changes in GSB and GST

166   activities occurred in response to the nitrogen status. A slight increase in GSB activity

167   was observed in the EK strain under nitrogen excess conditions, but this was not

168   apparently associated with changes in the expression of GS (*glnA*) itself (Fig. S2).

169   Taken together, these results confirm that the ability of the *nifA-E356K* mutant strain

170   to assimilate ammonia via GS is reduced compared to the wild type under diazotrophic

171   conditions. This is likely to be a consequence of increased adenylylation by the

172   bifunctional adenylyl transferase enzyme GlnE, which carries out post-translational

173   modification of GS in response to the nitrogen status. Since deletion of *glnE* prevents

174   adenylylation of GS in *A. vinelandii* (31), we introduced a *glnE* deletion into the *nifA-*

175   *E356K* background to generate the strain EKΔE. Under diazotrophic conditions, GSB

176   and GST activities in the EKΔE strain were similar to the those in the wild type strain

177   (DJ) and clearly distinct from the activities in the *nifA-E356K* strain (EK) (compare

178   green shaded plots in Fig. 2A-B and Fig. 2D-E). However, as anticipated from the

179   absence of adenylyl transferase activity, addition of ammonium to the media did not

180   lead to either reduction of GSB or increased GST activity in the EKΔE strain when

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181   compared to the DJ strain (compare yellow shaded plots in Fig. 2A-B and Fig. 2D-E).

182   As expected from the increased GS biosynthetic activity exhibited by the EKΔE strain

183   under nitrogen excess conditions, ammonia excretion was ablated in this strain (Fig.

184   2F and Fig. S3). These results therefore imply that the ability of the *nifA-E356K* (EK)

185   strain to excrete ammonia is dependent upon feedback regulation, resulting in

186   constitutive adenylylation of GS and hence decreased ammonia assimilation.

187

188   **Carbon signalling dominates regulation of *nif* gene expression in the *nifA-***

189   ***E356K* strain**

190   All the above experiments were conducted in media containing sucrose, a

191   carbon source enabling a high flux through the TCA cycle that evidently maintains

192   sufficient 2-oxoglutarate to activate NifA in *A. vinelandii*. Although the potential for

193   carbon regulation, signalled via binding of 2-oxoglutarate to the NifA GAF domain, has

194   been well established *in vitro,* the physiological relevance of 2-oxoglutarate in NifL-

195   NifA regulation has not been clearly demonstrated *in vivo*. NifA-E356K requires 2-

196   oxoglutarate in order to escape inhibition by NifL in the presence of GlnK *in vitro* (14,

197   21), suggesting that its ability to bypass nitrogen regulation *in vivo* might be regulated

198   by carbon source availability. To facilitate correlation of nitrogenase activity with

199   expression of the nitrogenase structural genes when strains were grown on different

200   carbon sources we constructed strains containing a translational *nifH::lacZ* fusion

201   located at a neutral site in the chromosome (see Supplementary Material and Table

202   S1). Initial screening for the ability of the NifA-E356K protein to escape nitrogen

203   regulation in several carbon sources (Figure S4) revealed that this variant protein

204   supported strong activation of the *nifH* promoter under nitrogen excess conditions

205   when grown in sucrose, glucose or glycerol. In contrast, only approximately 20-40%

206   of the maximum activity was observed when cells were grown in succinate, fumarate,

207   malate, pyruvate or acetate as the sole carbon source. Further comparisons of Av-

208   NifA-E356K activity were performed comparing sucrose and acetate as carbon

209   sources given that the growth penalty difference between the DJ and EK strains was

210   significantly alleviated in acetate (Figure S5).

211   When wild type *A. vinelandii* was subjected to a carbon shift from sucrose to

212   acetate, a significant reduction in both *nifH* expression (Fig. 3A) and nitrogenase

213   activity (Fig. 3B) was observed and as expected, both activities were repressed in the

214   presence of ammonium (+N). However, the ability of the EK strain to escape regulation

215   by fixed nitrogen (+N) was severely compromised when grown on acetate (Fig. 3D

216   and Fig. 3E). These results demonstrate that NifA-E356K responds to carbon status

217   regulation *in vivo*, as anticipated from the *in vitro* characterization experiments (14,

218   21). Since 2-oxoglutarate is required to activate NifA only when NifL is present, we

219   examined nitrogen and carbon regulation in the previously characterised strain

220   AZBB163 in which *nifL* is disrupted by a kanamycin resistance cassette. (19). In this

221   case, in contrast to NifA-E356K, nitrogenase activity was constitutive and not strongly

222   influenced by the carbon source (Fig. S6B). However, this strain exhibited unexpected

223   patterns of *nifH* expression when grown on sucrose that did not correlate with

224   nitrogenase activity (Fig. S6A), potentially because *nifA* expression is not driven by

225   the native   *nifL* promoter in strain AZBB163 (19).   Taken together, these results

226   demonstrate that nitrogenase expression and activity is suppressed in the EK strain

227   when grown under nitrogen excess conditions with acetate as the sole carbon source.

228   Since the NifA-E356K protein is unable to escape nitrogen regulation mediated by NifL

229   and GlnK when 2-oxoglutarate is limiting *in vitro*, this metabolite is likely to provide the

230   physiological signal that triggers the carbon source response. Quantification of internal

231   2-oxoglutarate levels in strains grown on the different carbon sources (Fig. 3C and

232   Fig. 3F) supports previous evidence that the levels of this metabolite are sensitive to

233   the carbon and nitrogen supply (15, 32). In the wild type strain (DJ), 2-oxoglutarate

234   levels dropped significantly in acetate compared to sucrose and a further decrease

235   was observed when excess fixed nitrogen was present regardless of the type of

236   carbon source (Fig. 3C). 2-oxoglutarate levels in the *nifA-E356K* strain were generally

237   higher than in the wild type, but were influenced in a similar manner in relation to

238   carbon and nitrogen source availability (Fig. 3F). Overall, the fluctuations in the 2-

239   oxoglutarate levels correlated well with nitrogenase activity and expression for both

240   the wild type (compare Fig. 3, panels A-C) and the *nifA-E356K* mutant (compare Fig.

