

1 **Creation and multi-omics characterization of a genomically hybrid** 2 **strain in the nitrogen-fixing symbiotic bacterium *Sinorhizobium meliloti***

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12 **Abstract**

13 Many bacteria, often associated with eukaryotic hosts and of relevance for biotechnological applications,
14 harbour a multipartite genome composed by more than one replicon. Biotechnologically relevant phenotypes
15 are often encoded by genes residing on the secondary replicons. A synthetic biology approach to developing
16 enhanced strains for biotechnological purposes could therefore involve merging pieces or entire replicons from
17 multiple strains into a single genome. Here we report the creation of a genomic hybrid strain in a model
18 multipartite genome species, the plant-symbiotic bacterium *Sinorhizobium meliloti*. In particular, we moved
19 the secondary replicon pSymA (accounting for nearly 20% of total genome content) from a donor *S. meliloti*
20 strain to an acceptor strain. The *cis*-hybrid strain was screened for a panel of complex phenotypes
21 (carbon/nitrogen utilization phenotypes, intra- and extra-cellular metabolomes, symbiosis, and various
22 microbiological tests). Additionally, metabolic network reconstruction and constraint-based modelling were
23 employed for *in silico* prediction of metabolic flux reorganization. Phenotypes of the *cis*-hybrid strain were in
24 good agreement with those of both parental strains. Interestingly, the symbiotic phenotype showed a marked
25 cultivar-specific improvement with the *cis*-hybrid strains compared to both parental strains. These results
26 provide a proof-of-principle for the feasibility of genome-wide replicon-based remodelling of bacterial strains
27 for improved biotechnological applications in precision agriculture.

28 **Key words:** replicon independence; genome coadaptation; experimental transplantation; accessory genome;
29 *Sinorhizobium meliloti*

30 INTRODUCTION

31 Interest in large-scale genome modification and synthetic bacterial chromosome construction has strongly
32 increased over the last decade (for instance see ¹) with a goal of engineering bacterial strains with new or
33 improved traits. However, phenotypes are often the result of the coordinated function of many genes acting
34 together in a defined genome architecture ². Hence, the ability to predict the phenotypic outcomes of large-
35 scale genome modification requires a precise understanding of the genetic and regulatory interactions between
36 each gene or gene product in the genome. As such, there is a need for integrated approaches, combining
37 experimental evidences with computational-based methods, to interpret and potentially predict the outcomes
38 of genome-wide DNA manipulations.

39 In this context, multipartite (or divided) genomes (i.e., genomes possessing more than one informational
40 molecule) are particularly interesting. The genome of bacteria with a multipartite structure is typically
41 composed of a principal chromosome that encodes the core housekeeping and metabolic genes essential for
42 cellular life, and one (or more) secondary replicons (termed chromids and megaplasmids). More than 10% of
43 the presently sequenced bacterial genomes are characterized by the presence of a multipartite architecture ^{3,4}.
44 The secondary replicons can account for up to half of the total genome size, and their level of integration into
45 cellular regulatory and metabolic networks is variable ^{5, 6, 7}{González, 2006 #3279}. In some cases, strong replicon-
46 centric transcriptional networks have been suggested ^{8,9}. The apparently functional modularity of secondary
47 replicons is particularly attractive from both ecological and biotechnological viewpoints. Indeed, secondary
48 replicons might act as plug-and-play functional modules, potentially allowing the recipient strain to obtain
49 previously untapped genetic information ¹⁰. This, in turn, might allow the emergence of novel phenotypic
50 features leading, for example, to the colonization of a new ecological niche ¹¹. Moreover, such modularity
51 paves the way for large-scale, genome-wide manipulations of bacterial strains with multipartite genome
52 structure, by synthetically merging complex biotechnologically important traits in the same strain ¹².

53 However, it remains unclear to what extent complex phenotypes can be directly transferred into a recipient
54 strain, as secondary replicons are in part co-adapted to the host genome, for example, through regulatory
55 interaction and/or inter-replicon metabolic cross-talk ^{8, 13}. We are aware of only one study examining the
56 phenotypic consequences of replacing a large (> 800 kb) native secondary replicon with a homologous replicon
57 of closely related strains or species. In that study, the third replicon of *Burkholderia cepacia complex* strains
58 was mobilized and the effects on various phenotypes including virulence was examined ¹⁴. It was found that
59 in some cases, phenotypes were dependent solely on the secondary replicon, whereas in other cases, the
60 phenotypes depended on genetic/regulatory interactions with the other replicons ¹⁴. However, additional
61 studies are required to examine the generalizability of those observations.

62 To further test the feasibility, the stability, and the predictability of secondary replicon shuffling on the
63 phenotype(s) of the cell, here we have performed experimental and *in silico* replicon transplantation between
64 two bacterial strains. We used the symbiotic nitrogen-fixing bacterium *Sinorhizobium meliloti* as the model,

65 given that it has a well-studied multi-replicon genome structure ^{11, 15}. Additionally, *S. meliloti* represents a
66 highly valuable microorganism in agriculture, as its symbiosis with crops like alfalfa is estimated to be worth
67 more than \$70 million/year in the U.S.A. ¹⁶. The genome of the mostly commonly studied *S. meliloti* reference
68 strains (Rm1021 and Rm2011) is composed by a chromosome (~ 3.7 Mb), and two secondary replicons: a
69 chromid (~ 1.7 Mb, called pSymB, carrying several genes involved in rhizosphere colonization) and a
70 megaplasmid (~ 1.4 Mb, called pSymA, carrying most of classical symbiosis genes). *S. meliloti* large replicons
71 have recently been proposed as scaffolds for novel shuttle vectors for synthetic biology ¹⁷. Furthermore,
72 genome reduction experiments previously performed have led to the complete removal of one or both of the
73 two secondary replicons^{11, 18}, and an *in silico* genome-scale metabolic model has been reconstructed ¹⁹, paving
74 the way for massive genome-scale remodelling of *S. meliloti*.

75 Here, we constructed a hybrid strain containing the chromosome and the chromid of the laboratory *S. meliloti*
76 Rm2011 strain with the pSymA replicon from the wild isolate *S. meliloti* BL225C. The genome of BL225C is
77 290 kbps larger than that of Rm2011 (which has a genome highly similar to Rm1021 strain) ^{15, 20}, and 1,583
78 genes are present in only one of these strains²¹ are present in only one of these strains ²². Furthermore, the
79 BL225C strain has been shown to have several interesting biotechnological features, including plant growth
80 promotion, and nodulation efficiency ^{15, 23}. The majority of the genetic differences between Rm2011 and
81 BL225C strains is associated with the symbiotic pSymA homolog megaplasms; 836 of the 1,583 variable
82 genes are located on this replicon²¹. We can then expect that creating a hybrid strain between Rm2011 and
83 BL225C, by moving the pSymA-equivalent from BL225C to the Rm2011 derivative lacking pSymA, will
84 provide a good testing ground for i) feasibility of large replicon shuffling between strains, and ii) stability and
85 predictability of the phenotypes linked to such replicons. We term this novel hybrid strain as *cis*-hybrid since
86 it derives from *cis*-genic manipulation, indeed it contains genetic material from the pangenome pool of the
87 same species (in contrast to a *trans*-genic strain that would contain genes from a distinct species). *Cis*-hybrid
88 strains could be an important way to promote environmental-friendly and regulatory compliant biotechnology
89 and synthetic biology in bacterial species of interest in agricultural and environmental microbiology ¹².

90 RESULTS AND DISCUSSION

91 We report here the creation of a *cis*-hybrid *S. meliloti* strain, where the symbiotic-related megaplasmid
92 pSINMEB01, homologous to pSymA (~ 1.6 Mb in size, accounting for nearly 23% of total genome) was
93 transferred from the natural strain BL225C to the laboratory strain Rm2011. *In silico* metabolic reconstruction
94 and a large set of phenotypic tests, including Nuclear Magnetic Resonance (NMR)-based metabolomic
95 profiling, Phenotype Microarray™, and symbiotic assays with different host plant cultivars have been
96 performed, as described in the following paragraphs.