241   3, panels D-F), reinforcing the importance of carbon signalling in the regulation of

242   nitrogen fixation. Notably, when the 2-oxoglutarate level decreased below 350 µM in

243   the *nifA-E356K* strain (Fig. 3F, acetate +N condition) nitrogen regulation was less

244   effectively bypassed *in vivo* (Fig. 3, panels D-E), commensurate with previous *in vitro*

245   biochemical data (14, 33). Consequently, lower levels of ammonia excretion were

246   detectable when the *nifA-E356K* strain was grown on acetate (Fig. 3G). In accordance

247   with this, the growth rate penalty observed in the EK (*nifA-E356K*) strain in the

248   presence of sucrose was significantly reduced when acetate was the carbon source

249   (Fig S5).

250

251   **NifA-E356K is a prototype for engineering conditional ammonia excretion in**

252   **diazotrophic Proteobacteria**

253   As the NifL-NifA operon is widely distributed in Proteobacteria (reviewed in 3)

254   and the glutamate residue at position 356 in *A. vinelandii* NifA is highly conserved in

255   other Proteobacteria (Fig. 4 panels A and B), we sought to evaluate if introduction of

256   the reciprocal amino acid substitution in NifA proteins from other Proteobacteria, would

257   yield the same regulation profile as in *A. vinelandii*. Using a previously established

258   two-plasmid system to study the *A. vinelandii* NifL-NifA system in an *E. coli*

259   background (11) we evaluated the activity of NifA variants from *Pseudomonas stutzeri*

260   A1501 (22, 23) and *Azoarcus olearius* DQS-4 (34, 35). Both of these species are

261   thought to be well adapted for the endophytic lifestyle and therefore are attractive

262   model organisms for engineering ammonia excretion to benefit plant growth. The

263   activities of wild type *P. stutzeri* NifA and *A. olearius* NifA in the presence of their

264   corresponding NifL partners were higher than wild type *A. vinelandii* NifA under

265   nitrogen-limiting conditions (-N) but as expected, were strongly inhibited in the

266   presence of fixed nitrogen (+N) (Fig. 4, panels C-E). In contrast, the reciprocal NifA-

267   E356K substitutions in *P. stutzeri* NifA (Ps-NifA-E356K) and *A. olearius* NifA (Ao-NifA-

268   E351K) gave rise to constitutive activation of the *nifH* promoter in nitrogen replete (+N)

269   conditions in *E. coli* (Fig. 4 panels D-E, respectively).

270   To examine the properties of the Ps-NifA-E356K substitution in its endophytic

271   host, we introduced the corresponding *nifA* mutation into the chromosome of *P.*

272   *stutzeri* A1501. However, contrary to *A. vinelandii,* this single mutation (in strain Ps-

273   EK) did not result in constitutive *nifH* transcription (Fig. S7), presumably because

274   expression of the *nifLA* operon itself is regulated by nitrogen availability in *P. stutzeri*

275   (23, 36). In order to remove this second layer of nitrogen regulation, we replaced the

276   native *P.stutzeri* *nifL* promoter with the *A. vinelandii nifL* promoter. Although this

277   replacement (in the strain Ps_nifLA[C]) suppressed nitrogen control of *nifA* transcription

278   as anticipated, constitutive *nifH* transcription was not observed, confirming that the *P.*

279   *stutzeri* wild-type NifL-NifA system remains responsive to nitrogen regulation when

280   expressed constitutively (Fig. S7). To examine the intrinsic ability of Ps-NifA-E356K to

281   escape nitrogen control, we combined the *A. vinelandii nifL* promoter replacement with

282   the *nifA-E356K* mutation in *P. stutzeri* (Fig. 5A). Although this strain (Ps-EK[C])

283   expressed relatively low levels of *nifLA* transcripts under diazotrophic (-N) conditions

284   (Fig. S7 F-G), direct correlation between the levels of *nifLA* and *nifH* transcripts was

285   observed in excess nitrogen (+N) conditions, confirming that the E356K substitution

286   enables Ps-NifA to escape nitrogen regulation mediated by NifL and GlnK in *P. stutzeri*

287   (Fig. S7, panels F-H).

288   The Ps-EK^C strain was able to activate *nifH* transcription on a variety of carbon

289   sources when grown under nitrogen excess conditions (+N) with lactate yielding the

290   highest level of *nifH* activation followed by glucose, malate and glycerol (Fig 5B). This

291   carbon source-dependent activation of *nifH* transcription by Ps-NifA-E356K correlated

292   directly with the level of nitrogenase activity in each condition (compare Fig. 5B and

293   5C). As anticipated from the relatively high level of *nifH* transcription and nitrogenase

294   activity conferred by growth on lactate, ammonia excretion was only observed when

295   the Ps-EK^C strain was grown on this carbon source (Fig. 5D). Finally, we observed no

296   growth penalty for the Ps-EK^C strain when grown on complex media (LB) or in minimal

297   media supplemented with glucose, malate, lactate or glycerol as carbon sources (Fig

298   S8), which implies that the relatively moderate activation of *nif* gene transcription in

299   the Ps-EK^C strain, allows carbon regulated ammonia excretion without severe impacts

300   to bacterial fitness. Altogether, these observations suggest that introducing the

301   reciprocal E356K substitution into NifA proteins from diazotrophic Proteobacteria, may

302   be broadly applicable for engineering new bacterial strains with carbon-controlled

303   excretion of ammonia.

304

305   **Discussion**

306   In order to cope with the energetic cost of biological nitrogen fixation,

307   diazotrophic bacteria require sophisticated signal transduction mechanisms ensuring

308   efficient adaption to changing conditions whilst successfully competing in the

309   environment. Achieving an appropriate balance between carbon and nitrogen

310   metabolism is particularly challenging for organisms that fix nitrogen, requiring

311   diversion of ATP and reducing equivalents from central metabolism to ensure

312   nitrogenase catalytic rates that meet the nitrogen demands required for growth. Hence

313   the ability to sense carbon availability in addition to the nitrogen status, is paramount

314   to resource allocation and to resolve conflicting metabolic demands.

315   The physiological signal for carbon status control is most likely to be 2-

316   oxoglutarate given the correlation observed here between the level of this metabolite

317   with nitrogen regulation *in vivo*, together with our previous biochemical demonstration

318   of the importance of this ligand in NifL-NifA regulation (14, 37). We propose that this

319   additional level of metabolite regulation provides a mechanism to integrate signals of

320   the carbon and nitrogen status to ensure that sufficient carbon resources are available

321   to support diazotrophy. The *in vitro* data indicate that when 2-oxoglutarate is limiting,

322   NifL, forms a binary complex with NifA, which inhibits its activity, even under nitrogen-

323   limiting conditions when GlnK is fully uridylylated and unable to interact with nifL (Fig.

324   6A). However, when sufficient levels of 2-oxoglutarate are available (Fig. 6B) the NifL-

325   NifA complex dissociates, enabling NifA to activate *nif* transcription (2, 4, 14, 37). Upon

326   a switch to excess nitrogen conditions, GlnK becomes de-uridylylated allowing the

327   formation of a ternary GlnK-NifL-NifA complex that inactivates NifA irrespective of the

328   level of 2-oxoglutarate (Fig. 6C and 6D). Hence in the wild-type NifL-NifA system, the

329   nitrogen status signal overrides the metabolic signal of the carbon status, when excess

330   fixed nitrogen is available. In contrast, in the variant NifA-E356K protein studied here,

331   the integration between nitrogen and carbon control is disrupted. Although this

332   substitution in the AAA+ domain of NifA (red star, Fig. 6E) perturbs the interaction with

333   NifL, the GlnK-NifL-NifA-E356K ternary complex still forms if 2-oxoglutarate is limiting

334   (Fig. 6E). In contrast, when 2-oxoglutarate levels are sufficient, conformational

335   changes triggered by its binding to the GAF domain disrupt the ternary complex,

336   enabling NifA-E356K to be active in the presence of excess fixed nitrogen (Fig. 6F).

337   Therefore, although the E356K substitution escapes nitrogen control, conferred by

338   resistance to the GlnK bound form of NifL, this is contingent upon the binding of 2-

339   oxoglutarate to the GAF domain of this variant protein. The response of the NifL-NifA

340   system to 2-oxoglutarate thus emphasises the key role of this metabolite as a master

341   signalling molecule (15). Consequently, the ability of the NifA-E356K variant to bypass

342   nitrogen regulation *in vivo* in both *A. vinelandii* and *P. stutzeri* is dependent on the

343   carbon status. The crucial role of carbon-mediated signalling in the regulation of

344   nitrogen fixation was evident from reduced *nifH* transcripts and activity of nitrogenase

345   when the *A. vinelandii nifA-E356K* strain was cultured under nitrogen excess

346   conditions with acetate as sole carbon source, which correlated with a significant

347   decrease in the level of 2-oxoglutarate and a 6-fold reduction in ammonium excretion

348   compared with sucrose as carbon source. Similarly, in *P. stutzeri* where lactate

349   appears to be a preferred carbon source to support nitrogen fixation in comparison to

350   glucose, the *nifA-E356K* mutant exhibited the highest level of *nifH* transcripts and

351   nitrogenase activity under nitrogen excess conditions when lactate was provided as a

352   carbon source. Not surprisingly, amongst the carbon sources tested, the *P. stutzeri*

353   EK[C] strain only excreted ammonia when provided with lactate under our experimental

354   conditions. The capacity for runaway expression of *nif* genes, constitutive nitrogenase

355   activity and ammonia excretion is therefore dependent on the nature of the carbon

356   source. Our studies with *A. vinelandii* and *P. stutzeri* therefore demonstrate the

357   potential to exploit the intrinsic carbon-sensing mechanism of the NifL-NifA system to

358   provide conditional release of fixed nitrogen and hence alleviate the fitness penalty

359   associated with constitutive expression of nitrogenase.

360   Bypassing nitrogen regulation of the NifL-NifA system to activate constitutive

361   expression of nitrogenase would not by itself be anticipated to promote ammonia

362 release, if the excess ammonia can be assimilated by the GS-GOGAT pathway. We

363 have demonstrated that overexpression of nitrogenase in the *A. vinelandii nifA-E356K*

364 strain leads to feedback regulation of GS activity via co-valent modification by the

365 adenylyl transferase activity of GlnE. This reduction of nitrogen assimilation via post-

366 translational modification of glutamine synthetase is a key factor in enabling ammonia

367 excretion, which is not observed in the *nifA-E356K* strain when the *glnE* gene is

368 deleted. Hence, in *A. vinelandii*, ammonia excretion is also dependent on the native

369 feedback regulation of GS activity, exacerbated by higher rates of nitrogen fixation in

370 the *nifA-E356K* strain when grown on a carbon source that sustains high levels of 2-

371 oxoglutarate under excess nitrogen conditions. This uncoupling of nitrogen fixation

372 from ammonium assimilation is somewhat analogous to what is observed in

373 differentiated nitrogen-fixing bacteroids in the legume-rhizobium symbiosis, where the

374 flux through the ammonia assimilation pathway is severely restricted to enable release

375 of most of the nitrogen fixed by the symbiont (38, 39). Analogous strategies to

376 decrease the activity of GS in non-symbiotic bacteria have resulted in ammonia

377 excretion (40–42), but to date have not been combined with mutations that express

378 high levels of nitrogenase on a conditional basis as deployed here.