97

98 Experimental creation of a *cis*-hybrid strain

99 Starting with the derivative of the *S. meliloti* Rm2011 strain that lacks pSymA replicon, herein referred to as
100 Δ pSymA²⁴, we produced a *cis*-hybrid strain that contains the Rm2011 chromosome and pSymB chromid, and
101 the pSymA replicon from a genetically and phenotypically distinct *S. meliloti* strain, BL225C (all strains used
102 in this work are listed in Table 1)^{15,23}. The *cis*-hybrid strain was produced through a series of conjugations as
103 described in the Methods section. Briefly, a plasmid for over-expressing *rctB*²⁵ was transferred to BL225C; as
104 RctB is a negative regulator of RctA²⁵, which in turn is a negative regulator of the pSymA conjugal genes²⁶,
105 this step was necessary to promote pSymA transfer without mutating the replicon. Concurrently, a plasmid
106 carrying an antibiotic resistance gene marker (gentamicin, plasmid pMP7605) was transferred to the Δ pSymA
107 strain to allow for the use of the gentamicin resistance marker in the selection of *cis*-hybrid transconjugants in
108 the next step. Finally, a mating mixture of the Rm2011 acceptor strain and the BL225C donor strain was
109 prepared, and *cis*-hybrid transconjugants were isolated on a medium selective for the gain of the pSymA
110 replicon (see Methods). Correct construction of the *cis*-hybrid strains was initially confirmed through PCR
111 amplification on specific unique genes on the Rm2011 chromosome and pSymB, and the pSymA homolog of
112 BL225C (pSINMEB01) (see Table S1). Subsequently, whole genome sequencing (Figure 1) confirmed the
113 complete transfer of pSymA, and the banding pattern observed in pulse-field gel electrophoresis (PFGE)
114 (Supplemental Figure S1) was consistent with pSINMEB01 being present as an independent replicon (i.e., not
115 integrated into the chromosome or pSymB).

116

117 ***In silico* metabolic network reconstruction**

118 In addition to the experimental creation of the *cis*-hybrid strain, we attempted to predict the metabolic outcomes
119 of the *cis*-hybrid strain by generating a new metabolic model which includes the genomic features present in
120 the *cis*-hybrid strain. The curated iGD1575 reconstruction (herein referred to as the Rm2011 reconstruction)
121 was used to represent metabolism of *S. meliloti* Rm2011²⁴; although iGD1575 is based on the strain Rm1021,
122 the genomic content of these strains are 99,9% identical, with the exception of numerous SNPs²⁰ that are not
123 considered in the process of metabolic reconstruction. Next, our recently described pipeline²⁷ was used to
124 build a representation of BL225C based on a draft reconstruction built with the Kbase webserver and enhanced
125 based on the iGD1575 model. An *in silico* representation of the *cis*-hybrid strain was then built by removing
126 all pSymA genes (and dependent reactions) from the Rm2011 model, followed by the addition of all pSymA
127 (pSINMEB01) genes (and associated reactions) from the BL225C model using our published pipeline²⁷.
128 Despite there being numerous (47 to 143 gene) differences in the gene contents of the metabolic
129 reconstructions, the Rm2011 model differed from the BL225C and the *cis*-hybrid models by no more than a
130 half dozen reactions (Table 2). The low reaction variability between models may i) reflect the difficulty in
131 predicting the function of the *S. meliloti* variable gene content, ii) suggest the presence of non-orthologous
132 genes encoding proteins catalyzing the same reaction(s), and/or iii) indicate that few metabolic features are
133 dependent on the accessory gene set. Not surprisingly, given the near identical reaction content of the
134 reconstructions, the outputs of flux balance analysis simulations for the different reconstructions were nearly
135 identical (data not shown); therefore, we do not describe these results further.

136

137 **Metabolic phenotypes and profiles of the *cis*-hybrid strain**

138 Phenotype MicroArray™ experiments were performed to test the growth of the *cis*-hybrid strain, as well as
139 the parental and wild type strains, with 192 different carbon sources and 95 different nitrogen sources. Previous
140 work has shown that the pSymA megaplasmid has little contribution to the metabolic capacity of *S. meliloti*¹¹,
141¹⁸. Consistent with this, only minor changes in the metabolic growth abilities were observed following the
142 introduction of the pSymA of BL225C (pSINMEB01) into the ΔpSymA strain (Figure 2/Table S2). This
143 confirms that transplantation of pSymA did not result in a major disturbance in the metabolic abilities of the
144 recipient strain. Additionally, the ΔpSymA strain lost the ability to use 3-methylglucose as a carbon source and
145 cytosine as a nitrogen source, and both abilities were restored upon the introduction of the pSymA replicon
146 from BL225C. This result helps validate the replicon transplantation approach by confirming that at least some
147 of the genes on the pSymA replicon of BL225C are properly expressed, and their gene productions functional,
148 in the Rm2011 background.

149 A metabolomic analysis through ¹H nuclear magnetic resonance (NMR) was performed to further define the
150 metabolic consequences of pSymA transplantation. Using an untargeted approach, both cellular lysates and
151 spent growth media were analysed to identify the fingerprint of the endo- and exo-metabolomes of the two
152 parental strains, the *cis*-hybrid strain, and the ΔpSymA recipient.

153 PCA was used to generate an initial overview of the metabolome differences among the four strains (Figures
154 3.a and 3.b), followed by PCA-CA to obtain the best discrimination among the strains by maximizing the
155 differences among their metabolomic profiles (Figures 3.c and 3.d). In both the PCA and PCA-CA score plots
156 (Figure 3), the *cis*-hybrid strain clustered very close to both the ΔpSymA recipient strain and to the parental
157 strain Rm2011, whereas the parental donor strain BL225C clustered separately. These results are consistent
158 with previous data indicating that pSymA has little contribution to the metabolome⁶, proteome⁷, or
159 transcriptome²⁸ of *S. meliloti* Rm2011 in laboratory conditions. Importantly, these results confirmed that the
160 synthetic large-scale horizontal gene transfer performed here to produce the *cis*-hybrid strain did not result in
161 a major perturbation of the cellular metabolism.

162 In addition to the multivariate analysis of the metabolic NMR fingerprints described above, the signals of 25
163 and 19 metabolites were unambiguously assigned and integrated in the ¹H-NMR spectra of the cell lysates and
164 growth media, respectively (Figure S2). The metabolites that are characterized by statistically significant
165 differences in concentration levels in at least one strain with respect to the two other strains are indicated in
166 Supplementary Table S3 and are also reported in Supplemental Figure S3. Validating the ability of this
167 approach to detect metabolic differences between the strains, it was noted that the ΔpSymA strain exported
168 cytosine unlike the wild type Rm2011 or the *cis*-hybrid strain, consistent with the inability of this strain to
169 catabolize cytosine as shown by the Phenotype MicroArray™ data.

170

171

172 **Assessment of the phenotypes of the *cis*-hybrid strain.**

173 *Growth profiles in synthetic laboratory media*

174 Growth profiles of the *cis*-hybrid and parental strains in complex (TY) and defined (M9-succinate) media are
175 reported in Figure 4. In the complex TY medium (Figure 4.a), growth of the *cis*-hybrid strain was impaired
176 relative to the recipient (Δ pSymA), and to the Rm2011 and BL225C parental strains. Or in other words, gain
177 of the BL225C pSymA replicon by the Δ pSymA strain resulted in a decrease in the growth rate in TY medium.
178 Although we cannot provide a definitive explanation for this phenomenon, it may be that the simultaneous
179 gain of hundreds of new genes not integrated into cellular networks imposes a high metabolic cost to the cell,
180 resulting in impaired fitness. In contrast, little to no difference was observed in the growth rate of any of the
181 strains in the defined M9-succinate medium (Figure 4.b). The lack of an observable growth impairment of the
182 *cis*-hybrid strain in the M9-succinate medium may be due to the growth impairment being masked by the
183 general decrease in growth rate of all strains in this medium. Moreover, the similarity of the growth profiles of
184 all strains in the minimal medium suggest that, at least in artificial laboratory conditions, the primary growth
185 characteristics of these strains are primarily dependent on the core, not accessory, genome.

186

187 *Growth using root exudates as a nutrient source*

188 Root exudates can be considered a proxy of the nutritional conditions of the plant rhizosphere²⁹. We therefore
189 evaluated the ability of the three strains to grow on M9 mineral medium supplemented with root exudates of
190 *Medicago sativa*, a *S. meliloti* symbiotic partner. None of the strains were able to grow when the root exudate
191 was used as the sole carbon source; this was likely due to the root exudate being too dilute for use as a carbon
192 source. In contrast, all strains could utilize the root exudate as the sole nitrogen source when provided succinate
193 as a carbon source, and differential growth patterns were observed (Figure 4.c). In particular, BL225C
194 displayed the highest growth among all four strains when grown with root exudates as a sole nitrogen source.
195 Plating for viable colony forming units confirmed the differences in the final population densities (data not
196 shown). As the robust growth of BL225C with root exudates did not transfer to the *cis*-hybrid strain, it is likely
197 that this phenotype is primarily dependent on the chromosome and/or pSymB of BL225C, as was suggested
198 by previous studies^{11, 21, 24, 30}. This observation would further suggest that the adaptation of the tested strains
199 to growth in the rhizosphere occurred prior to the gain of pSymA and symbiotic abilities, consistent with recent
200 work indicating that the majority of *S. meliloti* rhizosphere growth-promoting genes are chromosomally
201 encoded³¹. Finally, considering that there are relatively few differences in the nitrogen metabolic capacity of
202 Rm2011 and BL225C²³, and that FBA (flux balance analysis) simulations for the metabolic model
203 reconstructions were nearly identical (data not shown), we hypothesize that the growth differences observed
204 between these strains is primarily related to regulatory differences, and less so to differences in metabolic
205 genes.

206

207 *Biofilm formation*

208 Biofilm formation is a key factor in root colonization and plant invasion for many Proteobacteria³². In the light
209 of producing a novel strain with good biotechnological features, biofilm formation was measured for the *cis*-
210 hybrid strain. Biofilm production (estimated as the total biofilm-to-biomass ratios) by the *cis*-hybrid and the
211 parental strains was similar (Figure S4). Interestingly, the Δ pSymA recipient strain showed a higher ($p < 0.005$)
212 level of biofilm production compared to the other three strains. This led us to hypothesize that at least under
213 the tested conditions, there is a pSymA mediated negative regulation of biofilm formation in both Rm2011 and
214 BL225C strains. In contrast to these results, previous studies have suggested that deletion of pSymA³³, or just
215 the pSymA-encoded common *nod* genes³⁴, results in a major reduction of biofilm formation. Future work is
216 required to understand why the biofilm production phenotypes of *S. meliloti* strains lacking pSymA differed
217 so dramatically between this study and those by Fujishige *et al.*⁴².

218

219 **Symbiotic phenotypes of the *cis*-hybrid strain**

220 Many of the key genes required for symbiotic abilities (e.g. nodule formation and nitrogen-fixation) are present
221 on pSymA of *S. meliloti* Rm2011³⁵ and the homologous megaplasmid pSINMEB01 of BL225C¹⁵. These
222 replicons additionally contain non-essential genes that promote improved symbiotic abilities³⁶. While many of
223 the symbiotic genes are conserved between these strains, relevant differences between pSymA and
224 pSINMEB01 are present. The 482 genes exclusive to pSINMEB01 included symbiotic (e.g. *nws*,
225 *hemA* homolog, *C P450*¹⁵) and nonsymbiotic functions (e.g. *acdS*)³⁷. For these reasons, this replicon swapping
226 study was initiated in large part to evaluate whether swapping the symbiotic megaplasmid could promote
227 differential symbiotic abilities.

228 To test the robustness of symbiotic abilities following replicon transplantation, *in vitro* symbiotic assays were
229 performed on a panel of alfalfa cultivars, as alfalfa is the main host legume of *S. meliloti*³⁸ (Figure 5). In
230 particular, the *cis*-hybrid strain and the two parental strains (Rm2011, BL225C) were tested in combination
231 with seven alfalfa cultivars (Table S4). These cultivars belong to the species *M. sativa*, *Medicago x varia*, and
232 *Medicago. falcata*, and they are representative of the variability of cultivars and germplasm mainly used as
233 crops in Europe. Moreover, BL225C was originally isolated on the *M. sativa* cultivar “Lodi” at the CREA-
234 FLC institute (Italy) during a long-course experiment³⁹. The percentage of nodulated plants (Figure 5.a), the
235 number of nodules per plant (Figure 5.b), the shoot dry weight (Figure 5.c), the plant aerial part length (Figure
236 5.d), and nodule colonization abilities (Supplemental Figure S5) were recorded using standard procedures²³,
237⁴⁰. Not surprisingly, for each strain there was high variability in the symbiotic phenotypes observed with the
238 different cultivars. The symbiotic interaction is a multistep developmental process which involves a tight
239 exchange of signals between the bacterium and the plant root at both rhizospheric and endophytic levels^{12,41}.
240 Earlier works have demonstrated strain and cultivar specificities in this process, which result in *S. meliloti*
241 strains displaying differential symbiotic effectiveness with various plant genotypes^{39,42}.

242 The *cis*-hybrid strain performed very poorly in symbiosis with some cultivars, such as in the cv. “Prosementi”,
243 “Camporegio” and “Verbena”, in particular in the number of nodules per plant and the length of the aerial part
244 (Figure 5b;c, Table S5) ($p < 0.005$), indicating that the pSymA and pSINMEB01 megaplasmids are not always
245 interchangeable. This could reflect the importance of the genomic context of the symbiotic megaplasmid and
246 hypothetically the importance of inter-replicon regulatory networks^{8,43}. Alternatively, pSINMEB01 may lack
247 important (but still unknown) symbiotic genes with their function replaced by chromosomal genes in BL225C
248 but not by chromosomal genes in Rm2011.

249 Strikingly, the *cis*-hybrid strain displayed clearly improved symbiotic capabilities during symbiosis with the
250 cultivar “Lodi” compared to both Rm2011 and BL225C (Figure 5). This was true for several key measures of
251 symbiosis, including nodule number per plant, shoot dry weight, and length of the aerial part of the plant.
252 These data suggest the presence of nonlinear and genomic context dependent genic interactions in the
253 establishment of symbiotic abilities. Such interactions may resemble (at the logic level) those present in some
254 eukaryotic genomes that result in the so called “hybrid vigour”, i.e., the tendency for hybrids to be superior to
255 the parental genotypes⁴⁴. However, since hybrid vigour is related to heterozygosis, in our case we may
256 speculate that strain-by-strain variability of regulons⁸, as well as metabolic redundancy of *S. meliloti* genome
257^{4,45,46} (which could in some way mimic the presence of multiple alleles) could be the contributor to the increase
258 in the observed symbiotic-related phenotype.

259 Summing up, these data highlight the potential of a large-scale genome manipulation approach to obtain highly
260 effective, and cultivar specific, rhizobial strains. This provides a rational basis for the use of similar approaches
261 in the development of elite bio-inoculants for use in precision agriculture^{12,47}.