379   Since introduction of the reciprocal E356K substitution into NifA proteins from

380 other members of the Proteobacteria also results in nitrogen-insensitive activators

381 when analysed either in a heterologous chassis, *E. coli* (Fig. 4), or in *P. stutzeri* (Fig.

382 5), this strategy may allow generation of new diazotrophic strains with conditional

383 excretion of ammonia. The carbon responsive control mechanism could present an

384 opportunity for activation of ammonium excretion contingent upon carbon sources

385 provided by root exudates of crops, a feature highly desirable in the engineering of a

386 synthetic symbiosis (43, 44). However, given the regulatory complexities associated

387   with fine-tuning nitrogen regulation in diverse Proteobacteria, additional manipulations

388   to disrupt transcriptional control of NifL-NifA expression itself or the coupling between

389   nitrogen fixation and assimilation may be needed to achieve ammonia excretion (3,

390   45). One such example explored in this study was the need to remove native nitrogen

391   regulation from the *P. stutzeri* A1501 *nifLA* promoter. Serendipitously, we

392   demonstrated that providing relatively low levels of the *nifA-E356K* transcripts in *P.*

393   *stutzeri* generated a strain with carbon-regulated ammonia excretion without the

394   severe growth penalties observed in *A. vinelandii*. Furthermore, we demonstrated that

395   the levels of ammonia excretion are directly correlated with specific nitrogenase

396   activity rates in both organisms analysed. Under optimal conditions of carbon and

397   oxygen supply, the *A. vinelandii nifA-E356K* mutant sustained a very high rate of

398   nitrogenase activity (200-300 nmol C_2H_4.mg protein^{-1}.min^{-1}) under nitrogen excess

399   conditions. On the other hand, in *P. stutzeri* the levels of nitrogenase activity conferred

400   by *nifA-E356K* were at least 100-fold lower under our experimental conditions. Hence,

401   *A. vinelandii* excreted millimolar levels of ammonia in contrast to the micromolar levels

402   observed in *P. stutzeri*. As the ammonium excreting strain from *P. stutzeri* was not

403   subject to the same growth penalty observed in the *A. vinelandii* counterpart, we

404   anticipate that these studies will guide future efforts to define more precise trade-offs

405   to engineer nitrogen releasing strains that do not have a competitive disadvantage in

406   the rhizosphere. Moreover, the addition of multi-layered regulatory control of ammonia

407   excretion by expressing activator variants under the control of promoters that respond

408   to specific signalling molecules exchanged between the plant and the bacteria, may

409   deliver the required level of specificity for the establishment of an efficient synthetic

410   symbiosis (46, 47).

411

Materials and Methods

Detailed methods are available in the Supplementary Material. Bacterial stains are listed in Table S1, plasmids are listed in Table S2 and primers are listed in Table S3.

Author Contributions

M.B.B, Y-P.W and R.D designed research, M.B.B, P.B. and C.A-A. performed experiments and M.B.B, Y-P.W and R.D. wrote the manuscript