262

263 **Conclusions**

264 The work presented here provides a proof-of-principle for the feasibility of using a large-scale genome
265 manipulation approach that makes use of the species’ pangenome (i.e. the extended gene set present in a group
266 of microbial strains belonging to the same species⁴⁸) to produce daughter strains with improved
267 biotechnologically relevant (i.e., nitrogen fixing symbiosis) characteristics¹². In the current work, the large-
268 scale genome manipulation was based on the transplantation of the primary symbiotic megaplasmid of a
269 bacterial multipartite genome, a genome organization commonly found in the rhizobia. Although an entire
270 replicon accounting for more than 20% of the total genome content was replaced with a homologous replicon
271 of a closely related species, resulting in the gain of 482 new genes (in addition to numerous SNPs) and the loss
272 of 354 genes, most of the core metabolic phenotypes appeared largely resilient to modification with this
273 approach. However, others phenotypes, particularly complex (i.e. multigenic) phenotypes such as the
274 symbiotic phenotypes, gave interesting features which support the validity of this approach to improve
275 biotechnologically relevant properties.

276 MATERIAL AND METHODS

277

278 **Microbiological and genetic methods.** Strains and plasmids used in this study are described in Table 1.
279 Conjugation between *E. coli* and *S. meliloti* were performed as described in ⁴⁹. All growth media (LB, LBmc,
280 TY, M9) and antibiotic concentrations were as previously described in ^{11, 45}.

281 **Cis-hybrid strain construction.** First, a triparental mating between the wild type strain BL225C (the future
282 donor), the helper strain *E. coli* MT616 (carrying pRK600 that has the RK2 *tra* genes) ⁵⁰, and *E. coli* with the
283 pTE3rctB vector (replicative plasmid overexpressing the *R. etli rctB* gene and carrying a tetracycline resistance
284 marker) ²⁵ was performed to create the BM848 (BL225C-rctB) strain. Secondly, a biparental mating between
285 *S. meliloti* Rm3498 (Δ pSymA) ¹¹ and an *E. coli* S17-1 strain carrying the pMp7605 vector (carrying a
286 gentamicin resistance marker) ⁵¹ was performed to generate the strain BM826. Lastly, the *cis*-hybrid strain
287 BM806 was created through a biparental mating between the strain BM848 (BL225C-rctB) as the donor and
288 the strain BM826 (Δ pSymA +pMp7605) as the acceptor. Selection for the *cis*-hybrid transconjugant strain
289 (which had the pSymA replicon of the donor strain) was performed on M9 medium containing 1 mM MgSO₄,
290 0.25 mM CaCl₂, 0.001 mg/ml biotin, 42 μ M CoCl₂, 76 μ M FeCl₂, 10 mM trigonelline, streptomycin, and
291 gentamycin. Streptomycin and gentamycin were used to select for the recipient strain, while the presence of
292 trigonelline as the sole carbon source selected for the gain of pSINMEB01, as the trigonelline catabolic genes
293 are located on pSymA/ pSINMEB01 ⁵².

294 **Validation of the transplanted strain.**

295 Pulsed-Field Gel Electrophoresis (PFGE) was performed to verify the successful uptake of pSymA via
296 restriction digestion of genomic DNA with *PmeI*. The applied PFGE protocol was modified from Herschleb
297 et. al 2007⁵³ and Mavingui et al. 2002⁵⁴, and a protocol from Sharon Long's research group (Stanford
298 University, available at
299 <http://cmgm.stanford.edu/biology/long/files/protocols/Purification%20of%20S%20meliloti.pdf>). *S. meliloti*
300 cultures were grown to an OD600 of 1.0 in TY medium supplemented with suitable antibiotics and harvested
301 by centrifugation (3000 g, 15 min, 4°C). All following steps were carried out either on ice or at 4°C.
302 Sedimented cells were washed with TE buffer (10mM Tris-HCl, 1mM EDTA) supplemented with 0.1% (w/v)
303 N-Lauroylsarcosine, and a second time with TE buffer. Washed cell pellets were then resuspended in TE buffer
304 and mixed (1:1) with 1.6% (w/v) low-melt agarose (50°C), thereby resulting in a final concentration of $\sim 8 \times 10^8$
305 cells/ml. Two hundred μ l of each suspension was casted into a moistened mold and gelatinized at 4°C. The
306 resulting agar plugs were subsequently incubated at 37°C for 3 h in lysis buffer (6mM Tris-HCl, 1M NaCl,
307 100mM EDTA, 0.5% (w/v) Brij-58, 0.2% (w/v) Sodium deoxycholate, 0.5% (w/v) N-Lauroylsarcosine)
308 supplemented with 1.5 mg/ml lysozyme (SERVA Electrophoresis GmbH, Germany). Treated agar plugs were
309 then washed in H₂O, followed by incubation at 50°C for 48 h in Proteinase K buffer (100mM EDTA, 10mM
310 Tris-HCl, 1% (w/v) N-Lauroylsarcosine, 0.2% (w/v) Sodium deoxycholate, pH 8.0) supplemented with 1

311 mg/ml Proteinase K (AppliChem GmbH, Germany). Finally, agar plugs were sequentially washed in four steps,
312 1 h per wash. After incubation in washing buffer (10mM Tris-HCl, 50mM EDTA), plugs were washed in
313 washing buffer supplemented with 1 mM Phenylmethylsulfonyl fluoride, then in washing buffer, and finally
314 in 0.1x concentrated washing buffer.

315 For restriction digestion with *PmeI* (New England Biolabs, USA), the prepared agar plugs were incubated in
316 1 ml of restriction enzyme buffer (1x concentrated) for 1 h with gentle agitation at room temperature. Then,
317 the plugs were transferred into 300 μ l of fresh enzyme buffer supplemented with *PmeI* (50 units per 100 μ l
318 agar plug). Restriction digestions were incubated over night at 37°C. After overnight incubation, agar plugs
319 were washed in 1x washing buffer for 1 h. For PFGE analysis, 1/8th of each agar plug was used. PFGE was
320 performed using the Rotaphor® System 6.0 (Analytik Jena, Germany) following the manufacturer's instructions.
321 Separation of DNA fragments was achieved using a 0.5% agarose gel (Pulse Field Certified Agarose, Bio-Rad,
322 USA) and 0.5x TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA). The following settings
323 were applied: step 1 – 18 h, 130V-100V (logarithmic decrease), angle: 130°-110° (logarithmic decrease),
324 interval: 50sec-175sec (logarithmic increase); step 2 – 18 h, 130V-80V (logarithmic decrease), angle: 110°,
325 interval: 175sec-500sec (logarithmic increase); step 3 – 40 h: 80V-50V (logarithmic decrease), angle: 106°,
326 interval: 500sec-2000sec (logarithmic increase). Buffer temperature was adjusted to 12°C.

327 For whole genome sequencing, a Nextera XT DNA library was constructed⁵⁵ and sequenced using the Illumina
328 MiSeq platform which generated 2,504,130 paired-end reads. After trimming, assembly was performed with
329 SPAdes 3.9.0⁵⁶, which produced 399 contigs. Contigs were aligned against the genomes of *S. meliloti* 2011
330 and BL225C. The assembly has been deposited to the GenBank database under the BioProject ID
331 PRJNA434498.

332 Finally, several PCR primer pairs for amplification of unique genes of Rm2011 and BL225C (Supplementary
333 Table S3), selected based on a comparative genome analysis with Roary⁵⁷, were routinely used to ensure the
334 correct identification of strains during all experiments.

335 **Growth curves.** Growth curves were initiated by diluting overnight cultures to an OD₆₀₀ of 0.1 in TY medium
336 or in M9 medium supplemented with succinate as a carbon source. Incubation was performed in 150 μ l
337 volumes in a 96 well microtiter plate. The microplates were incubated without shaking' at 30°C and growth
338 was measured with a microplate reader (Tecan Infinite 200 PRO, Tecan, Switzerland).

339 **Growth with root exudates.** The ability to colonize plant roots was tested using growth on root exudates as a
340 metabolic proxy for colonization. Root exudates were produced from seedlings of *M. sativa* (cv. Maraviglia)
341 as previously described in³⁷. Strains were grown on TY plates, following which a single colony was
342 resuspended in 0.9% NaCl solution to a final OD₆₀₀ of 0.5 (1×10^9 CFU/ml). Then, each microplate well was
343 inoculated with 75 μ l of either M9 without a carbon source or a nitrogen-free M9 composition with succinate
344 as a carbon source, 20 μ l of root exudate, and 5 μ l of the culture. The microplates were incubated without
345 shaking' at 30°C and the growth was measured on a microplate reader (Tecan Infinite 200 PRO, Tecan,

346 Switzerland). At the end of the incubation period, aliquots from each well were diluted and viable titres of *S.*
347 *meliloti* cells were estimated after incubation on TY plates at 30°C.

348 **Plant symbiotic assays.** Symbiotic assays were performed in microcosm conditions in plastic pots containing
349 a 1:1 mixture of sterile vermiculite and perlite, supplemented with 200 ml of Fahraeus N-free liquid plant
350 growth medium⁵⁸. *S. meliloti* strains were grown in liquid TY medium at 30°C for 48 h. Cultures were then
351 washed three times in 0.9% NaCl solution and resuspended to an OD₆₀₀ of 1.0. Approximately 1×10⁷ cells
352 were added to each pot, corresponding to ~ 4 × 10⁴ cells/cm³. Washed cell-suspensions were then directly
353 spread over the roots of one-week old seedlings that were directly germinated in the pots, and grown in a
354 growth chamber maintained at 26°C with a 16 h photoperiod (100 microeinsteins/m² /s) for 5 weeks. Nodule
355 counts were performed after the 5 weeks, then the shoots dried at 50°C for 7 days. The number of bacterial
356 genome copies per nodule was determined with qPCR as previously reported⁴⁰. The alfalfa cultivars (*M. sativa*,
357 *M. falcata*, *Medicago x varia*) used and their main features are reported in Supplemental Table S4.

358 **Biofilm assays.** Strains were inoculated in 5 ml of TY and grown for 24 h with shaking. After growth, cultures
359 were diluted to an OD₆₀₀ of 0.02 in fresh TY medium and 100 µl of the diluted culture was inoculated into a
360 microtiter plate. The plates were incubated at 30°C for 24 h, after which the OD₆₀₀ was measured to determine
361 the cell biomass. Each well was then stained with 20 µl of crystal violet solution for 10 minutes. The medium
362 containing the planktonic cells was gently removed and the microtiter plate wells were washed three times
363 with 200 µl of PBS (0.1 M, pH 7.4) buffer and allowed to dry for 15 min. The crystal violet in each well was
364 then solubilized by adding 100 µl of 95% EtOH and incubating for 15 min at room temperature as described
365 in⁵⁹. The plate was then read at 560 nm using a microtiter plate reader (Tecan Infinite 200 PRO, Tecan,
366 Switzerland).

367 **Phenotype Microarray.** Phenotype MicroArray™ experiments using Biolog plates PM1 (carbon sources),
368 PM2A (carbon sources), and PM3 (nitrogen sources) were performed largely as described previously²³. All
369 bacterial strains used in this study (parental and transplanted) are listed in Table 1. Data analysis was performed
370 with DuctApe⁶⁰. Activity index (AV) values were calculated following subtraction of the blank well from the
371 experimental wells. Growth with each compound was evaluated with AV values from 0 (no growth) to 4
372 (maximal growth), after elbow test calculation (Table S3 c;d).

373 **NMR metabolomics of the cell lysates and media.** Overnight cultures were washed, resuspended, and diluted
374 in 100 ml of fresh M9 medium (41 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.6 mM NaCl, 18.7 mM NH₄Cl, 4.1 µM
375 biotin, 42 nM CoCl₂, 1 mM MgSO₄, 0.25 mM CaCl₂, 38 µM FeCl₃, 5 µM thiamine-HCl, 10 mM succinate)¹¹.
376 For cell lysates, when cultures reached OD 1, 50 ml of each culture was pelleted by centrifuging for 25 minutes
377 at 15000 g. For the media, 1 ml of supernatant of each culture was collected. For cell lysate analysis, each
378 pellet was resuspended in 500 µL of PBS, and sonicated for 20 minutes with cycles of 1 second of activity and
379 9 seconds of rest (292.5 W, 13 mm tip), with contemporary cooling on ice. After cell lysis, the samples were
380 centrifuged for 25 min at 4°C at 8000 g. For each strain, four independent experiments were performed. NMR

381 samples were prepared in 5.00 mm NMR tubes (Bruker BioSpin) with 55 μ L of a $^2\text{H}_2\text{O}$ solution containing 10
382 mM sodium trimethylsilyl[2,2,3,3- $^2\text{H}_4$] propionate (TMSP) and 500 μ L of sample.

383 ^1H NMR spectra were acquired for both the cell lysates and the growth media. High reproducibility between
384 samples was seen (Supplemental Figure S2), as expected based on previous studies with eukaryotic cells ⁶¹.
385 NMR spectra were recorded using a Bruker 900 MHz spectrometer (Bruker BioSpin) equipped with a CP TCI
386 $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ probe. Before measurement, samples were kept for 5 minutes inside the NMR probe head for
387 temperature equilibration at 300 K. ^1H -NMR spectra were acquired with water peak suppression and a standard
388 Carr–Purcell–Meiboom–Gill (CPMG) sequence (cpmgpr; Bruker BioSpin srl), using 192 or 256 scans (for cell
389 lysates and growing media, respectively) over a spectral region of 18 kHz, 110 K points, an acquisition time
390 of 3.07 s, and a relaxation delay of 4 s. This pulse sequence ⁶² was used to impose a T_2 filter that allows
391 selective observation of small molecular weight components in solutions containing macromolecules.

392 The raw data were multiplied by a 0.3 Hz exponential line broadening before applying Fourier transformation.
393 Transformed spectra were automatically corrected for phase and baseline distortions and calibrated (chemical
394 shift was referenced to the doublet of alanine at 1.48 ppm for cell lysates, and to the singlet of TMSP at 0.00
395 ppm for growth media) using TopSpin 3.5 (Bruker BioSpin srl). Multivariate and univariate analyses were
396 performed on the obtained data using R software. For multivariate analysis, each spectrum in the region 10-
397 0.2 ppm was segmented into 0.02 ppm chemical shift bins, and the corresponding spectral areas were integrated
398 using the AMIX software (Bruker BioSpin). Binning is a mean to reduce the number of total variables and to
399 compensate for small shifts in the signals, making the analyses more robust and reproducible. The area of each
400 bin was normalized to the total spectral area, calculated with exclusion of the water region (4.50 – 5.15 ppm),
401 in order to correct the data for possible differences in the cell count of each of the NMR samples.

402 Unsupervised Principal Component Analysis (PCA) was used to obtain a preliminary overview of the data
403 (visualization in a reduced space, cluster detection, screening for outliers). Canonical analysis (CA) was used
404 in combination with PCA to increase supervised data reduction and classification. Accuracy, specificity, and
405 sensitivity were estimated according to standard definitions. The global accuracy for classification was
406 assessed by means of a leave-one-out cross-validation scheme. The metabolites, whose peaks in the spectra
407 were well defined and resolved, were assigned and their levels analyzed. The assignment procedure was
408 performed using an internal NMR spectral library of pure organic compounds, public databases such as the *E.*
409 *coli* Metabolome Database ⁶³ storing reference NMR spectra of metabolites, and spiking NMR experiments ⁶⁴.
410 The relative concentrations of the various metabolites were calculated by integrating the corresponding signals
411 in the spectra ⁶⁵, using a home-made program for signal deconvolution. The nonparametric Wilcoxon-Mann-
412 Whitney test was used for the determination of the meaningful metabolites: a p-value of 0.05 was considered
413 statistically significant. The molecule 1,4-dioxane was used as a standard to perform the quantitative NMR
414 analysis with the aim of obtaining the absolute concentrations (μM) of the analyzed metabolites.