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Figures
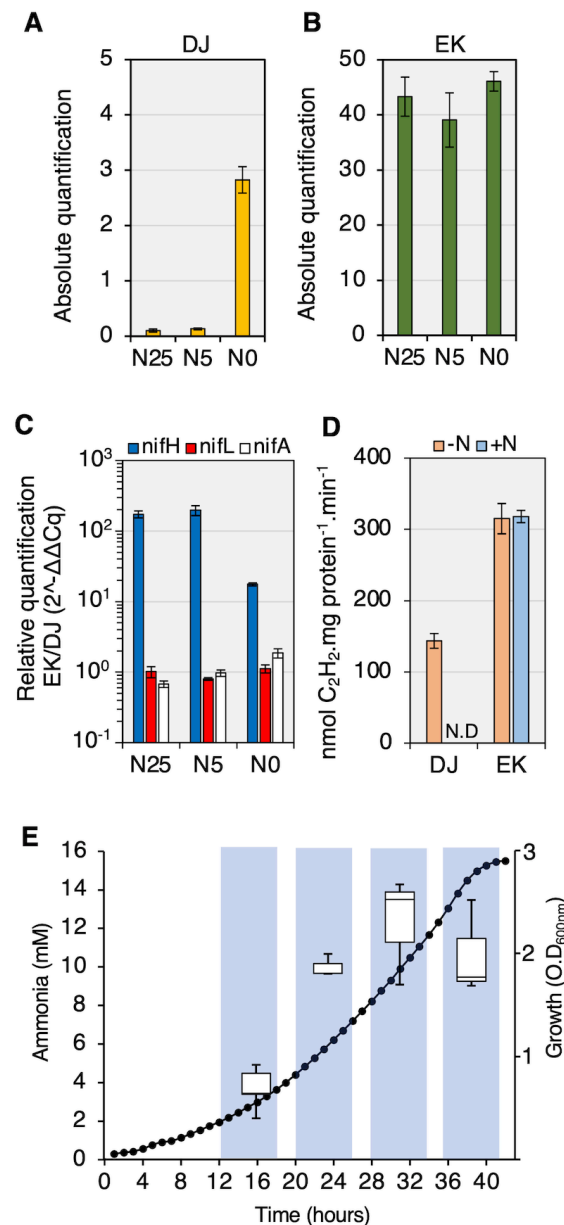
Fig. 1 – Constitutive expression and activity of nitrogenase results in ammonia excretion by the *nifA-E356K* strain (EK) with sucrose as the carbon source. (A) Absolute levels of *nifH* transcripts in the wild type (DJ) and (B) *nifA-E356K* (EK) under three different nitrogen regimes: 25 mM (N25), 5 mM (N5) or 0 mM (N0) ammonium acetate. (C) Relative levels of *nifH*, *nifL* and *nifA* transcripts between the strains EK and DJ. The graph is presented on a *log₁₀* scale to emphasize that the relative levels of *nifL* and *nifA* transcripts are close to 1 in all conditions. (D) *In vivo* nitrogenase specific activities in the absence (-N) or presence (+N) of 20 mM ammonium chloride. Activity was determined by the acetylene reduction assay using cultures grown to an $O.D_{600nm}$ between 0.3-0.4 as described in the methods. N.D: not detected. (E) Ammonia from the culture supernatant was quantified in the EK strain (left y axis, box blots) in the growth phases indicated by the bars shaded in blue (right y axis, closed circles).
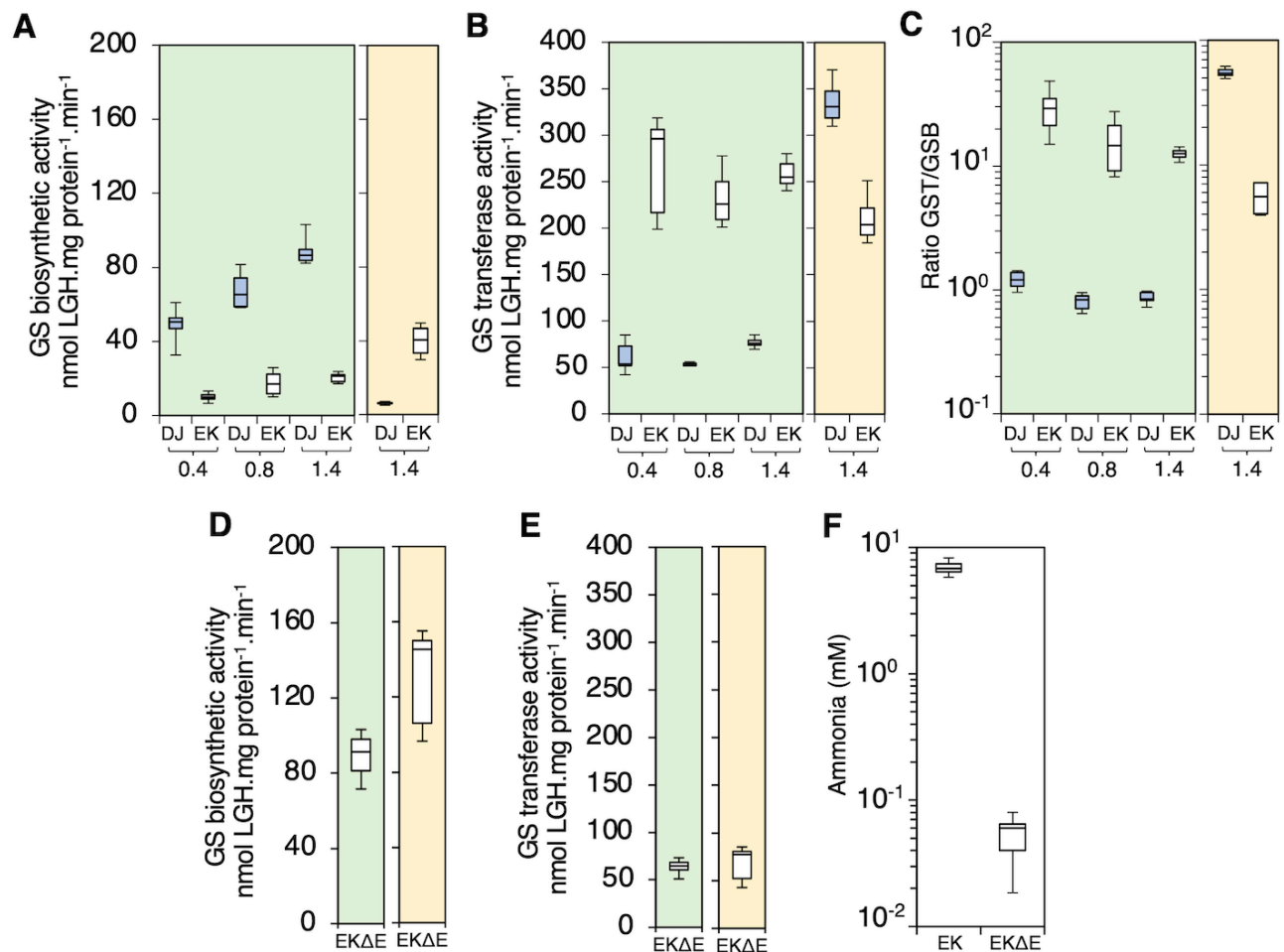
Fig. 2 – Ammonia excretion is dependent upon lower glutamine synthetase biosynthetic activity in the *nifA-E356K* strain (EK). (A) Glutamine synthetase (GS) biosynthetic and (B) transferase activities were measured in the wild type (DJ, blue box plots) and E356K (EK, white box plots) strains in three different phases of growth, corresponding to an $O.D_{600\ nm}$ of 0.4, 0.8 and 1.4 as indicated. Ratio between GS transferase (GST) and GS biosynthetic (GSB) activities is presented in (C) on a *log_{10}* scale to emphasize that the ratio between GST and GSB activities are close to 1 in the wild type (DJ) in all growth phases. (D) Glutamine synthetase (GS) biosynthetic and (E) transferase activities measured in the EKΔE strain (*nifA-E356K* with a *glnE* deletion) at an $O.D_{600\ nm}$ of 0.8. The charts shaded in green represent the activities under diazotrophic conditions (-N), while those shaded in yellow represent the activity in the presence of excess fixed nitrogen, (20 mM NH_4Cl, +N). (F) Ammonia from the culture supernatant was quantified in both EK and EKΔE strains grown under diazotrophic conditions.
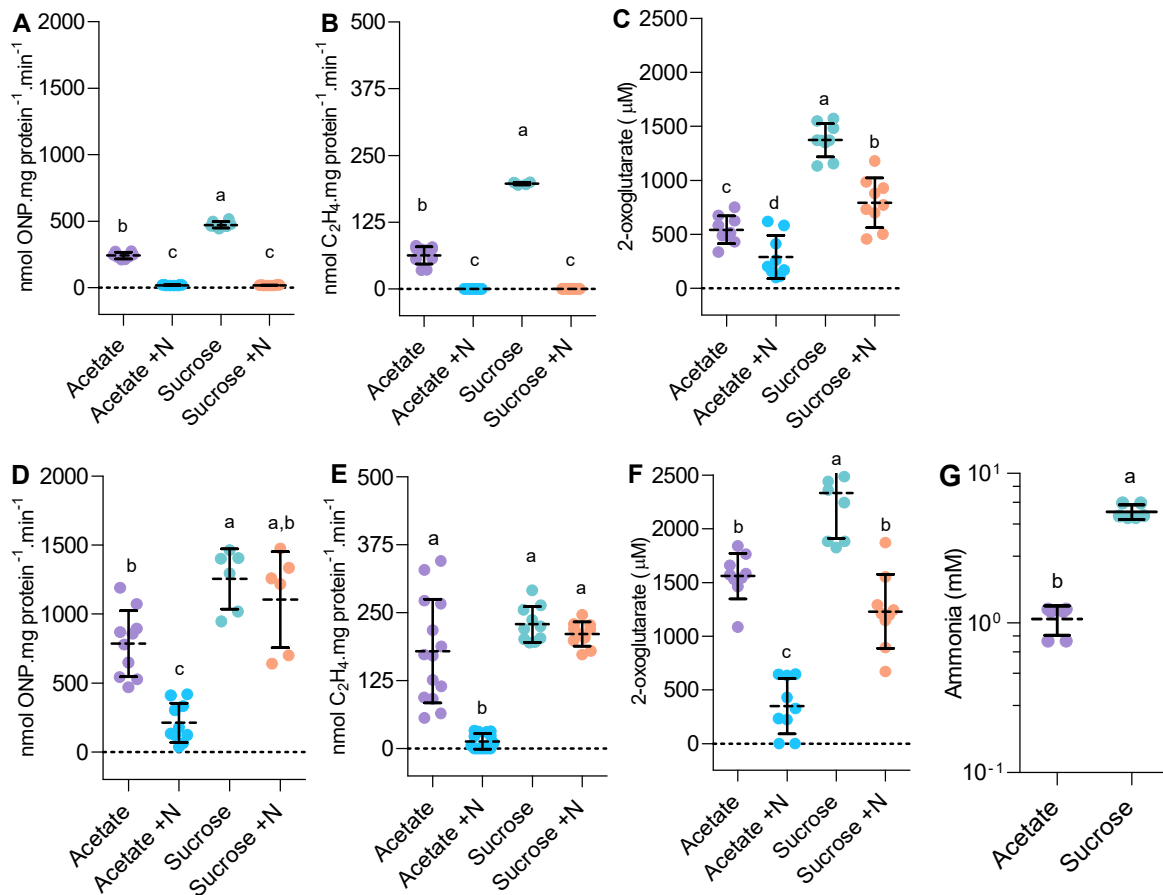
Fig. 3 – Carbon status regulation of nitrogenase expression and ammonia excretion in *A. vinelandii*. (A) Nitrogenase expression reported from a *nifH::lacZ* fusion, (B) nitrogenase activity and (C) internal 2-oxoglutarate levels are compared in the wild type (DJ) in acetate and sucrose in the absence (-N) or presence (+N) of 20 mM ammonium chloride. (D-F) The same comparisons as in (A-C) were done for the *nifA-E356K* strain (EK). For experiments in (A-F), strains were cultured to an O.D$_{600nm}$ between 0.2-0.3 in 30 mM acetate or 60 mM sucrose. To facilitate direct comparison of nitrogenase expression and activity, strain DJHZ was used in panels (A-B) whereas strain EKHZ was used in panels (D-E). These strains are isogenic to DJ and EK, respectively, except that they encode a *nifH::lacZ* fusion in the *algU* locus, a neutral site in the *A. vinelandii* genome (see Supplementary Material and Table S1). (G) Ammonia levels detected in the *nifA-E356K* (EK) strain when grown in either acetate or sucrose until cultures reached stationary phase. Plots followed by different letters are statistically different according to ANOVA with post-hoc Tukey's HSD or a paired t-test in (G).