415 NMR data were uploaded on the MetaboLights database (www.ebi.ac.uk/metabolights) with the accession
416 number MTBLS576.

417 **Generation of the metabolic models.** The manually curated iGD1575 reconstruction of *S. meliloti* Rm1021
418 ¹⁹ was modified to expand the composition of the biomass reaction through the inclusion of an additional 31
419 compounds, including vitamins, co-enzymes, and ions at trace concentrations as described elsewhere (Table
420 S6) ⁴⁶. Although iGD1575 is based on *S. meliloti* Rm1021, it is expected to accurately represent Rm2011
421 metabolism as these two strains are derived from the same field isolate (SU47) and have nearly identical gene
422 contents ²⁰; while there are numerous SNPs between the strains, SNPs are not considered during the process
423 of metabolic reconstruction.

424 All other metabolic models were constructed using our recently published protocols for template-assisted
425 metabolic reconstruction and assembly of hybrid bacterial models ²⁷. Briefly, a draft metabolic reconstructions
426 of *S. meliloti* BL225C was produced using the Kbase webserver (www.kbase.us) with gapfilling. The draft
427 model was enhanced using the curated Rm1021 model as a template according to ²⁷, using orthologous gene
428 sets between BL225C and Rm1021 produced with InParanoid ⁶⁶. Additionally, an appropriate *protein synthesis*
429 reaction was manually added to the model. Finally, replicon transplantation between the BL225C model and
430 the Rm1021 model was performed as described recently ²⁷, making use of the InParanoid generated orthology
431 data and the information contained within each model. All metabolic reconstructions used in this work are
432 provided in Supplementary File S1 in COBRA format within a MATLAB MAT-file. The enhancement and
433 transplantation pipeline is available at <https://github.com/TVignolini/replicon-swap>.

434

435 **Author contribution**

436 A. Checcucci created the strains, performed microbiological analyses. G. diCenzo contributed in metabolic
437 model creation and performed computation analyses on the metabolic modelling. V. Ghini, P. Turano, C.
438 Luchinat performed NMR analyses and contributed in NMR spectra interpretation. V. Ghini contributed in
439 preparing illustrations. A. Becker and J. Döhlemann contributed PFGE analysis and interpretation. T. Vignolini
440 and M. Fondi contributed the first draft of metabolic model and preliminary computational simulations. G.
441 diCenzo performed computational simulations. F. Decorosi and C. Viti contributed in Phenotype Microarray
442 analysis and interpretation. A. Checcucci and C. Fagorzi contributed to *in vitro* symbiotic assays. M.
443 Bazzicalupo and T. Finan provided data interpretation. A. Mengoni, M. Fondi, G. diCenzo, A. Checcucci
444 conceived the work. A. Checcucci, A. Mengoni, V. Ghini, M. Fondi, G. diCenzo prepared the manuscript. All
445 authors have read and approved the manuscript.

446 **Notes**

447 Author declare no competing financial interest

448

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660 **Figures**

661 **Figure 1. (a) Comparison of *cis*-hybrid strain genome sequence with 2011 chromosome and pSymB, and with**
662 **pSymA of the donor strain (BL225C)** (contigs alignment performed with Contiguator (Galardini, *et al.*, 2011); (b)
663 Percentage of identity of each replicon which composed the multipartite genome of *cis*-hybrid strain with those of the
664 donor strains (Rm2011 and BL225C).

665 **Figure 2. Metabolic phenotype of the *cis*-hybrid strain.** Heatmap of Phenotype Microarray profiles of the growth on
666 different carbon and nitrogen sources for Rm2011, BL225C, *cis*-hybrid and Δ pSymA strains. (a) heatmap with Euclidean
667 clustering; (b) values of pairwise Euclidean distances

668
669 **Figure 3. ¹H Nuclear Magnetic Resonance (NMR)-based metabolomic profiles of cellular lysates and growth media**
670 of Rm2011, BL225C, *cis*-hybrid and Δ pSymA strains. Score plot of PCA (a;b) and PCA-CA (c;d) analysis of cell lysates
671 (a;c) growing media (b;d). The confusion matrices and the discrimination accuracy values for PCA-CA analysis are also
672 reported. Ellipses in the score plots illustrate the 95% confidence level.

673
674 **Figure 4. Growth phenotypes of the *cis*-hybrid strain.** The growth of *S. meliloti* was examined in TY complex medium
675 (a) M9 minimal medium (b) and (c) M9 +succinate and root exudates as sole N source. Data points represent averages
676 from quadruplicate measurements. The letters on the curves represent the statistically significant differences among the
677 strains growth ($p < 0.005$, Tukey post-hoc contrasts).

678
679 **Figure 5. Symbiotic capabilities of the *cis*-hybrid strain.** Heatmaps of symbiotic performances profiles for Rm2011,
680 BL225C and *cis*-hybrid strains in a panel of seven alfalfa cultivars; (a) percentage of nodulated plants, (b) number of
681 nodules per plant, (c) plant aerial part length (cm) and (d) the shoot dry weight (mg).

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686 **Supplemental Material**

687 **Supplemental Table S1. List of primer used for the cis-hybrid strain creation**

688 **Supplemental Table S2. Differential utilization of carbon sources (a) and nitrogen sources (b) by cis-hybrid strain**
689 **respect to the wild type strains.** The differential growth was evaluated with the activity index values calculated with
690 DuctApe software (Galardini, *et al.*, 2014). Complete list of activity index values for all strains tested in carbon (c) and
691 nitrogen (d) sources.

692 **Supplemental Table S3. Concentration (μM) of each identified metabolite (a) in the cell lysates and (b) in the media.**

693 **Supplemental Table S4. List of alfalfa cultivars used for symbiotic assay.**

694 **Supplemental Table S5. Symbiotic capabilities of the strains.** Values and relatives standard deviation of symbiotic
695 performances profiles for Rm2011, BL225C and *cis*-hybrid strains in seven alfalfa cultivars.

696 **Supplemental Table S6. Biomass composition.**

697 **Supplemental File S1. Metabolic Model reconstruction in COBRA format within a MATLAB MAT-file.**

698

699 **Supplemental Figure S1. Pulse Field Gel Electrophoresis (PFGE)** performed on the *cis*-hybrid strains, the cured and
700 the parental strains. The banding profile of the *cis*-hybrid strain is detailed with band size and replicon origin. The
701 enzymatic digestion with PmeI was performed. Details: 0.5xTBE, 0.5% Agarose, M = PFGE marker *S. cerevisiae*
702 (Biorad), Cell density: $8 \times 10^8/\text{ml}$

703 **Supplemental Figure S2. NMR spectra of the endo (a) and exo (b) metabolic profiles.**

704 **Supplemental Figure S3. Boxplots of the interesting metabolites in cell lysates and media.**

705 **Supplementary Figure S4. Biofilm-to-biomass ratio** (OD560 / OD600) for the wild type, recipient, and *cis*-hybrid
706 strains. The values are calculated on the mean values of 4 replicates. Error bars indicate standard deviation (calculated on
707 the error propagation); ($p < 0.005$, Tukey post-hoc contrasts).

708 **Supplemental Figure S5. Nodule colonization efficiency in *M. sativa* cv Maraviglia.** Nodule colonization (bacterial
709 genome copies cells inside nodule, from qPCR, $n=10$); the experiment was performed in different biological replicate.
710 Values indicate means and standard deviation ($p < 0.005$, Tukey post-hoc contrasts).

711

712 **References**

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716 OmniLog™ Phenotype Microarray data. *Genomics* **103**: 1.