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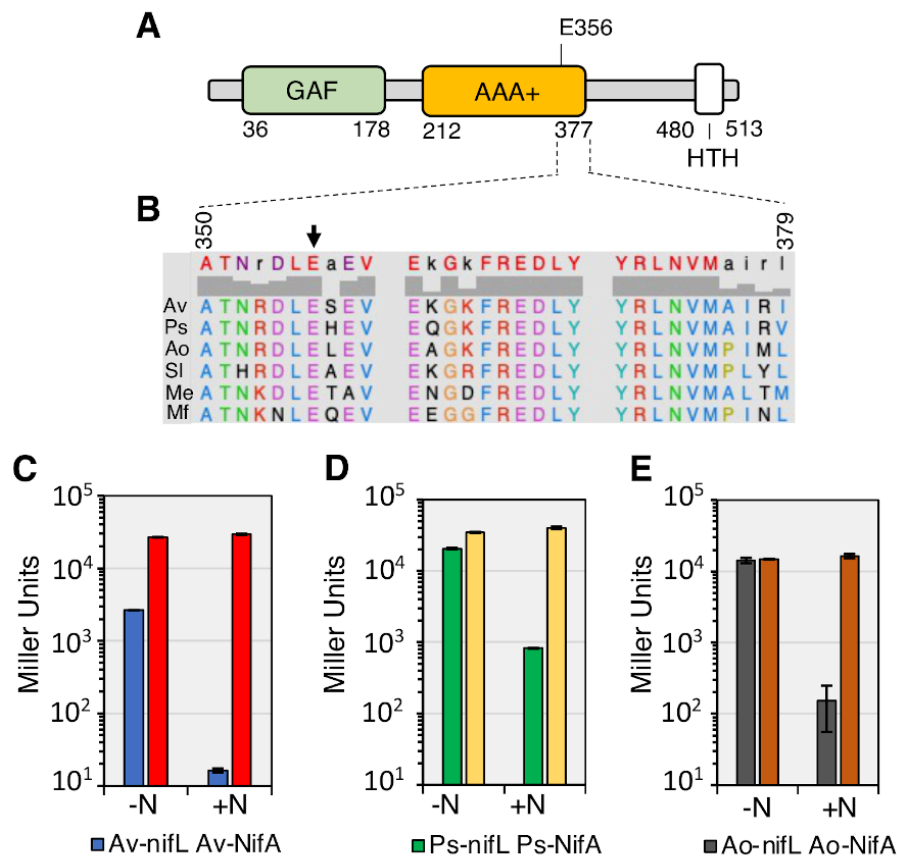
Fig. 4 - Reciprocal amino acid changes (related to *nifAE356K* in *A. vinelandii*) yield constitutively active NifA in Proteobacteria. (A) Diagram of the *A. vinelandii* NifA domains. (B) Alignment of residues close to E356 (black arrow) in the central AAA+ domain of NifA proteins. Sequence numbers refer to *A. vinelandii* NifA. Sequences used in the alignment are Av: *A. vinelandii* DJ, Ps: *Pseudomonas stutzeri* A1501 (Gammaproteobacteria), Ao: *Azoarcus olearius* DQS4, Sl: *Sideroxydans lithotrophicus* ES-1 (Betaproteobacteria), Me: *Martelella endophytica* YC6887 (Alphaproteobacteria) and Mf: *Mariprofundus ferrooxydans* M34 (Zetaproteobacteria). Panels C to E show β-galactosidase activities in the *E. coli* ET8000 chassis resulting from activation of a *nifH::lacZ* fusion (plasmid pRT22) by wild type and variant NifL-NifA systems from three different diazotrophs. Plasmids used to express NifL-NifA variants are as follows. (C) pPR34: Av-NifL-NifA, pPMA: Av-NifL-NifA-E356K; (D) pMB1804: Ps-NifL-NifA, pMB1805: Ps-NifL-NifA-E356K; (E) pMB1806: Ao-NifL-NifA, pMB1807: Ao-NifL-NifA-E351K. The assays were performed in NFDM media supplemented with 2% glucose in either nitrogen-limiting (200 µg/ml of casein hydrolysate, -N) or nitrogen excess (7.56 mM ammonium sulphate, +N) conditions.

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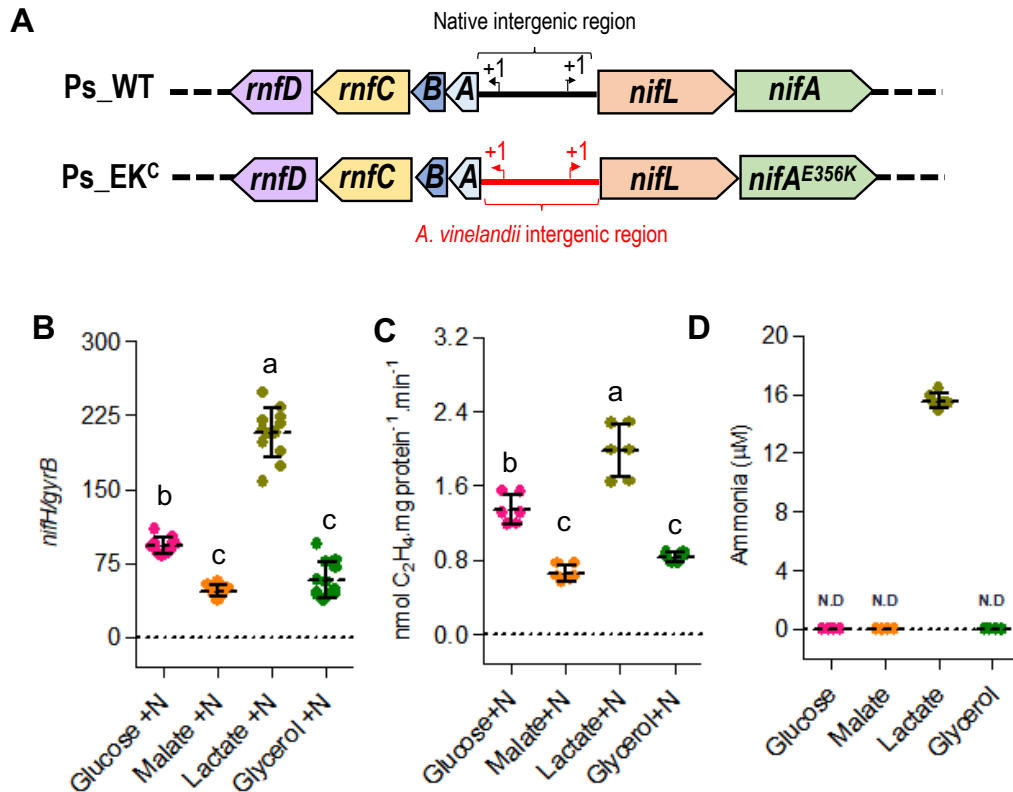
Fig. 5 – The Ps-NifA-E356K protein is able to escape NifL inhibition in *P. stutzeri* leading to carbon-dependent ammonia excretion. (A) Diagram depicting the modifications in the *P. stutzeri nifA-E356K* strain (Ps_EK^c) compared to wild type *P. stutzeri* (Ps_WT). Drawings are not to scale. (B) Levels of *nifH* transcripts in the *P. stutzeri* Ps_EK^c strain in the presence of 5 mM NH$_4$Cl (+N). (C) Nitrogenase activity in the *P. stutzeri* strain (Ps_EK^c) in the presence of 5 mM NH$_4$Cl (+N) . (D) Comparison of ammonia excretion profiles in different carbon sources. In all cases, the Ps_EK^c strain was grown in UMS-PS supplemented with 30 mM glucose, 45 mM malate, 60 mM lactate or 60 mM glycerol (to provide balanced carbon equivalents). N.D: not detected. Plots followed by different letters are statistically different according to ANOVA with post-hoc Tukey's HSD.
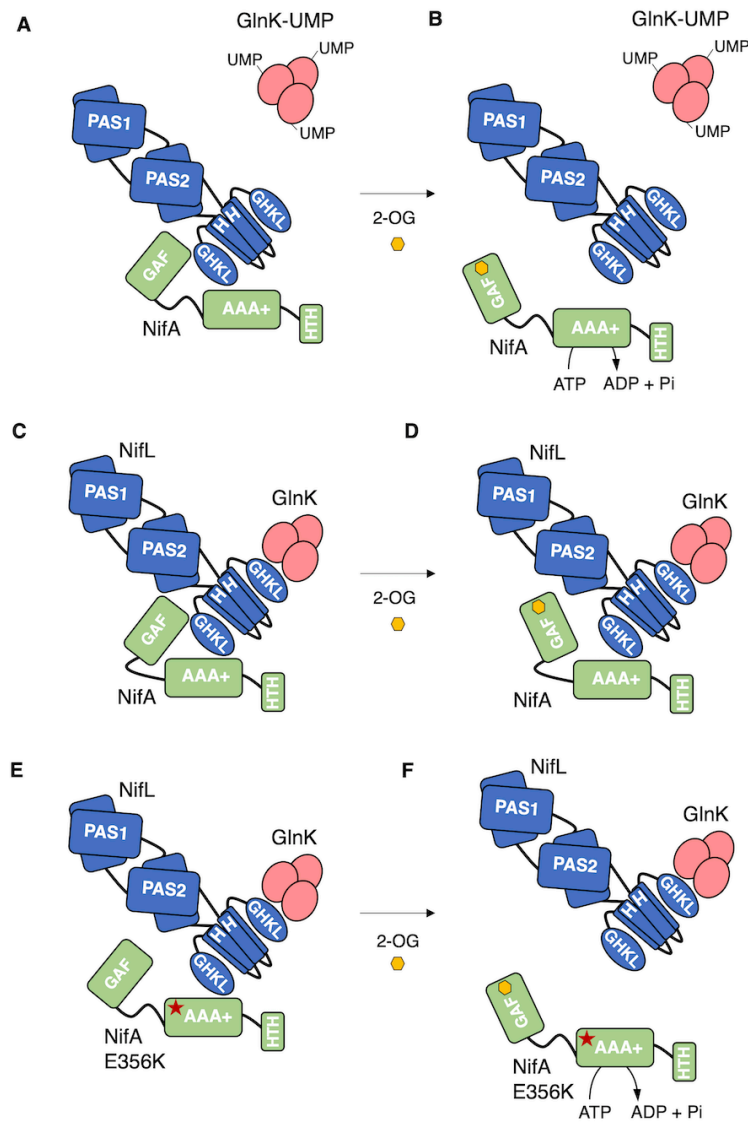
Fig. 6. Model for 2-oxoglutarate regulation of NifA activity based on genetic and biochemical experiments. (A) When 2-oxoglutarate levels are low, NifL can inhibit NifA even under nitrogen limiting conditions when GlnK is uridylylated (GlnK-UMP) and unable to interact with NifL. (B) Binding of 2-oxoglutarate (2-OG, yellow hexagon) to the GAF domain of NifA, disrupts the binary NifL-NifA interaction activating NifA. (C) Under nitrogen excess conditions, non-covalently modified GlnK, interacts with the GHKL domain of NifL, stimulating the formation of a ternary complex between GlnK, NifL and NifA that inhibits NifA activity. (D) The GlnK-NifL-NifA ternary complex is stable even if the GAF domain in NifA is saturated with 2-OG. (E) The E356K substitution in the AAA+ domain of NifA (red star), perturbs the interaction with NifL in the absence of the non-modified form of GlnK. However, under nitrogen excess conditions, the GlnK-NifL-NifA-E356K ternary complex is formed when 2-OG in limiting. (F) When 2-OG levels are sufficient, conformational changes triggered by 2-OG binding to the GAF domain disrupt the ternary complex, activating NifA-E356K. Physiologically, interactions depicted in (E), may arise upon growth *in vivo* under carbon limiting conditions. A switch to a preferred carbon source, will lead to an increase in 2-OG, triggering the conformation change that allows NifA-E356K to escape from GlnK-NifL inhibition (F).