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721 **Table 1. Strains and plasmids used in this study***

Strain or plasmid	Description	Reference
<i>Sinorhizobium meliloti</i>		
Rm2011	Wild type SU47 derivative; Sm ^R	20
BL225C	Wild isolate from <i>Medicago sativa</i> in Lodi (Italy)	15
RmP3498	Rm2011 ΔpSymAB with engA and tRNA into the chromosome; Sm ^R Sp ^R	11
BM 826	RmP3498 with pMp7605; Sm ^R Sp ^R Gm ^R	This work
BM 835	BM 826 with pSymA from BL225C; Sm ^R Sp ^R Gm ^R	This study
BM 848	BL225C with pTE3rctB; Tc ^R	This work
<i>Escherichia coli</i>		
MT616	Helper strain carrying pRK600 that has the RK2 <i>tra</i> genes; Cm ^R	50
Plasmids		
pMp7605	Broad host range vector constitutively expressing the <i>mCherry</i> gene; Gm ^R	51
pTE3rctB	Broad host range vector over-expressing the <i>Rhizobium etli rctB</i> gene; Tc ^R	25

722

723 * Code of strains and plasmid is reported. A succinct description of the main phenotypic features is shown;

724 Sm^R, streptomycin resistance, Sp^R, spectinomycin resistance, Gm^R gentamycin resistance, Tc^R tetracyclin

725 resistance, Cm^R, chloramphenicol resistance.

726

727 **Table 2. Comparison of *S. meliloti* metabolic network reconstructions.** The gene and reaction content of
 728 the four *S. meliloti* metabolic reconstructions used in this work are shown. For each cell, the values are a
 729 comparison of the strain indicated on the left with the strain indicated along the top. Three values are provided
 730 in each cell, and these correspond to the following. The first value is the number of genes or reactions in
 731 common between the models. The second value is the number of genes or reactions present in the
 732 reconstruction on the left but not in the one along the top. The third value is the number of genes or reactions
 733 present in the reconstruction along the top but not in the one on the left.
 734

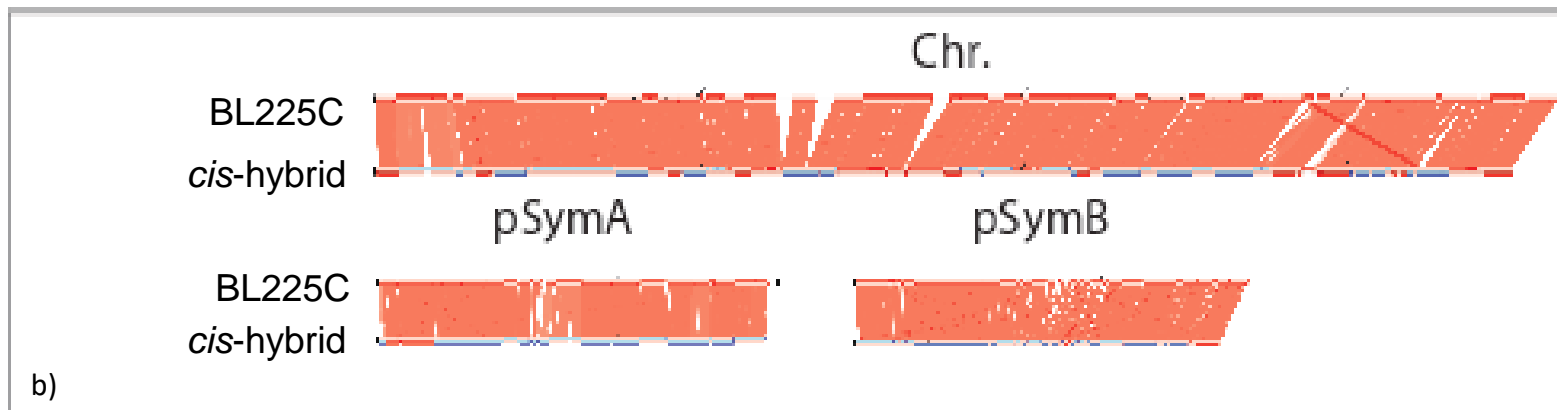
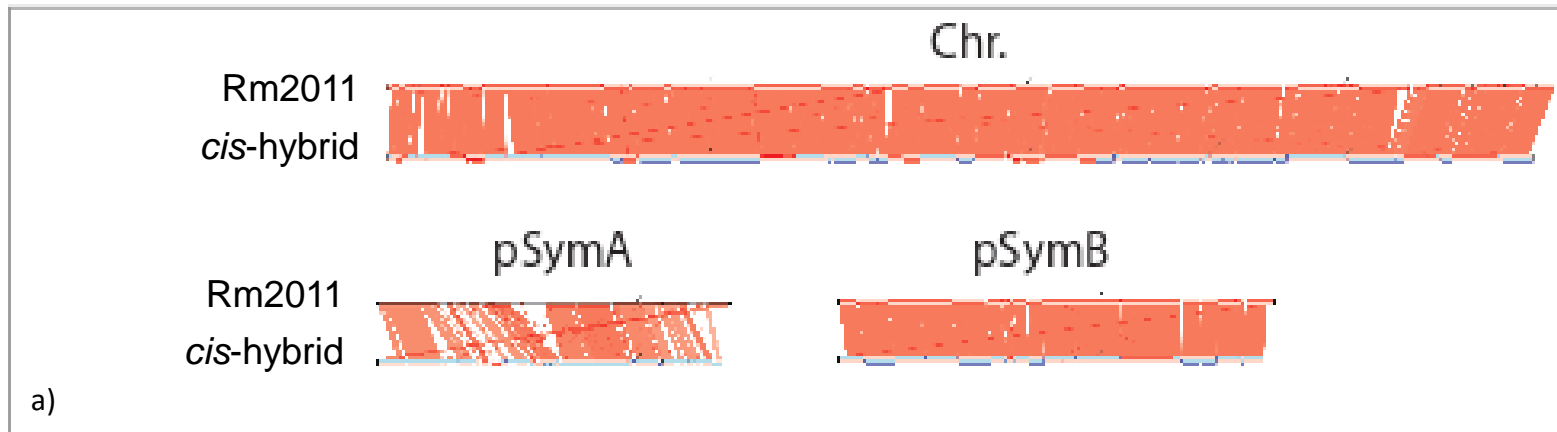
Strain	Genes			Reactions		
	BL225C	cis-hybrid	Δ pSymA	BL225C	cis-hybrid	Δ pSymA
Rm2011	1525 / 52 / 91	1551 / 26 / 76	1336 / 241 / 0	1821 / 6 / 6	1823 / 4 / 6	1755 / 72 / 0
BL225C	-	1598 / 18 / 29	1308 / 308 / 28	-	1827 / 0 / 2	1753 / 74 / 2
cis-hybrid	-	-	1336 / 291 / 0	-	-	1755 / 74 / 0

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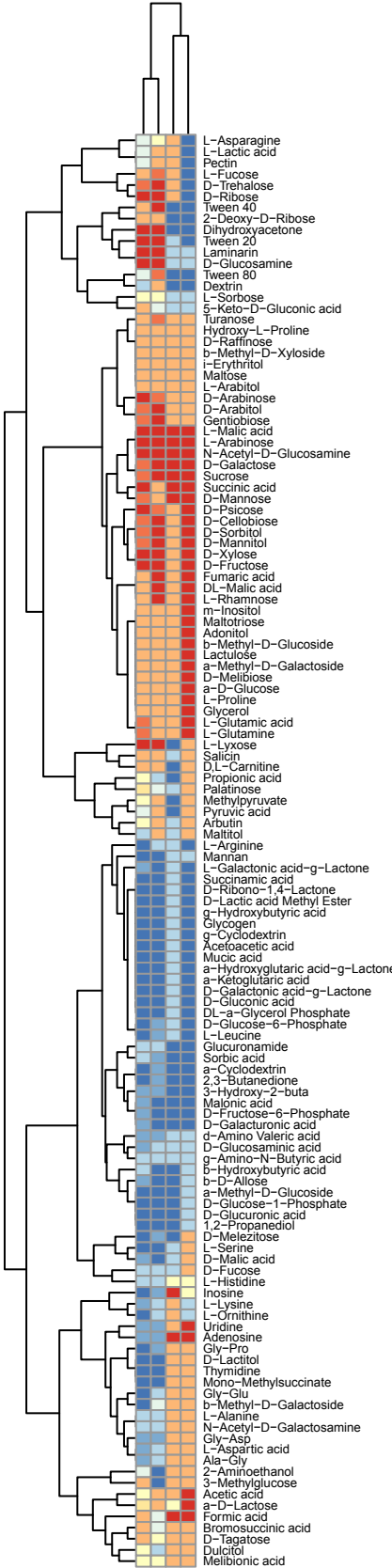
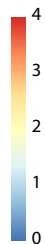
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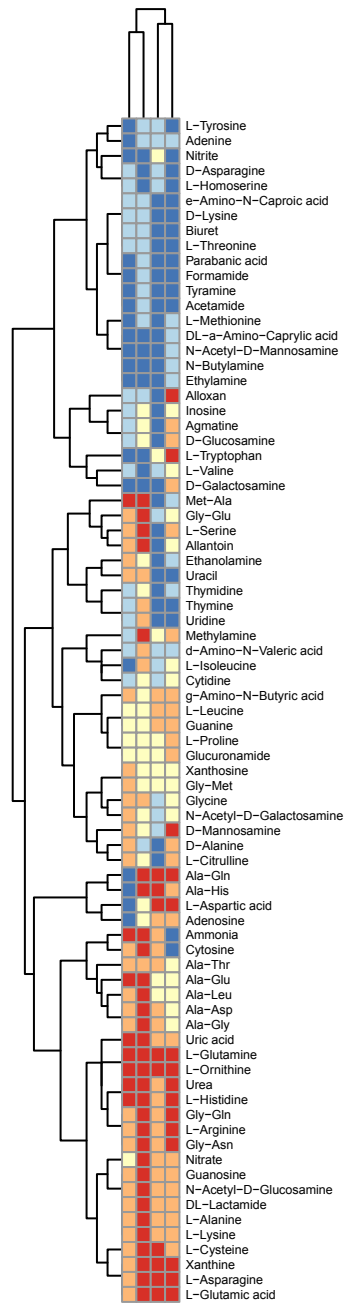
c)

<i>cis</i> -hybrid	Rm2011	BL225C
Chromosome	100%	99.97%
pSymA	98.62%	100%
pSymB	100%	99.19%

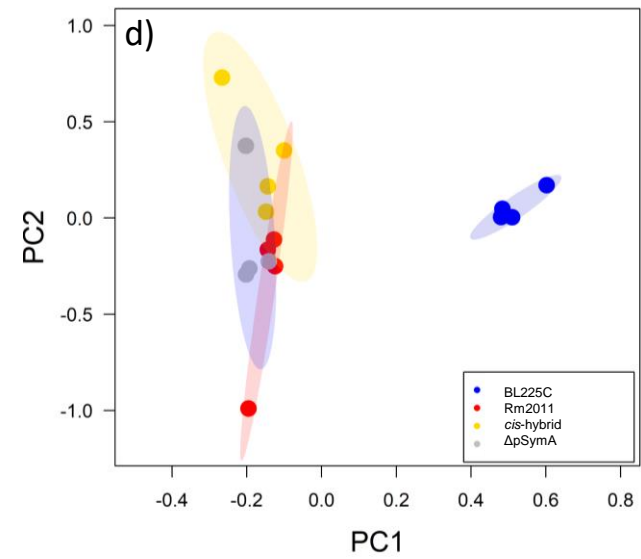
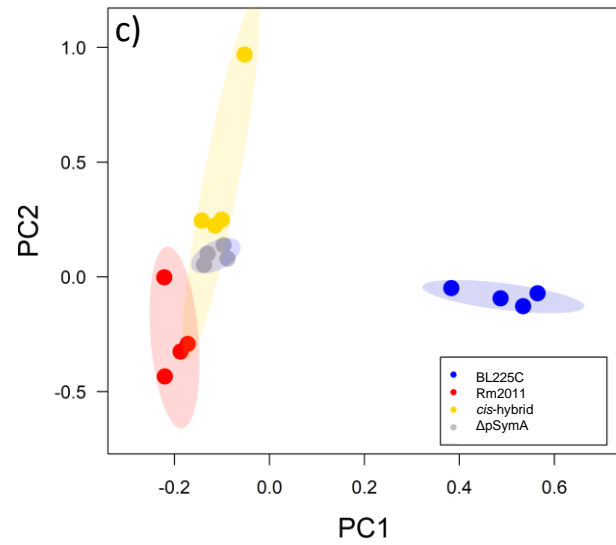
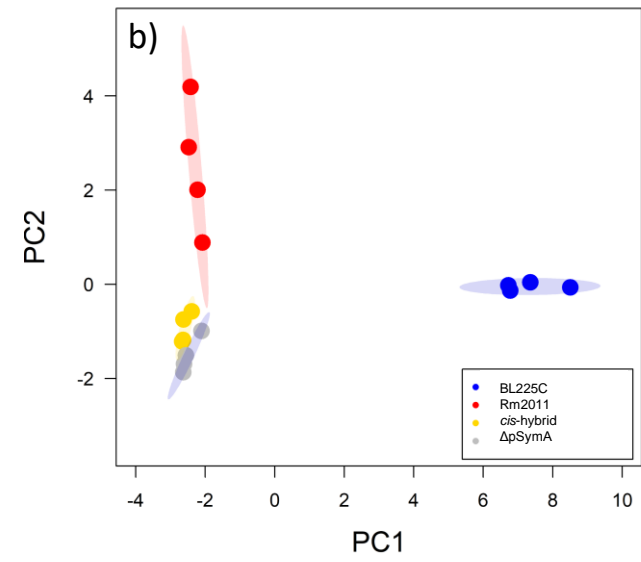
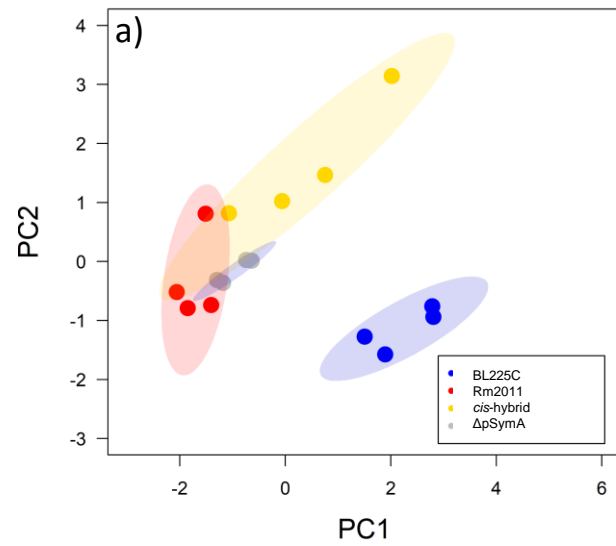
Activity index



A



B



	BL225C	Rm2011	<i>cis</i>-hybrid	ΔpSymA
BL225C	1	0	0	0
Rm2011	0	1	0	0
<i>cis</i> -hybrid	0	0	1	0
ΔpSymA	0	0.25	0	0.75

Discrimination accuracy
93.7%

	BL225C	Rm2011	<i>cis</i>-hybrid	ΔpSymA
BL225C	1	0	0	0
Rm2011	0	0.75	0.25	0
<i>cis</i> -hybrid	0	0	0.75	0.25
ΔpSymA	0	0	0	1

Discrimination accuracy
87.5%

